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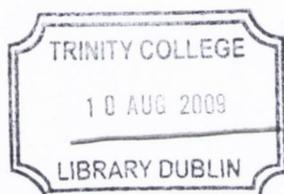
An investigation into the relative adhesion of
Candida dubliniensis and *Candida albicans* to
human cells and extracellular matrix proteins

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

Rachael P. C. Jordan, B. Sc. (Hons)

May 2009

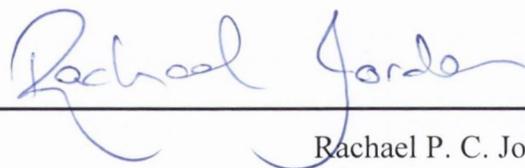
Microbiology Research Unit, Division of Oral Biosciences,
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DECLARATION

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Rachael P. C. Jordan, B. Sc. (Hons)

January 2009



*This thesis is dedicated to my Granda
“If at first you don’t succeed, try and try again”*

SUMMARY

Candida dubliniensis is a germ tube-positive, chlamyospore-producing yeast species originally recovered from the oral cavities of HIV-infected individuals and AIDS patients and first described in 1995. It is closely related to *Candida albicans*. Despite their close phenotypic, genotypic and phylogenetic relationship, epidemiological and infection model data indicate that *C. dubliniensis* is significantly less pathogenic than *C. albicans*. As adherence to host cells and tissue is an essential early step in the establishment of disease, the comparative ability of *C. dubliniensis* and *C. albicans* to adhere to human buccal epithelial cells (BECs), monolayers of the TR146 human epithelial cell line and extracellular matrix (ECM) proteins was investigated in order to obtain insights into the differential pathogenicity of the two species. The role of the agglutinin-like sequence (*ALS*) genes in the adhesion of both species to BECs was also investigated.

The ability of six reference strains of *C. albicans*, including SC5314, and 21 *C. dubliniensis* isolates from each of the four known genotypes to adhere to pooled samples of BECs was examined by microscopy and crystal violet staining. When *Candida* isolates were pre-cultured overnight (~16 h) in YEPD at 30°C, *C. dubliniensis* genotype 1 isolates adhered to BECs in significantly greater numbers than *C. albicans* and the other *C. dubliniensis* genotypes ($P < 0.001$). Similarly, when *Candida* isolates were pre-cultured in YEPGal at 37°C overnight, *C. dubliniensis* genotype 1 isolates adhered to BECs in significantly greater numbers than *C. albicans* and *C. dubliniensis* genotypes 2-4 ($P < 0.001$). Conversely, when *Candida* isolates were pre-cultured overnight in YEPD at 37°C, there was no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* isolates ($P > 0.05$) and both *C. dubliniensis* genotype 1 and *C. albicans* adhered to BECs in significantly greater numbers than the other *C. dubliniensis* genotypes ($P < 0.001$). *C. dubliniensis* genotypes 1, 2 and 4 (all $P < 0.05$) adhered to BECs in significantly greater numbers when pre-cultured in YEPD at 30°C compared to 37°C which may reflect its predominant association with the oral cavity, the temperature of which ranges between 29-32°C, lower than body temperature.

The ability of eight *C. albicans* isolates and 21 *C. dubliniensis* isolates from each of the four *C. dubliniensis* genotypes to adhere to monolayers of TR146 cells grown in wells of 96-well tissue culture plates was investigated and percentage candidal adhesion was elucidated using XTT dye and spectrophotometric analysis. When the

Candida isolates were pre-cultured in YEPD at 37°C overnight, *C. dubliniensis* genotype 4 isolates adhered in significantly greater numbers than isolates of the other *C. dubliniensis* genotypes (1, $P < 0.01$; 2, 3 and 4v, $P < 0.001$). There was no significant difference between the adhesion of *C. dubliniensis* genotype 4 and *C. albicans* ($P > 0.05$). Because BEC adhesion assay and TR146 adhesion data differed, it is likely that candidal adhesion is also influenced by the type of cell being adhered to.

The ability of 12 *C. albicans* isolates and 9 *C. dubliniensis* isolates from each of the four genotypes to adhere to six ECM proteins was investigated using surface plasmon resonance technology. The *Candida* isolates were pre-cultured in YEPD at 30°C overnight and the mean adhesion of *C. albicans* and *C. dubliniensis* to all six ECM proteins was determined. *C. dubliniensis* genotype 1 isolates adhered to all six ECM proteins in significantly greater amounts than isolates of the other *C. dubliniensis* genotypes and *C. albicans* isolates ($P < 0.001$). Mean adhesion of 21 isolates of both *C. albicans* ($n = 12$) and *C. dubliniensis* ($n = 9$) indicated that *Candida* adhered to laminin in significantly greater amounts than to the other five ECM proteins tested ($P < 0.001$). These findings reflect data obtained from the BEC adhesion assays when *Candida* are pre-cultured under similar conditions, supporting the BEC assay results.

The expression of the *ALS* genes of *C. albicans* and *C. dubliniensis* in adherent, stationary phase *Candida*, non-adherent, mid-exponential phase *Candida* and *Candida* grown in hyphal inducing medium were examined using quantitative real time RT-PCR. The results showed that the majority of the *ALS* genes of both species are involved in the adhesion of *Candida* to BECs as greater expression of the majority of *ALS* genes in *Candida* adherent to BECs occurred compared to non-adherent *Candida*. *CaALS3* was the most up-regulated gene in *C. albicans* SC5314 when pre-cultured under hyphal inducing conditions. *CdALS1* was the most up-regulated and *CdALS7* was the most down-regulated gene in all *C. dubliniensis* isolates tested when pre-cultured under hyphal inducing conditions. *C. dubliniensis* lacks an ortholog for the invasin *CaALS3*, which may contribute to its lower virulence compared to *C. albicans*.

In conclusion, *C. dubliniensis* appears to have a selective advantage in its ability to adhere to BECs when pre-cultured at 30°C compared to 37°C which may be the reason why it is primarily associated with the oral cavity. Candidal adhesion also appears to be influenced by host cell type. Despite the increased ability of *C. dubliniensis* to adhere to human cells at 30°C compared to 37°C, its lower virulence in comparison to *C. albicans* could be due in part to the lack of an ortholog for *CaALS3*.

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POSTER PRESENTATIONS ORIGINATING FROM THIS STUDY

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ABBREVIATIONS

| | |
|-----------------------|--|
| A _x |absorbance at x nm |
| AIDS |acquired immunodeficiency syndrome |
| ALS |agglutinin-like sequence |
| ANOVA |analysis of variance |
| ACT |Artemis Comparison Tool |
| APECED |autoimmune polyendocrinopathy, candidosis and ectodermal dysplasia |
| bp |base pair(s) |
| B. Sc. (Hons). |Bachelor of Science, Honours Degree |
| BEC(s) |buccal epithelial cell(s) |
| <i>CaALS</i> | <i>C. albicans</i> agglutinin-like sequence |
| <i>CdALS</i> | <i>C. dubliniensis</i> agglutinin-like sequence |
| CA |California |
| <i>C.</i> | <i>Candida</i> |
| CO ₂ |carbon dioxide |
| CSH |cell surface hydrophobicity |
| Co. |Company |
| cDNA |complementary deoxyribonucleic acid |
| Corp. |Corporation |
| Ct |cycle threshold |
| Coenzyme Q |2,3-Dimethoxy-5-methyl- <i>p</i> - benzoquinone |
| dATP |deoxy-adenosine triphosphate |
| dCTP |deoxy-cytidine triphosphate |
| dGTP |deoxy-guanosine triphosphate |
| dTTP |deoxy-thymidine triphosphate |
| dNTPs |deoxynucleotide triphosphates |

| | |
|-----------------------|--|
| DNase |deoxyribonuclease |
| DNA |deoxyribonucleic acid |
| DEPC |diethyl pyrocarbonate |
| DMSO |dimethyl sulfoxide |
| Dip. Appl. Biol. |Diploma in Applied Biology |
| Dr. |Doctor |
| <i>et al.</i> |and others |
| EF |elongation factor |
| EDTA |ethylenediaminetetraacetic acid |
| ExpPASy |Expert Protein Analysis System |
| ECM |extracellular matrix |
| e.g. |for example |
| EDC |1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride |
| Fig(s). |figure(s) |
| FC(s) |flow cell(s) |
| FUN-1 |2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide |
| GPI |glycosylphosphatidylinositol |
| g |grams |
| <i>H.</i> | <i>Helicobacter</i> |
| h |hour(s) |
| HIV |human immunodeficiency virus |
| HWP |hyphal wall protein |
| Inc. |Incorporated |
| ITS |internal transcribed spacer |
| I.U. |International units |

| | |
|------------------------|--|
| <i>I</i> | <i>Ixodes</i> |
| <i>in vitro</i> |Latin, meaning within the glass. Refers to the technique of performing an experiment in a controlled environment outside of a living organism |
| <i>in vivo</i> |occurring or made to occur within a living organism |
| i.e. |that is |
| kb |kilobase(s) |
| kDa |kilodaltons |
| Ltd. |Limited |
| L |litre |
| L ⁻¹ |per litre |
| MA |Massachusetts |
| M. B. D. |Masters in Biomedical Diagnostics |
| Mb |megabase |
| mg |milligram |
| ml |millilitre(s) |
| mm |millimetre(s) |
| mM |millimolar |
| min |minute(s) |
| MO |Missouri |
| M |molar |
| MLST |multilocus sequence typing |
| ml ⁻¹ |per millilitre |
| mm ⁻² |per millimetre squared |
| NHS |N-hydroxysuccinimide |
| ng |nanograms |
| nm |nanometers |
| nM |nanomolar |

| | |
|----------------|--|
| NCPF |National Collection of Pathogenic Fungi |
| NCYC |National Collection of Yeast Cultures |
| NJ |New Jersey |
| NTC |non-template control |
| N/A |not applicable |
| N/Av |not available |
| NMR |nuclear magnetic resonance |
| n |number |
| ON |Ontario |
| ORFs |open reading frames |
| OR |Oregon |
| pH |cologarithm of the activity of dissolved hydrogen ions |
| Ph. D. |Doctor of Philosophy |
| pI |isoelectric point |
| P |p value |
| PA |Pennsylvania |
| PBS |phosphate buffered saline |
| pmoles |picomoles |
| PCR |polymerase chain reaction |
| PDA |potato dextrose agar |
| Prof. |Professor |
| PRPs |proline rich peptides |
| QRT |quantitative real time |
| QRTALS |quantitative real time agglutinin-like sequence |
| QRTCaALS |quantitative real time <i>C. albicans</i> agglutinin-like sequence |
| QRTCdALS |quantitative real time <i>C. dubliniensis</i> agglutinin-like sequence |

| | |
|--------------------|---|
| QLD |Queensland |
| RGD |Arginine-Glycine-Asparagine |
| RHE |reconstituted human epithelial/epithelium |
| RU(s) |resonance unit(s) |
| RT |reverse transcription |
| RT-PCR |reverse transcription polymerase chain reaction |
| rpm |revolutions per minute |
| RNase |ribonuclease |
| RNA |ribonucleic acid |
| rRNA |ribosomal RNA |
| RAT agar |Rice Agar Tween agar |
| SDA |sabouraud dextrose agar |
| <i>Sa.</i> | <i>Saccharomyces</i> |
| s |seconds |
| SAP(s) |secreted aspartyl proteinase(s) |
| $S_{AB}(s)$ |similarity coefficient(s) |
| SD |standard deviation |
| SEM |standard error of the mean |
| <i>S.</i> | <i>Streptococcus</i> |
| SPR |surface plasmon resonance |
| Triton X-100 |t-octylphenoxypolyethoxyethanol |
| TX |Texas |
| TIR |total internal reflection |
| TBE |Tris-Borate-EDTA |
| UV |ultraviolet |
| UK |United Kingdom |
| USA |United States of America |
| U |units |

| | |
|--------------------------|---|
| VASES |Valine-Alanine-Serine-Glutamic acid-Serine |
| VB1/2 |variable block 1/2 |
| v. |version |
| v/v |volume per volume |
| WA |Washington |
| w/v |weight per volume |
| WI |Wisconsin |
| XTT sodium salt |2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt |
| YEPD |yeast extract peptone dextrose |
| YEPGal |yeast extract peptone galactose |
| α |alpha |
| & |and |
| ~ |approximately |
| β |beta |
| $^{\circ}\text{C}$ |degrees Celsius |
| Δ |delta |
| = |equals/equal to |
| $\times g$ |gravitational force |
| > |greater than |
| \geq |greater than or equal to |
| < |less than |
| μg |microgram(s) |
| μl |microlitre(s) |
| μm |micrometer |
| μM |micromolar |
| % |percent |

| | |
|-----------|-----------------------------------|
| %id |percentage of identification |
| ± |plus or minus |
| ' |prime |
| ® |registered |
| IV |Roman numeral four |
| I |Roman numeral one |
| III |Roman numeral three |
| II |Roman numeral two |
| 2° |secondary |
| × |times/multiplication/by |
| ™ |Trade mark |
| 4v |4 variant |
| 5FC |5-flucytosine |

Chapter 1

General Introduction

1.1 GENERAL INTRODUCTION

1.1.1 Candidiasis and *Candida* species

Since the 1980s, the prevalence of fungal infections has increased significantly. This increased incidence is likely to be directly attributable to the increased number and longer survival of immunocompromised individuals, including human immunodeficiency virus (HIV)-infected and acquired immunodeficiency syndrome (AIDS) patients. It is also likely to be attributable to the number of immunosuppressed patients such as those undergoing chemotherapy or immunosuppressive therapy associated with bone marrow or solid organ transplantation. There has also been an increase in the use of invasive medical procedures and in-dwelling catheters and an increase in the number of patients using corticosteroids and broad-spectrum antibiotics, all of which are believed to contribute to infection with *Candida* species (McCourtie & Douglas, 1981; Pfaller, 1996; Pinjon *et al.*, 2005b). In addition, a variety of nutritional factors, including deficiency in iron, folic acid and vitamins and/or a diet rich in carbohydrates may predispose an individual to oral candidal infections (Farah *et al.*, 2000).

Candida species are frequently referred to as the most important causative agents of fungal infection in immunocompromised and immunosuppressed patients and rank fourth as the most common cause of nosocomial blood stream infection in the United States with an associated mortality rate of 35% (Wenzel, 1995; Pfaller *et al.*, 1998a; Edmond *et al.*, 1999). In one hospital in the United Arab Emirates a mortality rate of 50% occurred following candidemia (Ellis, 2002). Oral candidiasis is the most frequently encountered opportunistic infection in HIV-infected individuals and AIDS patients and it is often one of the earliest clinical symptoms detected, affecting 90% – 95% of subjects during HIV disease progression (Coleman *et al.*, 1997a). Although several species of *Candida* form part of the normal oropharyngeal and gastrointestinal flora (Odds, 1988), they can become opportunistic pathogens and establish infection if the balance of the normal flora is disrupted or the host's immune defences are impaired (Naglik *et al.*, 2003; Pinjon *et al.*, 2005b). The physiological status of the host is the primary factor governing the etiology of candidiasis and only slight alterations in the host's immune system may allow infection by these normally commensal organisms

(Sweet, 1997). Of the approximately two hundred *Candida* species known, approximately 20 have been associated with human disease (Odds, 1988). *Candida albicans*, which is polymorphic and can grow as yeast, pseudohyphae or true hyphae, is the most pathogenic species of the genus and is the most frequently associated with candidiasis (Odds, 1988; Cannon & Chaffin, 1999; Farah *et al.*, 2000). However, there has also been an increase in the prevalence of infection caused by other less pathogenic *Candida* species such as *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida dubliniensis* (Wingard *et al.*, 1991; Pfaller, 1994; Samaranayake & Samaranayake, 1994; Wingard, 1995; Coleman *et al.*, 1998; Gilfillan *et al.*, 1998). Many non-*C. albicans* *Candida* species including *C. glabrata* and *C. krusei* are inherently less susceptible to antifungal drugs than *C. albicans* and these could be selected following antifungal drug therapy (Coleman *et al.*, 1997a).

1.1.2 Superficial candidiasis

Candida species cause a wide range of human disease including superficial mucosal infections such as vulvovaginal or oropharyngeal candidiasis in immunocompetent individuals (Jones *et al.*, 2004). Approximately 75% of women, regardless of their HIV status will experience at least one episode of vaginal infection caused by *Candida* species during their lifetime (Ruhnke, 2002) and while most women suffer from sporadic mild to moderate vaginitis, approximately 10% of women suffer from more severe and recurrent symptoms. Women with predisposing factors such as immunosuppression or uncontrolled diabetes are at a higher risk from this kind of infection (Fidel *et al.*, 1999).

Oral candidiasis may present in a number of forms including acute/chronic erythematous candidiasis, pseudomembranous candidiasis, median rhomboid glossitis, angular cheilitis (which can occur as a bilateral or unilateral infection), hyperplastic candidiasis, denture stomatitis, candidal leukoplakia and chronic mucocutaneous candidiasis (and autoimmune polyendocrinopathy, candidosis and ectodermal dysplasia (APECED); Scully *et al.*, 1994; Farah *et al.*, 2000). The frequency of oral candidiasis increases with HIV disease progression and infection is often recurrent and unresponsive to therapy (Klein *et al.*, 1984; Samaranayake & Holmstrup, 1989; Samaranayake, 1992; Coleman *et al.*, 1993). Of the many forms of oral candidiasis, three manifestations are prevalent in HIV-infected individuals (Samaranayake, 1992).

Erythematous candidiasis is the most common manifestation of candidiasis in the HIV-infected population (Sullivan *et al.*, 1993), which presents as red lesions affecting the palate, buccal mucosa and dorsum of the tongue, the latter of which often becomes depapillated. Erythematous candidiasis usually precedes pseudomembranous candidiasis, which presents as whitish-yellow semi adherent patches or confluent membranes that can be removed from the mucosa leaving a red bleeding surface. Angular cheilitis (a co-infection with *Streptococcus* and *Staphylococcus* species) is the least common of the three manifestations and presents at the angles of the mouth as red, fissured, crusty lesions which may be ulcerated (Samaranayake & Holmstrup, 1989; Samaranayake, 1992). The onset of these manifestations of candidiasis are usually preceded by oral colonisation with *Candida* and it has been noted that many HIV-infected individuals have substantially higher levels of oral *Candida* than the normal healthy population (Tylenda *et al.*, 1989). It has also been noted that HIV-infected individuals are usually colonised and infected by one or a small number of *C. albicans* strains, which are characteristic for that individual and usually persist through symptomatic and asymptomatic periods, even following antifungal treatment and subsequent development of antifungal drug resistance (Coleman *et al.*, 1997a).

Ill-fitting dentures, poor denture hygiene and inadequate oral hygiene are chronic local irritants in the oral cavity. It is likely that constant irritation by dentures may contribute to denture stomatitis. That, combined with a diet rich in carbohydrates often eaten by denture wearers may allow for over growth of *Candida* species allowing initiation of infection (Farah *et al.*, 2000).

1.1.3 Invasive candidiasis

Invasive candidiasis is the term used for infections caused when *Candida* cells penetrate through the epithelial barrier. Invasive candidiasis includes candidemia which is defined as colonisation of the bloodstream by *Candida* species. *Candida* species can cause life-threatening invasive and disseminated infections in those with severely immunocompromised immune systems (Jones *et al.*, 2004). Systemic candidiasis involves haematogenous spread of *Candida* to multiple organs including the brain, kidneys, heart, liver and lungs (Parker *et al.*, 1976) and has a mortality range of 33% to 56% with high morbidity in those who survive (Todeschini, 1997). Systemic candidiasis may also develop following direct inoculation during surgery. Risk factors for systemic

candidiasis may include neutropenia, the use of in-dwelling catheters, abdominal surgery, burns and the use of broad-spectrum antibiotics and/or corticosteroids (Pfaller, 1996; Sullivan *et al.*, 2004).

1.2 *Candida dubliniensis*

1.2.1 Epidemiology of *C. dubliniensis*

Candida dubliniensis is a germ tube-positive, chlamyospore-producing yeast species which was first recovered from the oral cavities of HIV-infected individuals and AIDS patients with oral candidiasis in Ireland in 1995 (Sullivan *et al.*, 1995). The oldest confirmed isolate of the species was identified retrospectively and was recovered in Holland in 1952 and was originally identified as *C. albicans* (Meis *et al.*, 1999). *Candida dubliniensis* has a world-wide distribution and has been isolated from a wide range of anatomical sites and clinical samples including sputum, vaginal flora, faeces, urine, wounds, the respiratory tract and blood (Mahrous *et al.*, 1990; Asakura *et al.*, 1991; Schmid *et al.*, 1992; Sullivan *et al.*, 1993; McCullough *et al.*, 1994; Anthony *et al.*, 1995; Boerlin *et al.*, 1995; Le Guennec *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Tietz *et al.*, 1995; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds *et al.*, 1998; Pfaller *et al.*, 1998b; Pinjon *et al.*, 1998; Meiller *et al.*, 1999; Polacheck *et al.*, 2000; Boyle *et al.*, 2002; Peltroche-Llacsahuanga *et al.*, 2002; Al Mosaid *et al.*, 2005).

While *C. albicans* is the most common *Candida* species isolated from the oral cavity, *C. dubliniensis* is most commonly associated with oropharyngeal candidiasis in HIV-infected individuals and AIDS patients. Oral *C. dubliniensis* was recovered from 32% of AIDS patients presenting with clinical candidiasis and from 25% of asymptomatic AIDS patients (Coleman *et al.*, 1997a). A smaller number (10%) of AIDS patients with symptoms of oral candidiasis harboured *C. dubliniensis* only. *Candida dubliniensis* was also recovered from 27% of symptomatic and 19% of asymptomatic HIV-infected individuals. Similarly, a small number (6%) of HIV-infected patients with oral candidiasis harboured *C. dubliniensis* only indicating that *C. dubliniensis* can cause disease independently of other *Candida* species (Coleman *et al.*, 1997a; Coleman *et al.*, 1997b; Sullivan & Coleman, 1998). Seventy six percent of HIV-infected individuals

and AIDS patients and 83% of HIV-negative individuals who harbour oral *C. dubliniensis* also harboured other *Candida* species, most commonly *C. albicans* (Coleman *et al.*, 1997b).

Although *C. dubliniensis* is associated with oral candidiasis in HIV-infected individuals and AIDS patients (Sullivan *et al.*, 1995; Jabra-Rizk *et al.*, 2001b; Sullivan *et al.*, 2004), it has also been identified as a cause of oral disease in non-HIV-infected insulin-dependant diabetes mellitus patients and cancer patients (Willis *et al.*, 2000; Sebti *et al.*, 2001; Al Mosaid *et al.*, 2005) and from 14.6% of HIV-negative individuals with denture-associated oral candidiasis (Coleman *et al.*, 1997b). *Candida dubliniensis* was also found to reside as a normal commensal organism in a small minority (3.5%) of the normal healthy population (Pontón *et al.*, 2000; Sullivan *et al.*, 2004; Sullivan *et al.*, 2005). The prevalence of *C. dubliniensis* in normal healthy individuals may be underestimated however due to insufficiently sensitive techniques for differentiating *C. dubliniensis* from *C. albicans* (Sullivan *et al.*, 2005). *Candida dubliniensis* has also been isolated from a small number of cases of vulvovaginal candidiasis; however *C. albicans* is the primary causative agent of this form of candidiasis (Pontón *et al.*, 2000; Acikgoz *et al.*, 2004).

The prevalence of *C. dubliniensis* in blood cultures is extremely low (2%; Moran *et al.*, 2004) compared to *C. albicans* (65%; Kibbler *et al.*, 2003). *Candida dubliniensis* has been isolated from cases of fungaemia from six HIV-negative individuals and one HIV-infected individual, all with severe underlying medical conditions, especially in patients who are neutropenic following bone marrow or solid organ transplantation (Meis *et al.*, 1999; Brandt *et al.*, 2000; Gottlieb *et al.*, 2001; Sebti *et al.*, 2001). Despite its isolation from blood samples, *C. dubliniensis* is only rarely identified as a source of systemic disease (Kibbler *et al.*, 2003; Sullivan *et al.*, 2004) suggesting that *C. dubliniensis* may be less virulent than *C. albicans*. This indicates that despite the phenotypic similarity, *C. albicans* may have a competitive advantage in colonising and infecting humans over *C. dubliniensis* (Sullivan *et al.*, 2004).

Recently, *C. dubliniensis* was isolated from *Ixodes uriae* tick samples from a seabird colony on the Great Saltee Island demonstrating that *C. dubliniensis* is not solely confined to humans (Nunn *et al.*, 2007). The fungal isolates were associated with the surface of the *I. uriae* ticks which were collected from cracks in cliffs filled with common guillemot (*Uria aalge*) guano indicating that the most likely source of the fungus is seabird excrement and it is likely that *C. dubliniensis* inhabits the digestive

tracts of seabirds. Analysis of the fungal isolates determined that all isolates belonged to the predominant *C. dubliniensis* genotype, i.e. genotype 1.

1.2.2 *C. albicans* and *C. dubliniensis* are closely related

Candida dubliniensis is very closely related to *C. albicans* and consequently they are phenotypically very similar (Sullivan *et al.*, 1995). The similarity between the two species has, in the past, resulted in difficulties in accurate differentiation between them (Sullivan *et al.*, 1999) and *C. dubliniensis* has often been misidentified as *C. albicans*. Coleman *et al.* (1997a) reported that upon re-examination of two stored collections of oral isolates presumptively identified as *C. albicans*, 1.82% of isolates recovered from asymptomatic normal healthy individuals and 16.46% of oral isolates recovered from HIV-infected individuals were in fact *C. dubliniensis*. In a further study by Odds *et al.* (1998), 2,589 isolates in a yeast stock collection were examined and 2.1% of the isolates which has previously been identified as *C. albicans* were re-identified as *C. dubliniensis*.

While all *C. dubliniensis* genes analysed to date share more than 90% identity at the nucleotide sequence level with the orthologous *C. albicans* genes, Moran *et al.* (2004) found that 4.4% of *C. albicans* sequences analysed by comparative genomic hybridisation studies were likely to be absent or highly divergent in *C. dubliniensis*. While the vast majority of *C. albicans* genes are conserved in *C. dubliniensis*, indicating that the two species have only relatively recently diverged and are likely to inhabit similar host sites (Moran *et al.*, 2004), the overall organisation of the two genomes differs substantially (Sullivan *et al.*, 1995; Magee *et al.*, 2008). The *C. dubliniensis* genome contains twice the number of copies of the repetitive RPS sequence compared to *C. albicans* and these undergo reorganisation twice as frequently (Joly *et al.*, 2002). There may be some advantage for an organism to exhibit high levels of recombination such as rapid adaptation to environmental changes, however, it may also create instability and interfere with the normal functioning of the organism and this may contribute to the lower success rate of *C. dubliniensis* as a pathogen compared to *C. albicans* (Joly *et al.*, 2002). Despite the similarity between the two species and the prevalence of recovery from the oral cavity of HIV-infected individuals and AIDS patients (Sullivan *et al.*, 1995; Sullivan & Coleman, 1997; Sullivan *et al.*, 1999), the

reason for the relatively poor virulence of *C. dubliniensis* in comparison with *C. albicans* is unclear.

1.2.3 *C. dubliniensis* is comprised of four distinct genotypes

In 1999, Joly *et al.* developed a DNA fingerprinting probe specific for *C. dubliniensis*. The probe, Cd25, is homologous to sequences dispersed throughout the *C. dubliniensis* genome and produces no detectable signal when used to probe other related species. Southern blot analysis with the Cd25 *C. dubliniensis*-specific probe indicated that *C. dubliniensis* is comprised of two distinct populations, termed group I and group II (Joly *et al.*, 1999). Subsequent analysis by Gee *et al.* (2002) confirmed the results of Joly *et al.* (1999) and extended it by identifying 4 separate genotypes within *C. dubliniensis* based on nucleotide sequence analysis of the internal transcribed spacer (ITS) region of the small ribosomal RNA (rRNA) operon. Gee *et al.* (2002) found that the Cd25 group I isolates were primarily associated with carriage and infection in HIV-infected patients and consisted of a single genotype (genotype 1), whereas the Cd25 group II isolates were mainly recovered from non-HIV-infected individuals and consisted of three more loosely related genotypes (genotypes 2, 3 and 4; Fig. 1.1).

In 2005, a study by Al Mosaid *et al.* investigated the population structure of 30 *C. dubliniensis* oral isolates from Saudi Arabia and Egypt. Fingerprinting analysis of these isolates with the *C. dubliniensis*-specific probe, Cd25, revealed two distinct populations, the first of which consisted of 10 closely related genotype 1 isolates. The second population of 20 isolates was much more heterogeneous and consisted of two distinct subpopulations, one of which consisted of genotype 3 isolates and the other of genotype 4 isolates (Fig. 1.2). A mixed dendrogram generated from fingerprint data of 30 Saudi Arabian and Egyptian isolates, 5 Israeli isolates (Al Mosaid *et al.*, 2005) and 51 independent isolates from 13 other countries around the world (Gee *et al.*, 2002) was carried out and the results showed that these isolates could be divided into three distinct populations or clades. The first corresponded to the previously described Cd25 group I and contained all the Saudi Arabian, Egyptian, and Israeli genotype 1 isolates mixed with international isolates. The second clade corresponded to the previously described Cd25 group II and contained three Israeli isolates mixed with international isolates. The third clade termed Cd25 group III had not been previously described and consisted solely of the 20 Saudi Arabian and Egyptian genotype 3 and 4 isolates identified in the

study by Al Mosaid *et al.* (2005) and a previously described genotype 4 Israeli isolate (Fig. 1.3). All 20 Cd25 group III isolates exhibited high-level resistance to 5-flucytosine (5FC) unlike Cd25 group I and II isolates. Clade-specific 5FC resistance has previously been reported for *C. albicans* and the molecular basis of resistance was shown to be due to point mutations in *FCA1* and *FUR1* which encode enzymes involved in the pyrimidine salvage pathway (Dodgson *et al.*, 2004; Hope *et al.*, 2004). As no amino acid substitutions were found in the corresponding *C. dubliniensis* proteins, it is likely that an alternative mechanism of 5FC resistance is present in *C. dubliniensis* (Al Mosaid *et al.*, 2005; Sullivan *et al.*, 2005).

1.2.4 Phenotypic characteristics of *C. dubliniensis* and differences from *C. albicans*

Candida dubliniensis shares many phenotypic traits with its close relative, *C. albicans* and while this has hindered differentiation between the two species in the clinical laboratory in the past, exploitation of the subtle differences between the phenotypes of *C. albicans* and *C. dubliniensis* can allow rapid, easy and cost efficient differentiation between these two species. In order to fully assess the clinical importance of *C. dubliniensis* it is necessary to be able to differentiate between *C. dubliniensis* and *C. albicans* in an accurate and rapid fashion.

Both *C. dubliniensis* and *C. albicans* have the ability to produce germ tubes and chlamydospores (Sullivan *et al.*, 1995), traits which had previously been used for the definitive identification of *C. albicans* (Odds, 1988). *Candida dubliniensis* often produces large numbers of chlamydospores on Rice Agar Tween (RAT) agar, which are arranged in contiguous pairs, triplets or larger multiples and are attached to a single suspensor cell which is attached to short pseudohyphae with abundant lateral branching (Sullivan *et al.*, 1995). While *C. albicans* can also form contiguous pairs of chlamydospores on RAT agar, this happens infrequently compared to *C. dubliniensis* (Sullivan *et al.*, 1995) and the more usual formation of chlamydospores by *C. albicans* is singly at the ends of hyphae or pseudohyphae (Odds, 1998).

CHROMagar® *Candida* is a solid medium containing chromogenic substrates which allows for presumptive identification of clinically important *Candida* species upon initial isolation on the basis of colony colour (Odds & Bernaerts, 1994). *Candida dubliniensis* colonies appear dark green while *C. albicans* colonies appear light blue-green on this agar. However, the ability of *C. dubliniensis* to produce dark green

Average S_{AB} values

Cd25 group I = 0.8 ± 0.06

Cd25 group II = 0.57 ± 0.2

Cd25 group I and II = 0.54 ± 0.3

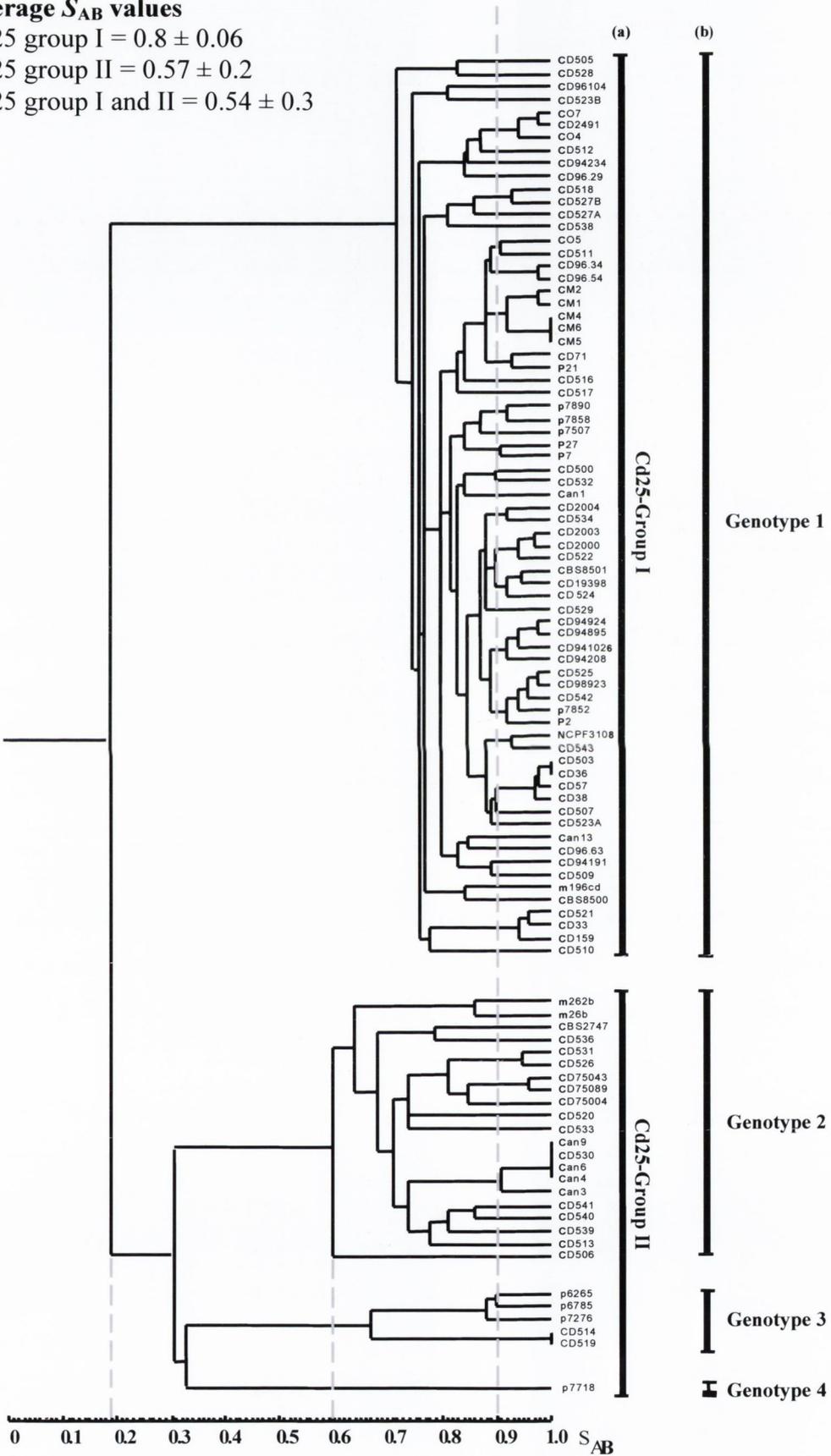




Figure 1.1. Dendrogram generated from the similarity coefficients (S_{AB}) computed for every possible pairwise combination of 98 isolates from 94 patients fingerprinted with Cd25. At an S_{AB} of 0.19 (short dashed vertical line), the isolates are divided into two main populations, termed Cd25 groups I and II. At an S_{AB} of 0.6 (short dashed vertical line), four distinct clades are evident; one clade corresponds to Cd25 group I, whereas the other three clades are found within Cd25 group II. Multiple isolates of the same *C. dubliniensis* strain recovered from the same clinical specimen for each of eight separate individuals yielded S_{AB} values of ≥ 0.93 ; the S_{AB} value of 0.9 (dashed vertical line) was therefore chosen as an arbitrary threshold value for describing clusters. The Cd25 groups (a) and the ITS genotypes (b) of the isolates are shown to the right of the dendrogram. All 27 of the Cd25 group II isolates and 43 of 71 of the Cd25 group I isolates had their genotypes determined by genotype-specific polymerase chain reaction (PCR; taken from Gee *et al.*, 2002).

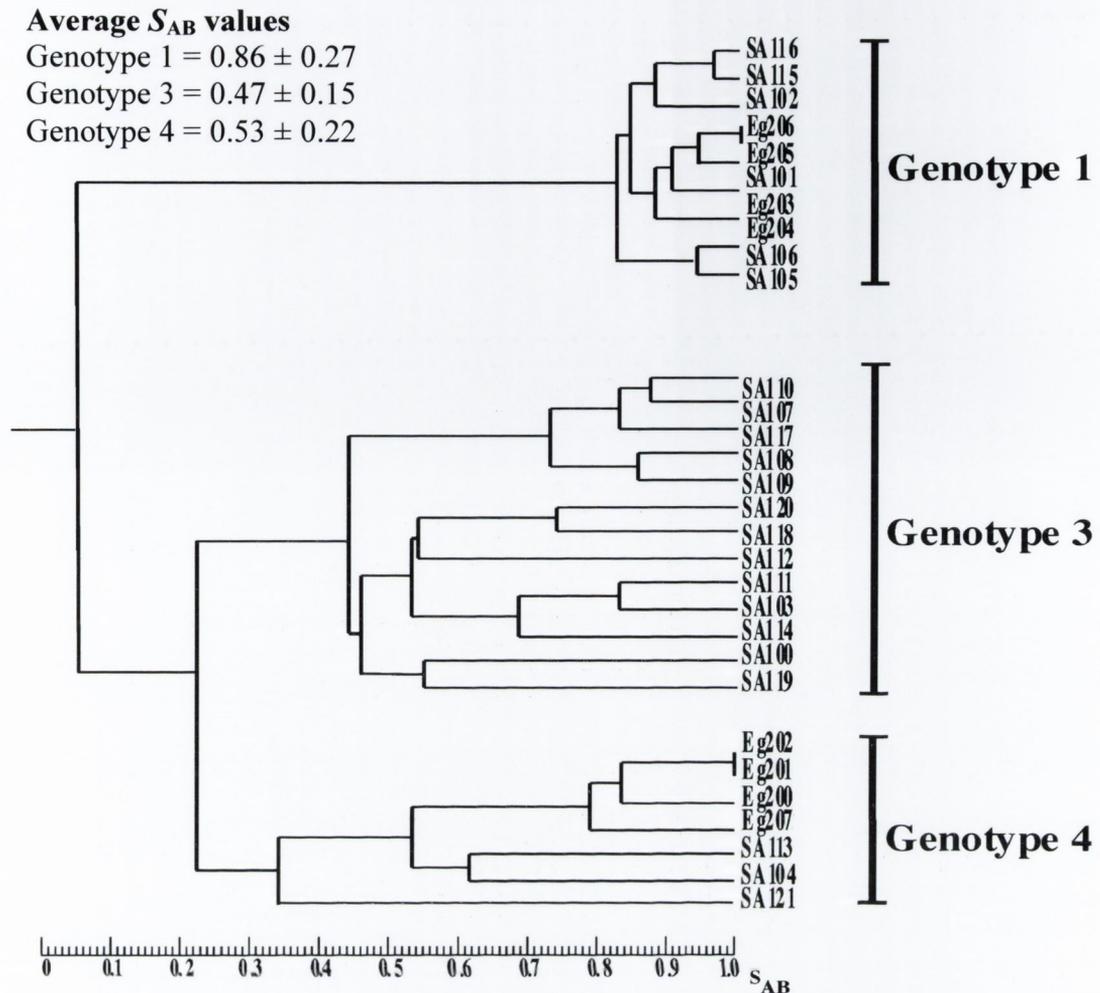


Figure 1.2. Dendrogram generated from the S_{ABS} computed for every pairwise combination of 30 *C. dubliniensis* isolates recovered from individual patients in Saudi Arabia and Egypt fingerprinted with Cd25. At an S_{AB} node of 0.05, the isolates are divided into two main populations. The first of these populations consists solely of genotype 1 isolates and are closely related, with an average S_{AB} value of 0.86 ± 0.27 . The second population consists of genotype 3 and genotype 4 isolates and are less closely related to each other than are genotype 1 isolates and have an average S_{AB} value of 0.35 ± 0.19 . At an S_{AB} node of 0.22, the second population is divided into two subpopulations consisting of genotype 3 and genotype 4 isolates, respectively (taken from Al Mosaid *et al.*, 2005).

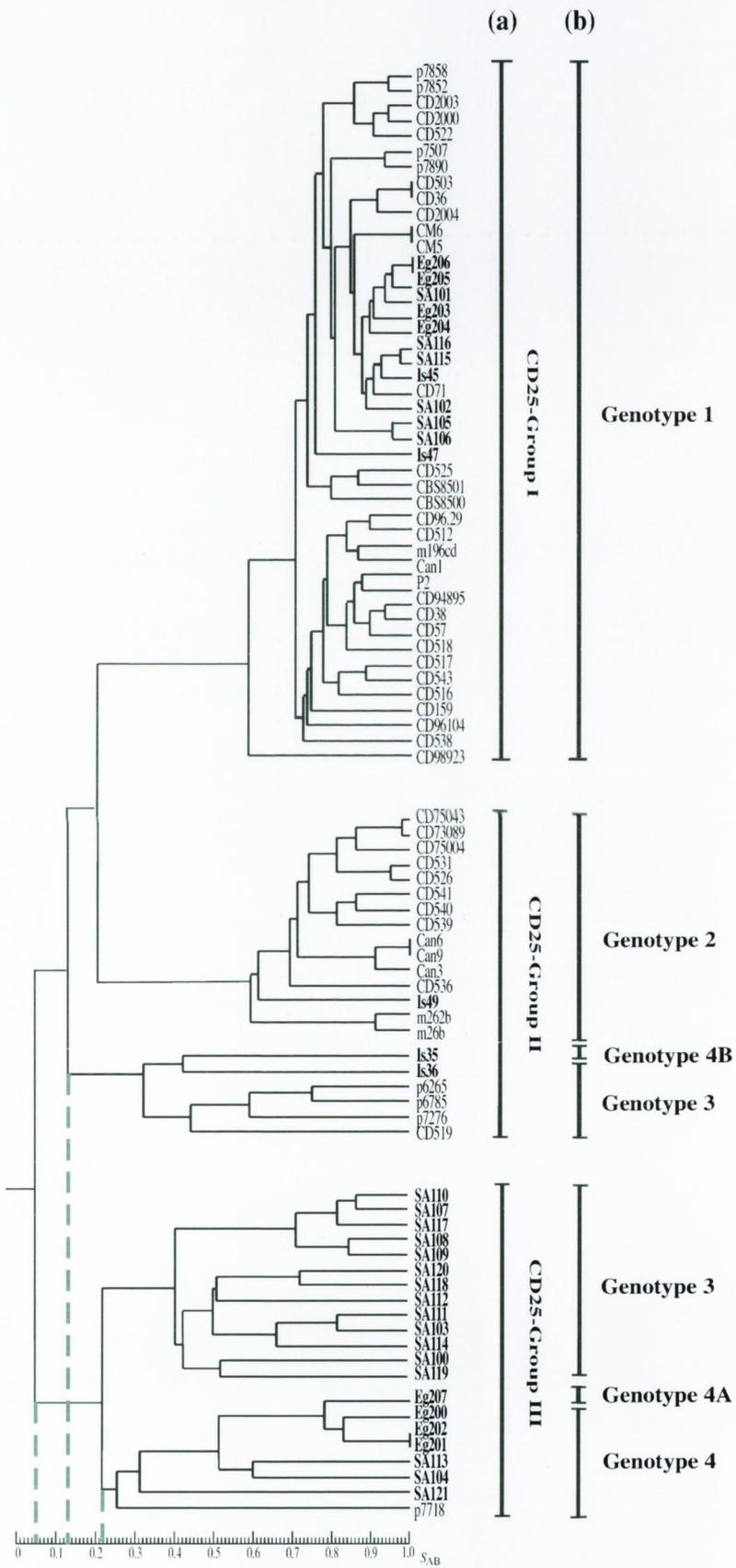




Figure 1.3. Dendrogram generated from the S_{AB} s computed for every possible pairwise combination of independent *C. dubliniensis* isolates from Saudi Arabia and Egypt (n = 30), Israel (n = 5) and 13 other countries (n = 51) from the study of Gee *et al.* (2002) fingerprinted with Cd25. At an S_{AB} node of 0.05 (short dashed vertical line), the isolates are divided into three main populations, the first and second of which correspond to the major clades Cd25 group I and Cd25 group II described previously by Joly *et al.* (1999) and Gee *et al.* (2002). The third main population (Cd25 group III) corresponds to a third major clade identified in a study by Al Mosaid *et al.* (2005) and contains $^{20}/_{30}$ of the Saudi Arabian and Egyptian isolates investigated. The Cd25 group I major clade consists solely of closely related genotype 1 isolates with an average S_{AB} value of 0.63 ± 0.12 . At an S_{AB} node of 0.13 (short dashed vertical line), the Cd25 group II major clade can be divided into two minor clades, the first of which consists solely of genotype 2 isolates. The second minor clade consists of genotype 3 isolates and the 4 variant (4v) isolate Is35. At an S_{AB} node of 0.22 (short dashed vertical line), the Cd25 group III major clade can also be divided into two minor clades, the first of which consists solely of genotype 3 isolates and the second solely of genotype 4 isolates, including the genotype 4v isolate Eg207. The Cd25 groups (a) and the ITS genotypes (b) of the isolates are shown to the right of the dendrogram (taken from Al Mosaid *et al.*, 2005).



colonies on CHROMagar® *Candida* medium is not always reproducible following subculture and storage (Schoofs *et al.*, 1997) and as such this medium should only be used for presumptive identification of *Candida* species upon primary isolation from clinical specimens. Similarly, following primary isolation from clinical samples, *C. dubliniensis*, unlike *C. albicans*, does not exhibit fluorescence when grown on methyl blue-Sabouraud agar under Wood's light however this result may not be reproducible upon repeated subculture and storage of the isolates (Schoofs *et al.*, 1997).

When grown on Staib agar (Staib & Morschhäuser, 1999; Al Mosaid *et al.*, 2001), Pal's agar (Al Mosaid *et al.*, 2003) or Tobacco agar (Khan *et al.*, 2004), 97.7%, 100% and 100% of *C. dubliniensis* colonies, respectively, were readily distinguishable by their rough appearance or hyphal fringe which consisted of hyphae, pseudohyphae and/or chlamydospores while *C. albicans* colonies were smooth and consisted solely of blastospores. A modification of Pal's agar to make it transparent has allowed direct observation of chlamydospore production by *C. dubliniensis* through the petri dish (Adou-Bryn *et al.*, 2003) thus making differentiation of the two species easier.

Candida albicans and *C. dubliniensis* both grow well on standard mycological media at 30°C and 37°C however, while *C. albicans* grows well at 42°C and the majority of *C. albicans* isolates grow at 45°C (Kirkpatrick *et al.*, 1998), *C. dubliniensis* grows poorly or not at all at 42°C and does not grow at 45°C (Sullivan *et al.*, 1995; Coleman *et al.*, 1997b; Pinjon *et al.*, 1998).

Candida dubliniensis expresses different carbohydrate-source assimilation profiles to those of *C. albicans* determined using commercially available kits such as API ID 32C and while these carbohydrate assimilation profiles are distinct from those of *C. albicans*, the results had been difficult to interpret until improvements were made in the carbohydrate assimilation test databases which have helped the differentiation between these two species (Pincus *et al.*, 1999).

Another phenotypic difference between *C. albicans* and *C. dubliniensis* is the inability of *C. dubliniensis* to express β -glucosidase activity which was discovered by Boerlin *et al.* (1995) using the technique of multilocus enzyme electrophoresis. A rapid assay based on this finding has been described and can effectively differentiate between these two species (Boerlin *et al.*, 1995; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997).

Other methods of differentiation between the two species include an immunochromatographic assay and an immunofluorescence assay (Bikandi *et al.*, 1998; Marot-Leblond *et al.*, 2004). The immunochromatographic assay (Marot-Leblond *et al.*,

2004) differentiates between *C. dubliniensis* and *C. albicans* on the basis of immunochromatographic analysis and involves the use of two distinct monoclonal antibodies; one reacts with a protein moiety that is specifically expressed in the cell wall of both *C. albicans* and *C. dubliniensis* (Marcilla *et al.*, 1999) and the other reacts with a glycoprotein component expressed uniquely on the surfaces of *C. albicans* hyphae (Marot-Leblond *et al.*, 2000). In the presence of *C. albicans* two lines appear while in the presence of *C. dubliniensis* only one line appears (Marot-Leblond *et al.*, 2004). Bikandi *et al.* (1998) produced an anti-*C. dubliniensis* serum which, after adsorption with *C. albicans* blastospores, was found to differentially label *C. dubliniensis* isolates in an indirect immunofluorescence test. The antiserum reacted with blastospores and germ tubes of *C. dubliniensis* but did not react with the blastospores of *C. albicans*. The antiserum did however react with *C. albicans* germ tubes (Bikandi *et al.*, 1998).

1.2.5 Phylogenetic characteristics of *C. dubliniensis*

The phenotypic and genotypic characteristics of *C. dubliniensis* show it is a separate species from *C. albicans*, however they can not estimate the genetic relatedness of these two organisms (Sullivan & Coleman, 1998). An accurate method for determining the measure of genetic relationships between individual species involves the comparison of the nucleotide sequence of the genes encoding for rRNA (Olsen & Woese, 1993). The rRNA genes are highly conserved and are present in eukaryotic genomes in multiple copies. Analysis of a 500 bp fragment of the V3 variable region of the large rRNA subunit genes showed that *C. dubliniensis* formed a homogenous cluster that was phylogenetically distinct from other *Candida* species and showed 2.25% sequence divergence from *C. albicans*. For comparison, DNA fragments were also amplified from the corresponding region of the rRNA genes from one isolate each of five other *Candida* species and it was noted that the sequence data for each species differed considerably from the *C. albicans* sequence. Using these data a phylogenetic tree was generated and this grouped *C. dubliniensis* and *C. albicans* as separate species (Fig. 1.4; Sullivan *et al.*, 1995). Comparison of the sequences of the entire small rRNA genes of *C. dubliniensis* and *C. albicans* revealed a difference of 1.4% between the two species (Gilfillan *et al.*, 1998). Analysis of the *ACT1* genes from both species showed that the coding sequences differed by 2.1% between conserved *ACT1* associated introns (Donnelly *et al.*, 1999), indicating that although *C. albicans* and *C. dubliniensis* are

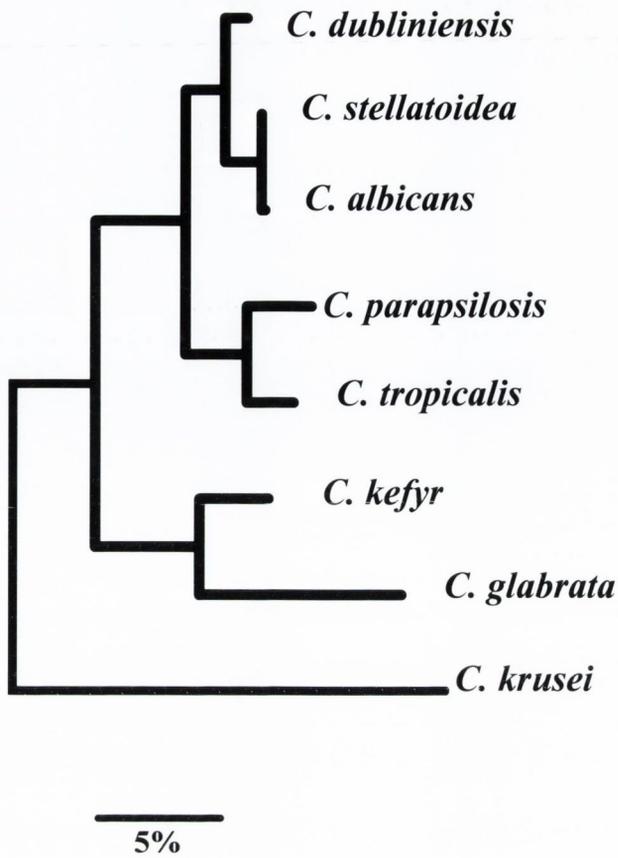


Figure 1.4. An unrooted phylogenetic neighbour-joining tree generated from the alignment of a 500 bp region of the V3 region of the large ribosomal genes from various *Candida* species. The scale bar represents a 5% difference in nucleotide sequence (taken from Sullivan *et al.*, 1995).



separate species, they are closely related. Analysis of the sequence of the large rRNA gene of *C. dubliniensis* indicated the presence of a self splicing group I intron. This sequence displays two regions of major sequence divergence from the sequences of the homologous loci in *C. albicans* (Boucher *et al.*, 1996). Boerlin *et al.* (1995) used the multilocus enzyme electrophoresis technique to demonstrate that the allelic makeup of *C. dubliniensis* isolates was distinct from that of *C. albicans*, further confirming their unique taxonomic position.

1.2.6 Genotypic characteristics of *C. dubliniensis* and differences from *C. albicans*

Genetic analysis of *C. dubliniensis* confirmed that it was a separate species from *C. albicans*. DNA fingerprint analysis of restriction endonuclease *Eco*R1-digested chromosomal *C. dubliniensis* DNA with the *C. albicans* specific mid-repeat sequence probe, 27A (Scherer & Stevens, 1988) yielded weak hybridisation profiles composed of four to seven bands compared to between 10 and 15 strongly hybridising bands obtained with *C. albicans* genomic DNA (Coleman *et al.*, 1993; Sullivan *et al.*, 1995). This probe corresponds to a repetitive DNA sequence which is dispersed throughout the *C. albicans* genome. These findings suggested that the genomic organisation of *C. dubliniensis* is quite different to that of *C. albicans*. *Candida dubliniensis* can also be distinguished from *C. albicans* on the basis of significant differences in the *Hinf*I-generated restriction fragment length polymorphisms pattern in agarose gels (Sullivan *et al.*, 1995). The use of five synthetic oligonucleotide probes and random amplified polymorphic DNA analysis employing a range of oligonucleotide primers also showed that the overall fingerprint profiles of *C. dubliniensis* were very similar to each other but distinctive from *C. albicans* (Sullivan *et al.*, 1995). Karyotype analysis (analysis of chromosome banding patterns following separation by pulsed-field gel electrophoresis) showed that *C. albicans* gave seven distinct chromosome-sized bands, while *C. dubliniensis* yielded nine to 10 chromosome sized DNA bands with one or more bands of < 1 Mb (Kwon-Chung *et al.*, 1988; Sullivan *et al.*, 1995). Despite extensive chromosome rearrangements, karyotypic differences between *C. dubliniensis* and *C. albicans* are unlikely to affect gene expression. However, karyotypic instability may account for the diminished pathogenicity of *C. dubliniensis* (Magee *et al.*, 2008).

Multilocus sequence typing (MLST) is a technique based on the analysis of the nucleotide sequences of a set of housekeeping genes and was initially developed for

population analysis of several bacterial species (Maiden *et al.*, 1998). This technique has also been applied to the analysis of *C. albicans* (Bougnoux *et al.*, 2002 & 2003; Tavanti *et al.*, 2003) and other *Candida* species (Dodgson *et al.*, 2003; Tavanti *et al.*, 2005b; Jacobsen *et al.*, 2007). MLST analysis has been shown to be as sensitive as DNA fingerprinting (Robles *et al.*, 2004) and in the case of *C. albicans*, it has been shown that the strain groupings identified by MLST correlate with the clades of *C. albicans* identified using the species specific DNA fingerprinting probe, Ca3 (Tavanti *et al.*, 2005a). The MLST scheme in use for *C. albicans* examines seven loci and when applied to *C. dubliniensis* this scheme demonstrated poor levels of discrimination between *C. dubliniensis* isolates (McManus *et al.*, 2008). Using extended loci, studies of the population structure of both species showed that the *C. albicans* clades are more divergent than those observed in *C. dubliniensis* and three distinct major clades were identified in *C. dubliniensis* (McManus *et al.*, 2008). These clades correlated with those previously determined using the fingerprinting probe, Cd25 and on the basis of ITS genotypes (Joly *et al.*, 1999; Gee *et al.*, 2002; Al Mosaid *et al.*, 2005) suggesting that MLST may be applied as a less time consuming, more reliable method of studying the population structure of *C. dubliniensis* (McManus *et al.*, 2008).

Because of the difficulties in using methods based on phenotypic characteristics to differentiate between *C. dubliniensis* and *C. albicans*, both of which may show intra-species variation, molecular methods which exploit genetic differences between these two species have been developed for more rapid and accurate differentiation. PCR has been used to differentiate between *C. dubliniensis* and *C. albicans*. This technique has advantages over other molecular based technologies in that it is fast, reproducible and allows for a high sample throughput. As the differences between *C. dubliniensis* and *C. albicans* are most pronounced at the genetic level, such differences should provide the basis for a rapid and accurate identification test.

1.2.6.1 *C. dubliniensis*-specific PCR based on the *ACT* gene of *C. dubliniensis* and *C. albicans*

ACT1 encodes a protein that is present in all eukaryotic cells and due to structural constraints the amino acid sequence of actin proteins from different eukaryotic species is highly conserved (Hightower & Meagher, 1986; Pollard, 1990; Hennessey *et al.*, 1993; Welch *et al.*, 1994). As *C. dubliniensis* and *C. albicans* are

closely related, the *ACT1* sequences should be highly conserved. The *ACT1* genes from both species contain a single class IV intron which should be subject to less evolutionary conservation than the actin protein coding exons (Losberger & Ernst, 1989; Donnelly *et al.*, 1999). *Candida dubliniensis* coding and intron sequences differ from corresponding *C. albicans* sequences by 2.1% and 16.6%, respectively. *Candida dubliniensis*-specific PCR primers were designed which amplified a 228 bp product from *C. dubliniensis* DNA and as only *C. dubliniensis* isolates should yield the 228 bp product, this method is a rapid and definitive technique for identification of *C. dubliniensis* (Donnelly *et al.*, 1999).

1.2.6.2 *C. dubliniensis* genotype-specific PCR

A genotype-specific PCR assay was designed on the basis of sequence difference in the ITS region between the four genotypes of *C. dubliniensis*. Each genotype specific primer pair amplified a product of approximately 330 bp from *C. dubliniensis* template DNA. The PCR reactions also contained the fungal universal primers which amplify a product of approximately 610 bp from the fungal large subunit rRNA gene and serve as an internal control. Amplification using these primers will result in two bands of 330 and 610 bp when DNA of the correct *C. dubliniensis* genotype is amplified (Gee *et al.*, 2002).

1.3 VIRULENCE FACTORS

Virulence factors are described as traits required to establish disease that interact directly with mammalian host cells and are components of a pathogen that damage its host (Furman & Ahearn, 1983; Casadevall & Pirofski, 2001; Odds *et al.*, 2001). In order to establish an infection, a pathogen must evade the hosts' immune system, survive in the host environment and spread to new tissues (Yang, 2003). While an impaired host immune system is a common risk factor for severe *Candida* infection (Casadevall & Pirofski, 1999), the fungus must be able to make the transition from a harmless commensal to an aggressive pathogen with the ability to colonise different host niches (Hube *et al.*, 2000). *Candida* species cause disease at several anatomically distinct sites which have a unique physiological environment (Yang, 2003) indicating that *Candida*

species have the capacity to adapt to very different environmental conditions and may express many virulence factors.

1.3.1 Proteinase secretion

The secreted aspartyl proteinases (SAPs) are encoded by 10 *SAP* genes in *C. albicans* and their proteolytic activity is associated with tissue invasion. Unlike Sap1 – Sap8, Sap9 and Sap10 have C terminal sequences typical for glycosylphosphatidylinositol (GPI) proteins indicating that Sap9p and Sap10p are incorporated into the cell wall of *C. albicans* (Monod *et al.*, 1998). Sap1 – Sap10 account for all the extracellular proteolytic activity of *C. albicans* and distinct differences in pH optima are evident between these heterologously expressed genes; Sap1 – Sap3 have higher activity at lower pH values and Sap4 – Sap6 have higher activity at higher pH values (Smolenski *et al.*, 1997; Borg-von Zepelin *et al.*, 1998). This range in pH optima most likely allows for functionality of these proteins in different niches of the host.

Saps have the ability to degrade many human proteins at lesion sites to provide nutrition for *Candida* cells, to aid penetration and invasion and to evade immune responses of the host (Hube *et al.*, 1998). Isolates that produce more proteolytic activity *in vitro* are generally more virulent (Rüchel, 1992); isolates from symptomatic patients with vaginal candidiasis were more proteolytic than isolates from asymptomatic vaginal carriers (Cassone *et al.*, 1987; De Bernardis *et al.*, 1990; Agatensi *et al.*, 1991). Similarly, isolates from HIV-infected women with symptomatic candidal vaginitis produced significantly more Sap proteins and were more pathogenic in mouse and rat vaginitis models than isolates from either HIV-infected asymptomatic carriers or HIV-negative patients with candidal vaginitis (De Bernardis *et al.*, 1996; de Bernardis *et al.*, 1999). *Candida albicans* isolates from HIV-infected patients with oropharyngeal candidiasis produced more proteinase activity than isolates from HIV-negative patients with or with out oropharyngeal candidiasis (Ollert *et al.*, 1995) indicating that the production of these proteinases is associated with disease.

Sap2p has a broad specificity with the ability to cleave a variety of proteins. Sap2p is known to degrade mucin, immunoglobulin A and proteins of the extracellular matrix (ECM) which may provide nitrogen for candidal growth and may enhance attachment, colonisation and invasion of host tissues and dissemination of the organism

(Rüchel, 1986; Ray & Payne, 1990; Ogrydziak, 1993; Goldman *et al.*, 1995; Colina *et al.*, 1996; Hube, 1996; de Repentigny *et al.*, 2000). Sap proteins are localised to the cell wall during *C. albicans* infection; Sap1 to Sap3 are present on the cell wall during the early stages of infection and while Sap1 to Sap3 are present on the surface of both yeast and hyphal cells, Sap4 to Sap6 are found predominantly on hyphal cells and different *SAP* expression patterns are associated with oral and vaginal candidiasis (Stringaro *et al.*, 1997; Schaller *et al.*, 1998; Felk *et al.*, 2002; Kretschmar *et al.*, 2002; Naglik *et al.*, 2003). Mutants lacking *SAP1*, *SAP2* or *SAP3* were less able to cause tissue damage than strains with functional *SAP1*, *SAP2* or *SAP3* genes in both oral and vaginal models of reconstituted human epithelium (RHE) while mutants lacking *SAP4 – SAP6* showed an equal level of tissue damage when compared to the wild type strain (Schaller *et al.*, 1999 & 2003) indicating the role of *SAP1 – SAP3* in mucosal infections (Naglik *et al.*, 2003). Similarly, mutants lacking one of *SAP1*, *SAP2* or *SAP3* or *SAP4 – SAP6* were used to infect guinea pig or murine models and these animals had longer survival times than those infected with the wild type strain (Hube *et al.*, 1997; Sanglard *et al.*, 1997a). The *SAP4 – SAP6* mutant was the most attenuated, suggesting that *SAP4 – SAP6* play a role in systemic infections. This mutant also showed reduced invasiveness in a model of murine peritonitis (Kretschmar *et al.*, 1999; Felk *et al.*, 2002). Isolates that are strongly proteolytic adhere in greater numbers to human buccal epithelial cells (BECs; Ghannoum & Abu Elteen, 1986) indicating their role in adhesion. Watts *et al.* (1998) found that while the *SAP1*, *SAP2* and *SAP3* mutants were moderately less adherent to human BECs than the wild type strain, the *SAP4 – SAP6* mutant had increased adherence to human BECs and endothelial cells (Ibrahim *et al.*, 1997).

Eight *SAP* genes which are homologous to the *SAP* family of *C. albicans* were detected in *C. dubliniensis* (Gilfillan *et al.*, 1998; Moran *et al.*, 2004). Although *C. dubliniensis* also encodes *SAP* genes, phenotypic studies to determine the levels of proteinase production have yielded contradictory results (Sullivan *et al.*, 2004). Hannula *et al.* (2000) found that *C. dubliniensis* has less frequent production of proteinase than *C. albicans* while McCullough *et al.* (1995) and Gilfillan *et al.* (1998) found that proteinase activity of *C. dubliniensis* was greater than that of *C. albicans*. The discrepancies between the two studies could be due to the small number of isolates used in each study and the fact that the isolates from the studies of McCullough *et al.* (1995) and Gilfillan *et al.* (1998) were from HIV-infected patients while the isolates from the study by Hannula *et al.* (2000) were from non-HIV-infected individuals. As it is known

that isolates from HIV-infected patients have higher proteinase production, these studies are not comparable.

1.3.2 Secreted lipases

Lipases are characterised by their ability to hydrolyse ester bonds at the interface between insoluble triacylglycerols and the aqueous phase in which the enzymes are dissolved. Several of these enzymes also display activity towards mono- and diacylglycerols or phospholipids (Anthonsen *et al.*, 1995). Anti-lipase antibodies have been detected in patients suffering from *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections suggesting that lipases are being expressed by these bacteria during infection (Rollof *et al.*, 1988). Lipase activity seems to be critical for *Hortea werneckii* to cause tinea nigra and is essential for viability of the facultative pathogenic yeast *Malassezia furfur* (Ran *et al.*, 1993; Göttlich *et al.*, 1995). Extracellular lipase activity of *C. albicans* was first described in 1965 by Werner (Hube *et al.*, 2000) and *LIP1* (Fu *et al.*, 1997) encodes for a lipolytic enzyme in *C. albicans*.

The *LIP* genes constitute a large family of at least ten genes which have similar three-dimensional and isozyme structures and with the exception of Lip7p, all Lip proteins contain a putative N terminal signal sequence indicating that these genes encode for proteins that are secreted. Southern blot analysis using *LIP* probes suggested that these genes are present in other pathogenic *Candida* species (Hube *et al.*, 2000). The fact that a family of genes is present in *C. albicans* suggests that different genes are required during different stages or types of infection or that certain genes are partially or wholly functionally redundant such as with the *SAP* or agglutinin-like sequence (*ALS*) families (Kretschmar *et al.*, 1999; Schaller *et al.*, 1999; Hoyer, 2001). Several *SAP* genes were expressed along with the *LIP* genes indicating that *C. albicans* can express proteinases and lipases under the same conditions (Hube *et al.*, 2000). Expression of some of the *LIP* genes appears to be constitutive and less regulated than the expression of the *SAP* genes. The lipases of *C. albicans* may have several functions; the ability to hydrolyse lipids in order to use fatty acids and/or glycerols as substrates allowing *Candida* to colonise and persist on skin or the intestinal tract in the absence of carbohydrate. The release of fatty acids may alter the pH of the microenvironment, allowing for other processes, such as the production of proteinases, to occur. *LIP2* and *LIP9* were expressed in media that did not contain lipids indicating that their

transcription is lipid independent suggesting that these genes may function in other ways than hydrolysing lipids (Hube *et al.*, 2000). Non-specific hydrolysis of phospholipids may damage host cell membranes allowing for further invasion of *Candida* (Ibrahim *et al.*, 1995) and lipolytic activity may also trigger local inflammatory disorders which may in turn damage the host tissue (Heller *et al.*, 1998) allowing for further invasion by *Candida* species. Comparison of the *C. albicans* and *C. dubliniensis* genomes reveal that a similar repertoire of *LIP* genes is present in the *C. dubliniensis* genome.

1.3.3 Phospholipase production

Phospholipases hydrolyse phospholipids which are a major constituent of the host cell envelope. Production of phospholipases is part of the virulence arsenal of *Candida* species. Phospholipases play a role in damaging cell membranes allowing invasion of the host by *Candida* cells, causing mucosal and disseminated infections. All *C. albicans* and *C. dubliniensis* isolates tested in a study by Bennett *et al.* (1998) possessed sequences homologous to *CaPLC1*, the *C. albicans* phospholipase C gene. Despite this, *C. dubliniensis* was reported not to produce phospholipases in contrast to *C. albicans*, which produces phospholipases in varying degrees (Hannula *et al.*, 2000; Fotedar & Al-Hedaithy, 2005). The lack of detectable phospholipase activity in *C. dubliniensis* may further reduce its capacity to initiate infection.

1.3.4 Antifungal drug resistance

The increasing incidence of infection with non-*C. albicans* species is attributable in part to the increasing use of antimycotics such as fluconazole. Despite the fact that the majority of *C. dubliniensis* isolates are susceptible to commonly used antifungal agents, repeated and lengthy exposure of severely immunocompromised patients to antifungal therapies has led to an increase in azole resistance. One study found that the minimal inhibitory concentration for fluconazole in *C. dubliniensis* was significantly higher than for a matched group of *C. albicans* indicating that *C. dubliniensis* may persist following antifungal treatment which has successfully removed other more susceptible *Candida* species (Odds *et al.*, 1998; Pfaller *et al.*, 1999 & 2004). Martinez *et al.* (2002) described the replacement of *C. albicans* with fluconazole susceptible

C. dubliniensis in HIV-infected patients receiving fluconazole treatment and fluconazole resistant *C. dubliniensis* isolates have been recovered from HIV-infected individuals and AIDS patients treated with fluconazole for prolonged periods of time and have replaced susceptible strains (Ruhnke *et al.*, 2000; Perea *et al.*, 2002).

Stable fluconazole resistant *C. dubliniensis* derivatives can be generated more easily than fluconazole resistant *C. albicans* derivatives following exposure of susceptible isolates to fluconazole *in vitro* (Sullivan & Coleman, 1998). Fluconazole resistant *C. albicans* derivatives were not stable and fluconazole resistance was readily lost following *in vitro* subculture (Calvet *et al.*, 1997). One study showed that exposure of *C. dubliniensis* to fluconazole increased adherence to epithelial cells and levels of proteinase secretion, possibly aiding this species to colonise HIV-infected patients. The same conditions caused a decrease in the adhesion of *C. albicans* to epithelial cells (Borg-von Zepelin *et al.*, 2002).

Failure to accumulate azole drugs has been shown to be a major factor involved in azole resistance in clinical *C. albicans* isolates and is associated with an up-regulation in genes encoding multi-drug efflux transporters (Sanglard *et al.*, 1995; Albertson *et al.*, 1996; Sanglard *et al.*, 1997b). The Mdr1 drug efflux pumps are fluconazole specific and encoded by *MDR1*, while the Cdr1 pumps can transport a range of azole drugs and are encoded by *CDR1*. Up-regulation of *CDR1* is associated with fluconazole resistance in *C. albicans* while fluconazole resistance in *C. dubliniensis* is associated with the up-regulation of *CdMDR1* (Pinjon *et al.*, 2005b). A study by Moran *et al.* (2002) discovered that 35% of *C. dubliniensis* isolates harboured a defective *CdCDR1* gene. These isolates all belong to genotype 1 and were mainly recovered from HIV-infected individuals, many of whom had been receiving fluconazole treatment (Gee *et al.*, 2002). In contrast, *C. dubliniensis* genotype 3 isolates showed decreased susceptibility to fluconazole and this was associated with up-regulation of *CdCDR1* and *CdCDR2* while expression of *CdMDR1* was not detected (Pinjon *et al.*, 2005a). Point mutations in *ERG11* of *C. albicans* and *C. dubliniensis* are also involved in fluconazole resistance showing that resistance to fluconazole is multifactorial (Sullivan *et al.*, 2004).

Itraconazole is a substrate of *CdCDR1* so *C. dubliniensis* isolates with a non-functional *CdCDR1* gene should be susceptible to itraconazole. However, 10% of clinical isolates tested exhibited reduced susceptibility to itraconazole indicating that although the patients from whom they were taken were treated with fluconazole only, these isolates may be developing resistance to itraconazole in tandem with fluconazole.

Derivatives in which resistance to itraconazole was induced were also resistant to fluconazole and ketoconazole. All derivatives showed increased levels of *CdERG11* expression indicating that the mechanisms of drug resistance in *Candida* species are complex (Pinjon *et al.*, 2003).

1.3.5 Dimorphism

The ability to switch between the yeast and hyphal form is thought to be an important virulence factor for both *C. albicans* and *C. dubliniensis* and is known as dimorphism (Lo *et al.*, 1997; Saville *et al.*, 2003). While *C. dubliniensis* is the only other *Candida* species which can form true hyphae, it forms hyphae at a slower rate than *C. albicans* (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). This reduced rate of hyphal formation may negatively affect the ability of *C. dubliniensis* to invade host tissue and may contribute to its lower virulence compared to *C. albicans*. It is likely that *C. dubliniensis* is defective in the environmental sensing and signalling programs that are involved in the formation of hyphae. *Candida dubliniensis* does not produce hyphae nor invade epithelial tissue in a RHE model while *C. albicans* produces abundant hyphae which penetrated deep into the tissue, further showing the major differences in the regulation of hyphal formation between the two species (Stokes *et al.*, 2007).

1.3.6 Phenotypic switching

Candida albicans can switch, spontaneously and reversibly, among different phenotypes including smooth, rough, star, ring, stippled, hat, irregular wrinkle and fuzzy at high frequency (10^{-4} to 10^{-1} ; Slutsky *et al.*, 1985) however, the involvement of this ability to switch phenotypes in virulence is not clear. Switching in *C. albicans* is pleiotropic affecting its morphology and a number of virulence traits (Soll, 1992). Smooth, white colonies with round-ovoid cells (white) can switch to flat, grey colonies with elongated cells (opaque) with *SAP1* being expressed in conjunction with the switch (opaque) phase. *SAP3* may also be regulated by phenotypic switching (Morrow *et al.*, 1993; White *et al.*, 1993) but is only detected when *SAP2* is expressed (Hube *et al.*, 1994; White & Agabian, 1995; Smolenski *et al.*, 1997). *SAP2* is expressed in both white and opaque cells but only in the presence of exogenous protein (Hube *et al.*, 1994; White & Agabian, 1995). Opaque cells have a greater ability to colonise the skin in a

cutaneous model than white cells but are less virulent in a systemic animal model (Soll, 1997; Kvaal *et al.*, 1999). *Candida dubliniensis* switches more frequently than *C. albicans* (Hannula *et al.*, 2000) and small petite colonies are often observed, especially after prolonged storage (Sullivan & Coleman, 1998).

1.3.7 Cell surface hydrophobicity

Cell surface hydrophobicity (CSH) is characterised by the presence of hydrophobic proteins embedded in the yeast cell wall matrix beneath an outer fibrillar layer and exposure of these hydrophobic proteins results in CSH. CSH is believed to contribute somewhat to the adherence of *C. albicans* to human BECs (Hazen, 1989). Initial attachment of *C. albicans* to various surfaces is mediated by mannoprotein adhesins on the outermost fibrillar layer of the yeast cell surface and hydrophobic proteins embedded in the matrix of the cell wall beneath the fibrillar layer provide the hydrophobic interactions needed to strengthen adhesion. Growth temperature changes are known to affect the hydrophobicity of *C. albicans* yeast cells by influencing the length of the fibrils of the cell wall outermost layer, which mask the hydrophobic proteins in the cell wall matrix. *Candida albicans* cells are hydrophobic when grown at 25°C and were found to have better adherence to human epithelial cells than *C. albicans* which were grown at 37°C, and were consequently hydrophilic. Growth temperature had no effect on *C. dubliniensis* CSH and the cell surface fibrils remained at a constant length when grown at either 25°C or 37°C, permanently expressing the hydrophobic phenotype (Hazen *et al.*, 2001; Jabra-Rizk *et al.*, 1999). However, other studies (Samaranayake *et al.*, 2003) have found no correlation between adhesion and CSH.

Hazen *et al.* (2001) suggested that CSH is the manifestation of several surface molecules that are involved in pathogenesis and lack of one of these molecules may not detectably reduce CSH but could affect virulence. Expression of a hydrophobic phenotype does not predict successful virulence or dissemination of *Candida*. *Candida dubliniensis* constitutively displays a hydrophobic phenotype but is not as successful a pathogen as *C. albicans*, which has the ability to cause disseminated and systemic disease more readily. CSH status in *C. albicans* is mediated by a combination of protein and post-translational modifications within the cell wall (Singleton & Hazen, 2004). The hydrophobic surface protein Csh1p, which was described by Singleton *et al.* (2001), is expressed in *C. albicans*. This protein has a homologue in *C. dubliniensis* and

is influenced by growth temperature. In both species, greater adhesion correlates with greater expression of this protein, which occurs when *Candida* cells are grown at 37°C compared to 23°C (Hazen, 2004), thus indicating that it is not the hydrophobic phenotype of *Candida* which affects CSH, and in turn adhesion, but regulation of cellular protein components of the cell. The level of cell surface mannosylation is also known to directly affect CSH (Masuoka & Hazen, 1997; Singleton *et al.*, 2005).

1.3.8 Adhesion

Adhesion of a pathogen to its hosts' cells is seen as an essential step in the establishment of disease preventing the pathogen from being washed away by salivary or blood flow and allowing it to initiate an infection. Although pathogenicity is positively correlated with adherence, the molecular basis of candidal adhesion is poorly understood (Calderone & Braun, 1991; Yang, 2003). *Candida* species not only adhere to host cells, but can also adhere to mucin, catheters, polystyrene and acrylic (McCourtie & Douglas, 1981; Hawser & Douglas, 1994; de Repentigny *et al.*, 2000; Sánchez-Sousa *et al.*, 2001; Tamura *et al.*, 2003). Mucins are produced and secreted from mucous cells of the salivary glands, the oesophagus, the stomach and the small and large intestines and play an important role in lubrication of epithelial surfaces and host defence (Forstner & Forstner, 1994). Adhesion of a pathogen to this mucosal layer may result in penetration by the pathogen and access to the hosts' epithelial cells, to which the pathogen may then adhere. Adherence of *Candida* species to purified small intestinal mucin showed a correlation between adherence and pathogenicity (de Repentigny *et al.*, 2000).

1.3.8.1 Biofilms

The number of critically ill patients requiring catheters is increasing and a study by Wey *et al.* (1989) found that the presence of catheters was a risk factor for candidemia. The mortality rate associated with these blood infections is high (41%) and drops to almost half (21%) when the catheter is removed (Nguyen *et al.*, 1995). The ability of *Candida* to adhere to, and form biofilms on, the plastic and acrylic components of catheters, dentures and other in-dwelling medical devices may mean that

these devices act as a reservoir for microorganisms thus increasing the chance that a patient will develop candidiasis.

Biofilms are defined as microbial communities encased in a matrix of extracellular polymeric substances that display phenotypic features that differ from their planktonic or free floating counterparts (Costerton *et al.*, 1995). *Candida* biofilms are widespread and have been observed in many medical devices such as shunts, pacemakers, implants and catheters (Ramage *et al.*, 2006). A biofilm develops in four sequential steps; adhesion of a microorganism to a surface, colony formation, secretion of extracellular polymeric substances into a three dimensional structure and dissemination of progeny biofilm cells (Seneviratne *et al.*, 2008). There are numerous factors which affect biofilm formation. The properties of a substrate can influence candidal adhesion, for example soft lining materials of dentures are better for adhesion of *Candida* than acrylic surfaces (Radford *et al.*, 1998) and smooth surfaces minimise biofilm formation (Seneviratne *et al.*, 2008). The coating of a medical device in body fluid such as blood or saliva can act as a 'conditioning film' which may aid candidal biofilm development (Seneviratne *et al.*, 2008). The production of extracellular polymeric substances is unique to biofilms and has been proposed to play a pivotal role in biofilm formation. Extracellular polymeric substances may potentially provide defence against phagocytic cells, provide a scaffold to maintain biofilm integrity and/or limit active drug diffusion into the biofilm (Seneviratne *et al.*, 2008).

Candida biofilms are difficult to eradicate as they are highly resistant to antifungal drugs and often results in the need to remove an infected medical device if the biofilm can not be removed (Tunney *et al.*, 1996). *Candida* biofilms are 30 to 2,000 times more resistant than planktonic cells to various antifungal agents such as fluconazole, itraconazole and ketoconazole (Hawser & Douglas, 1995). *Candida* biofilms also show increased fluconazole resistance *in vivo* with a minimum inhibitory concentration for fluconazole 128 times higher than planktonic cells (Chandra *et al.*, 2001; Andes *et al.*, 2004). The extracellular polymeric substances associated with biofilm formation may act as a physical barrier that prevents access of antimicrobials to the cells embedded in the biofilm community, thus contributing to drug resistance. Microarray analysis showed that *MDR1*, *CDR1* and *CDR2* of *C. albicans* were up-regulated in the biofilm form of growth contributing to azole resistance in the early states of biofilm formation, whereas changes in sterol composition were involved in azole resistance in the mature biofilm (García-Sánchez *et al.*, 2004). Mature

C. dubliniensis biofilms resemble *C. albicans* biofilms and consist of yeast and filamentous forms embedded in exopolymeric material (Ramage *et al.*, 2001). *Candida dubliniensis* biofilms are also intrinsically resistant to fluconazole and show an increased resistance to amphotericin B (Ramage *et al.*, 2001).

1.3.8.2 Adhesin genes

HWPI (hyphal wall protein) encodes for an outer surface mannoprotein (Hwp1p) which contains a cell surface exposed N terminal domain which resembles mammalian transglutaminase substrates, suggesting that *HWPI* is involved in the formation of hyphal specific stable complexes with human BECs (Staab *et al.*, 1996; Staab & Sundstrom, 1998). A mutant lacking *HWPI* did not form stable attachments with human BECs and was also less virulent in a mouse model of systemic candidiasis (Staab *et al.*, 1999). The ability of hyphal cells to form stable attachments with human BECs indicates that hyphal cells may play more of a role in adhesion than yeast cells. A homologue for *HPWI* in *C. dubliniensis* was detected however this gene is 49% identical to the *C. albicans HWPI* gene (Moran *et al.*, 2004) indicating significant divergence between the two genes. Several large deletions in the N terminal of the *C. dubliniensis HWPI* gene were found compared to the *C. albicans HWPI* gene and this may explain the defect in the ability of *C. dubliniensis* to form stable complexes with oral epithelium. This may also be partly responsible for the reduced prevalence of *C. dubliniensis* in humans (Moran *et al.*, 2004).

Eap1p in *C. albicans* possesses homology to Hwp1p of *C. albicans* and Flo1 of *Saccharomyces cerevisiae*. Eap1p enhances adherence to human epithelial cells and polystyrene when expressed in *Sa. cerevisiae* indicating its role in adhesion. *EAP1* expression complements *flo11* mutations in *Sa. cerevisiae* restoring invasive growth to haploid *flo8Δ* and *flo11Δ* strains as well as filamentous growth to diploid *flo8Δ/flo8Δ* and *flo11Δ/flo11Δ* strains. When *EAP1* is expressed in *Sa. cerevisiae* it exhibits functional similarity to *FLO11* which encodes a cell wall protein required for both invasion and pseudohyphal formation, presumably enabling adhesion. *EAP1* is also expressed under non-hyphal inducing conditions indicating that *EAP1* may play a role in adhesion of yeast cells to mammalian cells before the formation of hyphae, as well as in the process of hyphal formation (Li & Palecek, 2003).

1.3.8.3 Comparative adhesion studies

Various adhesion studies have suggested that one or other of *C. albicans* or *C. dubliniensis* is more adherent (McCullough *et al.*, 1995; Vidotto *et al.*, 2003), while other studies show mixed results depending on the growth medium used (Gilfillan *et al.*, 1998). Difficulties in interpreting the data from these previous adhesion studies have arisen due to the small number of isolates used (McCourtie & Douglas, 1984; Enache *et al.*, 1996; Gilfillan *et al.*, 1998), the fact that the *C. dubliniensis* isolates were not delineated into their respective genotypes (Vidotto *et al.*, 2003) and the fact that there was more than one experimental variable in some studies (McCourtie & Douglas, 1984; Gilfillan *et al.*, 1998). These studies are described in more detail in chapter 3. Given the wide range of human cells that *Candida* species can adhere to, it is possible that these species possess a large number of adhesins.

1.3.9 Comparative virulence between *C. albicans* and *C. dubliniensis* in infection models

Although *in vitro* infection models have been developed, use of whole system models such as the mouse model, are necessary to elucidate the full effects of *Candida* virulence on its host. Using the mouse model of systemic infection, *C. albicans* has been shown to be more virulent than *C. dubliniensis* (Gilfillan *et al.*, 1998 Vilela *et al.*, 2002) and under certain growth conditions, *C. albicans* showed higher virulence in mice (McCourtie & Douglas, 1984). Mice infected with *C. albicans* had yeast and hyphal cells present in infected organs, while those that were infected with *C. dubliniensis* had only yeast cells present in infected organs (Vilela *et al.*, 2002). This suggests that different signals and pathways may be involved in hyphal induction and formation of both species. As hyphal cells are considered to be a more virulent phenotype of *Candida* compared to yeast cells, it can be assumed that *C. albicans* is better able to cause systemic disease in mice. In an oral-intragastric infant mouse infection model (Cole *et al.*, 1990; Cole *et al.*, 1993; Cole *et al.*, 1996), *C. dubliniensis* was found to be far less able to disseminate to the kidney and liver than *C. albicans* and *C. albicans* was found in both yeast and hyphal forms in the stomach, kidney and liver. *Candida dubliniensis* was only found in a small number of animals in the yeast phase in the stomach and kidney, although hyphal cells were observed in the liver (Stokes *et al.*, 2007). While

C. dubliniensis is able to form hyphae and pseudohyphae, the fact that several hyphal specific genes are absent and others are highly divergent could indicate that *C. dubliniensis* grows preferentially in the yeast phase in its natural environment. The lower virulence of *C. dubliniensis* is most likely to be multifactorial, however the lower capacity of *C. dubliniensis* to cause systemic infections is probably due to its limited ability to form hyphae and invade oral and gastrointestinal tissue (Stokes *et al.*, 2007). *Candida dubliniensis* was found to be more virulent than *C. albicans* in a systemic neutropenic mouse model compared with non-neutropenic mice (Curfs *et al.*, 2000) suggesting that *C. dubliniensis* may have a selective advantage in its ability to cause disease in immunocompromised hosts.

1.4 AIMS AND OBJECTIVES

Despite their close phenotypic, genotypic and phylogenetic relationship, epidemiological and infection model data suggest that *C. albicans* is significantly more pathogenic than *C. dubliniensis*. The reasons for this are currently unclear. The objectives of this study were to investigate the relative adhesion of *C. dubliniensis* and *C. albicans* to human cells and tissue at the phenotypic and molecular level and to determine whether differences in the ability to adhere to human cells may in part explain why *C. albicans* is a more successful pathogen than *C. dubliniensis*. This study aimed to:

- Compare the ability of an extensive and comprehensive selection of *C. dubliniensis* isolates and reference strains of *C. albicans* to adhere to a range of human cells including human BECs and an epithelial cell line when the *Candida* cells were grown under a wide range of temperature and media conditions.
- Attempt to develop a more uniform and reproducible model for investigating adhesion which may overcome some of the variability in adhesion associated with the use of a heterogenous population of human BECs.
- Investigate and quantify the adherence of *C. albicans* and *C. dubliniensis* to a specific range of host proteins such as those of the ECM which are known to be ligands for candidal adhesion using a BIAcore™ 3000 biosensor and surface plasmon resonance technology.
- Identify the homologues of known *C. albicans* adhesins (e.g. *ALS* genes) in *C. dubliniensis* and to examine the expression of these adhesins in both species using conventional and real time PCR when the *Candida* cells are grown under various temperature and media conditions.

Chapter 2

Materials and Methods



2.1 GENERAL MICROBIOLOGICAL METHODS

2.1.1 *Candida* strains and isolates, culture media and growth conditions

All *Candida* strains and isolates used in this study are detailed in Tables 2.1 and 2.2. Not all isolates were used in all experiments. All isolates of *C. dubliniensis* and *C. albicans* were routinely cultured on Potato Dextrose Agar (PDA; potato extract 4 g L⁻¹, glucose 20 g L⁻¹, agar 15 g L⁻¹; Oxoid Ltd., Basingstoke, Hampshire, UK or Lab M Ltd., Bury, Lancashire, UK) pH 5.6 ± 0.2 at 37°C and occasionally cultured on Sabouraud Dextrose Agar (SDA; peptone 10 g L⁻¹, glucose 40 g L⁻¹, agar 12 g L⁻¹; Lab M) pH 5.6 ± 0.2 at 37°C and re-plated onto fresh plates every week. For liquid culture, isolates were routinely grown in Yeast Extract Peptone Dextrose (YEPD) broth (1% (w/v) yeast extract (Sigma-Aldrich Ltd., Tallaght, Dublin, Ireland and Poole, Dorset, UK or Oxoid), 2% (w/v) bacterial peptone (Oxoid) and 2% (w/v) glucose (Sigma)) at 37°C overnight (~16 h) in an orbital incubator (Gallenkamp, part of Weiss-Gallenkamp, Epinal Way, Loughborough, UK) at 200 rpm. Isolates were also cultured in YEPD at 30°C and in Yeast Extract Peptone Galactose (YEPGal) broth (1% (w/v) yeast extract, 2% (w/v) bacterial peptone and 2% (w/v) galactose (Sigma)) at 30°C and 37°C. Stock cultures of *Candida* isolates were maintained on plastic beads in Microbank cryovials (Pro-lab Diagnostics, Richmond Hill, ON, Canada) at -80°C. Cells were activated by sub-culture onto PDA and incubated for 48 h at 37°C.

CHROMagar™ *Candida* medium for presumptive identification of clinically important *Candida* species was purchased from CHROMagar, Paris, France.

For hyphal induction, a reference *C. albicans* strain and reference strains of each genotype of *C. dubliniensis* were pre-cultured overnight (~16 h) in an orbital incubator at 200 rpm in Lee's medium (Lee *et al.*, 1975; Buffo *et al.*, 1984), pH 4.5 at 30°C and sub-cultured into pre-warmed 10% (v/v) filter-sterile new born bovine calf serum (Sigma) in Phosphate Buffered Saline (PBS; Oxoid) at 37°C.

2.1.2 Chemicals, buffers, dyes and solutions

Proteins and analytical grade, molecular biology grade or cell culture grade chemicals were purchased from Sigma and Oxoid. Custom synthesised primers were purchased from Sigma-Genosys Ltd., Haverhill, Suffolk, UK. DNA molecular weight markers, deoxynucleotide triphosphates (dNTPs), magnesium chloride and Oligo dT(15) Primer were purchased from The Promega Corporation, Madison, WI, USA. *Taq* reaction buffer and *Taq* DNA polymerase were purchased from Sigma. Green GoTaq® Flexi Buffer, colourless GoTaq® Flexi Buffer and GoTaq® DNA polymerase were purchased from Promega. Power SYBR® green PCR Master Mix for real time PCR was purchased from Applied Biosystems, Warrington, Cheshire, UK.

Tris-Borate-EDTA (TBE) buffer was prepared at 10× concentration and consisted of 0.89 M trizma base (Sigma), 0.89 M boric acid (Sigma) and 0.02 M ethylenediaminetetraacetic acid (EDTA; Sigma). This was diluted in ultra-purified water (18.2 MΩ-cm resistivity) at 25°C from a MilliQ water purification system (Millipore, Molsheim, France) to a final concentration of 0.5× and was used as the buffer for agarose gel electrophoresis. Agarose gels were prepared by diluting agarose (Sigma) to the required concentration in 0.5× TBE buffer and contained 0.013 M ethidium bromide (Sigma). Samples were loaded into agarose gels in 6× Blue/Orange Loading Dye (Promega), which is a commercially available marker dye containing 0.4% (w/v) orange G, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, 15% (w/v) Ficoll®400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0). It is provided in a premixed, ready-to-use form and was diluted 6-fold with the sample.

PBS was prepared at 1× concentration and contained 8 g L⁻¹ sodium chloride, 0.2 g L⁻¹ potassium chloride, 1.15 g L⁻¹ disodium hydrogen phosphate, 0.2 g L⁻¹ potassium dihydrogen phosphate, pH 7.3 ± 0.2 at 25°C.

Accustain® Gram stain, which consisted of crystal violet solution (2.3% (w/v) certified crystal violet, 0.1% (w/v) ammonium oxalate and 20% (w/v) ethyl alcohol), Gram's iodine solution (0.33% (w/v) iodine and 0.66% (w/v) potassium iodide) and decolouriser (75% (w/v) isopropyl alcohol and 25% (w/v) acetone) was purchased from Sigma Diagnostics (St. Louis, MO, USA).

Trypsin-EDTA at 1× concentration, Dulbecco's Modified Eagle's Medium (supplemented with 4.5 g L⁻¹ glucose, sodium pyruvate, L-glutamine and sodium bicarbonate), heat inactivated foetal bovine serum, penicillin-streptomycin and RPMI-

Table 2.1. *C. dubliniensis* strains and isolates used in this study

| Strain/isolate | Anatomical site ^a | Year isolated | Country of Origin | Genotype ^b | Reference |
|--------------------|------------------------------|---------------|-------------------|-----------------------|--------------------------------|
| CD36* ¹ | Oral | 1988 | Ireland | 1 | Sullivan <i>et al.</i> , 1995 |
| CD33 | Oral | 1989 | Ireland | 1 | Sullivan <i>et al.</i> , 1995 |
| Co4 | Oral | 1993 | Switzerland | 1 | Boerlin <i>et al.</i> , 1995 |
| Cm1 | Oral | 1991 | Australia | 1 | Sullivan <i>et al.</i> , 1995 |
| SA105 | Oral | 2002 | Saudi Arabia | 1 | Al Mosaid <i>et al.</i> , 2005 |
| CD539 | Oral | 1994 | UK | 2 | Pinjon <i>et al.</i> , 1998 |
| CD75004 | Oral | 1975 | UK | 2 | Pinjon <i>et al.</i> , 1998 |
| Can6 ² | Oral | 1995 | Canada | 2 | Pinjon <i>et al.</i> , 1998 |
| Can9 | Oral | 1996 | Canada | 2 | Pinjon <i>et al.</i> , 1998 |
| CD506 | Oral | 1989 | Ireland | 2 | Gee <i>et al.</i> , 2002 |
| P6785 | Urine | 1999 | Israel | 3 | Polacheck <i>et al.</i> , 2000 |
| CD519 ³ | Oral | 1997 | Ireland | 3 | Gee <i>et al.</i> , 2002 |
| DB305 | Oral | 2000 | New Zealand | 3 | Al Mosaid <i>et al.</i> , 2003 |
| P7276 | Respiratory tract | 1999 | Israel | 3 | Polacheck <i>et al.</i> , 2000 |
| CD514 | Oral | 1995 | Ireland | 3 | Gee <i>et al.</i> , 2002 |
| Eg200 | Oral | 2002 | Egypt | 4 | Al Mosaid <i>et al.</i> , 2005 |
| P7718 ⁴ | Wound | 1999 | Israel | 4 | Gee <i>et al.</i> , 2002 |
| Eg202 | Oral | 2002 | Egypt | 4 | Al Mosaid <i>et al.</i> , 2005 |
| Eg201 | Oral | 2002 | Egypt | 4 | Al Mosaid <i>et al.</i> , 2005 |
| Eg207 | Oral | 2002 | Egypt | 4v ^c | Al Mosaid <i>et al.</i> , 2005 |
| Is35 | Oral | 2001 | Israel | 4v | Al Mosaid <i>et al.</i> , 2005 |

^aAnatomical location from which the isolate was originally recovered

^bThe genotype of each isolate was assigned based on differences observed in the nucleotide sequence of the ITS region of the rRNA gene cluster (Gee *et al.*, 2002; Al Mosaid *et al.*, 2005)

^cThe ITS sequence of the 4v isolates was found to differ from the genotype 4 consensus sequence by 1 or 2 bases in the ITS region compared to genotype 4 (Al Mosaid *et al.*, 2005)

*Type strain (i.e. the nomenclatural type of a species) for *C. dubliniensis*

¹*C. dubliniensis* genotype 1 reference strain for *C. dubliniensis* genotype PCR

²*C. dubliniensis* genotype 2 reference strain for *C. dubliniensis* genotype PCR

³*C. dubliniensis* genotype 3 reference strain for *C. dubliniensis* genotype PCR

⁴*C. dubliniensis* genotype 4 reference strain for *C. dubliniensis* genotype PCR



Table 2.2. *C. albicans* strains and isolates used in this study

| Strain/isolate | Anatomical site ^a | Year isolated | Country of origin | Source/Reference |
|-----------------------|------------------------------|-------------------|-------------------|----------------------------------|
| SC5314* | Systemic | 1984 | N/Av ^b | Gillum <i>et al.</i> , 1984 |
| 132A | Oral | 1992 | Ireland | Gallagher <i>et al.</i> , 1992 |
| NCPF3153 ^c | Systemic | N/Av ^b | N/Av ^b | UK reference strain ^c |
| m182CA | Oral | 1990 – 95 | Ireland | Pinjon, 2003 |
| JM1 | Oral | 1997 | Ireland | Pinjon, 2003 |
| CA400 ⁺ | Oral | 1997 – 98 | Ireland | This study |
| CA401 ⁺ | Oral | 1997 – 98 | Ireland | This study |
| NCPF8317 ^c | N/Av ^b | 1998 | UK | UK reference strain ^c |
| NCPF8324 ^c | Oral | 1998 | UK | UK reference strain ^c |
| CA800 ⁺ | Oral | 2004 | Ireland | This study |
| CA801 ⁺ | Oral | 2004 | Ireland | This study |
| GRI681 ^d | Cervical | 1983 | Scotland | McCourtie & Douglas, 1984 |
| GDH2346 ^e | Oral | 1983 | Scotland | McCourtie & Douglas, 1984 |
| 6CA ⁺ | Oral | 1997 | Ireland | This study |
| CAN33 ^f | N/A ^g | N/A ^g | N/A ^g | Lo <i>et al.</i> , 1997 |
| CAF2-1 | N/A ^g | N/A ^g | N/A ^g | Fonzi & Irwin, 1993 |

^aAnatomical location from which the isolate was originally recovered

^bNot available

^cFrom the UK National Collection of Pathogenic Fungi (NCPF), Kingsdown, Bristol, UK

^dStored in the National Collection of Yeast Cultures (NCYC), Colney, Norwich, UK as NCYC 1472

^eStored in the NCYC as NCYC 1467

^fCalled HLC52 in Lo *et al.*, 1997

^gNot applicable

*Type strain (i.e. the nomenclatural type of a species) for *C. albicans*

⁺Oral isolates from the laboratory collection of the Microbiology Research Unit, Dublin Dental School and Hospital



1640 were purchased from Sigma. Dimethyl sulfoxide (DMSO) used in storage of the TR146 cell line under liquid nitrogen was purchased from Sigma.

Triton X-100 (t-octylphenoxyethoxyethanol) was purchased from Sigma.

XTT solution consisted of 0.6 mM XTT sodium salt (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt), minimum 90% (Sigma) and 0.27 mM Coenzyme Q (2,3-Dimethoxy-5-methyl-*p*-benzoquinone; Sigma) in 1× PBS solution. FUN-1 (2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide) cell stain, a 10 mM solution in anhydrous DMSO, was purchased from Molecular Probes Inc., Eugene, OR, USA. Trypan blue which was used to distinguish viable from non-viable cells was purchased from Sigma.

HBS-EP buffer which was used as a rinsing solution for the BIAcore™ 3000 biosensor (BIAcore AB, Uppsala, Sweden) consisted of 0.01 M hepes (Sigma) pH 7.4, 0.15 M sodium chloride (Sigma) and 3 mM EDTA. Sodium hydroxide (Fisher Scientific, Loughborough, Leicestershire, UK), 0.005% (v/v) surfactant P20 (BIAcore) and BIA Desorb solution (BIA Maintenance Kit; BIAcore) were used in the day to day running of the BIAcore™ 3000 biosensor. Sodium acetate buffers (10 mM), supplied in a range of pHs, and an amine coupling kit, which consisted of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as coupling solutions and ethanolamine hydrochloride as a deactivation solution, were purchased from BIAcore. Acetic acid was purchased from Fisher Scientific.

Tri reagent (molecular biology tested), chloroform (minimum 99%), isopropyl alcohol (2-propanol; for molecular biology, minimum 99%), absolute ethanol (200 proof for molecular biology) and diethyl pyrocarbonate (DEPC; approximately 97% NMR) were purchased from Sigma. First Strand buffer (5×), 0.1 M DTT and SuperScript™II Reverse Transcriptase were purchased from Invitrogen Corp., Carlsbad, CA, USA. RNase inhibitor and the TURBO DNA-*free*™ kit, which consisted of 10× TURBO DNase Buffer, TURBO DNase and DNase Inactivation Reagent, were purchased from Ambion Inc., An Applied Biosystems Business, Austin, TX, USA.

2.1.3 Kits

The API ID 32C yeast identification system was used to confirm the identity of *Candida* isolates to the species level using a series of carbohydrate-source assimilation tests contained in 32 separate cupules on a plastic strip and was purchased from bioMérieux SA, Marcy l'Etoile, France (Pincus *et al.*, 1999) and used according to the manufacturer's instructions.

PCR products were purified using either the GenElute™ PCR Clean-Up Kit (Sigma) or the QIAEX II Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions.

The MycoAlert™ Mycoplasma Detection Kit was purchased from Cambrex Corp., NJ, USA, and was used to detect the presence of mycoplasma contamination of the TR146 cell line.

2.1.4 Disposable laboratory plastic-ware

Microfuge tubes (0.2 ml, 0.5 ml and 1.5 ml) were purchased from Eppendorf AG, Hamburg, Germany. Screw cap tubes of 15 ml and 50 ml volume with a conical bottom, 5 ml, 10 ml and 25 ml pipettes, 96-well flat bottomed tissue culture plates with lid and petri dishes were purchased from Greiner Bio-One Ltd., Stonehouse, UK. Screw cap tubes of 2 ml capacity with a conical bottom and skirt were purchased from Sarstedt AG & Co., Nümbrecht, Germany. Pipette tips (0.1 – 10 µl, 1 – 12 µl, 1 – 200 µl and 101 – 1000 µl) with and without filters were purchased from Starlab GmbH, Ahrensburg, Germany. Treated tissue culture dishes of 90 mm diameter from the IWAKI range were purchased from Sterilin Limited, Bargoed, Caerphilly, UK.

2.1.5 PCR primer design

Primers for conventional PCR were designed using the web based program Primer3 v. 0.3.0 (<http://frodo.wi.mit.edu/>; Rozen & Skaletsky, 2000). Primers for real time PCR were designed using the TaqMan® Probe and Primer Design function of Primer Express version 1.5 (Applied Biosystems) on a Macintosh platform.

2.2 IDENTIFICATION OF *C. dubliniensis* ISOLATES

All *C. albicans* and *C. dubliniensis* strains and isolates used were from the collections of the Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School & Hospital, University of Dublin, Trinity College, Lincoln Place, Dublin 2, Ireland and The Department of Tissue Engineering & Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK. All isolates in both collections had previously been identified by standard identification methods. Several presumptive phenotypic and definitive molecular techniques were used to differentiate *C. dubliniensis* from *C. albicans*.

2.2.1 Growth on CHROMagar™ Candida medium

CHROMagar™ Candida is a commercially available agar medium containing chromogenic substrates that allow colonies of some medically important *Candida* species to be presumptively identified on the basis of colony colour following primary isolation from clinical specimens. These colony colours are produced by reactions of species-specific enzymes with the chromogenic substrates in the agar. Colonies of *C. albicans* (green colonies), *C. krusei* (rough, pale pink colonies), *C. tropicalis* (dark blue-grey or steel blue) and *C. glabrata* (dark pink colonies) can easily be distinguished from each other upon primary isolation, and the medium has been shown to be clinically useful in the presumptive identification of these species (Odds & Bernaerts, 1994; Pfaller *et al.*, 1996). Freshly isolated, putative *C. dubliniensis* isolates were inoculated on this medium along with control *C. albicans* isolates and incubated for 48 h at 37°C. *Candida dubliniensis* isolates could be distinguished from *C. albicans* isolates upon initial isolation on the basis of colour, with *C. albicans* colonies typically being light blue-green and *C. dubliniensis* colonies typically being dark green. The dark green colour produced by *C. dubliniensis* upon initial isolation may not be reproducible upon storage at -70°C and repeated subculture (Schoofs *et al.*, 1997).

2.2.2 Biotyping

The examination of carbohydrate assimilation profiles, or biotyping, was carried out using the API ID 32C yeast identification system. This system is used to identify *Candida* isolates to the species level using a series of carbohydrate-source assimilation tests contained in 32 separate cupules on a plastic strip. Tests were carried out according to the manufacturer's instructions whereby an inoculum was prepared for each test isolate by culture of the test isolate on PDA for 24 – 48 h. Several of these colonies were re-suspended in 2 ml API suspension medium to the turbidity of a 2 McFarland standard and 250 µl of this homogenised suspension was then used to inoculate an aliquot of API C medium which was supplied by the manufacturers. Each of the cupules in the strip was then inoculated with 135 µl of the homogenised API C medium suspension and incubated for 48 h at 30°C. Readings were made by visually assessing the growth of the test isolate in each of the cupules compared to a control which was also included in the strip. Any cupule that appeared to be more turbid than the control was recorded as positive for growth. The presence or absence of growth for each cupule was recorded on a results sheet which was supplied by the manufacturers, and the substrate assimilation profile of the isolate was converted into an eight-digit numerical profile. These profiles were then cross-referenced in the APILAB ID 32C analytical profile index. Each profile is listed along with a percentage of identification (%id), which is an estimate of how closely the profile corresponds to a taxon relative to all the other taxa in the database and the T index, which is an estimate of how closely the profile corresponds to the most typical set of reactions for a particular taxon. Based on these parameters, a set of reactions which closely resemble those of a particular taxon will be classed as 'excellent' or 'good', and will yield an identification to the species level, whereas atypical results will be classed as having 'poor' or 'low' discriminatory powers and are usually unable to yield a positive identification. This system is designed for the identification of yeasts included in the database, and no other microorganisms. Only pure cultures of single organisms should be used for the purposes of identifications using the API ID 32C yeast identification system. Following a database modification of trehalose assimilation as suggested by Pincus *et al.* (1999), more consistent results for the identification of *C. dubliniensis* were obtained.

2.2.3 Identification of *C. dubliniensis* by *C. dubliniensis*-specific PCR

Identification of *C. dubliniensis* by PCR was carried out using the *C. dubliniensis*-specific primer pair DUBF and DUBR, which amplifies a 288-bp portion of the *C. dubliniensis* *ACT1*-associated intron (Donnelly *et al.*, 1999). A single colony from a culture grown for 48 h at 37°C on PDA was suspended in 50 µl ultra-purified water. Cell suspensions were boiled for 10 min and the lysed cells were centrifuged for 8 min at 20,000 × *g* in an Eppendorf 5417C microfuge, fitted with a FA45-30-11 rotor (Eppendorf). Template DNA contained in 25 µl supernatant was used for PCR amplification, which was carried out in a final volume of 50 µl containing 1× *Taq* reaction buffer, 2.5 mM magnesium chloride, 200 nM each DUBF, DUBR, RNAF and RNAR, 200 µM each dATP, dTTP, dCTP, dGTP and 2.5 U *Taq* DNA polymerase. Cycling conditions consisted of initial denaturation for 6 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, followed by a final extension for 10 min at 72°C. Each reaction mixture contained the fungal universal primers RNAF and RNAR (Fell, 1993) as an internal positive control. These primers amplify a product of approximately 610 bp from the fungal large subunit rRNA gene. The positive control for *C. dubliniensis* is *C. dubliniensis* CD36.

Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in a Thermo Hybaid MBS 0.2G thermal cycler (Thermo Scientific, part of Thermo Fisher Scientific Inc., Waltham, MA, USA) using either 0.2 ml or 0.5 ml satellite blocks. PCR amplification products were separated by agarose gel electrophoresis through 2% (w/v) agarose gels containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm; Ultraviolet Products (UVP), Upland, CA, USA) and photographed using the Imagestore 7500, version 7.22, gel documentation system (UVP).

2.2.4 Identification of *C. dubliniensis* genotype by genotype-specific PCR

Identification of each of the 4 genotypes (1 – 4) of *C. dubliniensis* by PCR was performed using the following primer pairs, G1F/G1R, G2F/G2R, G3F/G3R and G4F/G4R, respectively (Gee *et al.*, 2002). These primers were designed based on differences observed in the nucleotide sequence of the ITS regions of the rRNA gene cluster of isolates belonging to each of the 4 *C. dubliniensis* genotypes. The genotype 4-

specific forward and reverse primers (G4F/G4R) were designed with a single base pair change from the other genotype primers at their 3' ends to increase their specificity (Gee *et al.*, 2002). Each PCR reaction was carried out with one of the genotype primer pairs, and the internal positive control primers, RNAF and RNAR, which were described in section 2.2.3 above.

Each 100 μ l PCR reaction for genotypes 1, 2 and 3 contained 1 \times *Taq* reaction buffer, 1.5 mM magnesium chloride, 300 nM each G1, G2, or G3 forward and reverse primers plus RNAF and RNAR, 200 μ M each dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* DNA polymerase. Template DNA was prepared by boiling a single 24-h old colony in 50 μ l sterile ultra-purified water for 10 min. After boiling the debris was removed by centrifugation for 8 min at 20,000 \times *g* in an Eppendorf 5417C microfuge and DNA contained in 25 μ l of supernatant was removed and used as the source of template DNA. The PCR cycling conditions were initial denaturation for 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, and 20 s at 72°C, and a final extension for 10 min at 72°C. PCR conditions for genotype 4 were as above with the exception that 20 μ M dNTPs were used and the annealing temperature was 55°C. Positive controls for each of the *C. dubliniensis* genotypes are described in Table 2.1

Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in a Thermo Hybrid MBS 0.2G thermal cycler using either 0.2 ml or 0.5 ml satellite blocks. Following PCR, a 5 μ l volume of the amplification mixture for G1, G2 and G3 specific PCR and a 10 μ l volume of the amplification mixture from G4 PCR were separated by electrophoresis through 2% (w/v) agarose gels containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm) and photographed using the Imagestore 7500, version 7.22, gel documentation system (Gee *et al.*, 2002).

2.3 RECOMBINANT DNA TECHNIQUES

2.3.1 Polymerase Chain Reaction (PCR)

Custom oligonucleotide primers were stored at a stock concentration of 100 μM in sterile ultra-purified water at -20°C . Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in one of the following thermal cyclers; a Biometra T Personal DNA thermal cycler (Biometra biomedizinische Analytik GmbH, Göttingen, Germany), a Thermo Hybaid MBS 0.2G thermal cycler using either 0.2 ml or 0.5 ml satellite blocks or a G-Storm thermal cycler (Gene Technologies Ltd., Braintree, Essex, UK) in 50 μl or 100 μl volumes. Constituent reagents, concentration of reagents, specific primers, amplification conditions and concentration of agarose gel for separation of PCR products are described in the relevant sections. Alternatively, if the PCR product was to be sequenced the reactions were carried out using either $1\times$ *Taq* reaction buffer and 2.5 U *Taq* DNA polymerase or $1\times$ colourless GoTaq® Flexi Buffer and 0.75 U GoTaq® DNA polymerase. PCR products that were to be sent for sequencing were purified using either the GenElute™ PCR Clean-Up Kit (for products of 100 bp – 10 kb) or the QIAEX® II gel extraction kit according to the manufacturer's instructions.

2.3.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Solutions containing 5 μg DNase-treated stock RNA in a volume of 11 μl were used as template for RT-PCR reactions. To each sample, 500 ng Oligo dT(15) Primer was added and the sample was denatured for 10 min at 70°C before being allowed to cool on ice for 1 min. To each sample on ice, 8 μl of master mix containing $2.5\times$ First Strand buffer, 25 mM DTT, 1.25 mM each dATP, dTTP, dCTP and dGTP and 40 U RNase inhibitor was added and the sample incubated for 2 min at 42°C . While holding the sample at 42°C , 200 U SuperScript™II Reverse Transcriptase was added to each sample. The samples were then incubated for a further 60 min at 42°C before the reaction was terminated by incubation for 15 min at 70°C . As this reaction was a $5\times$ reaction (i.e. there were 5 μg DNase-treated stock RNA used in the initial solution), the resultant cDNA was diluted 5-fold with ultra-purified water and stored at 4°C until

needed. Amplification reactions were carried out in 0.5 ml microfuge tubes in either a Biometra T Personal DNA thermal cycler or a G-Storm thermal cycler.

2.4 DNA SEQUENCE ANALYSIS

DNA sequencing was performed commercially by Cogenics (Takeley, Essex, UK) using the dideoxy chain termination method of Sanger *et al.* (1977), the 3730xl DNA Analyser (Applied Biosystems), a fully automated 96 capillary electrophoresis instrument, and the fluorescent dye-labelled terminators, BigDye™ Terminator Version 1.1 (Applied Biosystems). The BigDye™ Terminator Version 1.1 is recommended for specialty applications including the sequencing of short PCR fragments and other applications requiring optimal base calling next to the primer and for sequencing short PCR product templates with rapid electrophoresis run modules. These fluorescent dye labelled terminators, a different colour for each nucleotide base, allow sequences to be determined within a single lane. The template for sequencing in each case was 50 ng PCR product and the primer used for sequencing was 8 pmoles of the forward primer used in the amplification process of the PCR product. The sequence reaction products are detected as they electrophorese past a scanning argon laser, which excites the dyes causing them to fluoresce. This emitted fluorescence is collected, passed through four filters and directed onto a photomultiplier tube before the digitised output is processed using Sequence Analysis Software version 5.2 (Applied Biosystems), which uses an automated base-calling routine, and this data is then stored. The sequence information can be edited within this Sequence Analysis Software package (<http://dna-9.int-med.uiowa.edu/sequencing.htm>; personal communication, Stephen Foster, UK Scientific Director, Cogenics).

Results are provided in the form of a text file containing the sequence and a chromatogram. Chromatograms were analysed using Chromas, version 2.31 (Technelysium Pty Ltd., Tewantin, QLD, Australia). Searches of the EMBL, GenBank, DDBJ, PDB, SwissProt, PRF and GeneDB databases for nucleotide and amino acid sequence similarities were performed using the BLAST series of computer programs (Altschul *et al.*, 1990; Altschul *et al.*, 1997; Zhang *et al.*, 2000). Alignments of nucleotide sequences were carried out using the CLUSTAL W sequence alignment computer program (Thompson *et al.*, 1994).

2.5 EXTRACTION AND 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 DEPC. DEPC was dispersed 1 ml per L in all solutions, which were left to incubate at room temperature overnight (~16 h), before autoclaving to inactivate DEPC. Plastic-ware such as microfuge tubes and 50 ml screw cap tubes with a conical bottom, which were assumed to be free of RNase contamination, were handled only when wearing vinyl or synthetic gloves. Acid washed glass beads (Sigma) that were used in RNA extractions were 425 – 600 microns in diameter.

2.5.2 RNA isolation from *Candida* isolates

Yeast cells were harvested at mid exponential phase (A_{600} : 1) from 50 ml broth cultures for RNA extractions by centrifugation in an Eppendorf 5804 centrifuge which was fitted with a A-4-44 rotor (Eppendorf) for 3 min at $1,125 \times g$ at room temperature. Hyphal and pseudohyphal cells were harvested from 10% (v/v) filter-sterile new born bovine calf serum in PBS at 37°C for RNA extractions by centrifugation in an Eppendorf 5804 centrifuge for 3 min at $1,125 \times g$ at room temperature. Adherent *Candida* cells were harvested following adhesion to BECs by centrifugation in an Eppendorf 5804 centrifuge for 3 min at $1,125 \times g$ at room temperature. Pellets were re-suspended in 1 ml tri reagent and the re-suspended culture was transferred immediately to a 2 ml screw cap tube containing 0.3 g acid washed glass beads. The re-suspended pellet was processed in a FastPrep™ FP120 machine (Bio 101/Thermo Electron Corp., part of Thermo Fisher Scientific Inc.) at a speed rating of 6 for 2 periods of 30 s each and the samples were immediately frozen in a 2 ml screw cap tube at -80°C until needed.

2.5.3 RNA extraction from *Candida* isolates

Tri Reagent-suspended cells were thawed on ice and transferred to a 1.5 ml microfuge tube which had not been processed by autoclave. Following incubation for 10

min at room temperature, 200 μ l chloroform was added to the tri reagent-suspended cells which were vortexed for 15 s before being centrifuged at $12,000 \times g$ for 15 min at 4°C . The upper aqueous phase was removed to a fresh 1.5 ml microfuge tube which had not been processed by autoclave. This aqueous phase was incubated with 500 μ l isopropyl alcohol (2-propanol) for 10 min at room temperature before centrifugation at $12,000 \times g$ for 10 min at 4°C . The resultant pellet was washed in 1 ml 70% (v/v) ethanol, briefly air dried and re-suspended in $\sim 50 \mu$ l DEPC-treated water. The RNA samples were frozen at -80°C until needed.

2.5.4 DNase treatment of RNA

Frozen stock RNA was thawed on ice and 20 μ l of stock RNA was transferred to a 0.5 ml microfuge tube to which 2 μ l $10\times$ TURBO DNase Buffer plus 1 μ l TURBO DNase were added. This mixture was incubated for 30 min at 37°C before 2 μ l DNase Inactivation Reagent was added. The RNA mixture was then incubated for 2 min at room temperature with occasional shaking. Debris was removed by centrifugation for 1.5 min at $10,000 \times g$ and the clear supernatant was transferred to a fresh 0.5 ml microfuge tube and stored at -80°C until required.

2.5.5 Analysis of RNA

RNA was analysed after extraction from *Candida* culture and DNase treatment for degradation by separation of 1 μ g of each sample (diluted with DEPC-treated water) by electrophoresis through 1% (w/v) agarose gel containing 0.013 M ethidium bromide. TBE buffer used when working with RNA was made to $10\times$ concentration as described in section 2.1.2 and diluted to $0.5\times$ using DEPC-treated water. Samples were loaded onto the agarose electrophoresis gel in $6\times$ Blue/Orange Loading Dye which was diluted 6-fold with the sample and run at maximum voltage for 10 min. The samples were visualised on a high performance UV transilluminator (wavelength 345 nm) and photographed using the Imagestore 7500, version 7.22, gel documentation system. If the RNA is intact two bands will be present corresponding to the 26S and 18S units of the ribosomal RNA.

Purity of RNA was calculated as a ratio of $A_{260}:A_{280}$ and is required to be between 1.5 and 2 for RT-PCR. RNA concentrations were measured by absorbance at

260 nm (1 unit of optical density at 260 nm = 40 $\mu\text{g ml}^{-1}$ of water based on the extinction coefficient of RNA in water). A_{260} and A_{280} readings were taken when the sample was diluted in DEPC-treated water.



Chapter 3

**Comparative adhesion of *C. dubliniensis* and
C. albicans to human buccal epithelial cells**



3.1 INTRODUCTION

The mechanisms of microbial adherence to host cells are varied and complex (Sturtevant & Calderon, 1997) with one of the most important virulence factors considered to be the ability of the microorganism, including yeasts, to recognise and adhere to host cells and tissue (Sullivan *et al.*, 2004). This prevents these microorganisms being cleared by salivary flow. Adherence to host cells and tissue is seen as an essential early step in the establishment of disease (Yang, 2003) and generally, pathogenicity correlates positively with adherence to host cells, with the most adherent *Candida* species, *C. albicans*, being the most pathogenic (Calderone & Braun, 1991; Cannon & Chaffin, 1999). Filamentous forms of *Candida* adhere to a greater extent and variety of substances than yeast forms (Calderone & Braun, 1991) and mutants of *C. albicans* which are locked in the yeast form and are unable to form hyphae were found to be avirulent when tested using a mouse infection model (Lo *et al.*, 1997).

Growth media influences candidal adhesion *in vitro* to both epithelial cells and plastics. McCourtie & Douglas (1981) observed that *C. albicans* adhered to acrylics in greater numbers when grown in media containing high concentrations (500 mM) of various sugars compared to media containing a lower concentration (50 mM) of glucose, noting that there was more than a 10-fold increase in adherence to acrylic when yeast cells were grown in media containing 500 mM galactose compared to media containing 50 mM glucose. This group also observed that adherence increased linearly with increasing concentrations of sugar in the growth media for all sugars tested, indicating that individuals who consume carbohydrate rich diets may be more at risk of oral candidiasis than those who do not. A further study by McCourtie & Douglas (1984) confirmed that *C. albicans* isolates from active infection adhered to acrylic and also to human BECs in greater numbers when grown in media containing 500 mM galactose compared to media containing 50 mM glucose. The increased adherence to acrylic and human BECs when grown in media containing 500 mM galactose appears to be species specific; *C. albicans* and *C. tropicalis* adhered to acrylic and human BECs in greater numbers when grown in media containing 500 mM galactose whereas other species, including *Candida stellatoidea*, *C. parapsilosis* and *Candida pseudotropicalis* showed little or no increase in adhesion when cultured in this media (Critchley & Douglas,

1985). Enache *et al.* (1996) found that *C. albicans* cells adhered to various cell types including human BECs in greater numbers when grown in media containing galactose compared to media containing equimolar concentrations of glucose (500 mM). This adhesion assay was performed at 37°C however growth of *C. albicans* was enhanced when cultured at 30°C in broth containing glucose compared to broth containing galactose (Enache *et al.*, 1996). This result indicated that growth temperature had an effect on candidal growth and may also affect candidal adhesion to various substances, a theory that will be tested in the present study.

Results from a comparative study of adhesion of *C. dubliniensis* and *C. albicans* to human BECs by Gilfillan *et al.* (1998) found that *C. dubliniensis* isolates adhered to human BECs in greater numbers than *C. albicans* when grown in media containing glucose. There was no difference in the adhesion of *C. dubliniensis* and *C. albicans* to human BECs when grown in media containing galactose. McCullough *et al.* (1995) also found that *C. dubliniensis* adhered to human BECs in greater numbers than *C. albicans*. Vidotto *et al.* (2003) compared the adhesion of *C. dubliniensis* and *C. albicans* to both human BECs and human vaginal epithelial cells when grown in media containing galactose. Both species adhered to human vaginal epithelial cells in greater amounts than to human BECs. However, in contrast with the studies of McCullough *et al.* (1995) and Gilfillan *et al.* (1998), Vidotto *et al.* (2003) found that *C. albicans* adhered to both human cell types in significantly greater numbers than *C. dubliniensis*. Both *C. dubliniensis* and *C. albicans* have the ability to adhere to acrylic and hydroxyapatite, materials which are often used in dentures (Henriques *et al.*, 2004). Adhesion to dentures can act as a reservoir for *Candida* and as patients with dentures tend to consume soft, carbohydrate rich food, which can also promote adhesion of *Candida* to human BECs, the risk of oral colonisation is increased.

Growth temperature is known to affect the cell morphology of dimorphic yeasts such as *C. dubliniensis* and *C. albicans* thus affecting yeast cell surface composition and perhaps their ability to adhere. *Candida albicans* cells are hydrophobic when grown at 25°C and were found to have better adherence to human epithelial cells than *C. albicans* cells that were grown at 37°C, and consequently hydrophilic. Growth temperature had no effect on *C. dubliniensis* cell surface hydrophobicity (CSH) and the cell surface fibrils remained at a constant length when grown at either 25°C or 37°C, permanently expressing the hydrophobic phenotype (Hazen *et al.*, 2001; Jabra-Rizk *et al.*, 2001a).

Other studies (Samaranayake *et al.*, 2003) have found no correlation between adhesion and CSH.

The human BEC adherence assay has been extensively used to investigate the adhesion of a variety of microorganisms, including yeasts, to human BECs. The aim of this part of the present study was to investigate the adhesion of a wide range of well characterised strains and isolates of *C. dubliniensis* and *C. albicans* to human BECs under various growth conditions, and in particular to investigate whether the reduced virulence of *C. dubliniensis* relative to *C. albicans* may be associated with a reduced ability to adhere to human cells compared to *C. albicans*.

3.2 MATERIALS AND METHODS

The assay used to monitor adhesion of *Candida* cells to human BECs was based on a protocol described by Murphy & Kavanagh (2001). BECs were collected from healthy adult human volunteers using sterile swabs (Venturi Transystem, Brescia, Italy) by gently rubbing the inside of the buccal cavity with the swab. BECs from multiple volunteers were pooled in 10 ml sterile PBS (Oxoid), centrifuged in an Eppendorf 5804 centrifuge, which was fitted with rotor A-4-44 (Eppendorf), at $760 \times g$ for 5 min at room temperature and were washed twice with 10 ml sterile PBS. This cell suspension was adjusted to 2×10^5 cells ml^{-1} using a Neubauer improved bright line haemocytometer (Hausser Scientific, Horsham, PA, USA) and a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) which was fitted with a super high pressure mercury lamp (Nikon).

Yeast cultures (50 ml) were grown in 250 ml conical flasks (Duran Group GmbH, Mainz, Germany) in an orbital incubator (Gallenkamp) overnight (~16 h) under various temperature and media conditions at 200 rpm. These yeast cultures were harvested by centrifugation at $2,100 \times g$ for 5 min at room temperature, washed twice with 10 ml sterile PBS and re-suspended in sterile PBS at a final density of 1×10^7 cells ml^{-1} .

Yeast cell suspension (1 ml) and BEC suspension (1 ml), which were prepared freshly each day, were pooled, giving a ratio of 50:1 yeast cells: human BECs, and were incubated for 2 h at the same temperature as the yeast cells were grown at, in an orbital incubator at 200 rpm. Following incubation, human BECs with adherent yeast cells

were collected by filtering the sample through a hydrophilic, polycarbonate membrane containing 12 µm pores (Millipore, Cork, Ireland) and washed gently, twice with 10 ml sterile PBS in order to remove any non-adherent yeast cells. The polycarbonate membrane was removed from a Nalgene® polysulfone filter holder with receiver (upper chamber capacity 250 ml, receiver capacity 250 ml; Nalgene® Labware, part of Thermo Fisher Scientific Inc.) and human BECs were transferred to a glass microscope slide (25 × 75 × 1 mm) by placing the polycarbonate membrane face down on the glass microscope slide. Samples were allowed to air dry before being stained for 30 s with crystal violet solution and rinsed with decolouriser (Sigma Diagnostics). The number of yeast cells adhering to each of 100 single human BECs on at least three occasions for each candidal growth condition was determined microscopically.

Images of human BECs with adherent *Candida* were observed using a Nikon Eclipse E600 microscope fitted with a super high pressure mercury lamp and were recorded using a Nikon Eclipse E400 microscope and a Nikon DXM1200 digital camera. Images were edited using ACT-1 software (Nikon). Data from the human BEC experiments were correlated, analysed and graphically depicted using GraphPad Prism® Software Version 4.00 (GraphPad Software Inc., La Jolla, CA, USA) for Windows. Data was analysed using the mean, standard error of the mean (SEM), one-way analysis of variance (ANOVA) with Tukey's multiple comparison post test and unpaired 2-tailed t-test. Where samples were found to have unequal variances, Welch's unpaired 2-tailed t-test was used.

3.3 RESULTS

3.3.1 Comparative adhesion of *C. dubliniensis* and *C. albicans* to human BECs

The main purpose of this part of the present study was to investigate the hypothesis that *C. dubliniensis* has a lower virulence than *C. albicans* due to a reduced ability to adhere to human BECs. To investigate this, the adherence of 27 isolates of *C. dubliniensis* (n = 21) and *C. albicans* (n = 6) to human BECs was examined. Representative strains and isolates from each of the four known genotypes of *C. dubliniensis* (Table 3.1) and reference strains and clinical isolates of *C. albicans* (Table 3.2) were included in this study.

Table 3.1. *C. dubliniensis* strains and isolates used in the BEC adhesion assay

| Strain/isolate | Genotype ^a | Country of Origin | Source/Reference |
|--------------------|-----------------------|-------------------|--------------------------------|
| CD36* ¹ | 1 | Ireland | Sullivan <i>et al.</i> , 1995 |
| CD33 | 1 | Ireland | Sullivan <i>et al.</i> , 1995 |
| Co4 | 1 | Switzerland | Boerlin <i>et al.</i> , 1995 |
| Cm1 | 1 | Australia | Sullivan <i>et al.</i> , 1995 |
| SA105 | 1 | Saudi Arabia | Al Mosaid <i>et al.</i> , 2005 |
| CD539 | 2 | UK | Pinjon <i>et al.</i> , 1998 |
| CD75004 | 2 | UK | Pinjon <i>et al.</i> , 1998 |
| Can6 ² | 2 | Canada | Pinjon <i>et al.</i> , 1998 |
| Can9 | 2 | Canada | Pinjon <i>et al.</i> , 1998 |
| CD506 | 2 | Ireland | Gee <i>et al.</i> , 2002 |
| P6785 | 3 | Israel | Polacheck <i>et al.</i> , 2000 |
| CD519 ³ | 3 | Ireland | Gee <i>et al.</i> , 2002 |
| DB305 | 3 | New Zealand | Al Mosaid <i>et al.</i> , 2003 |
| P7276 | 3 | Israel | Polacheck <i>et al.</i> , 2000 |
| CD514 | 3 | Ireland | Gee <i>et al.</i> , 2002 |
| Eg200 | 4 | Egypt | Al Mosaid <i>et al.</i> , 2005 |
| P7718 ⁴ | 4 | Israel | Gee <i>et al.</i> , 2002 |
| Eg202 | 4 | Egypt | Al Mosaid <i>et al.</i> , 2005 |
| Eg201 | 4 | Egypt | Al Mosaid <i>et al.</i> , 2005 |
| Eg207 | 4v ^b | Egypt | Al Mosaid <i>et al.</i> , 2005 |
| Is35 | 4v | Israel | Al Mosaid <i>et al.</i> , 2005 |

^aThe genotype of each isolate was assigned based on differences observed in the nucleotide sequence of the ITS region of the rRNA gene cluster (Gee *et al.*, 2002; Al Mosaid *et al.*, 2005)

^bThe ITS sequence of the 4v isolates was found to differ from the genotype 4 consensus sequence by 1 or 2 bases in the ITS region compared to genotype 4 (Al Mosaid *et al.*, 2005)

*Type strain (i.e. the nomenclatural type of a species) for *C. dubliniensis*

¹*C. dubliniensis* genotype 1 reference strain for *C. dubliniensis* genotype PCR

²*C. dubliniensis* genotype 2 reference strain for *C. dubliniensis* genotype PCR

³*C. dubliniensis* genotype 3 reference strain for *C. dubliniensis* genotype PCR

⁴*C. dubliniensis* genotype 4 reference strain for *C. dubliniensis* genotype PCR



Table 3.2. *C. albicans* strains and isolates used in the BEC adhesion assay

| Strain/isolate | Source/Reference |
|-----------------------|----------------------------------|
| SC5314* | Gillum <i>et al.</i> , 1984 |
| 132A | Gallagher <i>et al.</i> , 1992 |
| NCPF3153 ^a | UK reference strain ^a |
| m182CA | Pinjon, 2003 |
| CA800 ⁺ | This study |
| CA801 ⁺ | This study |

^aFrom the National Collection of Pathogenic Fungi (NCPF), Kingsdown, Bristol, UK

*Type strain (i.e. the nomenclatural type of a species) for *C. albicans*

⁺Oral isolates from the laboratory collection of the Microbiology Research Unit, Dublin Dental School and Hospital



In order to determine if candidal growth conditions have an effect on the adhesion of each species, experiments were performed when *Candida* cells were grown overnight (~16 h) at 30°C and 37°C in media containing either glucose (YEPD) or galactose (YEPGal). YEPD is the usual growth media for *Candida*. Medium containing galactose had been used in previous studies and as such YEPGal was used here as a growth media to test if there was a difference in the adhesion of *Candida* to human BECs when *Candida* were grown in a medium containing another sugar source. The use of glucose and galactose in the growth media was used to reflect the experiments previously described by McCourtie & Douglas (1981 & 1984), Enache *et al.* (1996) and Gilfillan *et al.* (1998). To ensure reproducibility, each adhesion assay was performed on a minimum of three separate occasions.

3.3.2 Adhesion of *Candida* cells grown overnight (~16 h) at 30°C in YEPD to human BECs

In this experiment, the conditions used corresponded with the published adhesion protocol of Murphy & Kavanagh (2001). Murphy & Kavanagh (2001) investigated the adherence of the yeast *Sa. cerevisiae* to human BECs when *Sa. cerevisiae* was grown at 30°C overnight in YEPD. Here, 27 *Candida* isolates (*C. dubliniensis* (n = 21) and *C. albicans* (n = 6)) were tested for their ability to adhere to human BECs.

The results of this adhesion assay are shown as the mean number of adherent *Candida* ± SEM to human BECs. The adhesion of each isolate tested to 100 human BECs on a minimum of three separate occasions was used to obtain the mean number of adherent *Candida*. A wide variation in adherence of *Candida* to human BECs was observed (Fig. 3.1). The data indicated that *C. dubliniensis* genotype 1 isolates adhered to human BECs in greater numbers than the other isolates of *Candida* (Fig. 3.2). For *C. dubliniensis*, the results of the adhesion of each isolate within a particular genotype to human BECs were averaged to obtain the mean adhesion result to human BECs for each genotype. Similarly, the results of the adhesion of each isolate to human BECs within *C. albicans* were averaged to obtain the mean adhesion result to human BECs for *C. albicans* (Fig. 3.3).

The results in Fig. 3.3 showed that *C. dubliniensis* genotype 1 adhered in significantly greater numbers to human BECs than the other genotypes of

C. dubliniensis, the variants of *C. dubliniensis* genotype 4 and *C. albicans*. One-way ANOVA with Tukey's multiple comparison post test was performed and this showed that *C. dubliniensis* genotype 1 isolates adhered to human BECs in significantly greater numbers than the other genotypes of *C. dubliniensis*, the variants of *C. dubliniensis* genotype 4 and *C. albicans* ($P < 0.001$). There was no significant difference in adhesion to human BECs between genotypes 2, 3, 4, 4v and *C. albicans* ($P > 0.05$). *Candida dubliniensis* genotypes 1 ($P < 0.001$), 2 ($P < 0.01$) and *C. albicans* ($P < 0.001$) adhered to human BECs in significantly greater numbers than the numbers of yeast which were found to be naturally adherent to the control cells.

3.3.3 Adhesion of *Candida* cells grown overnight (~16 h) at 37°C in YEPD to human BECs

As 37°C is body temperature, and *Candida* is a human pathogen, the previous experiment was repeated when the yeast cells were grown in YEPD, but this time at 37°C. This allowed the effect of temperature on the adherence of *Candida* to human BECs to be investigated. The adhesion assays were carried out at 37°C following culture of the yeasts overnight (~16 h) in YEPD at 37°C.

The same 27 isolates were used in this as the previous experiment and are described in Tables 3.1 and 3.2. Yet again a wide variation in the ability of the isolates to adhere to human BECs was observed and the data indicated that the isolates of *C. dubliniensis* genotype 1 and *C. albicans* adhered to human BECs in similar numbers. The *C. dubliniensis* genotype 1 isolates tested had a smaller variation in their adherence to human BECs than *C. dubliniensis* genotype 2 (Fig. 3.4).

The mean adhesion result \pm SEM for each genotype of *C. dubliniensis* and of *C. albicans* are detailed in Fig. 3.5. A one-way ANOVA with Tukey's multiple comparison post test was performed. This showed that there was no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* to human BECs ($P > 0.05$) and that *C. dubliniensis* genotype 1 and *C. albicans* adhere to human BECs in significantly greater numbers than *C. dubliniensis* genotypes 2 – 4, 4v and the control ($P < 0.001$).

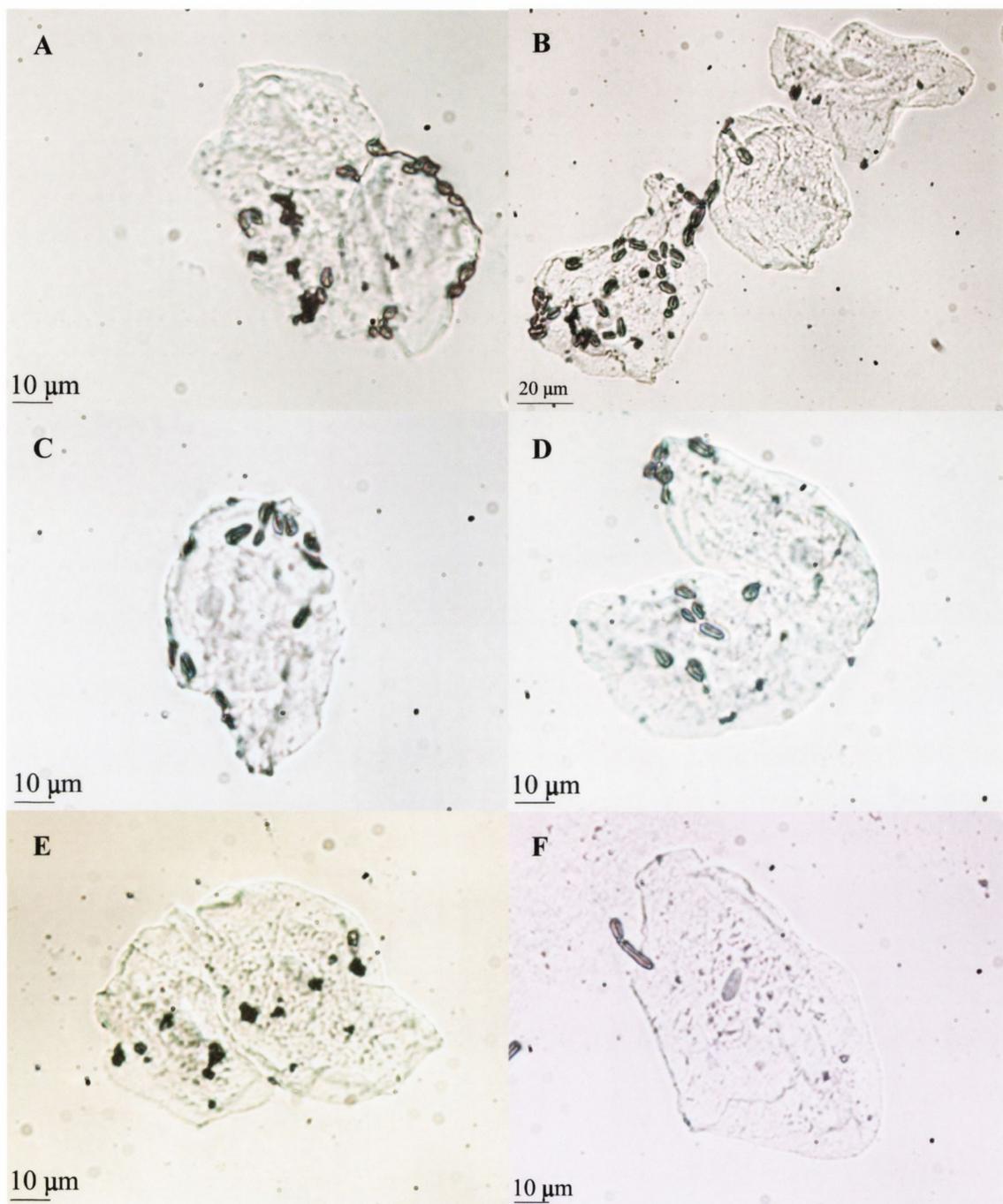


Figure 3.1. Light micrographs showing the relative adhesion of *C. dubliniensis* and *C. albicans* to human BECs when *Candida* cells were grown at 30°C overnight (~16 h) in YEPD. (A) *C. albicans* SC5314; (B) *C. dubliniensis* CD36 (genotype 1); (C) *C. dubliniensis* CD506 (genotype 2); (D) *C. dubliniensis* CD514 (genotype 3); (E) *C. dubliniensis* Eg200 (genotype 4) and (F) *C. dubliniensis* Is35 (genotype 4v).

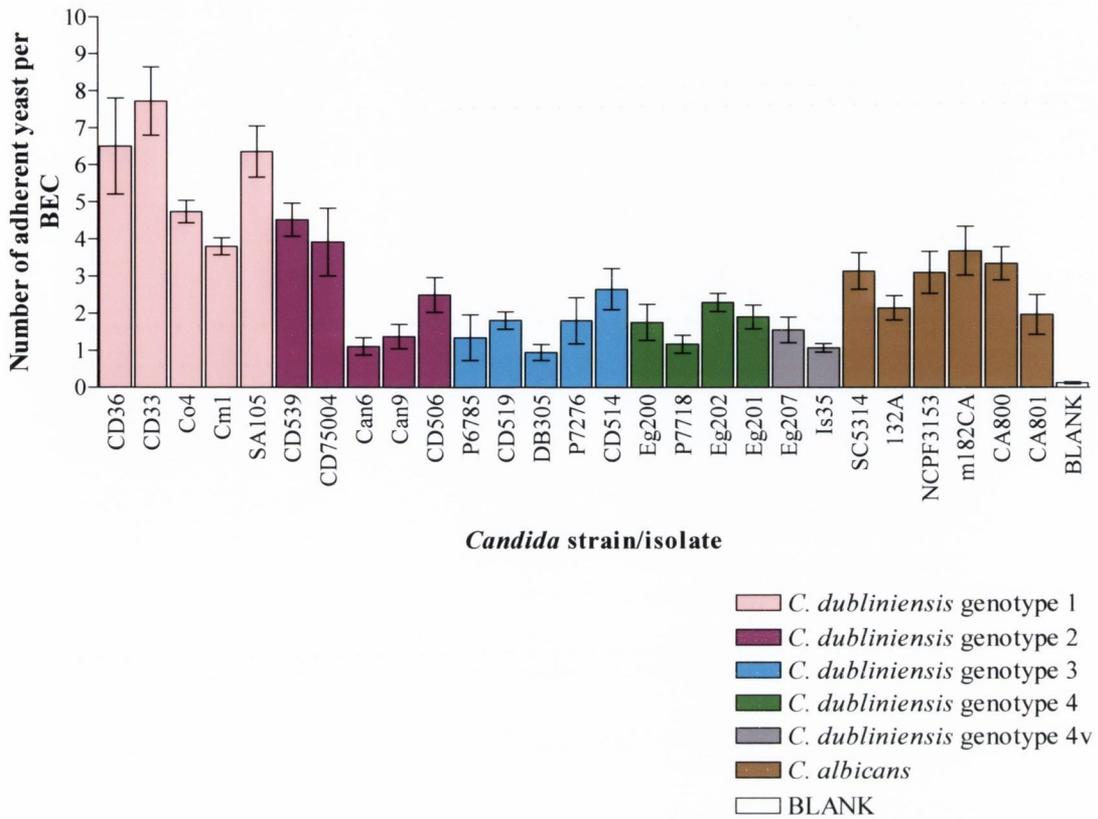


Figure 3.2. Number of adherent *C. dubliniensis* and *C. albicans* cells per human BEC at 30°C when the *Candida* strains and isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C. The results show the mean adhesion per BEC \pm SEM for each isolate of *C. dubliniensis* and *C. albicans* tested to 100 BECs on at least three separate occasions. The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.

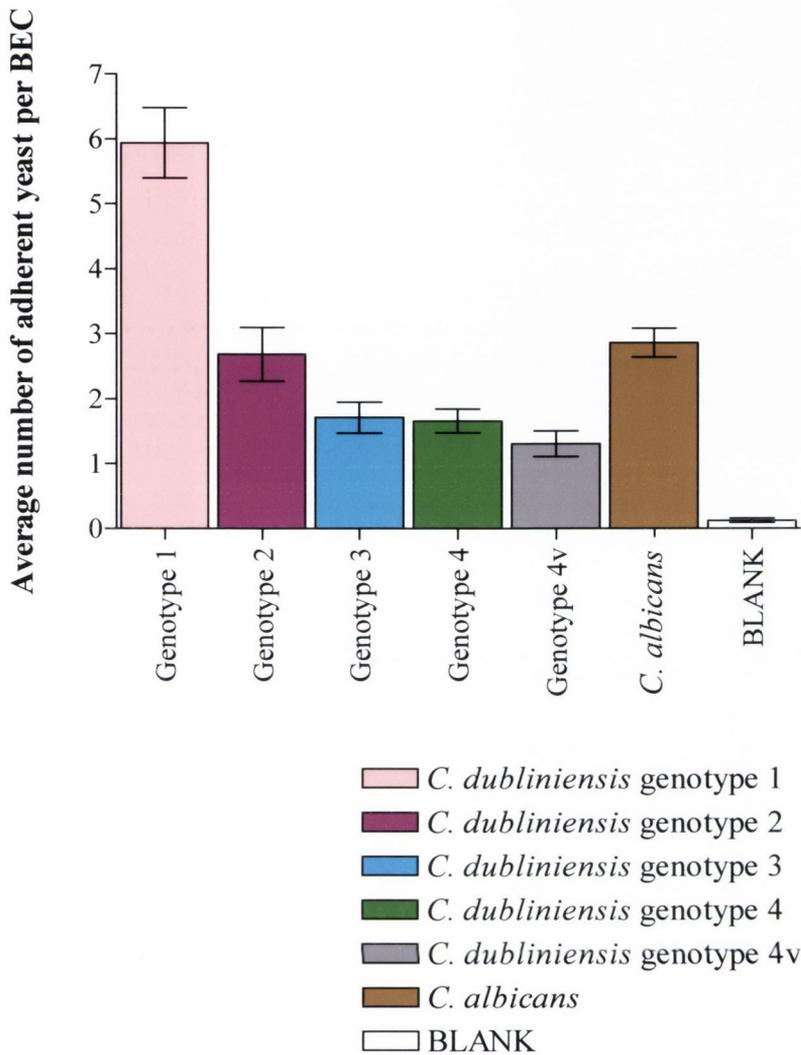


Figure 3.3. Mean number of adherent *C. dubliniensis* and *C. albicans* per human BEC at 30°C when *Candida* strains and isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to 100 BECs on at least three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 6) to give the mean number of adherent *Candida* ± SEM per human BEC across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 isolates adhered to human BECs in significantly greater numbers than *C. dubliniensis* genotypes 2, 3, 4, 4v and *C. albicans* ($P < 0.001$). The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.



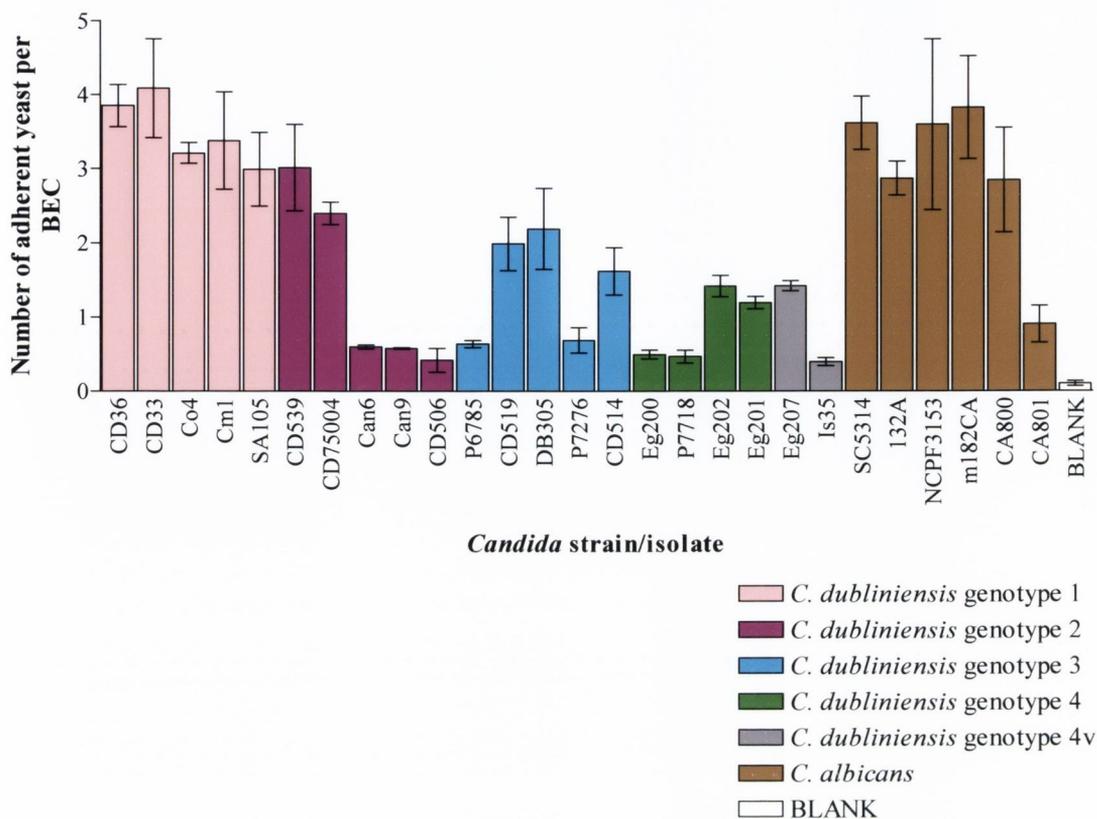


Figure 3.4. Number of adherent *C. dubliniensis* and *C. albicans* cells per human BEC at 37°C when the *Candida* strains and isolates used were pre-cultured in YEPD overnight (~16 h) at 37°C. The results show the mean adhesion per BEC ± SEM for each isolate of *C. dubliniensis* and *C. albicans* tested to 100 BECs on at least three separate occasions. The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.



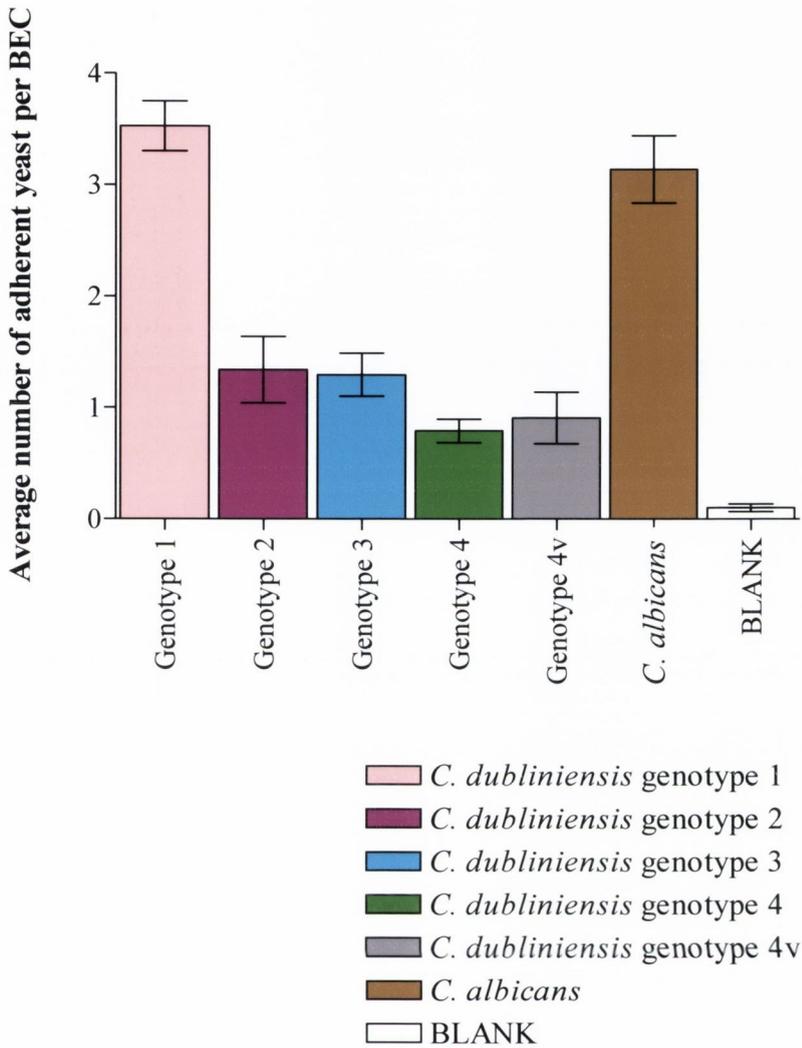


Figure 3.5. Mean number of adherent *C. dubliniensis* and *C. albicans* per human BEC at 37°C when *Candida* strains and isolates used were pre-cultured in YEPD overnight (~16 h) at 37°C. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to 100 BECs on at least three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 6) to give the mean number of adherent *Candida* ± SEM per human BEC across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 and *C. albicans* strains and isolates adhere to human BECs in significantly greater numbers than *C. dubliniensis* genotypes 2, 3, 4 and 4v ($P < 0.001$). The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.



3.3.4 Adhesion of *Candida* cells grown overnight (~16 h) at 37°C in YEPGal to human BECs

To investigate the effect of sugar source on the adhesion of *Candida* to human BECs, the yeast were grown in a medium containing a different sugar source from the one previously investigated. In this case the same 27 isolates of *Candida* as detailed in Tables 3.1 and 3.2 were grown in YEPGal at 37°C overnight (~16 h). As seen in the previous two experiments a variation in the ability of the isolates to adhere to BECs was observed (Fig. 3.6).

The mean adhesion result \pm SEM for each genotype of *C. dubliniensis* and *C. albicans* are detailed in Fig. 3.7. A one-way ANOVA with Tukey's multiple comparison post test was performed and this showed that *C. dubliniensis* genotype 1 adhered to human BECs in significantly greater numbers than the other genotypes of *C. dubliniensis* ($P < 0.001$), the variants of *C. dubliniensis* genotype 4 ($P < 0.01$) and *C. albicans* ($P < 0.001$). There was no significant difference between the adhesion of *C. dubliniensis* genotypes 2, 3, 4, the variants of genotype 4 and *C. albicans* ($P > 0.05$) to human BECs. With the exception of *C. dubliniensis* genotype 4 ($P > 0.05$) all genotypes of *C. dubliniensis* (genotype 1, $P < 0.001$, genotype 2, $P < 0.01$, genotype 3, $P < 0.05$), the variants of genotype 4 ($P < 0.05$) and *C. albicans* ($P < 0.001$) adhere to human BECs in significantly greater numbers than are found naturally adhering to the control.

3.3.5 Comparative adhesion of *Candida* to human BECs under varying temperature conditions

To compare the effect of growth temperature on the adhesion of *C. dubliniensis* and *C. albicans* to human BECs, mean adhesion \pm SEM of the 4 genotypes of *C. dubliniensis*, the variants of *C. dubliniensis* genotype 4 and *C. albicans* were compared when *Candida* were grown in YEPD overnight (~16 h) at either 30°C or 37°C. Unpaired, 2-tailed, t-tests were performed. Where samples were found to have unequal variances unpaired, 2-tailed, t-tests with Welch's correction were performed. The results showed there was a significant difference in the adhesion of *C. dubliniensis* genotypes 1 ($P < 0.05$; unpaired, 2-tailed, t-test with Welch's correction; greater adhesion to human BECs when grown at 30°C compared to 37°C), 2 ($P < 0.05$;

unpaired, 2-tailed, t-test; greater adhesion to human BECs when grown at 30°C compared to 37°C) and 4 ($P < 0.05$; unpaired, 2-tailed, t-test with Welch's correction; greater adhesion to human BECs when grown at 30°C compared to 37°C) to human BECs when *Candida* was grown in YEPD overnight (~16 h) at 30°C compared to 37°C (Fig. 3.8).

3.3.6 Comparative adhesion of *Candida* to human BECs under varying media conditions

To compare the effect of growth media on the adhesion of *C. dubliniensis* and *C. albicans* to human BECs, mean adhesion \pm SEM of the 4 genotypes of *C. dubliniensis*, the variants of *C. dubliniensis* genotype 4 and *C. albicans* were compared when *Candida* was grown in either YEPD or YEPGal overnight (~16 h) at 37°C. Unpaired, 2-tailed, t-tests were performed. Where samples were found to have unequal variances unpaired, 2-tailed, t-tests with Welch's correction were performed. The results showed that there was a significant difference in the adhesion of *C. dubliniensis* genotypes 4 ($P < 0.05$; unpaired, 2-tailed, t-test; greater adhesion to human BECs when grown in YEPGal compared to YEPD), 4v ($P < 0.05$; unpaired, 2-tailed, t-test; greater adhesion to human BECs when grown in YEPGal compared to YEPD) and *C. albicans* ($P < 0.05$; unpaired, 2-tailed, t-test; greater adhesion to human BECs when grown in YEPD compared to YEPGal) to human BECs when grown in different media at 37°C overnight (~16 h; Fig. 3.9).

3.4 DISCUSSION

The purpose of this part of the present study was to investigate whether a reduced ability of *C. dubliniensis* relative to *C. albicans* to adhere to human cells may be a contributory factor in the reduced virulence of *C. dubliniensis*. In order to compare adherence between species, the ability of both species to adhere to human BECs under a range of candidal growth conditions was investigated. A commonly used assay for determining the adherence of *Candida* cells is the human BEC adherence assay and thus the ability of representative strains and isolates of *C. dubliniensis* genotypes 1 – 4, the variants of *C. dubliniensis* genotype 4 and *C. albicans* (Tables 3.1 and 3.2) to adhere to

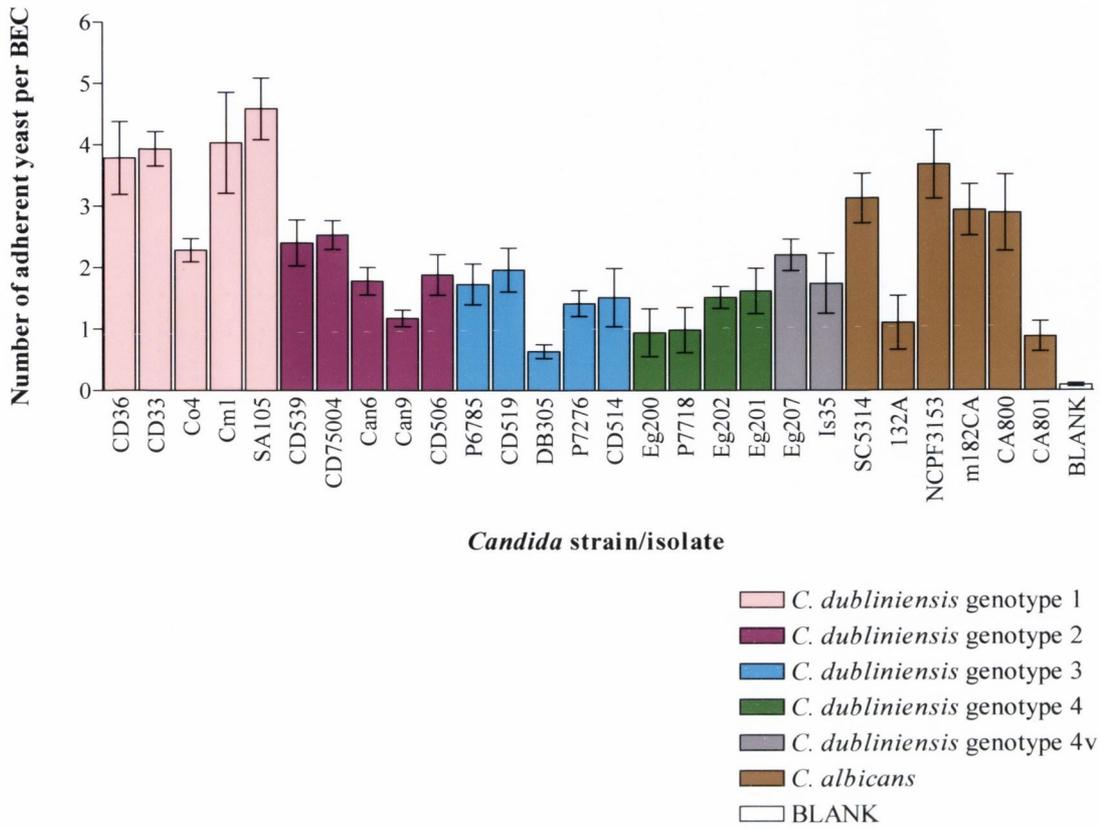


Figure 3.6. Number of adherent *C. dubliniensis* and *C. albicans* cells per human BEC at 37°C when the *Candida* strains and isolates used were pre-cultured in YEPGal overnight (~16 h) at 37°C. The results show the mean adhesion per BEC \pm SEM for each isolate of *C. dubliniensis* and *C. albicans* tested to 100 BECs on at least three separate occasions. The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.



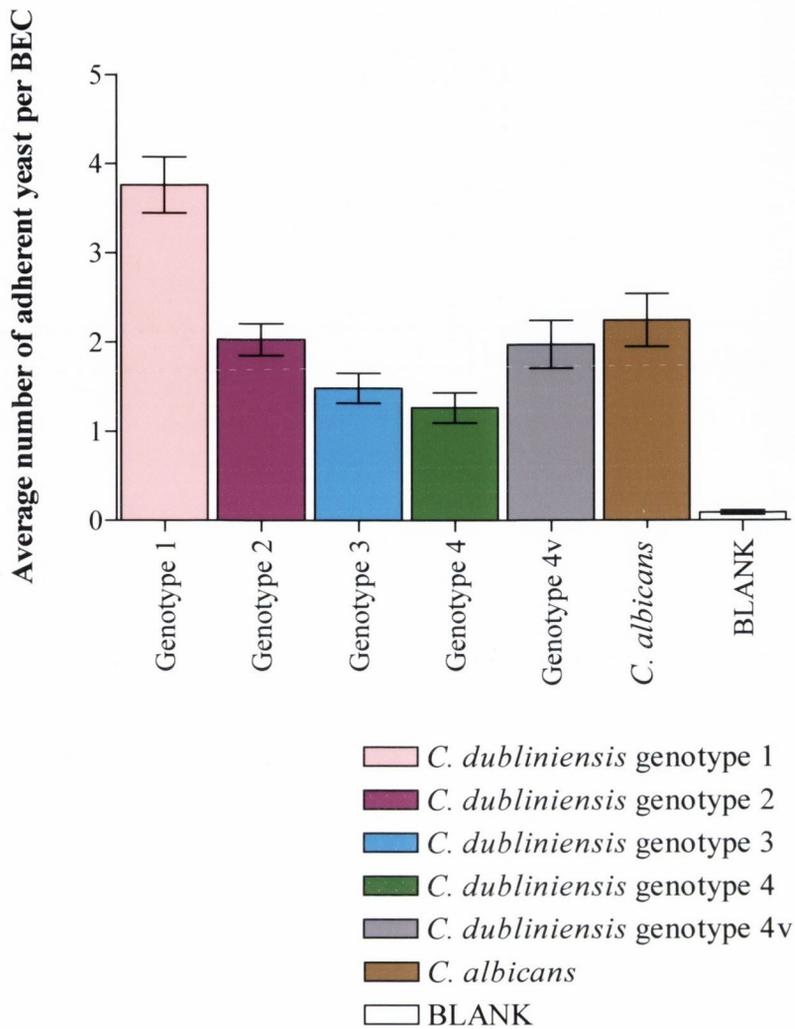


Figure 3.7. Mean number of adherent *C. dubliniensis* and *C. albicans* per human BEC at 37°C when *Candida* strains and isolates used were pre-cultured in YEPGal overnight (~16 h) at 37°C. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to 100 BECs on at least three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 6) to give the mean number of adherent *Candida* ± SEM per human BEC across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 adhered to human BECs in significantly greater numbers than *C. dubliniensis* genotypes 2, 3, 4 and *C. albicans* ($P < 0.001$) and *C. dubliniensis* genotype 4v ($P < 0.01$). The control, which is represented by white, indicates the number of yeast cells found to be naturally adherent to human BECs.



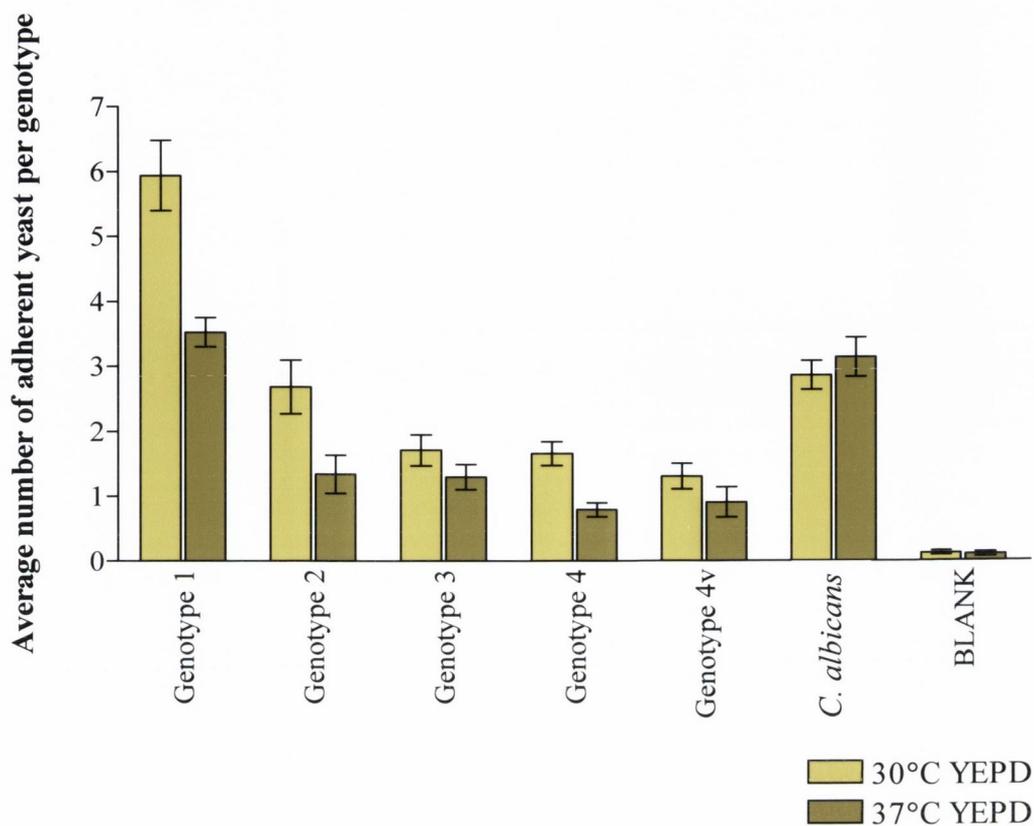


Figure 3.8. Comparative adhesion of *C. dubliniensis* and *C. albicans* to human BECs when *Candida* was pre-cultured in YEPD overnight (~16 h) at either 30°C or 37°C. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to 100 BECs, on at least three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 6) to give the mean number of adherent *Candida* ± SEM per human BEC across each genotype of *C. dubliniensis* and of *C. albicans*. Adhesion of *Candida* to human BECs when *Candida* cells were grown at either 30°C or 37°C in YEPD are compared here. *Candida dubliniensis* genotypes 1, 2 and 4 ($P < 0.05$) adhered to human BECs in significantly greater numbers when *Candida* cells were grown in YEPD overnight (~16 h) at 30°C compared to 37°C.

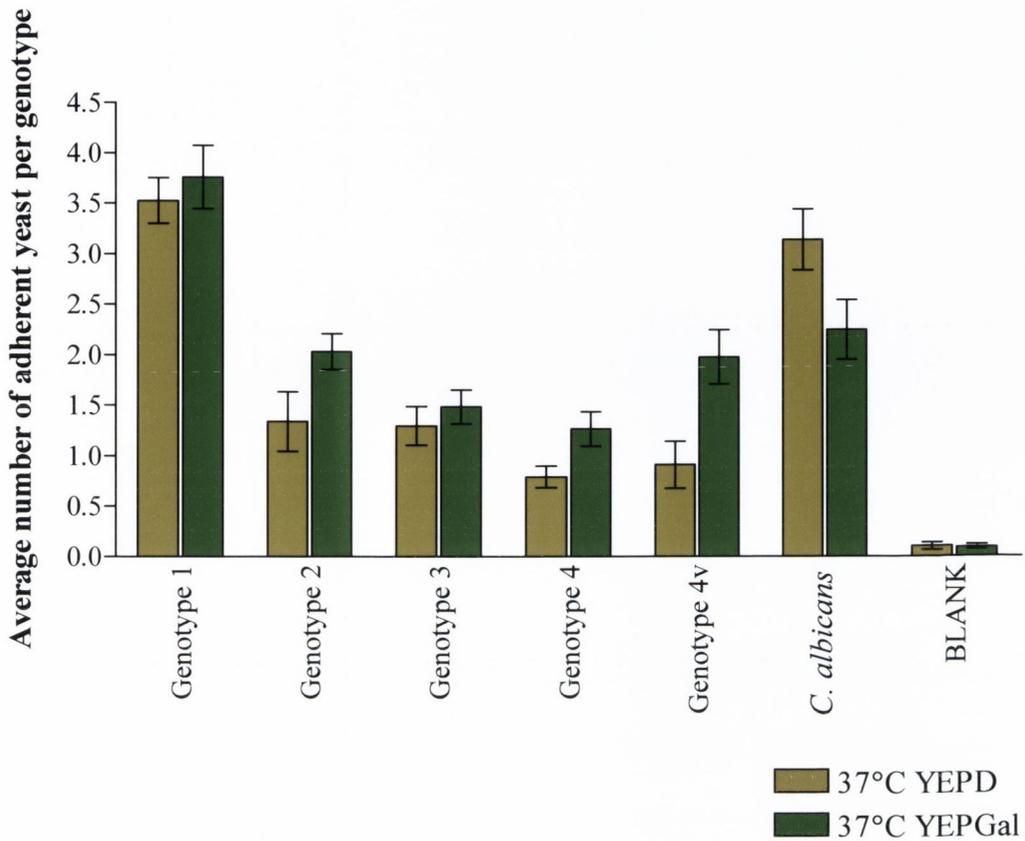


Figure 3.9. Comparative adhesion of *C. dubliniensis* and *C. albicans* to human BECs when *Candida* was pre-cultured in either YEPD or YEPGal overnight (~16 h) at 37°C. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to 100 BECs, on at least three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 6) to give the mean number of adherent *Candida* \pm SEM per human BEC across each genotype of *C. dubliniensis* and of *C. albicans*. Adhesion of *Candida* to human BECs when *Candida* cells were grown at 37°C in either YEPD or YEPGal are compared here. *Candida dubliniensis* genotypes 4 and 4v ($P < 0.05$) adhered to human BECs in significantly greater numbers when grown in YEPGal compared to YEPD at 37°C overnight (~16 h) and *C. albicans* ($P < 0.05$) adhered to human BECs in significantly greater numbers when grown in YEPD compared to YEPGal at 37°C overnight (~16 h).

human BECs were examined. One of the most significant limitations of previous studies that investigated the adherence of *Candida* to human cells concerned the use of a relatively small number of isolates (McCourtie & Douglas, 1984; Enache *et al.*, 1996; Gilfillan *et al.*, 1998). Although Vidotto *et al.* (2003) used 26 isolates of *C. dubliniensis* and 27 isolates of *C. albicans* in their study they did not group the *C. dubliniensis* isolates tested into their respective genotypes as was carried out in this part of the present study. The results here show a significant difference in the adhesion of the genotypes of *C. dubliniensis* to human BECs (Fig. 3.2 to Fig. 3.7) and not separating the isolates into their respective genotypes would skew the results. In these experiments, the *Candida* cells were grown overnight (~16 h) at 30°C and 37°C in YEPD and YEPGal. The results from these experiments confirmed that both *C. dubliniensis* and *C. albicans* adhere to human BECs (Fig. 3.1 to Fig. 3.7). *Candida dubliniensis* genotype 1 adhered to human BECs in greater numbers than genotypes 2, 3, 4 and 4v in all conditions (Fig. 3.3, Fig. 3.5 and Fig. 3.7) and under certain growth conditions *C. dubliniensis* genotype 1 isolates adhered to human BECs in greater numbers than *C. albicans* (Fig. 3.3 and Fig. 3.7; $P < 0.001$). While *C. albicans* is better equipped to cause systemic and disseminated disease (Odds, 1988; Cannon & Chaffin, 1999; Farah *et al.*, 2000), *C. dubliniensis* is mostly isolated from cases of oral candidiasis (Sullivan *et al.*, 1995; Sullivan & Coleman, 1997; Sullivan *et al.*, 1999). The greater adhesion of *C. dubliniensis* genotype 1 to the human cells used in this study is therefore probably due to the fact that the human cells were of oral origin.

It is also possible that an effect of the host cells was being observed. All *C. dubliniensis* genotype 4 and 4v isolates were isolated from an Arab ethnic group (Table 3.1). As the majority of donors of BECs for this study were Irish and/or Caucasian it is possible that the *C. dubliniensis* genotype 4 and 4v isolates do not adhere well to the pooled BECs used in this part of the present study as the isolates comprising these genotypes have adapted to adhere to host cells of Arab ethnic origin. All isolates used were clinical isolates so have the ability to adhere to human cells however *C. dubliniensis* genotypes 2, 3, 4 and 4v do not adhere as well to the pooled human BECs used in this part of the present study as *C. dubliniensis* genotype 1. The assumption that certain genotypes of *C. dubliniensis* have adapted to colonise particular ethnic groups and hence are primarily isolated from infection in these ethnic groups does not hold true for *C. dubliniensis* genotype 2 isolates. The majority of the *C. dubliniensis* genotype 2 isolates were from infection in what were assumed to be

Caucasian hosts (Table 3.1), yet have poor adhesion to a pooled sample of BECs which is primarily from Caucasian donors.

3.4.1 Effect of candidal growth temperature on the adhesion of *C. dubliniensis* and *C. albicans* to human BECs

To determine if candidal growth temperature had an effect on the ability of *Candida* to adhere to human BECs the results of the adhesion assays where *Candida* were pre-cultured in the same media at different temperatures were compared. *Candida dubliniensis* genotypes 1, 2 and 4 ($P < 0.05$; Fig. 3.8) adhered to human BECs in significantly greater numbers when *Candida* were grown in YEPD overnight (~16 h) at 30°C compared to 37°C. The lower growth temperature seems to increase the ability of these genotypes of *C. dubliniensis* to adhere to human BECs. In contrast, the same number of *C. albicans* cells adhered to human BECs at both growth temperatures. When grown at 30°C, the adhesion of *C. dubliniensis* genotype 1 to human BECs is significantly greater than when grown at 37°C and is also significantly greater than the adhesion of *C. albicans* to human BECs when *C. albicans* was grown at either 30°C or 37°C. As such, it appears that a lower growth temperature increases the ability of most of the genotypes of *C. dubliniensis* to adhere to human BECs but has no effect on the adhesion ability of *C. albicans* when cultured in the same media (Fig. 3.8). The increased ability of *C. dubliniensis* to adhere to human BECs when grown in YEPD at 30°C compared to 37°C is probably due to the fact that *C. dubliniensis* has adapted to colonise the oral cavity. The temperature of the oral cavity is lower than body temperature, with recorded temperatures in the upper trachea of between 29°C and 32°C (McFadden *et al.*, 1985).

3.4.2 Effect of candidal growth media on the adhesion of *C. dubliniensis* and *C. albicans* to human BECs

To determine if the media in which *Candida* cells were grown had an effect on the ability of *Candida* to adhere to human BECs, the results of adhesion assays where *Candida* were pre-cultured in different media at the same temperature were compared. *Candida dubliniensis* genotypes 4 and 4v and *C. albicans* showed a significant difference in their adhesion to human BECs when grown at 37°C in YEPD as compared

with YEPGal ($P < 0.05$). In the case of *C. dubliniensis*, the isolates grown in YEPD adhered less well to human BECs than those grown in YEPGal however for *C. albicans* the opposite effect was seen with isolates grown in YEPGal adhering less well to human BECs than those grown in YEPD ($P < 0.05$; Fig. 3.9). These results contradict published data. In previous studies (McCourtie & Douglas, 1984; Gilfillan *et al.*, 1998), the concentration of glucose in the media was lower than the concentration of galactose in the media. In this study the concentration of sugar in both media used was of equal concentration and as such this and previous studies (McCourtie & Douglas, 1984; Gilfillan *et al.*, 1998) are not directly comparable. McCourtie & Douglas (1981) found that adherence increased linearly with increasing concentration of sugar used, so it would be expected that had the same concentration of sugar been used in all media in previous studies (McCourtie & Douglas, 1984), such a dramatic increase in adhesion (5- to 11-times) of *C. albicans* to human BECs grown in media containing galactose compared to media containing glucose would not have been seen. Enache *et al.* (1996) used the same concentration of sugar in both media types however results from this part of the present study contradict the published results of Enache *et al.* (1996).

The data presented here go some way to explaining discrepancies in data obtained in previous studies. This study used an extensive range of *C. dubliniensis* isolates and the results show that there is a large degree of intra-species variation in the ability of both *C. dubliniensis* and *C. albicans* to adhere to human BECs. This study delineated *C. dubliniensis* isolates into their respective genotypes and examined the ability of each genotype of *C. dubliniensis* to adhere to human BECs. Unlike previous studies (McCourtie & Douglas, 1984; Gilfillan *et al.*, 1998) the method used to examine adhesion allows direct comparison of samples as there is only one differential variable per study. *Candida dubliniensis* genotype 1 isolates adhered to human BECs in greater numbers than genotypes 2, 3, 4 and 4v under all growth conditions, and under two of the three conditions tested, *C. dubliniensis* genotype 1 isolates adhered to human BECs in greater numbers than *C. albicans*. These results indicate that despite the lower virulence of *C. dubliniensis* in relation to *C. albicans*, *C. dubliniensis* genotype 1 isolates have a greater overall ability to adhere to human BECs and in conclusion the ability of a microorganism to adhere to human cells is not the sole method involved in pathogenicity.

Although the human BEC assay is a commonly used assay to examine adhesion of pathogens to human oral cells, it is time consuming, susceptible to operator error and

human BECs may affect the levels of adhesion due to intra- and inter-individual variation in the BEC population such as race, sex, hormone levels, health status such as the presence of xerostomia, anaemia or diabetes mellitus, medications, diet, donor *Candida* carrier status, the possibility of a high percentage of non-viable cells and varying degrees of enzymatic degradation. Human BECs are also likely to be contaminated with bacteria or proteins derived from saliva. Bacteria which are naturally adherent to human BECs and mucins which are present in human saliva may hinder the adhesion of *Candida* to human BECs thus altering the result. Depending on the form of *Candida* cells used in the BEC assay, for example, hyphal cells it may be difficult to enumerate adherent cells accurately and it is difficult to meaningfully compare the vast array of published data due to the wide range of cell types and culture medium and conditions used (Henriques *et al.*, 2006). Despite the fact that the BEC adhesion assay is a commonly used adhesion assay, there are many inconsistencies, with various studies suggesting that one or other species is more adherent (McCullough *et al.* (1995) found *C. dubliniensis* to be more adherent to human BECs whereas Vidotto *et al.* (2003) found *C. albicans* to be more adherent to human BECs), while other studies show mixed results depending on the growth medium used (results from a study by Gilfillan *et al.* (1998) showed that *C. dubliniensis* adhere to human BECs in greater numbers when pre-cultured in medium containing glucose whereas there was no difference in the adhesion of both *C. dubliniensis* and *C. albicans* to human BECs when *Candida* cells were pre-cultured in medium containing galactose). Although the present study has overcome many of the limitations of previous studies, such as using a wide range of *C. dubliniensis* isolates and delineating these *C. dubliniensis* isolates into their respective genotypes and examining one experimental variable per experiment, we aim to develop a new assay to examine adhesion. This new assay will use a more uniform population of human cells, aims to be less time consuming allowing for a higher through-put of samples and aims to reduce some of the source of operator bias or error.

Chapter 4

**Development and optimisation of an adhesion
assay to examine the relative adhesion of
C. dubliniensis and *C. albicans* to a
monolayer of the TR146 cell line**

4.1 INTRODUCTION

The human BEC assay is one of the most commonly used assays for assessing adhesion of pathogens to human cells, however it has some shortcomings in that it is time consuming, may be susceptible to operator error and levels of adherent *Candida* may vary due to the presence of a heterogeneous population of human BECs. Despite these shortcomings it has been widely used to assess microbial adhesion to human cells. The ease and speed with which human cells can be obtained and the ease with which variables, such as candidal growth temperature, can be altered makes it an easy adhesion assay to use.

Various cell types of uniform human tissue have been used to examine both adhesion and invasion of pathogens. Reconstituted Human Epithelium (RHE) has previously been used to examine adhesion (Schaller *et al.*, 1998; Li *et al.*, 2002; Zhao *et al.*, 2004, 2005 & 2007b) and invasion (Williams *et al.*, 2001; Zakikhany *et al.*, 2007) of *Candida*, however, RHE is expensive, requires long growth periods and specialist tools to manipulate. In this study, instead of using RHE to examine the adhesion of *Candida* to a uniform model of human tissue, a monolayer of TR146 cells was used. The TR146 cell line can be grown as a monolayer or as RHE. The use of a uniform model of human epithelium has some advantages over human BECs in assessing adhesion including the removal of intra- and/or inter-individual effects.

Previous studies have used monolayers of various cell lines to assess candidal adhesion. Enache *et al.* (1996) examined the adhesion of *C. albicans* to human BECs and to monolayers of a murine alveolar macrophage cell line and HET1-A cells. Although the cell line propagation technique for the adhesion assay differed between the two cell lines (the adhesion assay using HET1-A cells was carried out when the HET1-A cells were grown for 48 h in 6 well tissue culture dishes and the adhesion assay using the murine alveolar macrophage cell line was carried out when the macrophage cells were grown for 48 h on sterile glass coverslips) and the adhesion assays differed between the two cell lines and the human BECs, the results from the three adhesion assays concurred, showing that *C. albicans* grown in media containing galactose adhere to all cell types in greater amounts than *C. albicans* grown in media containing glucose (Enache *et al.*, 1996). Henriques *et al.* (2007) examined the differential ability of two isolates of *C. dubliniensis* and two isolates of *C. albicans* to adhere to the HeLa cell

line, which was derived from a cancer of the cervix. However as a small number of isolates were used in this study, it was difficult to interpret the results meaningfully. Sohn *et al.* (2006) examined the adhesion and subsequent transcriptional response of *C. albicans* SC5314 to two human cell lines; Caco-2 from the intestinal tract and A-431, a vulvovaginal cell line. After 4 h, the number of *C. albicans* SC5314 cells that had adhered to both cell lines was similar however the yeast appeared to adhere to the A-431 cell line at a slower rate. Other cell lines used for assessment of candidal adhesion include the human epithelial cell lines Hep-2 and A549 (Holmes *et al.*, 2002), the human epithelial cell line HSC-3 (Dorocka-Bobkowska *et al.*, 2003), the oral epithelial cell lines SCC4, SCC15 and OKF6/TERT-2 (Dongari-Bagtzoglou & Kashleva, 2003), human epidermal keratinocytes (Ollert *et al.*, 1993), epithelium from gingival tissues (Nikawa *et al.*, 2003) and gingival keratinocytes (Dongari-Bagtzoglou & Kashleva, 2003).

The TR146 cell line which was grown as a monolayer and used to assess candidal adhesion in this part of the present study is a human tumour-derived cell line which originated from a biopsy of a squamous cell carcinoma of buccal mucosa that had infiltrated a lymph node. TR146 cells have the ability to form stratified cultures and possess an epithelial-like morphology (Rupniak *et al.*, 1985). A study by Jacobsen *et al.* (1995) found that filter-grown TR146 had the potential to model human buccal epithelial permeation of drugs. The TR146 cell line possesses structural features similar to human buccal epithelium, shows better reproducibility of data with regard to bile salt enhancement studies and permits higher throughput of samples than a porcine buccal cell line, which had been routinely used as an *in vitro* model of human buccal epithelium (Nielsen & Rassing, 1999). Routine culture of the TR146 cell line forms undifferentiated, non-keratinised stratified epithelium sharing many characteristics of normal mucosa (Jacobsen *et al.*, 1995 & 1999) and as such could be a useful model by which to study the adhesion of pathogens.

Two cell stains were used in this part of the present study. FUN-1 cell stain fluoresces in the presence of protein or when complexed with DNA or RNA. Metabolically active cells sequester FUN-1 in their vacuoles giving rise to bright red-orange fluorescent cylindrical intra-vacuolar structures, whereas non-viable cells contain no cylindrical intra-vacuolar structures and stain uniformly with a bright yellow-green fluorescence (Millard *et al.*, 1997). XTT is readily reduced in viable cells

to a highly water soluble orange-coloured product (Scudiero *et al.*, 1988) which can be easily detected spectrophotometrically at 480 nm.

In order to examine the adhesion of *Candida* to a more uniform, and potentially more reproducible, model of human tissue than human BECs, adhesion to a monolayer of the human cell line, TR146 was examined. The aim of this part of the present study is to determine whether this model could be developed as a useful system for investigating adhesion of a large number of *Candida* isolates to human cells in a manner comparable with the BEC adhesion assay.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture propagation

The TR146 cell line was purchased from Cancer Research Technology, London, UK and was routinely cultured in 90 mm treated tissue culture dishes containing 10 ml complete tissue culture medium, which consisted of Dulbecco's Modified Eagle's Medium (supplemented with 4.5 g L⁻¹ glucose, sodium pyruvate, L-glutamine and sodium bicarbonate; Sigma), 10% (v/v) heat inactivated foetal bovine serum (Sigma), 100 I.U. ml⁻¹ penicillin (Sigma) and 100 µg ml⁻¹ streptomycin (Sigma). This cell line was maintained at 5% (v/v) CO₂ in a static incubator (RS Biotech Laboratory Equipment Ltd., Irvine, Ayrshire, Scotland) at 37°C in 98% relative humidity and the cells were fed by replacing 8 ml of complete tissue culture medium with 8 ml of fresh, pre-warmed complete tissue culture medium twice weekly. When confluent, the TR146 cells were passaged using 3 ml 1× Trypsin EDTA (Sigma). Briefly, all complete tissue culture medium was removed and the confluent monolayer was rinsed with 3 ml 1× Trypsin EDTA which was discarded before a further 3 ml 1× Trypsin EDTA was added to the confluent cell line. The TR146 cells were trypsinised for 10 min at 37°C in 5% (v/v) CO₂ in a static incubator, 98% relative humidity before aspiration of 1× Trypsin EDTA 10 times to remove any final adherent TR146 cells from the tissue culture dish. The TR146 cell line was found to be sensitive to trypsin and growth of the cell line was inhibited by trace amounts of it. In order to overcome this, the trypsinised TR146 cells were transferred to a 50 ml screw cap tube and centrifuged at 400 × g for 5 min to remove all 1× Trypsin EDTA. The pellet of TR146 cells was re-suspended in

complete tissue culture medium before re-plating. If the TR146 cells were for propagation of a new tissue culture dish, the pellet was re-suspended in 3 ml complete tissue culture medium and 1 ml of this solution was added to 9 ml of pre-warmed complete tissue culture medium in a new tissue culture dish. If the TR146 cells were to be stored, the pellet was re-suspended in 3 ml complete tissue culture medium containing 10% (v/v) DMSO (Sigma) and 3 screw cap tubes of 2 ml capacity containing 1 ml each high density cell solution were stored under liquid nitrogen. The cell culture propagation method was based upon a method detailed by Nielsen & Rassing (1999) however the TR146 cells used in this study were grown directly upon plastic tissue culture dishes as opposed to upon filters. Once the cell line had been passaged 30 times it was discarded and new cultures were seeded from frozen stock. The cell line was assessed on a regular basis for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Cambrex) according to the manufacturer's instructions.

4.2.2 Effect of Triton X100 on candidal growth

Candida cultures were grown in 50 ml YEPD in 250 ml conical flasks (Duran) at 37°C overnight (~16 h) in an orbital incubator (Gallenkamp) at 200 rpm before being diluted 20-fold into fresh YEPD and cultured for a further 2 h at 37°C in an orbital incubator at 200 rpm. These cultures were washed twice with sterile PBS (Oxoid), re-suspended in RPMI-1640 (Sigma) at 1×10^8 cells ml⁻¹ and serial dilutions of *Candida* were inoculated onto PDA (Oxoid or Lab M) plates. *Candida* cells that were to be treated with Triton X100 (Sigma) were processed as the non-Triton-treated cells before being re-suspended in 0.1% (v/v) Triton X100 in sterile ultra-purified water, aspirated to disrupt the pellet of *Candida* cells and left in 0.1% (v/v) Triton X100-solution for 8 min at room temperature. Serial dilutions of Triton-treated *Candida* which corresponded to the non-Triton-treated *Candida* dilutions were inoculated onto PDA plates. These plates were incubated for 48 h at 37°C and the colonies counted.

4.2.3 Effect of media, atmospheric conditions and Triton X100 on the integrity of the TR146 monolayer

In order to assess the integrity of a fully confluent TR146 monolayer following exposure to sterile PBS, filter-sterile PBS containing 2% (w/v) glucose (Sigma) and RPMI-1640 medium in air at 37°C for 3 h, the cell line was analysed with Trypan blue (Sigma), using the dye exclusion test. The dye exclusion test is based on the principle that live cells possess intact cell membranes and can exclude certain dyes, such as Trypan blue, whereas dead cells can not.

In order to assess the effect of Triton X100 on a fully-intact, confluent TR146 monolayer, the monolayer was exposed to 0.1% (v/v) Triton X100 in sterile ultra-purified water. The cells were incubated at room temperature for 8 min before repeated aspiration of the Triton X100-solution in an attempt to disrupt the cell line. The cell line was analysed using the dye exclusion test as described above.

4.2.4 TR146 adhesion assay

This adhesion assay is based on an assay described by Alberti-Segui *et al.* (2004). For the 96-well tissue culture plate adhesion assay, the cell line was seeded at various cell densities in a final volume of 100 µl complete tissue culture medium in each well of a 96-well tissue culture plate. A cell density of 4×10^5 cells ml⁻¹ in 100 µl final volume complete tissue culture medium was optimal to form a confluent monolayer in each well of a 96-well tissue culture plate when grown overnight (~16 h) at 37°C in 5% (v/v) CO₂ in a static incubator at 98% relative humidity. Individual cultures of *Candida* (Tables 4.1 and 4.2) were grown overnight (~16 h) at 37°C in 250 ml conical flasks containing 50 ml YEPD in an orbital incubator at 200 rpm. *Candida* cultures that were to be stained with FUN-1 cell stain (Molecular Probes Inc.) were diluted 20-fold into YEPD and grown for a further 2 h at 37°C in an orbital incubator at 200 rpm. *Candida* cells were washed twice in 10 ml sterile PBS. If the *Candida* cells were to be stained with FUN-1 cell stain for elucidation of adherent *Candida* numbers, the cultures were re-suspended in either 10 ml sterile PBS or 10 ml filter-sterile PBS containing 2% (w/v) glucose and re-adjusted to a cell density of 1×10^8 cells ml⁻¹. If the *Candida* cells were to be stained with XTT dye (Sigma) for elucidation of adherent *Candida* numbers, the cultures were re-suspended in sterile PBS and re-adjusted to a

cell density of 2×10^7 cells ml^{-1} . Each monolayer of TR146 cells was washed twice, gently with 100 μl sterile PBS and 100 μl *Candida*-suspension was added to each well in triplicate. Following incubation at 37°C for a range of time periods not exceeding 2 h in a static incubator in air, non-adherent *Candida* cells were removed by gently washing each well five times with 100 μl sterile PBS. The TR146 monolayers were lysed by the addition of 100 μl 0.1% (v/v) Triton X100 in sterile ultra-purified water and incubation at room temperature for 8 min before aspiration to disrupt the monolayer. The 96-well tissue culture plates were centrifuged at $1,783 \times g$ for 5 min in a Sorvall® Legend T centrifuge (Thermo Scientific) which was fitted with rotor TTH-750 and 4 microplate carriers (Thermo Scientific). The supernatant was discarded and the remaining *Candida* cells were stained with either FUN-1 cell stain or XTT-solution.

Candida cells that were to be stained with FUN-1 cell stain were re-suspended in 100 μl 10 μM FUN-1-solution in either sterile PBS or filter-sterile PBS containing 2% (w/v) glucose. Clear 96-well tissue culture plates with an empty column between each column of samples (to prevent fluorescent contamination between neighbouring samples) were incubated for 30 min at 37°C in the dark and read in a fluorescence microplate reader at an excitation wavelength of 485 nm and emission wavelengths of 535 nm, 590 nm and 620 nm (Tecan GENios basic; Tecan Group Ltd., Männedorf, Switzerland).

Candida cells that were to be stained with XTT dye were re-suspended in a solution of 400 $\mu\text{g ml}^{-1}$ XTT in sterile PBS containing 50 $\mu\text{g ml}^{-1}$ Coenzyme Q (Sigma). To each well, 200 μl XTT-solution was added and incubated for 30 min at 37°C before 100 μl XTT-solution was removed from each well and transferred to a new 96-well tissue culture plate which was read at 480 nm in a microplate reader. Any signal obtained with XTT-solution from wells that previously contained mammalian cells only was subtracted from the results values prior to data analysis.

4.2.5 Graphical depiction and statistical analysis of results

Images of TR146 monolayers were observed using a Nikon TMS-F inverted microscope. Results of the optimisation of FUN-1 cell stain and XTT dye, results of the TR146 adhesion assay and further comparisons of experimental conditions were recorded using a Tecan microplate reader and Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Table 4.1. *C. dubliniensis* strains and isolates used in the TR146 adhesion assay

| Strain/isolate | Genotype ^a | Source/Reference |
|----------------|-----------------------|--------------------------------|
| CD36* | 1 | Sullivan <i>et al.</i> , 1995 |
| CD33 | 1 | Sullivan <i>et al.</i> , 1995 |
| Co4 | 1 | Boerlin <i>et al.</i> , 1995 |
| Cm1 | 1 | Sullivan <i>et al.</i> , 1995 |
| SA105 | 1 | Al Mosaid <i>et al.</i> , 2005 |
| CD539 | 2 | Pinjon <i>et al.</i> , 1998 |
| CD75004 | 2 | Pinjon <i>et al.</i> , 1998 |
| Can6 | 2 | Pinjon <i>et al.</i> , 1998 |
| Can9 | 2 | Pinjon <i>et al.</i> , 1998 |
| CD506 | 2 | Gee <i>et al.</i> , 2002 |
| P6785 | 3 | Polacheck <i>et al.</i> , 2000 |
| CD519 | 3 | Gee <i>et al.</i> , 2002 |
| DB305 | 3 | Al Mosaid <i>et al.</i> , 2003 |
| P7276 | 3 | Polacheck <i>et al.</i> , 2000 |
| CD514 | 3 | Gee <i>et al.</i> , 2002 |
| Eg200 | 4 | Al Mosaid <i>et al.</i> , 2005 |
| P7718 | 4 | Gee <i>et al.</i> , 2002 |
| Eg202 | 4 | Al Mosaid <i>et al.</i> , 2005 |
| Eg201 | 4 | Al Mosaid <i>et al.</i> , 2005 |
| Eg207 | 4v ^b | Al Mosaid <i>et al.</i> , 2005 |
| Is35 | 4v | Al Mosaid <i>et al.</i> , 2005 |

^aThe genotype of each isolate was assigned based on differences observed in the nucleotide sequence of the ITS region of the rRNA gene cluster (Gee *et al.*, 2002; Al Mosaid *et al.*, 2005)

^bThe ITS sequence of the 4v isolates was found to differ from the genotype 4 consensus sequence by 1 or 2 bases in the ITS region compared to genotype 4 (Al Mosaid *et al.*, 2005)

*Type strain (i.e. the nomenclatural type of a species) for *C. dubliniensis*



Table 4.2. *C. albicans* strains and isolates used in the TR146 adhesion assay

| Strain/isolate | Source/Reference |
|-----------------------|----------------------------------|
| SC5314* | Gillum <i>et al.</i> , 1984 |
| 132A | Gallagher <i>et al.</i> , 1992 |
| NCPF3153 ^a | UK reference strain ^a |
| m182CA | Pinjon, 2003 |
| CA800 ⁺ | This study |
| CA801 ⁺ | This study |
| CAN33 ^b | Lo <i>et al.</i> , 1997 |
| CAF2-1 | Fonzi & Irwin, 1993 |

^aFrom the National Collection of Pathogenic Fungi (NCPF), Kingsdown, Bristol, UK

^bCalled HLC52 in Lo *et al.*, 1997

*Type strain (i.e. the nomenclatural type of a species) for *C. albicans*

⁺Oral isolates from the laboratory collection of the Microbiology Research Unit, Dublin Dental School and Hospital



Data for the optimisation of the cell stains used was correlated, analysed and graphically depicted using Excel and GraphPad Prism. Data was analysed in Excel using the mean and standard deviation (SD), and was graphically depicted using a standard curve with a linear (in the case of FUN-1) or logarithmic (in the case of XTT) slope. Final data for the optimisation of both cell stains was correlated, analysed and graphically depicted with GraphPad Prism using the mean, SEM and linear regression (polynomial of the 1st order).

Data for the number of lysis washes needed was correlated, analysed and graphically depicted with GraphPad Prism using the mean and SEM. Data on the effect of Triton X100 on candidal viability was correlated, analysed and graphically depicted using GraphPad Prism. Results were recorded on three separate occasions and analysed using the mean, SEM and two tailed, unpaired, t-tests.

Results of the adhesion of *Candida* to the TR146 monolayer were correlated, analysed and graphically depicted with GraphPad Prism using the mean, SEM and one-way ANOVA with Tukey's multiple comparison post test. Further comparisons of experimental conditions were correlated, analysed and graphically depicted with GraphPad Prism using the mean, SEM and two-way ANOVA with Bonferroni post tests.

4.3 RESULTS

4.3.1 Effect of Triton X100 on candidal growth

In order to assess the effect of the detergent, Triton X100, on candidal growth, *Candida* were treated with 0.1% (v/v) Triton X100 in sterile ultra-purified water as described in section 4.2.2 above. There was no significant difference between the number of colonies growing on Triton-treated and non-Triton-treated plates ($P > 0.05$), with the exception of *C. dubliniensis* Is35 ($P < 0.05$), where significantly more colonies grew on PDA plates from the Triton-treated *C. dubliniensis* Is35 sample than the non-Triton-treated sample (Fig. 4.1). These results showed that 0.1% (v/v) Triton X100 in sterile ultra-purified water had no effect on *Candida* viability.

4.3.2 Effect of media, atmospheric conditions and Triton X100 on the integrity of the TR146 monolayer

In order to determine the effect of experimental conditions on the integrity of the TR146 cell line, monolayers of the cell line were exposed to sterile PBS, filter-sterile PBS containing 2% (w/v) glucose and RPMI-1640 medium in air at 37°C and 0.1% (v/v) Triton X100 in sterile ultra-purified water in air at room temperature. After 30 min at 37°C in air in sterile PBS, less than 1% dead TR146 cells were observed using Trypan blue and the dye exclusion test and a Nikon TMS-F inverted microscope as described in section 4.2.3 above. After 2 h in sterile PBS the numbers of dead cells had not increased, however after 3 h the integrity of the cell line deteriorated; the cells were intact but the majority had detached from the tissue culture dish (data not shown). Similar results were observed when the TR146 cell line was exposed to filter-sterile PBS containing 2% (w/v) glucose. As the integrity of the cell line deteriorated after 2 h in both candidal cell suspension media, the adhesion assays were restricted to a maximum time of 2 h. After 2 h in air at 37°C, TR146 cells in RPMI-1640 medium appeared elongated presumably due to RPMI-1640 medium and therefore the use of RPMI-1640 medium was discontinued as a candidal cell suspension medium.

Following incubation of the TR146 cells with 0.1% (v/v) Triton X100 in sterile ultra-purified water at room temperature for 8 min and repeated aspiration, the TR146 cells were observed to have become detached from the base of the tissue culture dish. The integrity of the cells of the TR146 monolayer had been disrupted as was evident from the blue colour of the cells (data not shown). The results showed that this concentration of detergent was effective for lysis of the TR146 monolayer without having any effect on candidal viability.

4.3.3 Assessment of FUN-1 cell stain for determination of the number of adherent *Candida* to the TR146 human cell line

In an effort to remove some of the source of operator bias, use of a cell stain was employed to enumerate adherent *Candida*. Two substances, FUN-1 cell stain and XTT dye were compared for their usefulness in elucidating adherent *Candida*. In order to determine the optimal wavelength for recording the results of FUN-1 stained adherent *Candida* to the TR146 cell line, results obtained from the use of three filters were

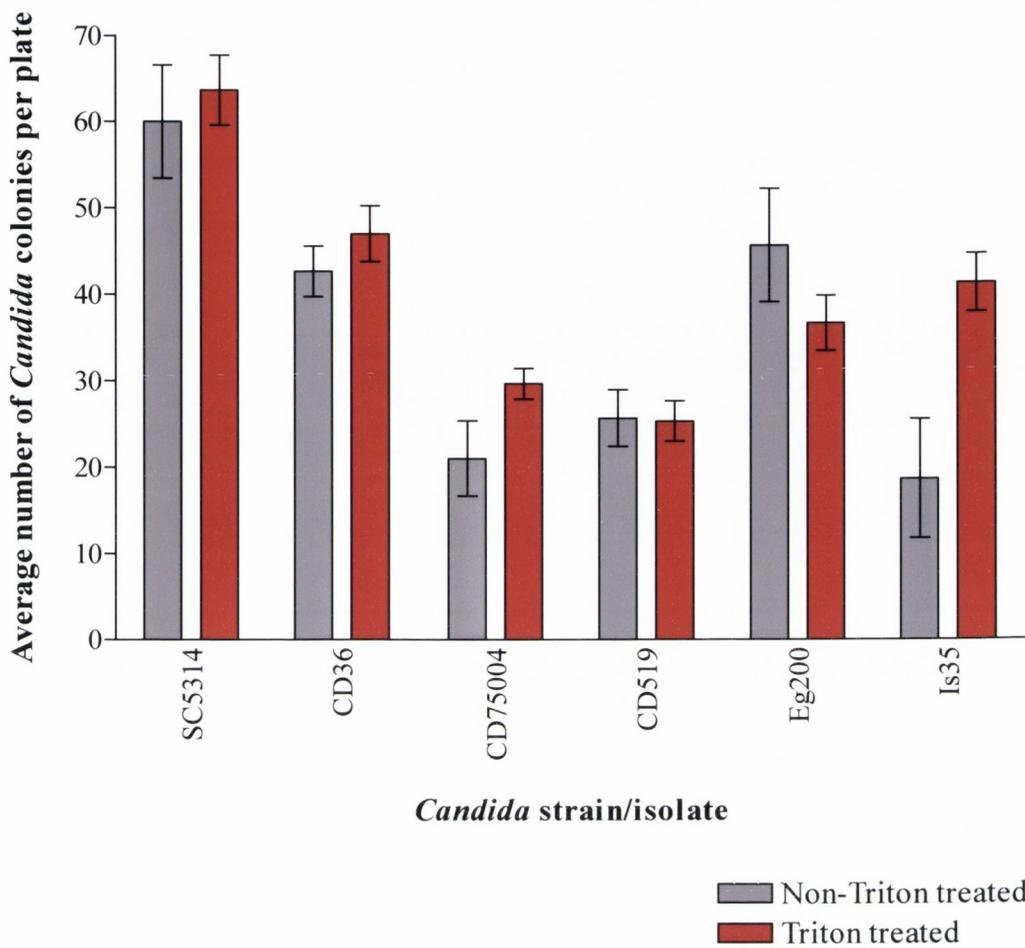


Figure 4.1. Effect of Triton X100 treatment on the growth of *Candida* strains and isolates. The results show the mean number of colonies which grew on PDA plates \pm SEM. The number of colonies for each isolate tested were observed on three separate occasions. Significantly more colonies of *C. dubliniensis* Is35 grew following Triton treatment compared with a non Triton-treated sample ($P < 0.05$).



compared. Green fluorescence was measured at 535 nm and red fluorescence was measured at both 590 nm and 620 nm. A study by Millard *et al.* (1997) showed that a higher fluorescence emission by FUN-1 was detected at 590 nm than 620 nm, results which concurred with the present study (data not shown), and therefore use of the 620 nm filter was discontinued.

Results obtained when using the 535 nm filter showed a higher level of fluorescence in wells that contained only TR146 monolayers compared to wells that contained TR146 monolayers and *Candida* cells and it was concluded that the monolayer was emitting green fluorescence. Similar results were obtained by Marr *et al.* (2001) who reported large amounts of non-specific background staining with FUN-1 green in intact macrophages. To overcome this problem and prevent interference from a FUN-1 signal from human cells, the TR146 monolayer was lysed, however, this step introduced intracellular proteins into solution, and as proteins in solution fluoresce in the presence of FUN-1 (Millard *et al.*, 1997) the use of the 535 nm filter was also discontinued.

Red fluorescence (at 590 nm) of adherent *C. albicans* SC5314 to TR146 monolayers was recorded after exposure of the yeast cells to FUN-1 cell stain for 30 min when FUN-1 cell stain was re-suspended in either sterile PBS, or filter-sterile PBS containing 2% (w/v) glucose. Millard *et al.* (1997) showed that addition of glucose to the staining solution increased the formation of red fluorescent cylindrical intravacuolar structures in *Sa. cerevisiae*. The addition of 2% (w/v) glucose to the staining solution had no effect on the red fluorescence of *C. albicans* SC5314 cells (Fig. 4.2) and therefore FUN-1 cell stain was re-suspended in sterile PBS for enumeration of adherent *Candida*.

Separate standard curves for each isolate used were elucidated as each isolate metabolised FUN-1 at a different rate (data for *C. albicans* SC5314 and *C. dubliniensis* CD36 are shown in Fig. 4.3). Replicates for each isolate were reproducible. Standard curves of each isolate were elucidated in the presence of, and following lysis of, the TR146 monolayer. Similar results were obtained for both sets of conditions when the results were recorded using the 590 nm filter (Fig. 4.3).

4.3.4 Assessment of XTT dye for determination of the number of adherent *Candida* to the TR146 human cell line

The usefulness of XTT in determining the number of adherent *Candida* to TR146 monolayers was investigated. As XTT is known to develop an orange colour in the presence of live epithelial cells, TR146 monolayers were lysed before results were recorded. The results showed that two or three lysis washes with 0.1% (v/v) Triton X100 in sterile ultra-purified water were optimal to fully lyse the TR146 monolayer (Fig. 4.4). XTT has the ability to detect a wider range of cell densities than FUN-1 so a standard curve based on a wider range of cell densities can be generated using XTT rather than FUN-1 and as such the use of FUN-1 cell stain for enumerating adherent *Candida* was discontinued. As with FUN-1 cell stain, each isolate metabolised XTT at a different rate, so individual standard curves for each isolate used were generated (Fig. 4.5). However, it was necessary to set up a standard curve using XTT in one 96-well tissue culture plate and carry out the experiment in another, and as differences in absorbance values were observed in corresponding cell numbers between the two 96-well tissue culture plates, the adhesion of *Candida* to TR146 monolayers was elucidated using percentage adhesion with XTT.

4.3.5 Adhesion of *Candida* cells grown overnight (~16 h) at 37°C in YEPD to a monolayer of TR146 cells

Percentage adherence of each isolate of *C. dubliniensis* (n = 21) and *C. albicans* (n = 8) to TR146 monolayers was elucidated using XTT dye (Fig. 4.6). Both 100% and 0% controls were included on each clear 96-well tissue culture plate for each isolate tested per plate. The results showed that *C. dubliniensis* genotype 4 isolates adhered to the TR146 monolayer in significantly greater numbers than the other genotypes of *C. dubliniensis* (*C. dubliniensis* genotype 1, $P < 0.01$; *C. dubliniensis* genotypes 2, 3 and 4v, $P < 0.001$; Fig. 4.7). There was no significant difference between the adhesion of *C. dubliniensis* genotype 4 and *C. albicans* to the TR146 monolayer ($P > 0.05$; Fig. 4.7). While there was no significant difference in the adherence of *C. dubliniensis* genotype 1 and *C. albicans* to the TR146 monolayer, both *C. dubliniensis* genotype 1 ($P < 0.05$) and *C. albicans* ($P < 0.01$) adhered to the TR146 monolayer in significantly greater numbers than *C. dubliniensis* genotype 3 only (Fig. 4.7).

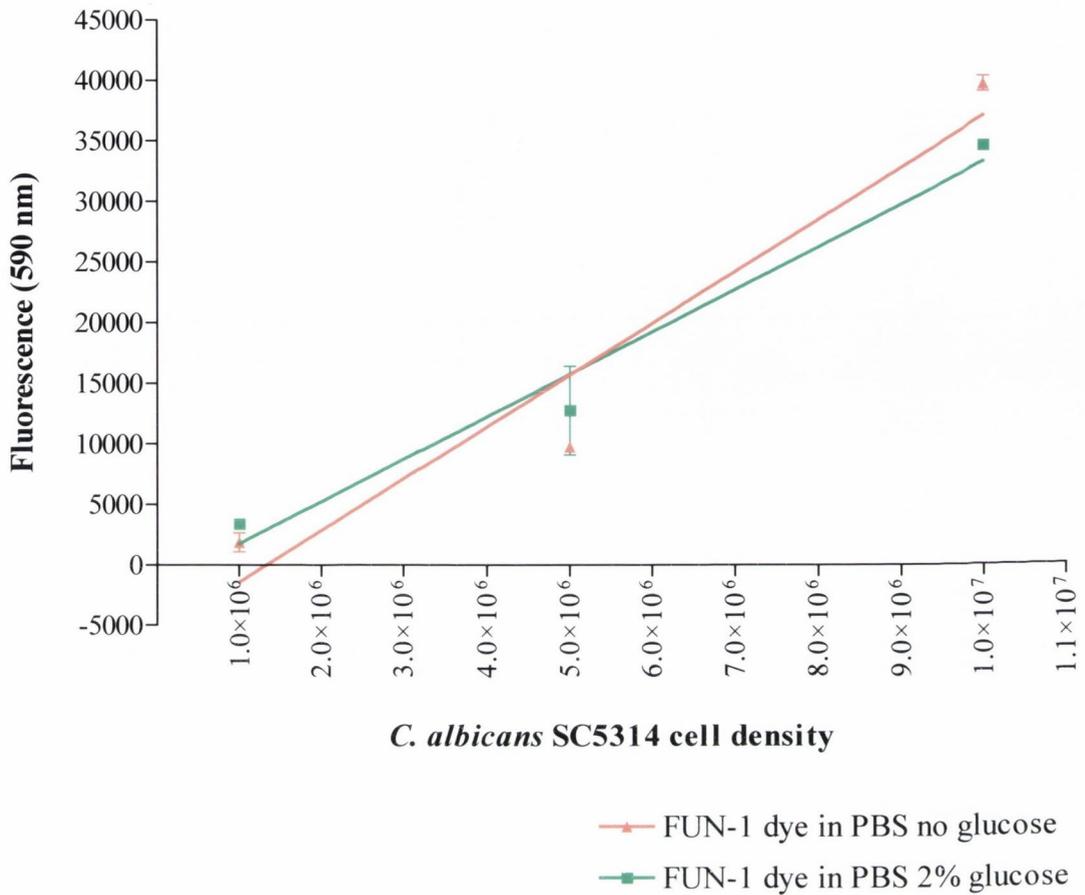


Figure 4.2. Relative fluorescence of *C. albicans* SC5314 when stained with FUN-1 cell stain in the presence and absence of 2% glucose. Standard curves of *C. albicans* SC5314 were elucidated with FUN-1 cell stain in the presence and absence of 2% glucose. Mean fluorescence \pm SEM for each data point on the graph was observed on two separate occasions. Standard curves were obtained using non linear regression (polynomial, first order).

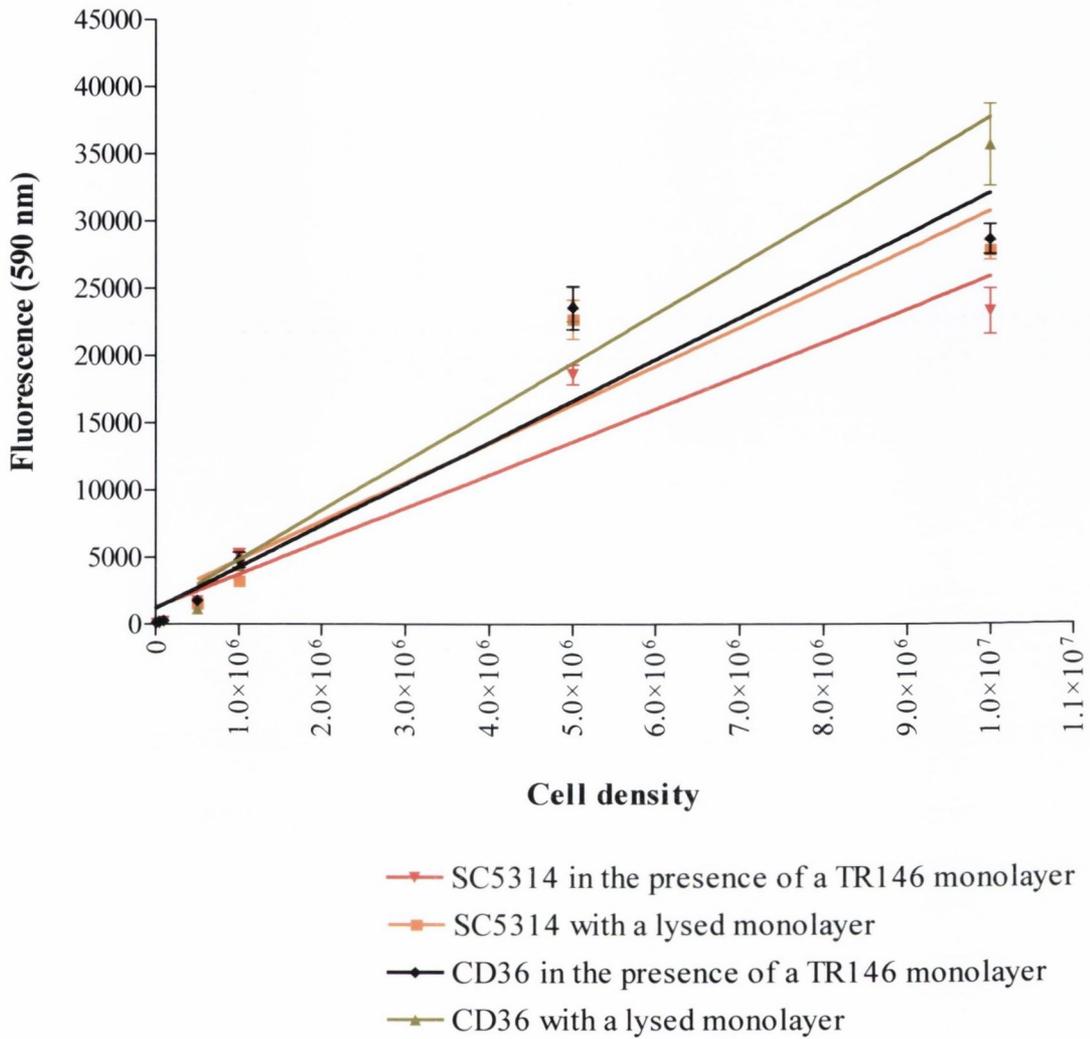
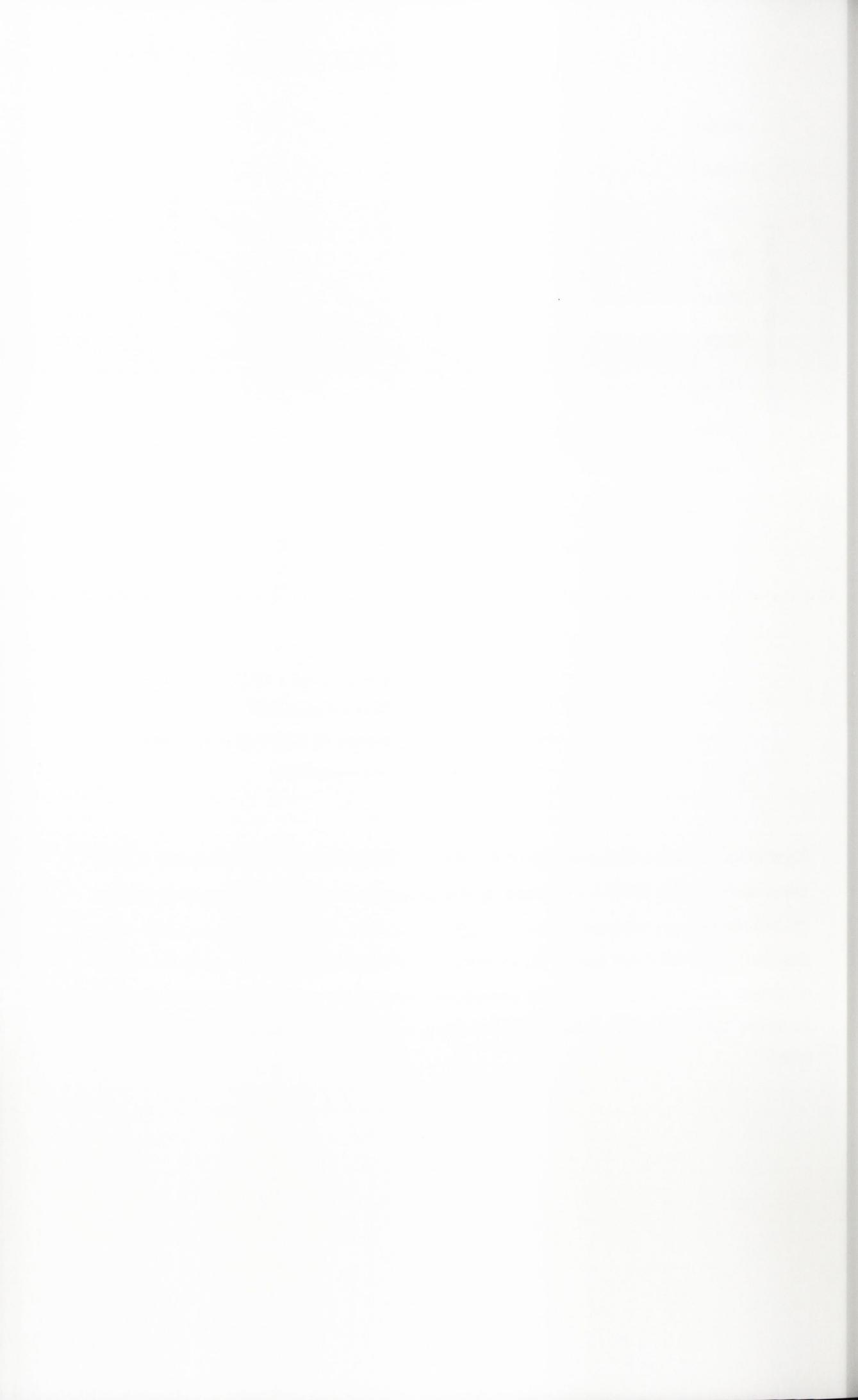


Figure 4.3. Relative fluorescence of *C. albicans* SC5314 and *C. dubliniensis* CD36 when stained with FUN-1 cell stain in the presence of and following lysis of the TR146 monolayer. Standard curves of *C. albicans* SC5314 and *C. dubliniensis* CD36 were elucidated with FUN-1 cell stain in the presence of, and following lysis of, a confluent TR146 monolayer. Mean fluorescence \pm SEM for each data point on the graph was observed on three occasions. Standard curves were elucidated using non-linear regression (polynomial, first order).



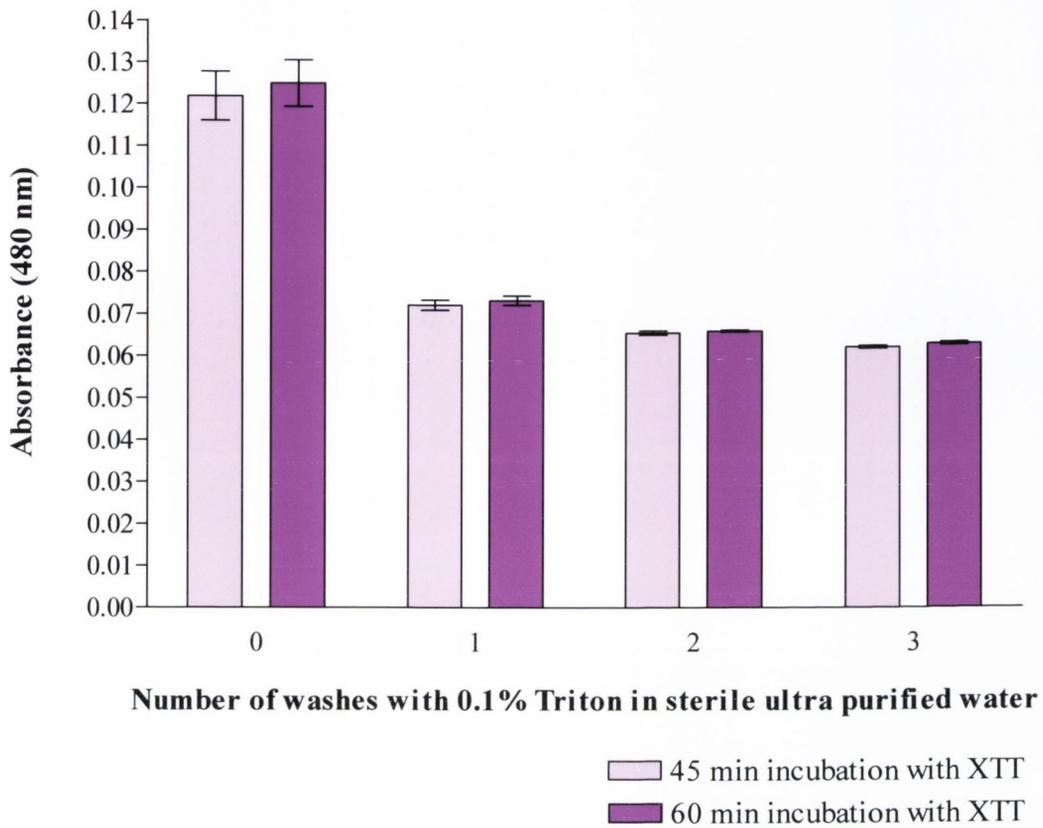


Figure 4.4. Determination of the number of washes with 0.1% Triton X100 in sterile ultra-purified water needed to fully lyse the TR146 monolayer. Cultures of the TR146 monolayer were washed with 0.1% (v/v) Triton X100 in sterile ultra-purified water in order to determine the optimal number of washes needed to fully lyse the monolayer. Mean absorbance values \pm SEM were obtained from six replicates. There was no significant difference between incubation times in XTT.

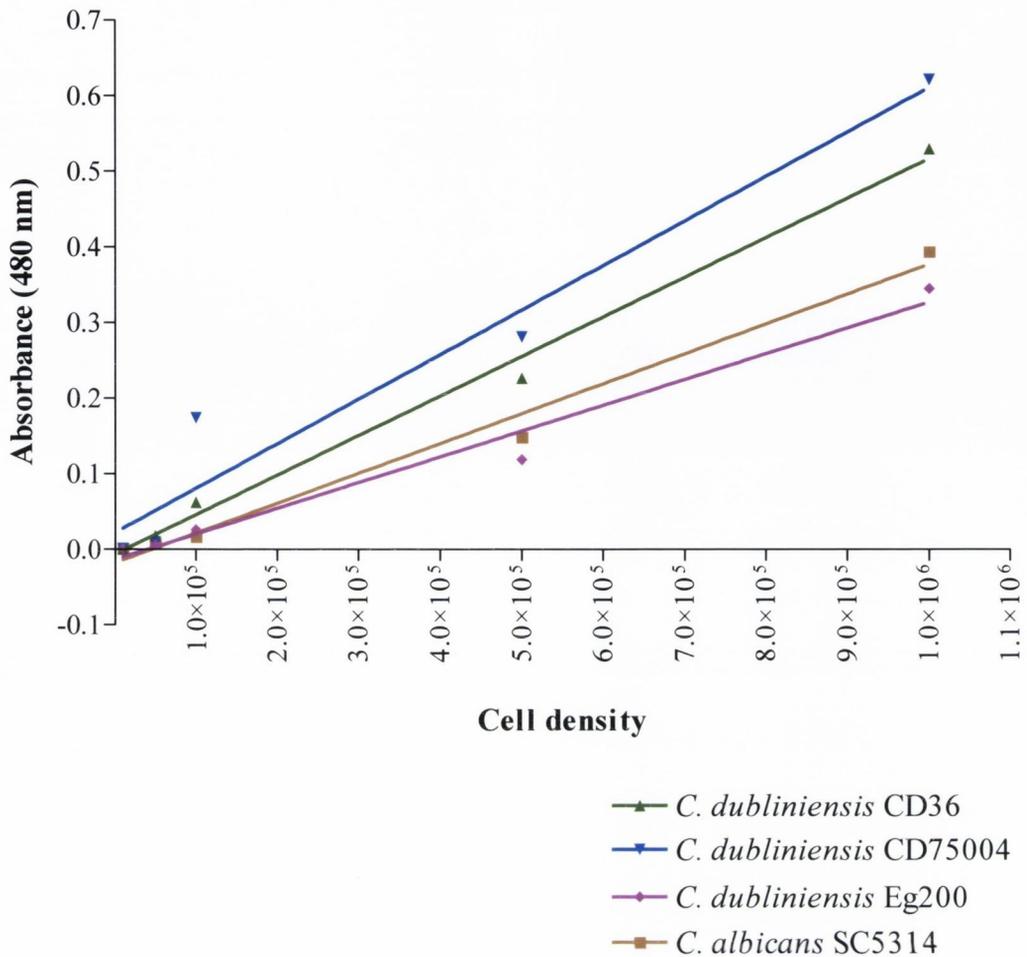
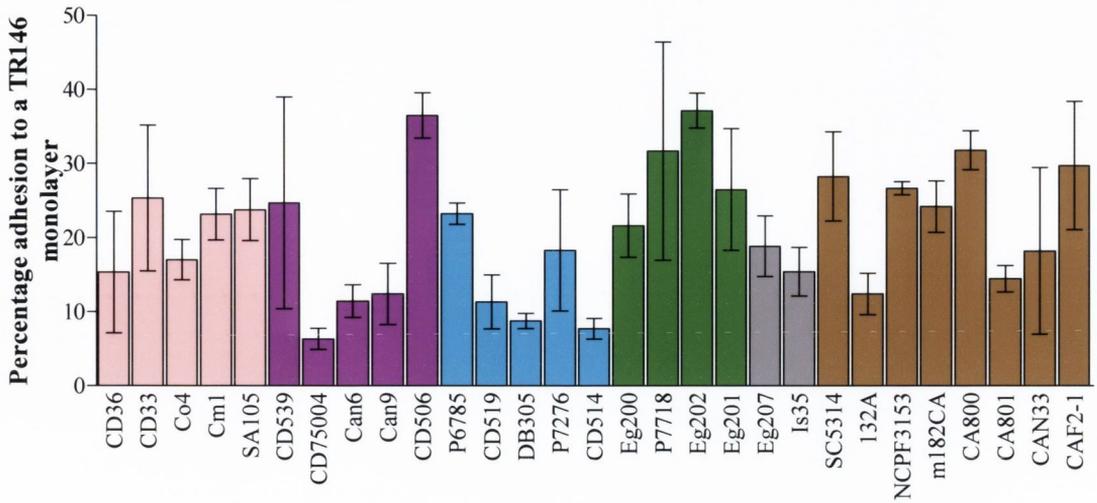


Figure 4.5. Standard curves of *C. albicans* SC5314, *C. dubliniensis* CD36, *C. dubliniensis* CD75004 and *C. dubliniensis* Eg200 elucidated in the presence of XTT. Standard curves of *C. albicans* SC5314, *C. dubliniensis* CD36, *C. dubliniensis* CD75004 and *C. dubliniensis* Eg200 were elucidated in the presence of XTT. Standard curves were obtained using non linear regression (polynomial, first order).



Candida strain/isolate

- C. dubliniensis* genotype 1
- C. dubliniensis* genotype 2
- C. dubliniensis* genotype 3
- C. dubliniensis* genotype 4
- C. dubliniensis* genotype 4v
- C. albicans*

Figure 4.6. Mean percentage adhesion of *C. dubliniensis* and *C. albicans* strains and isolates to a monolayer of TR146 cells. The results show the mean percentage adhesion of *Candida* from each isolate tested to the TR146 monolayer \pm SEM. Adherent *Candida* were observed on three separate occasions using XTT.

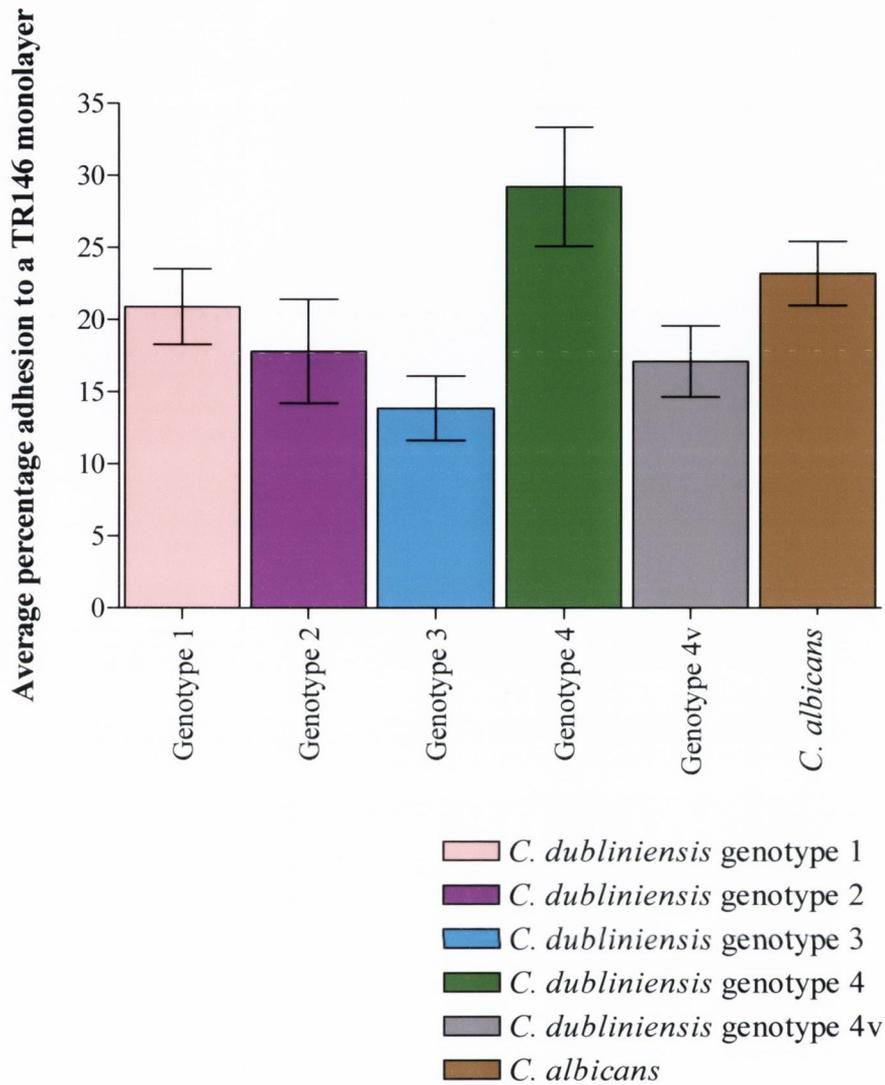


Figure 4.7. Mean percentage adhesion of the genotypes of *C. dubliniensis* and of *C. albicans* to a monolayer of TR146 cells. The results of the adhesion of each isolate recorded on three separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 5, *C. dubliniensis* genotype 2 = 5, *C. dubliniensis* genotype 3 = 5, *C. dubliniensis* genotype 4 = 4, *C. dubliniensis* genotype 4v = 2, *C. albicans* = 8) to give the mean percentage adherence of *Candida* ± SEM to a monolayer to TR146 cells. *Candida dubliniensis* genotype 4 isolates adhered to a monolayer of TR146 cells in significantly greater numbers than the other genotypes of *C. dubliniensis* (1 = $P < 0.01$; 2, 3 and 4v = $P < 0.001$). *Candida dubliniensis* genotype 1 ($P < 0.05$) and *C. albicans* ($P < 0.01$) adhered to a monolayer of TR146 cells in significantly greater numbers than *C. dubliniensis* genotype 3. Percentage adhesion was determined using XTT.

4.3.6 Effect of various incubation conditions on adhesion of *C. dubliniensis* and *C. albicans* to the TR146 monolayer

The adhesion of *C. albicans* SC5314, *C. dubliniensis* CD75004 (genotype 2) and *C. dubliniensis* Eg201 (genotype 4) to a monolayer of TR146 cells under varying adhesion conditions was examined. *Candida albicans* SC5314 was chosen as the numbers of cells adhering to human BECs in the human BEC adhesion assay did not change with changing assay conditions, so it was assumed that the assay conditions had no effect on the ability of *C. albicans* SC5314 to adhere to human BECs. *Candida dubliniensis* CD75004 was chosen as it is a constitutively pseudohyphal isolate and *C. dubliniensis* Eg201 was chosen as it adhered poorly to human BECs in the human BEC assay. Changes to the adhesion conditions included shaking the 96-well tissue culture plate after *Candida* cells had been added to the TR146 monolayer or re-suspending *Candida* cells in 50% (v/v) human saliva in sterile PBS before incubation with TR146 monolayers. *Candida albicans* SC5314 ($P < 0.01$) and *C. dubliniensis* Eg201 ($P < 0.001$) adhered to TR146 monolayers in significantly greater numbers when re-suspended in 50% (v/v) human saliva in sterile PBS under shaking as opposed to static conditions. *Candida dubliniensis* CD75004 was the only isolate of the three tested to show significantly more adhesion to TR146 monolayers when re-suspended in sterile PBS under shaking conditions compared to static conditions ($P < 0.05$). *Candida dubliniensis* Eg201 ($P < 0.001$) adhered in significantly greater numbers to TR146 monolayers when re-suspended in sterile PBS under static condition than when re-suspended in 50% (v/v) human saliva in sterile PBS under static conditions (Fig. 4.8).

Candida dubliniensis CD36 and *C. albicans* SC5314 were re-suspended in sterile PBS and incubated for 20 min, 40 min and 60 min with a monolayer of TR146, under either static or shaking conditions. The results show that neither the length of incubation nor the presence or absence of shaking during incubation of *Candida* with the TR146 monolayer had an effect on adhesion ($P > 0.05$ for all results).

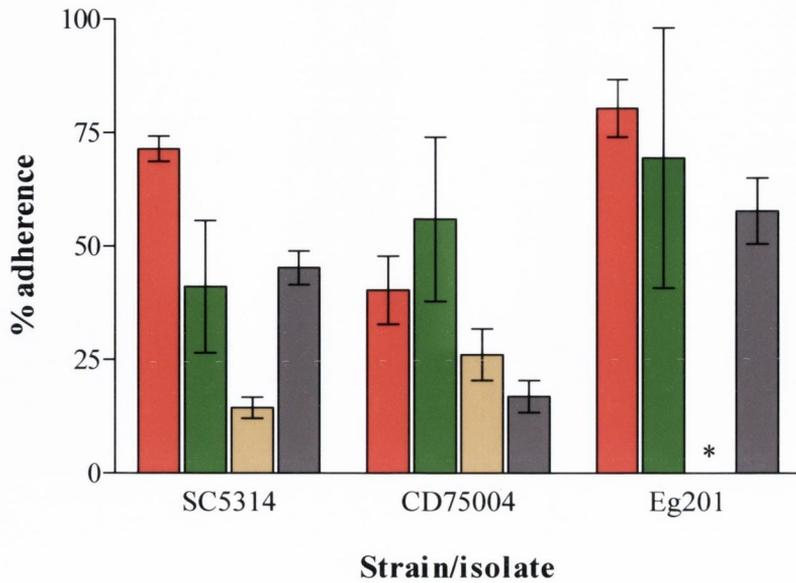
4.4 DISCUSSION

Yeast adhesion to epithelial cells varies considerably with the origin of the yeast and the epithelium. The use of a uniform model of human epithelium has some advantages over human BECs in assessing candidal adhesion. When using a uniform model of human epithelium, any source of variation in the data obtained can be assumed to be from differences in the ability of the test isolates to adhere to human tissue as the use of a cell line such as TR146 removes any intra- or inter-individual effects from the equation.

The RHE model has been widely used to study adhesion and invasion of yeast and other pathogens, however, the use of a monolayer of human epithelium has some advantages over RHE. Firstly, a cell line is a less expensive model of human epithelium than RHE. Once the cell line has been established and cells have been stored for further use, each monolayer of human epithelium can be passaged up to 30 times with the passaged cells being used to seed many tissue culture dishes and plates for experimentation; RHE can only be used once. Secondly, growth of a monolayer has a quicker turnover time than RHE (16 h compared to 30 days) thus allowing a higher through-put of samples. The aim of this part of the present study was to investigate whether monolayers of human oral epithelial cells could be used to develop a candidal adhesion assay that is comparable with the BEC adhesion assay.

4.4.1 Usefulness of FUN-1 dye and XTT dye in enumerating adherent candidal numbers

FUN-1 proved less useful for enumerating adherent *Candida* than XTT. The most common use for FUN-1 cell stain is in assessing cell viability. The difference between the starting and ending red to green ratios is used to determine changes in the live/dead yeast population. A single ratio can not be interpreted alone as a large initial fluorescence ratio may be indicative of dead yeast cells and so the use of FUN-1 was discontinued and adherent candidal numbers to the TR146 monolayer were assessed using XTT dye. While the adherence of hyphal *Candida* was not assessed using the TR146 adherence assay, the use of a dye such as XTT may be more practical in enumerating adherent hyphae, provided the number of hyphae can be accurately



■ 50% (v/v) human saliva, shaking conditions
■ PBS, shaking conditions
■ 50% (v/v) human saliva, static conditions
■ PBS, static conditions

Figure 4.8. Difference in adhesion of *C. albicans* SC5314, *C. dubliniensis* CD75004 and *C. dubliniensis* Eg201 to a monolayer of TR146 cells under various incubation conditions. Results from each treatment were recorded on three separate occasions.

**Candida dubliniensis* Eg201 did not adhere to a monolayer of TR146 cells when re-suspended in 50% (v/v) human saliva under static conditions.



determined with the dye by a method such as elucidation of a standard curve. Should this method prove useful, the number of adherent hyphal *Candida* to the TR146 monolayer could be assessed in comparison with adhesion of yeast cells to this monolayer.

4.4.2 Adhesion of *C. dubliniensis* and *C. albicans* to TR146 monolayers

Both *C. dubliniensis* and *C. albicans* adhered to the TR146 monolayer (Fig. 4.6). Adhesion results were obtained when the yeast cells were grown overnight (~16 h) in YEPD at 37°C, re-suspended in sterile PBS at 2×10^7 cells ml⁻¹ and incubated with a monolayer of TR146 cells for 2 h at 37°C in air. *Candida dubliniensis* genotype 4 adhered to the TR146 monolayer in significantly greater numbers than the other genotypes of *C. dubliniensis* (genotype 1, $P < 0.01$; genotypes 2, 3, and 4v, $P < 0.001$; Fig. 4.7). While there was no significant difference between the adhesion of *C. dubliniensis* genotype 4 and *C. albicans* to the TR146 monolayer, *C. albicans* adhered to the TR146 monolayer in significantly greater numbers than *C. dubliniensis* genotype 3 only ($P < 0.01$). There was also no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* to the TR146 monolayer and *C. dubliniensis* genotype 1 adhered to the TR146 monolayer in significantly greater numbers than *C. dubliniensis* genotype 3 ($P < 0.05$; Fig. 4.7). These adhesion results were compared with results from the BEC adhesion assay. Although the methods were not the same, the results from the two experiments with the most similar experimental conditions (i.e. when the yeast cells were pre-cultured in YEPD overnight (~16 h) at 37°C) were compared. It should be noted that there are four differences between the two adhesion assays. Firstly, the human cell type used in this part of the present study differed from the cells used in the human BEC adhesion assay. Secondly, in the human BEC assay, *Candida* and BECs are incubated together under shaking conditions, whereas *Candida* cells and the TR146 monolayer are incubated together under static conditions. Results from this part of the present study show that of three *Candida* isolates tested (*C. albicans* SC5314, *C. dubliniensis* CD75004 and *C. dubliniensis* Eg201), only one (*C. dubliniensis* CD75004) showed significantly greater adhesion to the TR146 monolayer when re-suspended in sterile PBS under shaking as opposed to static incubation conditions ($P < 0.05$; Fig. 4.8). It can be assumed, therefore, that shaking or static incubation conditions had an effect on the adhesion of a minority of

isolates of *Candida* to the TR146 monolayer. Thirdly, although the inoculum is different between the two assays, *Candida* cells are in excess (50:1 in the BEC adhesion assay, 25:1 in the TR146 adhesion assay) of the human cells. Fourthly, the results were recorded in a different way. The results from the human BEC adhesion assay were recorded as the mean number of adherent *Candida* per human BEC, whereas the results for the TR146 adhesion assay were recorded as mean percentage candidal adherence to the human monolayer. While the results have different units for adhesion between the two assays, the results can still be compared based on their relative values.

The results from the BEC adhesion assay when *Candida* were pre-cultured in YEPD overnight (~16 h) at 37°C showed that there was no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* to human BECs and that *C. dubliniensis* genotype 1 and *C. albicans* adhered to human BECs in significantly greater numbers than other genotypes (2, 3, 4 and 4v) of *C. dubliniensis*. Similarly to the BEC adhesion assay, there was no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* to TR146 monolayers. However, unlike the BEC adhesion assay where *C. dubliniensis* genotype 1 and *C. albicans* adhered to BECs in significantly greater numbers than all other genotypes of *C. dubliniensis*, *C. dubliniensis* genotype 1 ($P < 0.05$) and *C. albicans* ($P < 0.01$) adhered to the TR146 monolayer in significantly greater numbers than *C. dubliniensis* genotype 3 only (Fig. 4.7). The results from the adhesion of *Candida* to TR146 monolayers showed that there was less of a range of candidal adhesion to TR146 monolayers compared to adhesion of *Candida* to human BECs probably due to the more homogenous population of human cells in the TR146 cell line. Although there was no significant difference between the adhesion of *C. dubliniensis* genotype 4 and *C. albicans* to the TR146 monolayer, *C. dubliniensis* genotype 4 adhered to the TR146 monolayer in significantly greater numbers than the other genotypes (1, 2, 3 and 4v) of *C. dubliniensis*. This result has no correlation with the results of the BEC adhesion assay where *C. dubliniensis* genotype 4 adhered to human BECs in similar amounts to *C. dubliniensis* genotypes 2, 3, and 4v and adhered in significantly lower numbers to human BECs than *C. dubliniensis* genotype 1 and *C. albicans*. *Candida dubliniensis* genotype 4 isolates adhered in the greatest amount to TR146 monolayers while *C. dubliniensis* genotype 1 and *C. albicans* adhered to human BECs in the greatest amounts.

The results from the two studies indicate that there is little correlation between the adhesion of *Candida* to human BECs and to TR146 monolayers and as such it is

difficult to know which assay reflects candidal adhesion *in vivo*. There may be many reasons for this the poor correlation in results between the two assays. The difference in the way in which the adhesion assays were carried out may have an effect on candidal adhesion to human cells albeit a minor one. Inter- and/or intra-individual effects as mentioned above may influence adhesion; a population of human BECs from a different ethnic group may have given a different adhesion result which may have had better correlation with the TR146 adhesion assay. There may have been adherent oral bacteria present on the BECs which may influence the adhesion of yeast cells to BECs; these bacteria would not be present on the TR146 cell line. Human BECs are naturally exposed to saliva and this may also influence the adhesion of yeast cells to human BECs; the TR146 cell line would not normally be exposed to saliva. The human cells may be at different stages of the cell cycle; human BECs are assumed to be dead as they are easily shorn from the buccal cavity whereas TR146 cells are healthy and alive as if not, they would not grow and this may affect the way in which *Candida* adhere to them.

4.4.3 Effect of incubation conditions on the adhesion of *Candida* to the TR146 monolayer

The effects of various incubation conditions on the adhesion of *Candida* to a monolayer of TR146 cells were examined. Although changes to the adhesion conditions were carried out at various candidal cell densities, the results for 2×10^7 *Candida* cells ml^{-1} are reported only, as this was the cell density used in the TR146 adhesion assay. The results showed that under shaking conditions, when re-suspended in 50% (v/v) human saliva in sterile PBS, *C. albicans* SC5314 ($P < 0.01$) and *C. dubliniensis* Eg201, ($P < 0.001$) adhered to the TR146 monolayer in significantly greater numbers than under static conditions (Fig. 4.8). All other conditions have an isolate-by-isolate effect on adhesion to the TR146 monolayer (Fig. 4.8). In order to obtain a more meaningful result of the effect of various incubation conditions on the adhesion of *Candida* to human cells, adhesion of a greater range of *Candida* isolates to human cells should be examined.

A study by Holmes *et al.* (2002) showed that addition of pooled whole human saliva to adherence assays promoted the adhesion of *C. albicans* to monolayers of 3 epithelial cell lines, however, these results contradict results found here where no increase in adhesion of *C. albicans* SC5314 to TR146 monolayers was found when the

yeast cells were re-suspended in 50% (v/v) human saliva in sterile PBS compared to sterile PBS alone.

In conclusion, the adhesion of *Candida* to TR146 monolayers differs from the adhesion of *Candida* to human BECs, and this is most likely influenced by differences in the human cells rather than differences in the experimental conditions used in this study. Of these two adhesion assays used, adhesion of *Candida* to human BECs is probably more representative of adhesion to human oral cells *in vivo* and while the TR146 adhesion model is useful and can be used to assess adhesion it should not be thought of as a more reliable method for assessing adhesion than the BEC adhesion assay. Despite this, both assays have advantages and disadvantages which should be taken into account when designing an experiment to assess adhesion of a microorganism to human cells. Of previous studies investigated, only one (Enache *et al.*, 1996) examined the comparative adhesion of three isolates of *Candida* to human BECs and two cell lines, one murine and one human when *Candida* were grown in media containing either glucose or galactose. While the percentage adhesion of *Candida* to human BECs and both cell lines was greater when grown in media containing galactose compared to media containing glucose, it must be noted that only three isolates of *Candida* were used in this study and in order to gain more useful information from such a comparative study, a wider range of isolates would need to be investigated. In this part of the present study the adhesion of *Candida* to TR146 monolayers was only investigated when *Candida* were grown in YEPD at 37°C and as there was little correlation between the adhesion of *Candida* grown in YEPD at 37°C to TR146 monolayers and the adhesion of *Candida* grown in YEPD at 37°C to human BECs, further candidal adhesion conditions were not investigated.

In order to further examine candidal adhesion and to determine if *Candida* adhere to cellular ligands preferentially, the molecular interaction of *Candida* with extracellular matrix proteins will be investigated using a BIAcore™ 3000 biosensor.

Chapter 5

**Relative adhesion of *C. dubliniensis* and
C. albicans to extracellular matrix proteins
determined using surface plasmon resonance**

5.1 INTRODUCTION

Both the human BEC adhesion assay and the TR146 adhesion assay showed different results for candidal adhesion. There could be a number of reasons for this including the use of a heterogeneous population of human cells in the human BEC adhesion assay, the absence of oral bacteria and/or salivary proteins from the TR146 cell line and differences between the methods of the two adhesion assays. However, the mostly likely reason for differences in candidal adhesion is the difference in the human cell types used in the two assays. In an attempt to further investigate candidal adhesion and the specific interactions of *Candida* with human cells, the molecular adhesion of *Candida* to six extracellular matrix (ECM) proteins was investigated using a BIAcore™ 3000 biosensor.

The BIAcore™ 3000 biosensor measures, in real time, molecular interactions of very small amounts of analytical material using the phenomenon of surface plasmon resonance (SPR). SPR is an optical resonance effect that detects refractive index changes at the surface of the sensor chip. At a certain light angle, light is refracted parallel to the surface of the sensor chip. Beyond this angle, total internal reflection (TIR) occurs and light is reflected. Although no light passes through the surface of the sensor chip in TIR, an electronic field of photons extends about $\frac{1}{4}$ of a wavelength past the reflecting surface. This electronic field interacts with free electron constellations in the gold surface of the sensor chip. When this occurs, photons are converted into surface plasmons and SPR occurs. Plasmons create an electronic field, the evanescent wave, which extends from the gold film into the medium. The properties of the medium (in this case, HBS-EP buffer) determine in part, the way in which the evanescent wave travels through that medium and as such the velocity of the plasmons can change with the properties of the medium on either side of the conducting film. As the analyte concentration at the sensor chip changes, the velocity and momentum of the surface plasmon is affected, and the incident light angle at which resonance occurs, shifts (Markey, 1999; Sambrook & Russell, 2001).

SPR can be used to measure kinetic data such as rate constants for interactions between molecules (Lipschultz *et al.*, 2002; Nygren-Babol *et al.*, 2005; Pol & Wang, 2006). The BIAcore system has been used to measure interactions between proteins and other small molecules such as nucleic acids (Forde *et al.*, 2006; Law *et al.*, 2006; Di

Primo & Lebars, 2007), lipids (Sun *et al.*, 2003; Kölzer *et al.*, 2004; Levels *et al.*, 2005) and other proteins (Hargreaves & Al-Shamkhani, 2002; Keyhanfar *et al.*, 2007; Surinya *et al.*, 2008). The BIAcore™ 3000 biosensor has also been used to measure interactions, in real time, between whole cells and small molecules. Clyne *et al.* (2004) examined the interaction of the bacterium *Helicobacter pylori* with a human single domain trefoil protein. *Helicobacter pylori* has been designated a class 1 carcinogen by the World Health Organisation. It colonises only humans and non-human primates naturally and causes gastritis and duodenal ulcer disease in humans as it colonises gastric tissue only. Using the BIAcore™ 3000 biosensor, Clyne *et al.* (2004) showed that *H. pylori* bound specifically, with high affinity to a TFF1-coated sensor chip. TFF1, a trefoil peptide, is expressed in a site-specific manner in gastrointestinal epithelium, specifically in foveolar epithelial surface cells and the interaction of *H. pylori* with TFF1 enables binding to gastric mucin (Clyne *et al.*, 2004). Kinoshita *et al.* (2007) investigated the ability of lactobacilli, isolated from human intestinal tissue, to adhere to human colonic mucin and found that all isolates of lactobacilli tested adhered to human colonic mucin in greater amounts than to bovine serum albumin. Nobbs *et al.* (2007) investigated the adhesion of wild type *Streptococcus gordonii* and sortase A mutants of *S. gordonii* to salivary agglutinin and found that overall adhesion of the mutant was reduced by 97% indicating that the gene encoding for sortase A is required for functional expression of an *S. gordonii* adhesin(s) for salivary agglutinin. To date, no studies using whole *Candida* cells as an analyte for the BIAcore biosensor have been performed.

The ECM is a network of carbohydrates, proteins and lipids which provides support and protection to cells and performs a number of other very specific functions (e.g. cell-cell recognition and communication) depending on the type of cell it is associated with (Garrett & Grisham, 1999). The skin for example has an extensive ECM which keeps it elastic and plays an important role in the healing process as it provides a framework for cell adhesion, encouraging growth. Bone has extensive ECM protein associations being primarily derived from collagen and mineral deposits, creating a very solid, secure ECM (<http://www.wisegeek.com/what-is-an-extracellular-matrix.htm>).

Collagen type I is the predominant component of the collagens and is also part of the organic component of dentine (Makihira *et al.*, 2002a & 2002b). This form of collagen may be denatured to gelatine by acids and enzymes, particularly in caries. *Candida albicans* has the ability to bind to both the native and denatured form of collagen type I and although the mechanism of adhesion is different in both cases

(Makihira *et al.*, 2002a), both forms of collagen type I may be a target for candidal adhesion, particularly where dentine is exposed as would be the case in dental caries.

Collagen type IV is found only in basement membrane, which is an extracellular structure containing both collagenous and non-collagenous glycoproteins (Timpl *et al.*, 1979; Hudson *et al.*, 1993). Collagen type IV has a monomeric structure formed by a triple helix of three α chains which assemble to form a sheet like network which constitutes the basic structure of basement membrane (Hudson *et al.*, 1993). This network can bind to laminin either directly or indirectly allowing the association of these two major constituents of basement membrane (Alonso *et al.*, 2001). There are six types of α chain which assemble to allow different isoforms of collagen type IV. The six chains differ with regard to tissue distribution. Some appear to be ubiquitous, whereas others are restricted to certain sites (Hudson *et al.*, 1993). The results of a study by Gao *et al.* (1999) indicated that type IV collagen expression changed from a brown linear staining along the basement membrane to thin and discontinued in candidal leukoplakia. This type of change may be related to basement membrane destruction by *Candida*. It is probable that the *Candida* cells must adhere to collagen type IV before they can degrade it to such an extent and as such it is possible that collagen type IV may be a target for candidal adhesion, and may initiate systemic infection as these proteins are found throughout the body.

Laminin is a multifunctional, non-collagenous glycoprotein of high molecular weight which is made up of two components that are joined by disulphide bonds. It is a major component of basement membrane in normal tissue (Timpl *et al.*, 1979; Bouchara *et al.*, 1990) and although laminin is present in most tissues, it represents only a small portion of the total protein in the body. As laminin can bind to many of the components of basement membrane, including collagen type IV and itself, it is thought to have a role in organising and possibly initiating the formation of basement membrane. Laminin has potent biological activity in promoting nerve regeneration *in vivo* and increasing the survival of nerve grafts in the brain. Laminin also promotes cell migration and as such it may be useful in wound repair and prevention of disease (Malinda & Kleinman, 1996). Tumour cells can bind to laminin receptors on the basement membrane and subsequently produce metalloproteinases, which fragment and degrade the membrane and allow for tumour invasion (Souza *et al.*, 2007). Souza *et al.* (2007) concluded that there was decreased immunohistochemical expression of laminin in the basement membrane of high malignancy grade oral squamous cell carcinomas and this structural

change may affect basement membrane dynamism and favour tumour invasion. Such degradation of laminin may also favour candidal adhesion and invasion. *Candida albicans* possesses saturable and specific binding sites for laminin on the surface of germ tubes whereas non-germinating yeast, mycelial septa and 'mother cells' of *C. albicans* hyphae do not interact with soluble laminin (Bouchara *et al.*, 1990). As laminin is also found in basement membrane, it could be a source of adhesion for *Candida* hyphal cells during systemic infection.

Fibronectin is a polymorphic, non-collagenous glycoprotein of connective tissue. It is a major constituent of fibroblastic cells and is abundant in basement membrane and around smooth muscle cells and striated muscle fibres. Fibronectin is present in human plasma (Stenman & Vaheri, 1978) and interacts with cells promoting cell adhesion. Fibronectin can bind to itself as well as other proteins including fibrin and native and denatured collagen. The binding of fibrin to fibrinogen appears to direct fibrinogen into forming blood clots which are associated with wound healing. The main functional form of fibronectin is the insoluble protein which interacts with cells promoting cell adhesion (Ruoslahti, 1988). Adhesion of *Candida* to soluble fibronectin may be associated with *Candida* dissemination and systemic infection.

Vitronectin is a glycoprotein which is found circulating in serum and also in tissue. Unlike fibronectin, it binds in greater quantities to native collagen type I compared to gelatine (Ruoslahti, 1988; Limper & Standing, 1994). Vitronectin, like fibronectin, is one of the major cell adhesion proteins in plasma and although these two proteins have the same amino acid cell recognition sequence, they are structurally and immunologically distinct (Preissner, 1991). Vitronectin increases the adherence of *C. albicans* to cultured macrophages and as such could participate in the clearance of *C. albicans* during infection (Limper & Standing, 1994) however this could also cause systemic *Candida* infection in the immunocompromised host.

Proline Rich Peptides (PRPs) are synthesised in the acinar cells of the parotid glands which are located within a defined capsule in the cheek. There are three subsets of PRPs ranging in molecular weight from 6 to 36 kDa. PRPs are believed to have several roles in the oral cavity. The PRPs are present in the dental pellicle and have a high affinity for calcium ions and as such are believed to prevent the unwanted deposition of calcium on the tooth surface. PRPs have been shown to bind to tannins and large amounts of ingested tannins are associated with carcinomas. As a result, PRPs may therefore be the first line of defence against such dietary tannins. PRPs are also

known to play an important role in mediating bacterial adherence to oral surfaces (Price, 2004). If they have the ability to mediate bacterial adhesion to oral surfaces, it is possible they have an affinity for *Candida* species also. For this reason PRPs were examined to assess their adhesive abilities to *Candida*.

ECM proteins share a common cell attachment site consisting of the three amino acids arginine, glycine and asparagine (RGD). This RGD site within the ECM molecules is found within fibronectin (Nègre *et al.*, 1994), vitronectin (Limper & Standing, 1994), gelatine (Makihira *et al.*, 2002a & 2002b) and laminin (Ruoslahti, 1988) and may mediate the adhesion of *Candida* to ECM proteins.

The aim of this part of the present study was to elucidate the relative adhesion of a wide range of well characterised strains and isolates of both *C. dubliniensis* and *C. albicans* to ECM proteins.

5.2 MATERIALS AND METHODS

Binding of *C. dubliniensis* and *C. albicans* to human collagen type I (Duance, 1990), human collagen type IV (Duance, 1990), bovine fibronectin (Sigma), human laminin (Sigma), human vitronectin (Sigma) and PRPs isolated from human parotid saliva (Price, 2004) was measured using a BIAcore™ 3000 biosensor (BIAcore). Sensor chips (BIAcore) with short dextran matrices were selected (such as the CM3 sensor chip) as these sensor chips are known to be more beneficial when working with larger analytes such as whole cells. Each sensor chip housed 4 flow cells (FCs); FC1 and FC3 were chosen as control surfaces and FC2 and FC4 provided the test surface. The ligands used were the six ECM proteins described above and were individually coupled to the surface of the sensor chip. A single sensor chip therefore allowed two separate ligands to be covalently coupled to FC2 and FC4 with equivalent control surfaces (FC1 and FC3 respectively). During ligand immobilisation the control FCs were treated in the same manner as the test FCs with the exception of the ligand injection. The collagens used in this experiment were a gift from Prof. Vic Duance of the Connective Tissue Biology Laboratories, Cardiff School of Biosciences, Biomedical Building, Cardiff, UK. The PRPs used in this experiment had previously been isolated from human parotid saliva and coupled to a sensor chip by Dr. Claire Price, formerly of The Department of

Tissue Engineering and Reporative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK.

5.2.1 Immobilisation of a ligand to a CM3 sensor chip

5.2.1.1 Determination of the optimum pH at which protein ligands are immobilised to a CM3 sensor chip

The approach chosen to immobilise ligands onto the surface of the CM3 sensor chip involved amine coupling using reactive esters which covalently bind the ligand used to the dextran matrix of the CM3 sensor chip. Preliminary experiments involved identifying the appropriate pH buffer which would allow electrostatic interaction of the positive charges on the ligand with the negative charges on the dextran matrix surface of the CM3 sensor chip at pH values below the ligand pI. This process is termed pH scouting.

Ligands were diluted to a range of pH concentrations in 10 mM sodium acetate buffers (BIAcore). The ligands were injected over the surface of the sensor chip and the interaction was determined based on the change in resonance units (RUs). RUs are defined as arbitrary units that reflect the changes in refractive index, a function of mass, at the surface of the CM3 sensor chip. These changes occur as a result of the binding of solution-phase analyte to (or its release from) a surface-bound ligand on the sensor chip. RUs are arbitrarily defined as $1/3000$ of a degree in the BIAcore™ 3000 biosensor. Fig. 5.1 shows the result generated when pH scouting for the ligand bovine fibronectin was performed. This graph is representative of the results observed when pH scouting was carried out on all other ligands (data not shown). pH scouting for bovine fibronectin was carried out in 10 mM sodium acetate buffers at four different pH values. Fig. 5.1 showed that the greatest electrostatic interaction was observed at pH 4. This result determined the pH of the buffer that bovine fibronectin would be re-suspended in for immobilisation to the CM3 sensor chip.

The collagens were dissolved to 1 mg ml^{-1} in 0.5 M acetic acid (Fisher Scientific) before being diluted to various ligand concentrations in a pH range of 10 mM sodium acetate buffers (Table 5.1). Immobilisation of the ECM protein ligands onto the CM3 sensor chip was performed at various pH values as detailed in Table 5.1. The pH at which immobilisation of PRPs took place is unknown as the sensor chip on

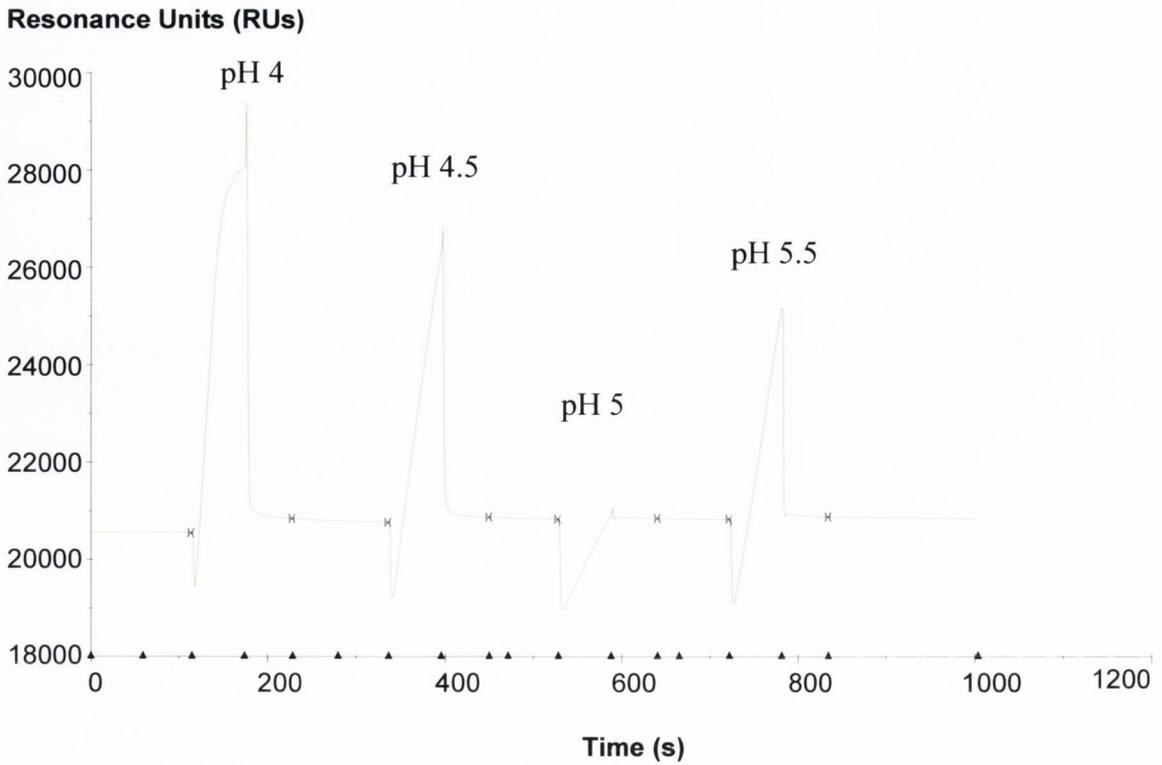


Figure 5.1. Determination of electrostatic interactions between bovine fibronectin and the surface matrix of a CM3 sensor chip, at a range of pH concentrations from 4 to 5.5 when diluted in 10 mM sodium acetate buffer. The greatest electrostatic interaction between bovine fibronectin and the surface matrix of a CM3 sensor chip was observed at pH 4. This result determined the pH of the buffer that bovine fibronectin would be re-suspended in for immobilisation to the sensor chip.

Table 5.1. Immobilisation conditions of extracellular matrix ligands for immobilisation to a CM3 sensor chip

| Protein | Ligand concentration ($\mu\text{g ml}^{-1}$) | Immobilisation pH | RUs adhering to the sensor chip | Protein bound to sensor chip (ng mm^{-2}) |
|------------------------|--|--------------------------|--|--|
| Human collagen type I | 100 | 4 | 1019.5 | 1.02 |
| Human collagen type IV | 40 | 5 | 1988.9 | 1.99 |
| Bovine fibronectin | 40 | 4 | 2229.2 | 2.23 |
| Human laminin | 20 | 4 | 2526.6 | 2.5 |
| PRPs from human saliva | 100 | Unknown | 1993.9 | 1.99 |
| Human vitronectin | 40 | 4 | 2129.3 | 2.1 |

which the PRPs from human parotid saliva were immobilised was provided as a gift by Dr. David Williams of The Department of Tissue Engineering and Reporative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK and Dr. Claire Price, formerly of The Department of Tissue Engineering and Reporative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK, and this information was unavailable.

5.2.1.2 Immobilisation via amine coupling

All reagents used for amine coupling of the chosen ligand to the CM3 sensor chips were supplied in an amine coupling kit (BIAcore). The carboxymethyl groups on the dextran matrix surface of the CM3 sensor chip were chemically activated by injecting a mixture of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) at a continuous flow rate of $5 \mu\text{l min}^{-1}$ (Fig. 5.2 (a)) to generate active ester groups. A $35\text{-}\mu\text{l}$ volume of NHS/EDC mix was injected over 7 min, followed by an injection of the protein ligand in the appropriate pH buffer (Fig. 5.2 (b)) as determined previously by pH scouting experiments. A targeted immobilisation level of 2,500 RUs was pre-selected using the BIAcore Wizard software (BIAcore) for bovine fibronectin, human laminin and human vitronectin. An immobilisation level of 2,000 RUs was aimed for in the case of human collagen type IV. Human collagen type I was immobilised using a timed injection over 4 min with a flow rate of $10 \mu\text{l min}^{-1}$. The procedure for immobilising PRPs from human parotid saliva to the CM3 sensor chip was unknown as this sensor chip was provided as a gift by Dr. David Williams of The Department of Tissue Engineering and Reporative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK and Dr. Claire Price, formerly of The Department of Tissue Engineering and Reporative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK, and this information was unavailable.

5.2.1.3 Deactivation

Once the ligand was covalently bound to the active ester groups on the dextran matrix surface of the CM3 sensor chip, deactivation of excess reactive groups on the surface of the CM3 sensor chip involved a 7 min pulse injection of 1 M ethanolamine hydrochloride pH 8.5 (amine coupling kit; Fig. 5.2 (c)).

Fig. 5.2 demonstrates a typical sensogram showing immobilisation of bovine fibronectin to a CM3 sensor chip using amine coupling. Interaction analysis determines if there is an electrostatic interaction between the ligand and the CM3 sensor chip, which is seen by a sharp peak in the sensogram. The surface of the sensor chip is then regenerated, where by any non-covalently bound ligand is removed. Carboxymethyl groups on the matrix surface of the CM3 sensor chip are chemically activated to generate active ester groups. This is followed by injection of the protein ligand in the appropriate pH buffer. The ligand binds covalently to the reactive ester groups on the surface of the sensor chip. Once the ligand has been coupled to the active ester groups on the dextran chip, deactivation of excess reactive groups on the surface of the sensor chip is carried out. The ligand is now covalently bound to the chip which can be seen by the higher RU value after immobilisation has been completed.

All protein ligands were successfully immobilised to the surface of CM3 sensor chips (data for other ligands not shown). The amount of protein ligand bound to each chip was taken as the difference in RUs between start and end of the immobilisation process and the results for each ECM protein ligand are shown in Table 5.1. Collagen type I was bound by timed injection, as there were not enough units to bind at 2,000 RUs.

5.2.2 Analyte binding to the immobilised ligand

In this study, the analyte used was whole *Candida* cells. *Candida* isolates (Tables 5.2 and 5.3) were cultured separately in 10 ml YEPD overnight (~16 h) in a static incubator at 30°C in 50 ml glass vials. After harvesting by centrifugation in a Denley BS400 centrifuge which was fitted with swing out rotor BS4402 (Denley Instruments Ltd., Billingshurst, UK) at $1,125 \times g$ for 10 min at room temperature, the cells were washed twice in HBS-EP buffer, and the yeast suspensions were adjusted to a concentration of 1×10^8 cells ml⁻¹ in HBS-EP buffer. The *Candida* suspensions were

Resonance Units (RUs)

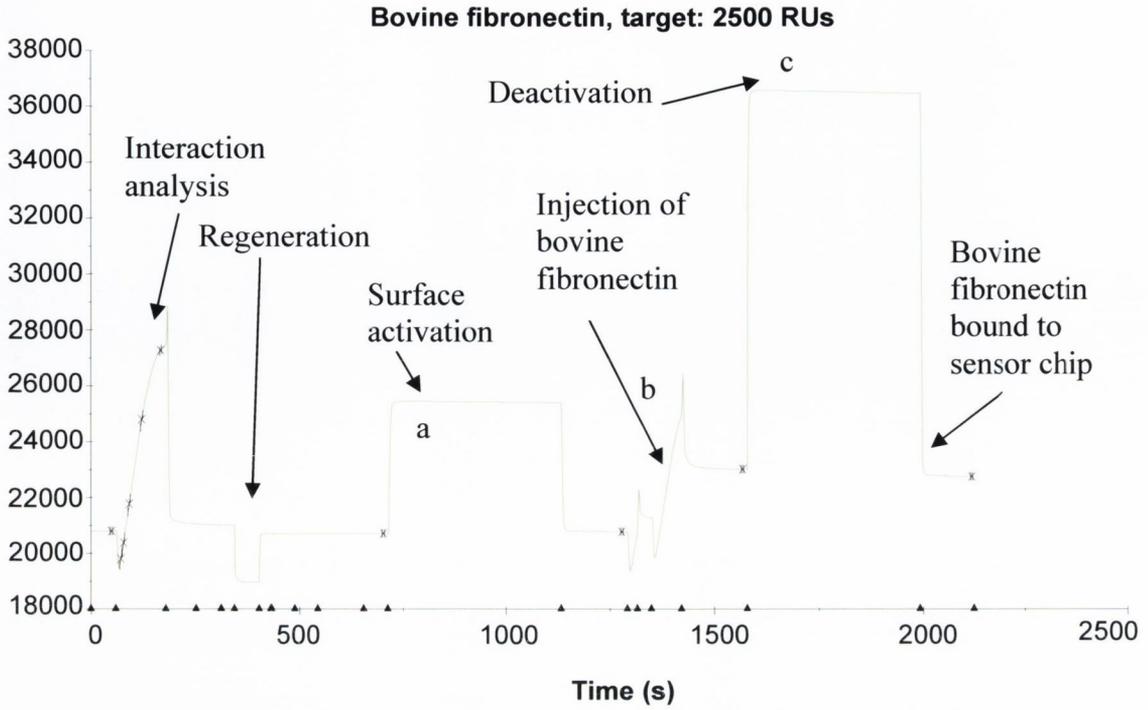


Figure 5.2. Sensogram demonstrating immobilisation of bovine fibronectin to a CM3 sensor chip. This sensogram demonstrates the immobilisation process for an extracellular matrix ligand to a CM3 sensor chip.

Table 5.2. *C. dubliniensis* strains and isolates used in the BIAcore™ 3000 assay

| Strain/isolate | Genotype ^a | Source/Reference |
|--------------------|-----------------------|--------------------------------|
| CD36* ¹ | 1 | Sullivan <i>et al.</i> , 1995 |
| CD33 | 1 | Sullivan <i>et al.</i> , 1995 |
| Co4 | 1 | Boerlin <i>et al.</i> , 1995 |
| CD539 | 2 | Pinjon <i>et al.</i> , 1998 |
| CD75004 | 2 | Pinjon <i>et al.</i> , 1998 |
| P6785 | 3 | Polacheck <i>et al.</i> , 2000 |
| CD519 ³ | 3 | Gee <i>et al.</i> , 2002 |
| Eg200 | 4 | Al Mosaid <i>et al.</i> , 2005 |
| P7718 ⁴ | 4 | Gee <i>et al.</i> , 2002 |

^aThe genotype of each isolate was assigned based on differences observed in the nucleotide sequence of the ITS region of the rRNA gene cluster (Gee *et al.*, 2002; Al Mosaid *et al.*, 2005)

*Type strain (i.e. the nomenclatural type of a species) for *C. dubliniensis*

¹*C. dubliniensis* genotype 1 reference strain for *C. dubliniensis* genotype PCR

³*C. dubliniensis* genotype 3 reference strain for *C. dubliniensis* genotype PCR

⁴*C. dubliniensis* genotype 4 reference strain for *C. dubliniensis* genotype PCR

The *C. dubliniensis* genotype 2 reference strain for *C. dubliniensis* genotype PCR (Can6) was not used in this part of the present study

Table 5.3. *C. albicans* strains and isolates used in the BIAcore™ 3000 assay

| Strain/isolate | Source/Reference |
|-----------------------|----------------------------------|
| SC5314* | Gillum <i>et al.</i> , 1984 |
| 132A | Gallagher <i>et al.</i> , 1992 |
| NCPF3153 ^a | UK reference strain ^a |
| m182CA | Pinjon, 2003 |
| JM1 | Pinjon, 2003 |
| CA400 ⁺ | This study |
| CA401 ⁺ | This study |
| NCPF8317 ^a | UK reference strain ^a |
| NCPF8324 ^a | UK reference strain ^a |
| GRI681 ^b | McCourtie & Douglas, 1984 |
| GDH2346 ^c | McCourtie & Douglas, 1984 |
| 6CA ⁺ | This study |

^aFrom the National Collection of Pathogenic Fungi (NCPF), Kingsdown, Bristol, UK

^bStored in the National Collection of Yeast Cultures (NCYC), Colney, Norwich, UK as NCYC 1472

^cStored in the NCYC as NCYC 1467

*Type strain (i.e. the nomenclatural type of a species) for *C. albicans*

⁺Oral isolates from the laboratory collection of the Microbiology Research Unit, Dublin Dental School and Hospital

injected over both control and test FCs at a rate of $5 \mu\text{l min}^{-1}$ for 4 min. After each injection, regeneration of the sensor chip was achieved by injection ($5 \mu\text{l min}^{-1}$ for 2 min) of 50 mM sodium hydroxide (Fisher Scientific) to remove the bound *Candida*. All *Candida* isolates were tested on a minimum of two separate occasions.

5.2.3 Proline rich peptides

In a study carried out by Dr. Claire Price during her Ph. D. at The Department of Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK, PRPs were isolated from human parotid saliva by gel filtration and resolved by electrophoresis. Samples of these PRPs immobilised to a CM3 sensor chip were provided for use in the present study. Confirmation of identity of the PRPs was by N-terminal amino acid sequence analysis (Price, 2004).

5.2.4 Data analysis

pH scouting and immobilisation data for each ligand from the BIAcore assay were recorded using BIAcore™ 3000 control software, version 3.2 (BIAcore). Binding results for each analyte tested to each ligand were also recorded using this software. This data was further analysed and graphically depicted using the BIAevaluation software, version 3.2 (BIAcore). Final overall binding data was correlated and graphically depicted using the BIAevaluation software, version 3.2 and correlated, analysed and graphically depicted using Microsoft Excel and GraphPad Prism. Data was analysed within GraphPad Prism using the mean, SEM and one-way ANOVA with Tukey's multiple comparison post test.

5.3 RESULTS

5.3.1 Comparative adhesion of *C. dubliniensis* and *C. albicans* to six ECM proteins

The molecular adhesion of *C. dubliniensis* and *C. albicans* was investigated by examining the relative adhesion of well characterised strains and isolates of *C. dubliniensis* (n = 9) and *C. albicans* (n = 12) to six ECM proteins. Results were recorded using a BIAcore™ 3000 biosensor and related software. Each isolate was tested on a minimum of two separate occasions. In this section not all the results are shown, but an example that is representative of each result is given. Fig. 5.3 shows a typical sensogram which showed the adhesion of *C. dubliniensis* CD36 and *C. albicans* GRI681 yeast cells to bovine fibronectin. The results showed that *C. dubliniensis* CD36 bound more strongly to bovine fibronectin than *C. albicans* GRI681. This result showed the adhesion of both isolates to bovine fibronectin on a single occasion. This sensogram is representative of the results of the adhesion of all isolates of both species to all six ECM proteins (data not shown). This experiment was carried out using the same isolate on at least two separate occasions, however replicates for each isolate were not reproducible (Fig. 5.4; not all data shown).

5.3.2 Adhesion of *Candida* cells grown overnight (~16 h) at 30°C in YEPD to each of six ECM proteins

Fig. 5.5 to Fig. 5.7 show the mean RUs indicating adhesion of each isolate of *C. dubliniensis* and *C. albicans* tested \pm SEM to each of the six ECM proteins tested. The results showed that *C. albicans* adhered poorly to collagen type I with some isolates failing to adhere (Fig. 5.5a). *Candida dubliniensis* genotype 1 isolates appear to have adhered to collagen type IV (Fig. 5.5b), fibronectin (Fig. 5.6a) and PRPs (Fig. 5.7a) in greater amounts than the other genotypes of *C. dubliniensis* and of *C. albicans*. The results also showed that there was a wide range in the adhesion of the isolates of *C. albicans* to laminin (Fig. 5.6b) and that all isolates of *C. dubliniensis* appear to have adhered in greater amounts to vitronectin than *C. albicans* (Fig. 5.7b).

For *C. dubliniensis*, the results from each isolate within a particular genotype were averaged to obtain the mean RU value indicating adhesion for each genotype.

Resonance Units (RUs)

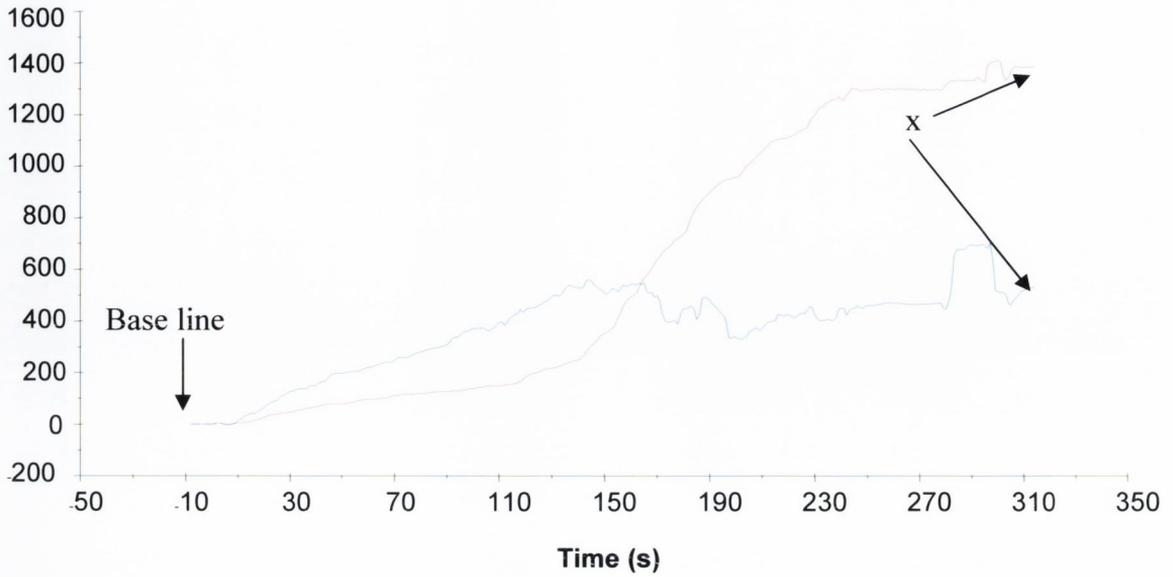


Figure 5.3. Adhesion of *C. dubliniensis* CD36 and *C. albicans* GRI681 to bovine fibronectin. The sensogram shows the adhesion of *C. dubliniensis* CD36 and *C. albicans* GRI681 to bovine fibronectin. The base line is the signal which is given before there is any adhesion to the ligand. *Candida dubliniensis* CD36 is represented by red and *C. albicans* GRI681 is represented by blue. The end point of the injection, at 315 s is marked as x, and the RUs recorded at this point are taken as the binding result.

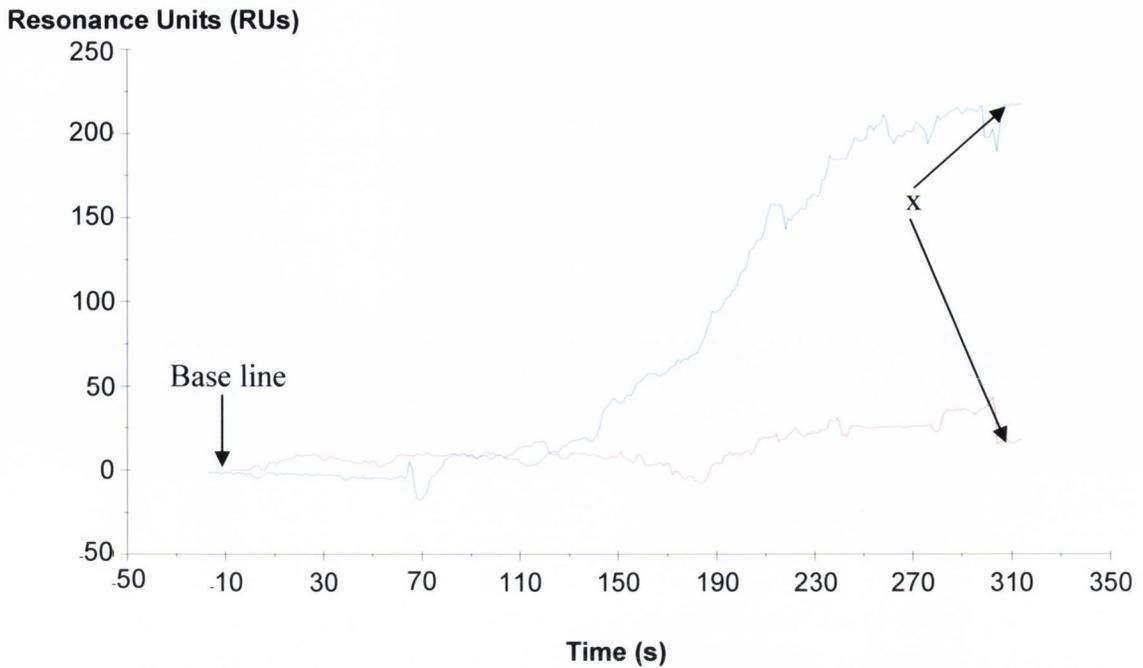


Figure 5.4. Adhesion of two replicates of *C. dubliniensis* Cm1 to bovine fibronectin.

The sensogram shows the adhesion of two replicates of *C. dubliniensis* Cm1 to bovine fibronectin. The base line is the signal which is given before there is any adhesion to the ligand. The first replicate is represented by red and the second replicate is represented by blue. The end point of the injection, at 315 s is marked as x, and the RUs recorded at this point are taken as the binding result.

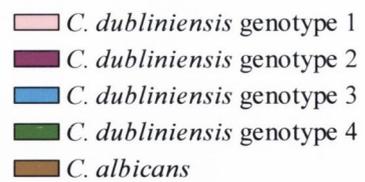
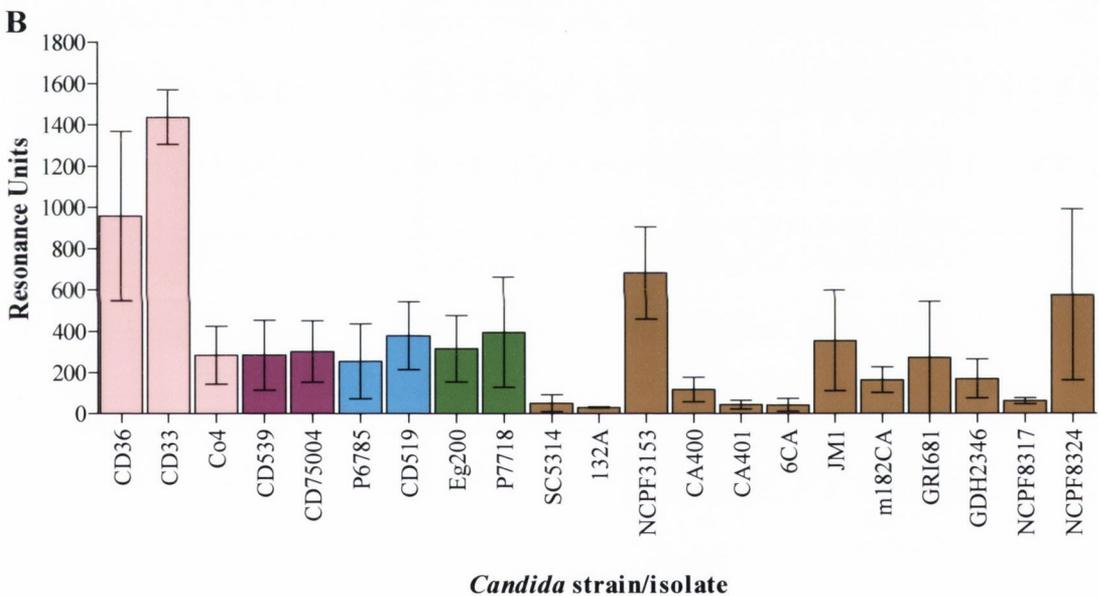
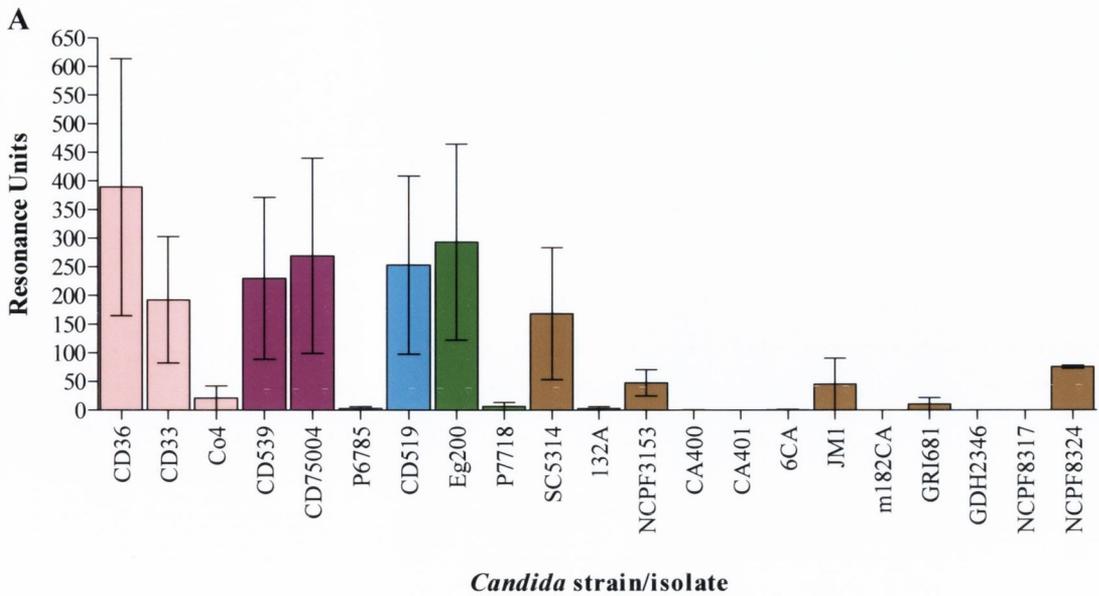


Figure 5.5. Resonance units indicating adhesion of *C. dubliniensis* and *C. albicans* to collagen type I (A) and collagen type IV (B) when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results show the average adhesion of each isolate of *C. dubliniensis* and *C. albicans* tested \pm SEM to collagen type I (A) and collagen type IV (B). Each isolate was tested on at least two separate occasions.

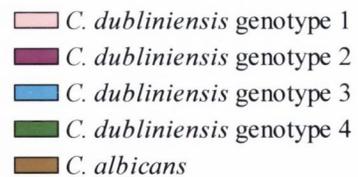
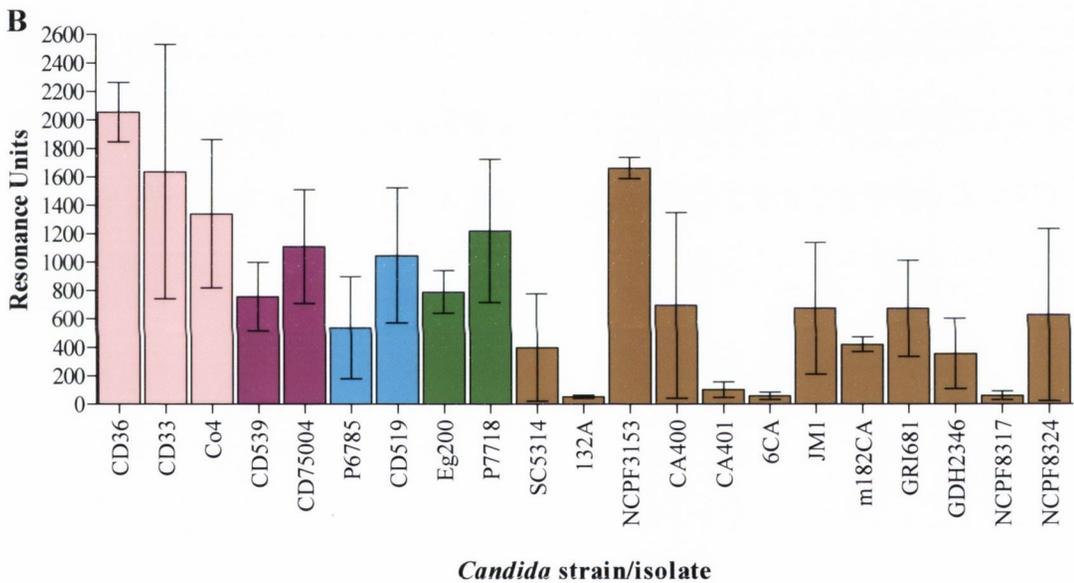
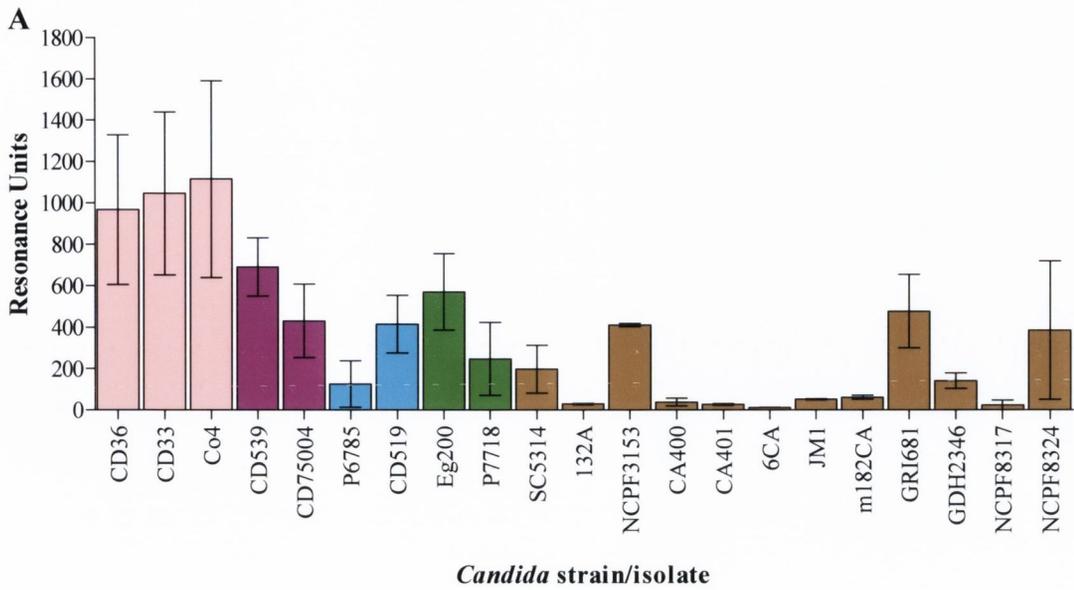


Figure 5.6. Resonance units indicating adhesion of *C. dubliniensis* and *C. albicans* to fibronectin (A) and laminin (B) when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results show the average adhesion of each isolate of *C. dubliniensis* and *C. albicans* tested \pm SEM to fibronectin (A) and laminin (B). Each isolate was tested on at least two separate occasions.

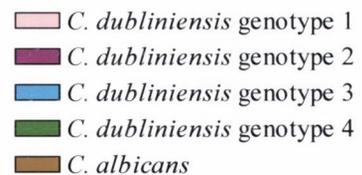
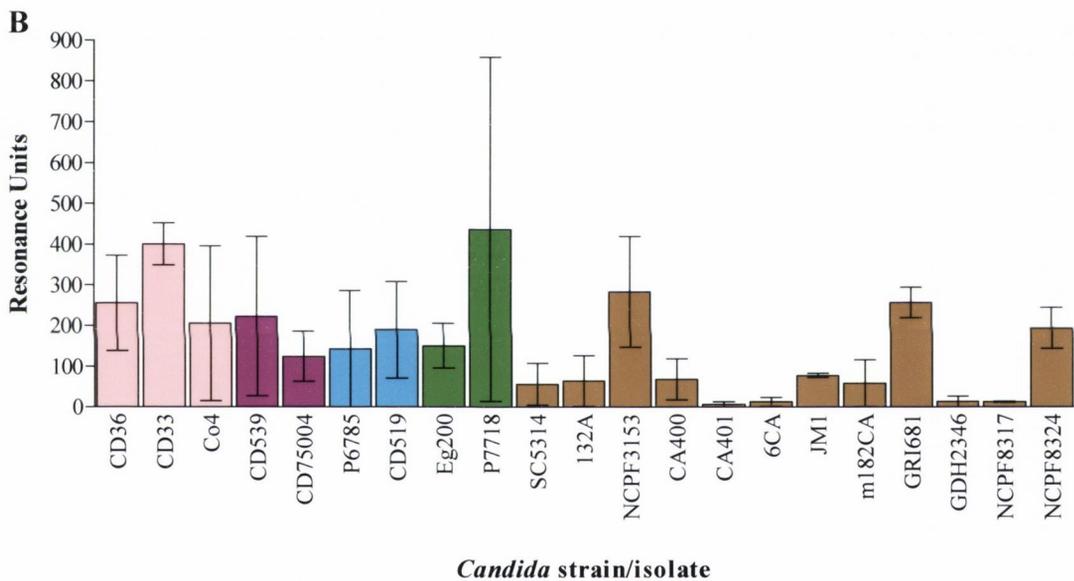
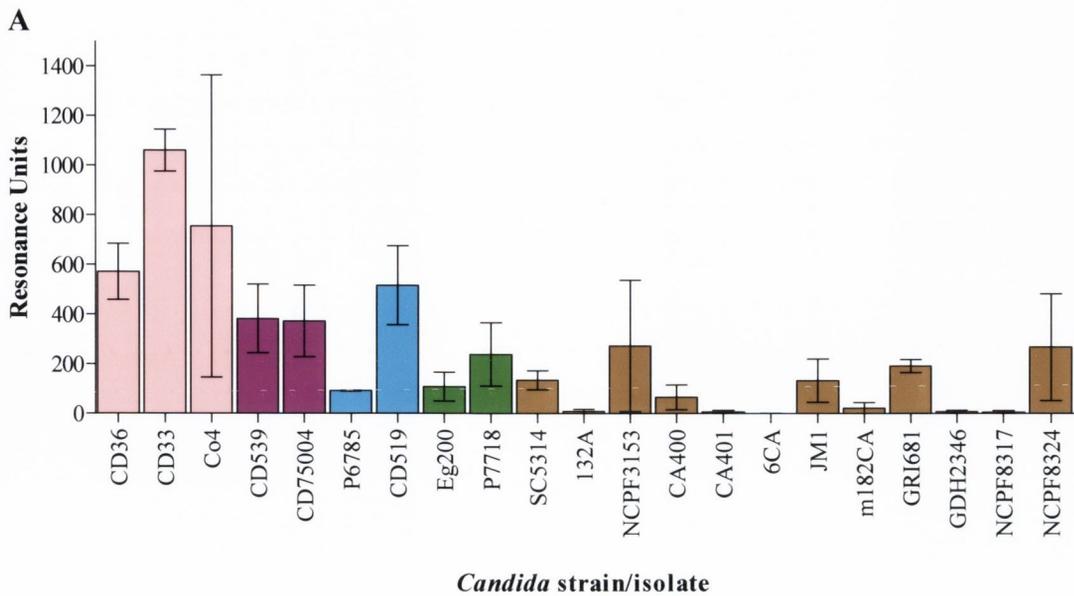


Figure 5.7. Resonance units indicating adhesion of *C. dubliniensis* and *C. albicans* to PRPs (A) and vitronectin (B) when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results show the average adhesion of each isolate of *C. dubliniensis* and *C. albicans* tested \pm SEM to PRPs (A) and vitronectin (B). Each isolate was tested on at least two separate occasions.

Similarly, the results from each isolate within *C. albicans* were averaged to obtain a mean RU value indicating adhesion for *C. albicans* (Figs. 5.8 to 5.13). One way ANOVA with Tukey's multiple comparison post test was performed and showed that there was no significant difference between the adhesion of *C. albicans* and each genotype of *C. dubliniensis* to collagen type I ($P > 0.05$; Fig. 5.8) or to vitronectin ($P > 0.05$; Fig. 5.13). *Candida dubliniensis* genotype 1 isolates adhered in significantly greater amounts to collagen type IV than *C. dubliniensis* genotype 2 and 3 ($P < 0.05$) and *C. albicans* ($P < 0.001$; Fig. 5.9). *Candida dubliniensis* genotype 1 isolates adhered in significantly greater amounts to fibronectin than *C. dubliniensis* genotype 2 and 4 ($P < 0.05$), *C. dubliniensis* genotype 3 ($P < 0.01$) and *C. albicans* ($P < 0.001$). *Candida dubliniensis* genotype 2 isolates adhered to fibronectin in significantly greater amounts than *C. albicans* ($P < 0.05$; Fig. 5.10). The only difference between adhesion of *C. dubliniensis* and *C. albicans* to laminin was between *C. dubliniensis* genotype 1 and *C. albicans* ($P < 0.001$) where *C. dubliniensis* genotype 1 adhered to laminin in significantly greater amounts than *C. albicans* (Fig. 5.11). *Candida dubliniensis* genotype 1 adhered to PRPs in significantly greater amounts than *C. dubliniensis* genotype 2 ($P < 0.05$), *C. dubliniensis* genotype 4 and *C. albicans* ($P < 0.001$). *Candida dubliniensis* genotype 2 also adhered to PRPs in significantly greater amounts than *C. albicans* ($P < 0.05$; Fig. 5.12).

5.3.3 Mean adhesion of *Candida* to all six ECM proteins

The mean RUs indicating adhesion of the genotypes of *C. dubliniensis* and of *C. albicans* to all six ECM proteins were shown (Fig. 5.14). The results showed that *C. dubliniensis* genotype 1 adhered to all six ECM proteins in significantly greater amounts than the other genotypes of *C. dubliniensis* and *C. albicans* ($P < 0.001$; Fig. 5.14). *Candida dubliniensis* genotype 2 adhered to ECM proteins in significantly greater amounts than *C. albicans* ($P < 0.05$; Fig. 5.14). These results showed that *C. dubliniensis* genotype 1 isolates were the most adherent of the *Candida* isolates tested to the ECM.

5.3.4 Mean adhesion of all isolates of *Candida* tested to each of six ECM proteins

The mean adhesion of the 21 isolates of *Candida* tested to each of the six individual proteins was shown (Fig. 5.15). The results showed that all isolates of *Candida* tested adhered to laminin in significantly greater amounts than to the other five ECM proteins ($P < 0.001$; Fig. 5.15). All isolates of *Candida* tested adhered to fibronectin in significantly greater amounts than to collagen type I and vitronectin ($P < 0.05$; Fig. 5.15).

5.4 DISCUSSION

While there have been numerous studies examining the adhesion of *Candida* to a variety of cell surfaces including BECs, RHE and monolayers of human epithelial cells, relatively little is known about the molecular basis of candidal adhesion. Some advantages of using the BIAcore system to assess candidal adhesion include the ability to measure the adhesion of *Candida* cells to small molecules such as proteins. This method may assist in the elucidation of the molecular pathways involved in adhesion. It is also a more objective method of measuring adhesion than, for example, the BEC adhesion assay and as such reduces any operator error that may occur.

Small changes in yeast binding numbers probably generate large RU differences in the BIAcore systems as RUs are arbitrary values and not the number of cells adhering. The uneven nature of the binding curves is probably due to random binding and release events of *Candida* cells and the potential for adhesion to the sensor chip of cell aggregates of *Candida*. The adhesion of the cells to the protein ligands can not be measured as pure 1:1 stoichiometry such as in protein-protein binding studies.

5.4.1 Relative adhesion of *C. dubliniensis* and *C. albicans* to ECM proteins

In an attempt to identify whether *C. dubliniensis* and *C. albicans* differ in their ability to adhere to specific ligands as they do to whole cells (chapters 3 and 4 of the present study), the BIAcore™ 3000 biosensor was used to assess the molecular adhesion of these microorganisms to six ECM proteins. The results showed that all *C. dubliniensis* isolates adhered to all ECM ligands tested, however, of the *C. albicans*

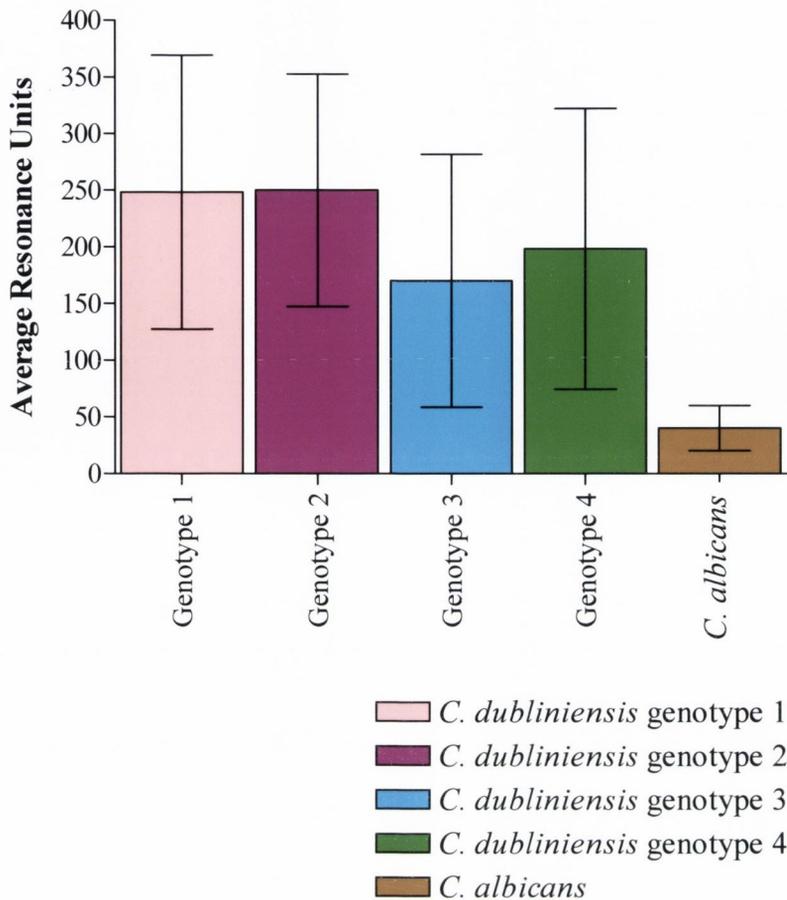


Figure 5.8. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to collagen type I when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to collagen type I on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to collagen type I across each genotype of *C. dubliniensis* and of *C. albicans*.

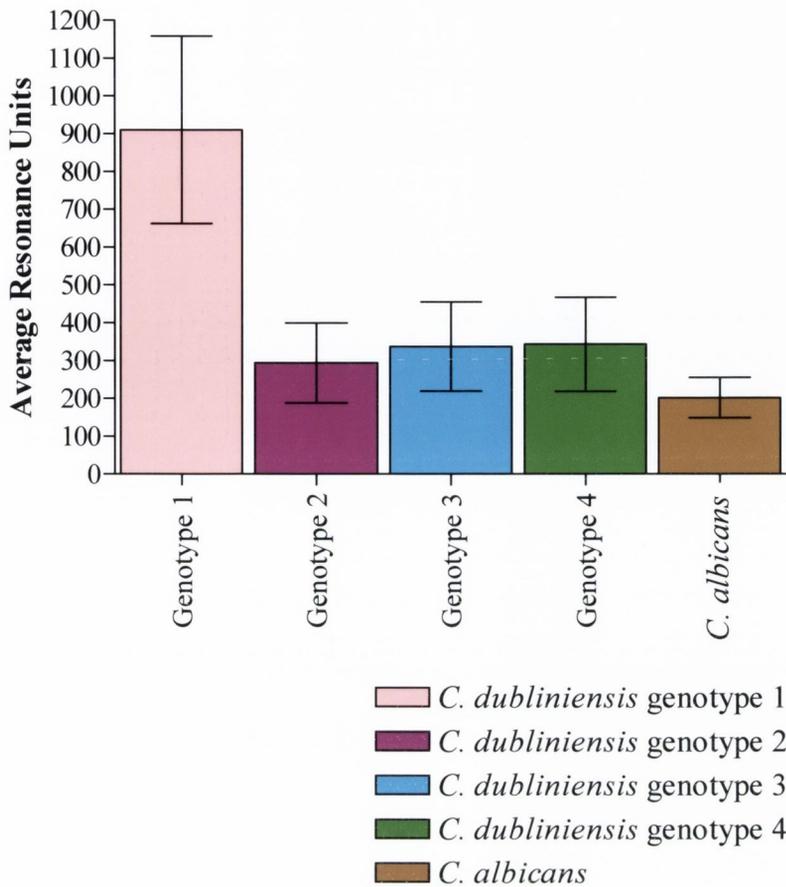


Figure 5.9. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to collagen type IV when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to collagen type IV on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to collagen type IV across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 isolates adhered in significantly greater amounts to collagen type IV than *C. dubliniensis* genotypes 2 and 3 ($P < 0.05$) and *C. albicans* ($P < 0.001$).

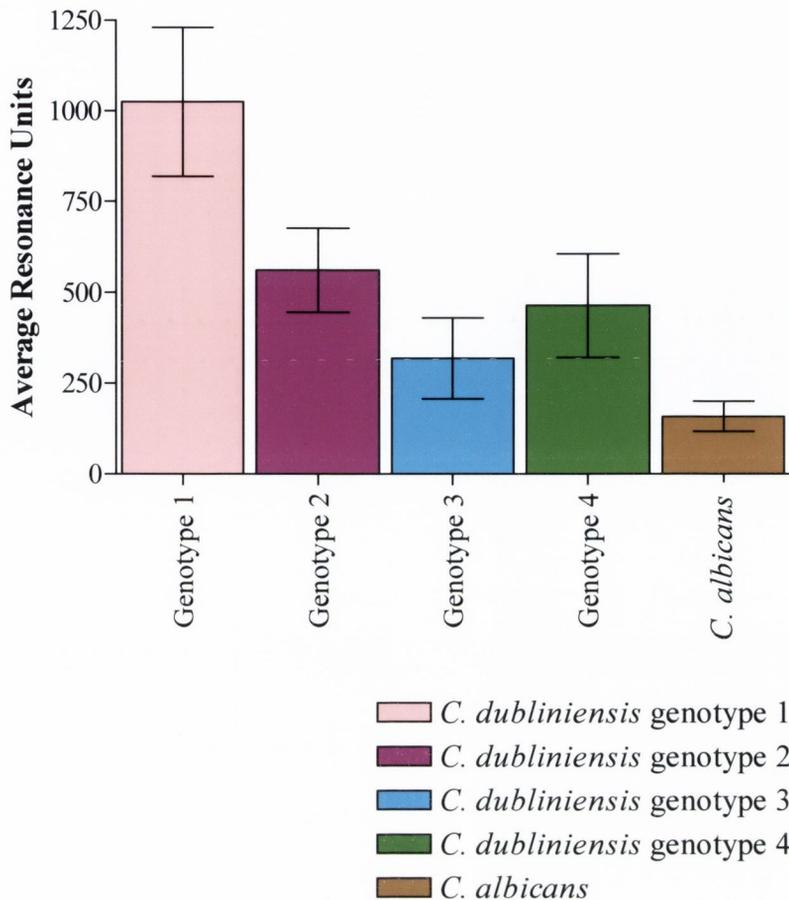


Figure 5.10. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to fibronectin when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to fibronectin on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to fibronectin across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 isolates adhered to fibronectin in significantly greater amounts than *C. dubliniensis* genotype 2 and 4 ($P < 0.05$), *C. dubliniensis* genotype 3 ($P < 0.01$) and *C. albicans* ($P < 0.001$).

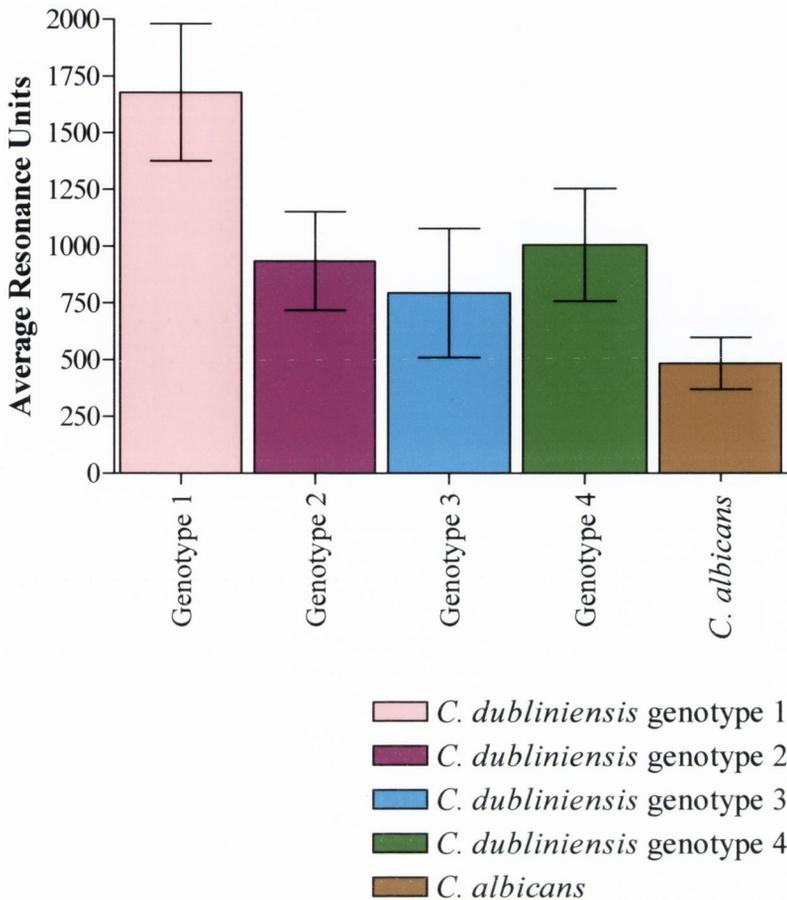


Figure 5.11. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to laminin when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to laminin on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to laminin across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 isolates adhered to laminin in greater amounts than *C. albicans* ($P < 0.001$).

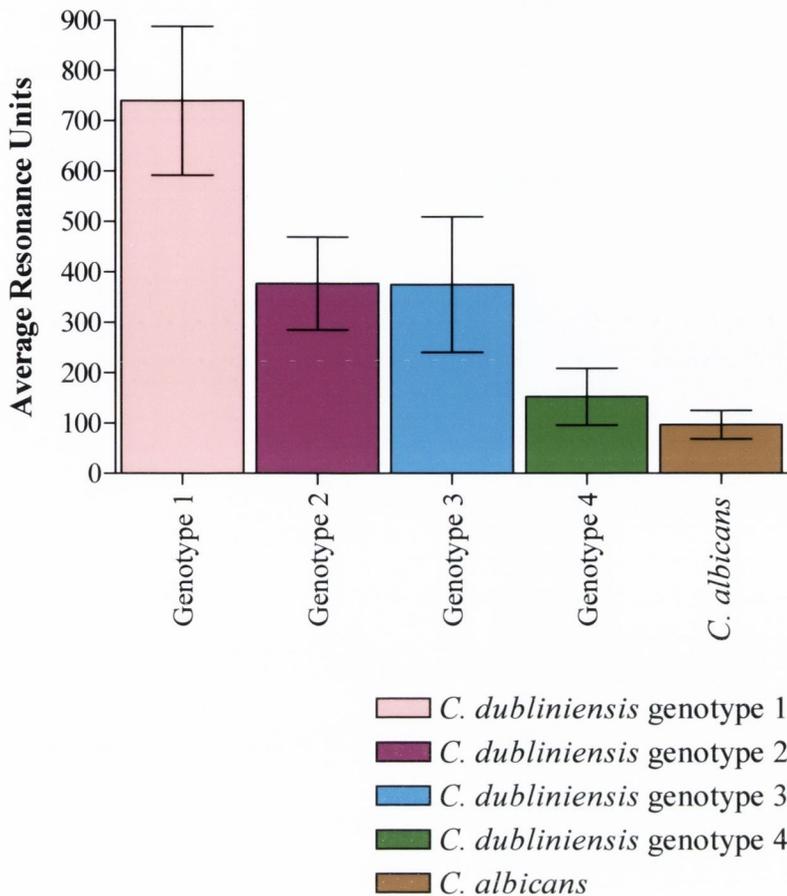


Figure 5.12. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to PRPs when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to PRPs on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to PRPs across each genotype of *C. dubliniensis* and of *C. albicans*. *Candida dubliniensis* genotype 1 isolates adhered to PRPs in significantly greater amounts than *C. dubliniensis* genotype 2 ($P < 0.05$), *C. dubliniensis* genotype 4 and *C. albicans* ($P < 0.001$). *Candida dubliniensis* genotype 2 isolates adhered to PRPs in significantly greater numbers than *C. albicans* ($P < 0.05$).

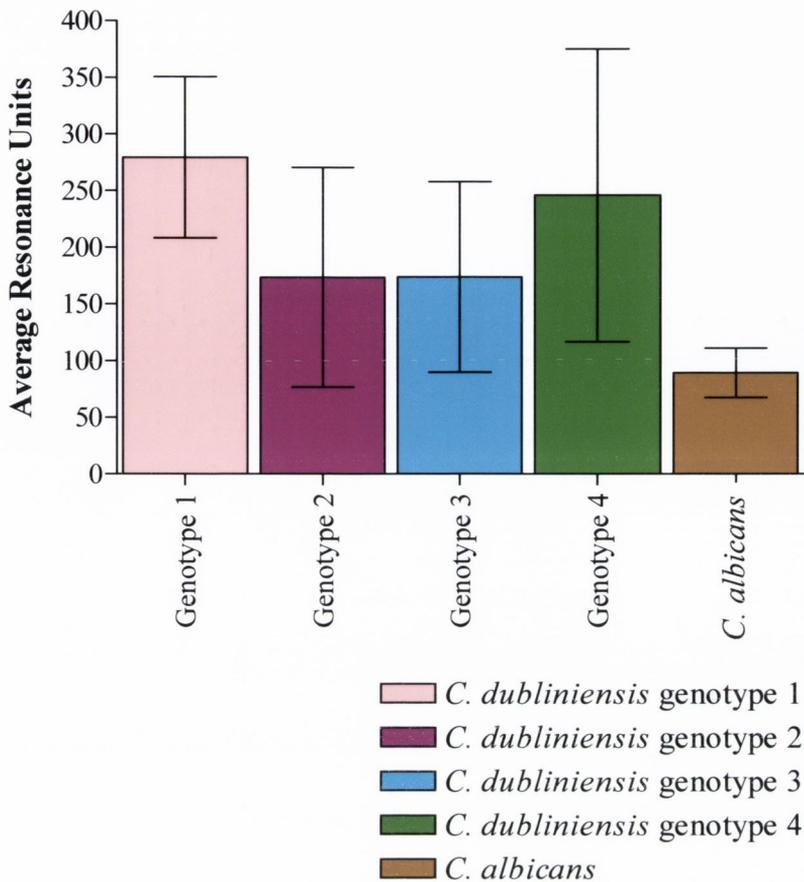


Figure 5.13. Mean resonance unit value indicating adhesion of *C. dubliniensis* and *C. albicans* to vitronectin when the *Candida* isolates used were pre-cultured in YEPD overnight (~16 h) at 30°C in a static incubator. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* to vitronectin on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of resonance units which indicate adhesion \pm SEM to vitronectin across each genotype of *C. dubliniensis* and of *C. albicans*.

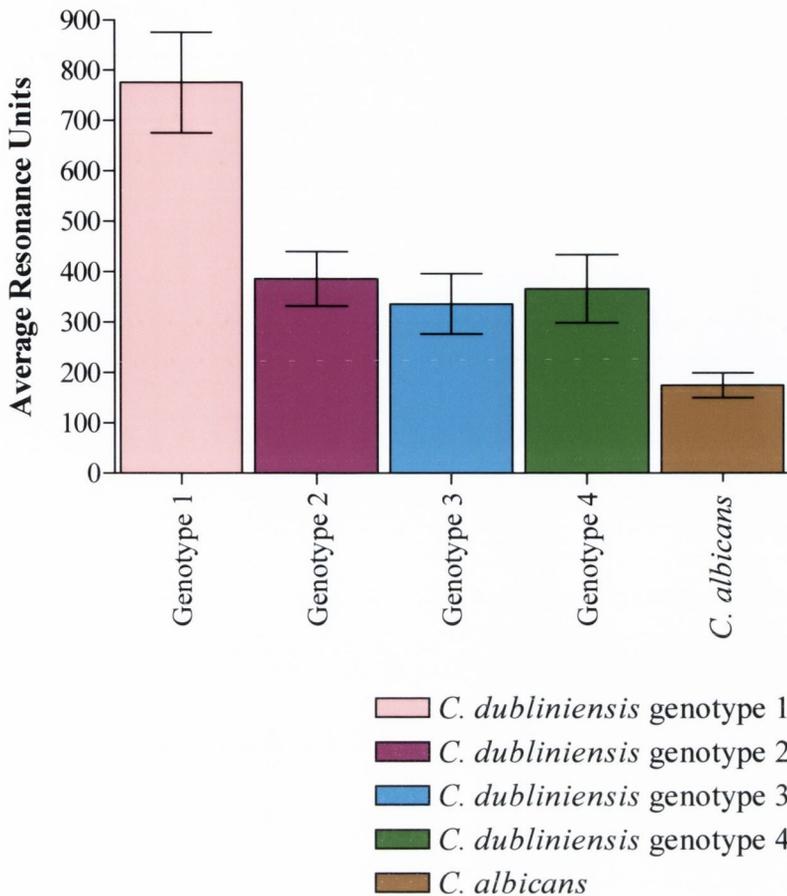


Figure 5.14. Mean adhesion of *C. dubliniensis* and *C. albicans* to all six extracellular matrix proteins. The results of the adhesion of each isolate of *C. dubliniensis* and *C. albicans* on at least two separate occasions within each genotype of *C. dubliniensis* and of *C. albicans* were averaged (*C. dubliniensis* genotype 1 = 3, *C. dubliniensis* genotype 2 = 2, *C. dubliniensis* genotype 3 = 2, *C. dubliniensis* genotype 4 = 2, *C. albicans* = 12) to give the mean number of adherent *Candida* ± SEM to all six extracellular matrix proteins. *Candida dubliniensis* genotype 1 isolates adhered to extracellular matrix proteins in significantly greater numbers than *C. dubliniensis* genotypes 2, 3 and 4 and *C. albicans* ($P < 0.001$). *Candida dubliniensis* genotype 2 isolates adhered to extracellular matrix proteins in significantly greater numbers than *C. albicans* ($P < 0.05$).

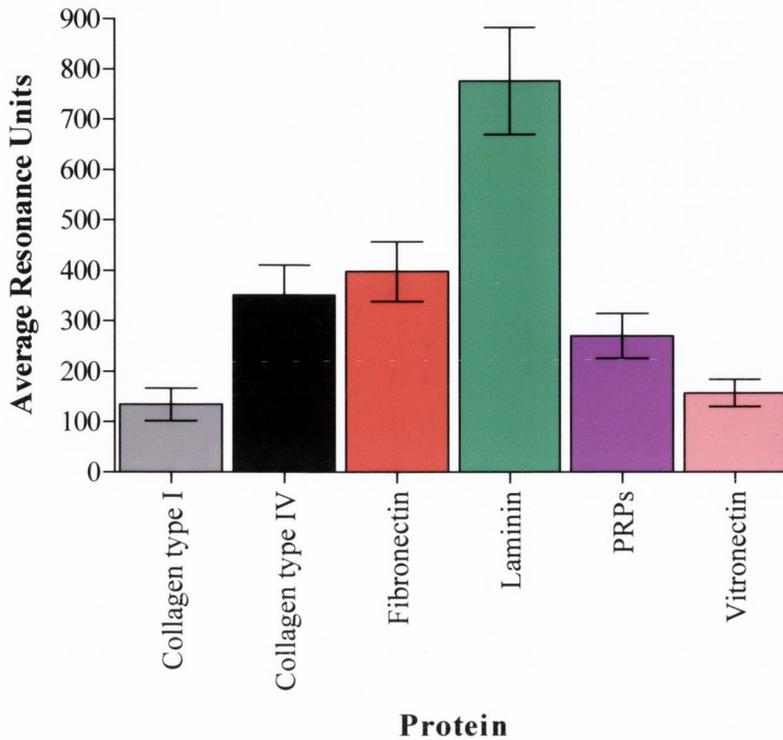


Figure 5.15. Mean adhesion of all isolates of *Candida* to six extracellular matrix proteins. The results of the adhesion of all isolates of *Candida* tested on at least two separate occasions to each of six extracellular matrix proteins was examined. All 21 isolates of *Candida* (*C. dubliniensis* = 9, *C. albicans* = 12) tested adhered to laminin in significantly greater numbers than the other extracellular matrix proteins ($P < 0.001$). All 21 isolates of *Candida* (*C. dubliniensis* = 9, *C. albicans* = 12) tested adhered to fibronectin in significantly greater numbers than vitronectin ($P < 0.05$).

isolates tested, CA401, m182CA, GDH2356 and NCPF8324 did not adhere to collagen type I (Fig. 5.5a). These isolates and all other isolates of *C. albicans* tested, adhered to the other five ECM proteins (Figs. 5.5b to 5.7b). The results also showed that there were inconsistencies, with variation evident between the replicate values in all cases.

In order to obtain a more meaningful result from the adhesion of the *Candida* isolates tested to ECM proteins, the adhesion of each isolate within each genotype of *C. dubliniensis* and of *C. albicans* was averaged to obtain a mean adhesion result. The results showed that although there is no significant difference in the ability of the two species to adhere to collagen type I (Fig. 5.8) or vitronectin (Fig. 5.13), *C. dubliniensis* genotype 1 adhered to the other four ECM protein ligands (i.e. collagen type IV ($P < 0.001$; Fig. 5.9), fibronectin ($P < 0.001$; Fig. 5.10), laminin ($P < 0.001$; Fig. 5.11) and PRPs ($P < 0.001$; Fig. 5.12)) in significantly greater amounts than *C. albicans*. The results also showed that *C. albicans* consistently had the lowest adhesion to the ECM proteins tested. When the overall adhesion of *C. dubliniensis* and *C. albicans* to all six ECM proteins was examined, *C. dubliniensis* genotype 1 was the most adherent, adhering to the ECM proteins in significantly greater amounts than the other genotypes of *C. dubliniensis* and *C. albicans* ($P < 0.001$; Fig. 5.14).

The adhesion of *C. dubliniensis* and *C. albicans* to all six ECM proteins was compared with the adhesion of *C. dubliniensis* and *C. albicans* to human BECs when the *Candida* isolates used were cultured under similar conditions (i.e. grown overnight (~16 h) at 30°C in YEPD). There were two main differences between the two adhesion assays; firstly, the *Candida* cells for the human BEC adhesion assay were grown under shaking conditions whereas *Candida* for the BIAcore assay were grown under static conditions and secondly, the incubation time of *Candida* with human BECs was 2 h whereas the exposure of *Candida* to the ECM proteins was 30 s. Despite the differences between the two adhesion assays, the results were similar. *Candida dubliniensis* genotype 1 isolates adhered to whole human BECs and on average, to all six ECM proteins tested, in greater amounts than the other genotypes of *C. dubliniensis* and of *C. albicans*.

Results from this section of the present study were not compared with the results of the TR146 adhesion assay as the yeast cells were grown at different temperatures in the two assays and as such the results were not directly comparable.

5.4.2 Overall adherence of *Candida* to ECM proteins

The mean adhesion of all 21 isolates of *Candida* tested to each of the six individual proteins was examined and the results showed that all isolates of *Candida* adhered to laminin in significantly greater amounts than to the other five ECM proteins ($P < 0.001$; Fig. 5.15). Most of the ECM proteins examined are not found in the mouth, except perhaps fibronectin, which may be present during inflammation such as in chronic erythematous candidiasis. Despite this all 21 isolates of *Candida* had a greater overall adherence to laminin. A different amount of ligand (i.e. ECM protein) had bound to each sensor chip (Table 5.1) and when this was taken into consideration, the greatest overall adherence of *Candida* was still to laminin. Bouchara *et al.* (1990) observed specific binding sites for laminin on the surface of germ tubes while non-germinating cells of *C. albicans* did not interact with soluble laminin however, the *Candida* cells used in the present study were in the yeast phase and adhered to laminin in significantly greater amounts than the five other ECM proteins tested. The fact that laminin was bound to a sensor chip in the present study, and not in solution, may be a contributing factor to these conflicting results.

Makihira *et al.* (2002a) found that *C. albicans* had the ability to bind to both the native and denatured form of collagen type I however results from this part of the present study showed that *C. albicans* adhered poorly, and in some cases not at all to collagen type I. Collagen type I is part of the organic component of dentine and may be denatured to gelatine by acids and enzymes, particularly in caries (Makihira *et al.*, 2002a & 2002b) so could be an important ligand for *Candida* in establishing an oral infection however, further studies would need to be performed in order to confirm these results.

In conclusion, all isolates of *C. dubliniensis* adhere to all ECM proteins tested. Four isolates of *C. albicans* did not adhere to collagen type I however, they and all other isolates of *C. albicans* adhered to the other five ECM proteins tested. *Candida dubliniensis* genotype 1 isolates were the most adherent, adhering to all six ECM proteins in significantly greater amounts than to the other genotypes of *C. dubliniensis* and *C. albicans*, results which concurred with the BEC adhesion assay. All 21 isolates of *Candida* examined adhered to laminin in significantly greater amounts than the other ECM proteins tested in the present study.

The results of the BIAcore experiments may be open to question due to the wide range of inter-experimental variation however, the observed trends may provide some insight into candidal adhesion. The reason for the lack of reproducibility of these experiments is likely due to the complexity of the interactions between the *Candida* cells and the ligands which were immobilised onto the surface of the sensor chips. While the BIAcore™ 3000 was originally developed to measure more specific interactions such as protein - protein or antibody - antigen interactions, previous studies (Clyne *et al.*, 2004; Kinoshita *et al.*, 2007) have used the BIAcore biosensor to examine the adhesion of whole bacterial cells to various ligands. It is likely there are multiple adhesins present on the candidal surface which result in a method of adhesion which is not suitable for assessment by the BIAcore system. In order to further investigate the molecular basis of adhesion of *Candida* to human cells, the role of the *ALS* gene family in candidal adhesion will be examined.

Chapter 6

**Comparative expression of the *ALS* genes of
C. dubliniensis and *C. albicans***

6.1 INTRODUCTION

As the use of the BIAcore™ 3000 biosensor was found to be an unsuitable method for examining the molecular adhesion of *Candida* to human cells, the comparative role of the *ALS* genes in the molecular adhesion of *C. dubliniensis* and *C. albicans* to human cells was examined. *Candida albicans* has several gene families which are involved in pathogenesis. These families include the *SAP* genes (Naglik *et al.*, 2003), the lipases (Hube *et al.*, 2000) and the *ALS* genes (Hoyer, 2001; Sheppard *et al.*, 2004; Hoyer *et al.*, 2008). *Candida dubliniensis* possesses families of genes which are orthologous to the pathogenic gene families in *C. albicans* such as the *SAP* family described by Gilfillan *et al.* (1998) and the *ALS* family described by Hoyer *et al.* (2001).

6.1.1 The Als family

The *ALS1* gene of *C. albicans* was initially isolated from a screen intended to identify genes transcribed in hyphal forms, but not in yeast forms (Hoyer *et al.*, 1995). Further experiments indicated that *C. albicans ALS1* was not a hyphal specific gene, but was transcribed in response to the components of growth medium (Hoyer *et al.*, 1995). *ALS1* of *C. albicans* encodes a protein that is similar to α -agglutinin, a cell-surface adhesion glycoprotein of *Sa. cerevisiae* that facilitates cell-cell contact during the mating of haploid yeast (Hauser & Tanner 1989; Lipke *et al.*, 1989). Sequence features of Als1p of *C. albicans* suggested it was a cell-surface glycoprotein and its similarities to α -agglutinin indicated that Als1p of *C. albicans* may have an adhesive function.

The *ALS* gene family of *C. albicans* (*CaALS*) encodes large cell surface glycoproteins which are implicated in the process of adhesion to host cells (Hoyer, 2001). The *ALS* gene family was first described in *C. albicans* (Hoyer *et al.*, 1995) and includes eight genes (Hoyer *et al.*, 2008). Each *CaALS* gene has a similar three domain structure including a 5' domain of 1,299 – 1,308 bp which is 55 – 90% identical across the family and of which the first 330 amino acids are predicted to be relatively free of glycosylation, a central domain of variable number of tandemly repeated copies of a 108-bp motif which encodes a serine/threonine rich amino acid sequence and a 3' domain that is relatively variable in length and sequence across the family and also encodes a serine/threonine rich amino acid sequence (Hoyer, 2001; Hoyer *et al.*, 2008;

Fig. 6.1). Both the tandem repeat section and the 3' domain are heavily glycosylated, unlike the 5' domain, and are expected to have an extended conformation. The N terminus contains a secretory signal sequence and the C terminus contains a GPI anchor addition sequence which is consistent with localisation of the CaAls protein to the cell wall (Hoyer *et al.*, 1998a & 1998b; Fu *et al.*, 2002; Zhao *et al.*, 2006; Hoyer *et al.*, 2008). Fu *et al.* (2002), Sheppard *et al.*, (2004) and Zhao *et al.* (2006 & 2007b) found that the ligand binding function of Als1p, Als3p, Als5p, Als6p and Als9p was localised to the N terminal domain and the variable length of the tandem repeat domain resulted in display of the N terminus either closer to, or at greater distance from the *C. albicans* cell surface (Jentoft, 1990; Kapteyn *et al.*, 2000).

6.1.2 ALS gene structure and organisation

The *CaALS* genes are found on three of the eight chromosomes of *C. albicans*. *CaALS1*, *CaALS2*, *CaALS4*, *CaALS5* and *CaALS9* are found on chromosome 6, *CaALS6* and *CaALS7* are found on chromosome 3 and *CaALS3* is found on chromosome R (Hoyer *et al.*, 1998a & 1998b; Hoyer & Hecht, 2000 & 2001; Zhao *et al.*, 2003). The *CaALS* family can be divided into subfamilies based on the central tandem repeat sequence where *CaALS1* – *CaALS4* hybridise with the *CaALS1* tandem repeat sequence (Hoyer *et al.*, 1998a & 1998b), *CaALS5* – *CaALS7* hybridise with the *CaALS5* tandem repeat sequence (Hoyer & Hecht, 2000) and *CaALS9* has a unique tandem repeat sequence (Zhao *et al.*, 2003). *CaALS7* is least like the other *CaALS* genes sharing only 55 – 60% nucleotide identity (Hoyer & Hecht, 2000). Generally, the 3' domain of the *CaALS* genes is less conserved across the ALS family however, the 3' sequence is highly conserved between certain pairs of genes such as *CaALS5*/*CaALS6* with 93% nucleotide similarity (Hoyer & Hecht, 2000) and *CaALS2*/*CaALS4* with greater than 95% nucleotide similarity (Hoyer *et al.*, 1998b) making them difficult to distinguish from each other. *CaALS2* and *CaALS4* share a long and highly similar tandem repeat domain followed by nearly identical 3' domains and flanking regions (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005). The 3' domain of *CaALS7* is the longest in the family (Hoyer & Hecht, 2000) and although this domain shares some sequence identity with *CaALS5* and *CaALS6*, *CaALS7* also encodes a 137 – 147 amino acid tandemly repeated motif within the 3' domain which in turn contains another internal five amino acid tandemly

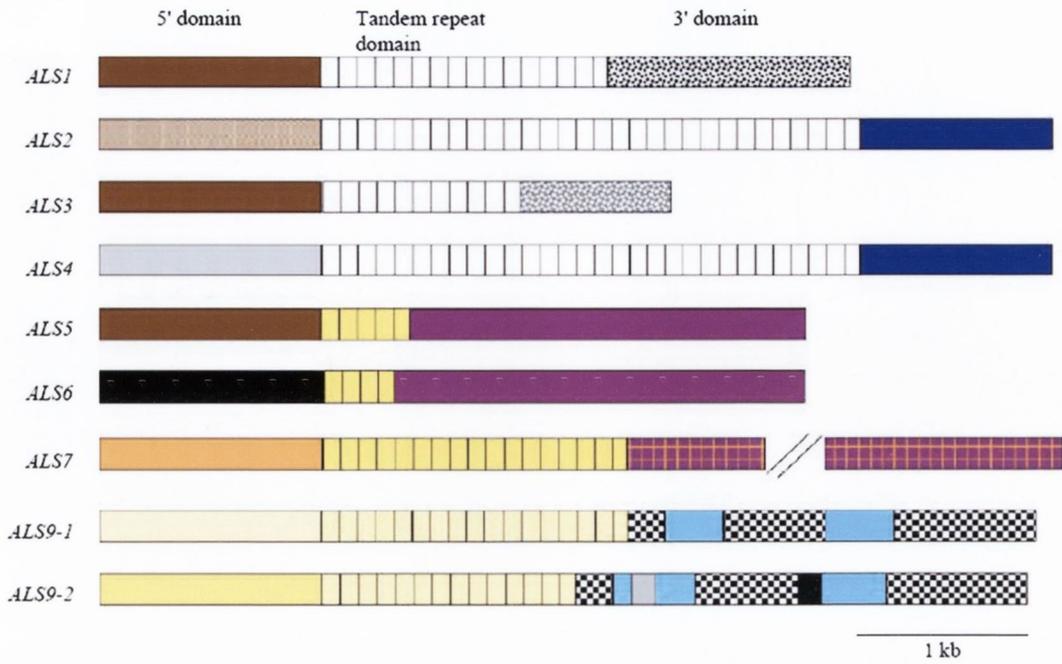


Figure 6.1. Line drawings showing selected features of the *C. albicans* *ALS* genes.

Colour coding is used to show regions of similarity between genes. The length of the 5' domain is similar in all *CaALS* genes (approximately 1.3 kb; Hoyer *et al.*, 1995). The tandem repeat domain is similar in all *CaALS* genes (approximately 1.3 kb; Hoyer *et al.*, 1995). The tandem repeat domain is drawn as individual repeated units to emphasise the composition of this portion of the coding region. The number of tandemly repeated copies in each *CaALS* gene varies by *C. albicans* strain, and often between alleles within the same strain. For all genes except *CaALS9*, the tandem repeat copy number depicted here represents the most common allele. For *CaALS9*, the two alleles from *C. albicans* SC5314 are shown (Zhao *et al.*, 2003 & 2007b). Length of the 3' domain is variable across the *CaALS* family and is drawn to scale. There is a repeated domain in the 3' end of *CaALS7* that is of variable length. The location of the repeated region is marked with parallel, diagonal lines (taken from Hoyer *et al.*, 2008). The scale bar represents 1 kilobase.

repeated VASES (Valine-Alanine-Serine-Glutamic acid-Serine) motif (Hoyer & Hecht, 2000).

6.1.3 *ALS* gene and allele variability

There is a great degree of variability between the *CaALS* genes indicating that some of these genes may have functions other than adhesion (Hoyer, 2001). The size of the same gene between strains and the size of alleles within the same strain can vary most commonly due to differences in the number of 108-bp tandem repeats present (Hoyer *et al.*, 2008). While *C. albicans* SC5314 possesses eight *CaALS* genes, *CaALS5* is absent from *C. albicans* strains B311 and B792 (Hoyer & Hecht, 2001) and *C. albicans* strain 1177 possesses an extra *CaALS6*-like sequence (Hoyer & Hecht, 2000). In *C. albicans* SC5314, a large degree of sequence variation (11% at the nucleotide level) was found within the 5' domain of the *CaALS9* alleles. Within the 3' domain, extra nucleotide blocks were present in two regions of *CaALS9-2* designated Variable Block 1 (VB1) and 2 (VB2). These regions are absent from the 3' domain of *CaALS9-1* (Zhao *et al.*, 2003). There appears to be an unequal contribution to adhesion to endothelial cells between these two alleles with strains containing only *CaALS9-2* adhering to endothelial cells at similar levels to a wild type strain and strains containing only *CaALS9-1* adhering to endothelial cells at similar levels to a mutant lacking *CaALS9*. Deletion of *CaALS9* had no effect on *C. albicans* adhesion to human BECs indicating that *CaALS9* was not involved in *C. albicans* adhesion to human BECs (Zhao *et al.*, 2007b). Both *CaALS9* alleles are widespread and conserved among a diverse range of *C. albicans* isolates and may have evolved independently for different functions (Zhao *et al.*, 2003 & 2007b). Some *C. albicans* strains possess two different sized *CaALS1* and *CaALS3* alleles indicating that two different sized CaAls1 and CaAls3 proteins will be produced (Hoyer *et al.*, 1998a). Variation in the number of tandem repeat copies was associated with Als protein function for alleles of *CaALS3*. Als3p proteins in *C. albicans* SC5314 have either nine or 12 copies of the tandem repeat sequence (Zhao *et al.*, 2004). Proteins with 12 copies of the tandem repeat sequence contribute more to *C. albicans* adhesion to endothelial or epithelial cells than those with nine copies. There is near perfect allelic sequence conservation in the 5' and 3' ends of *CaALS3* indicating that the difference in adhesion between the two alleles is due to the difference in the number of tandem repeat copies, indicating that this domain may have

a function in adhesion along with the N terminal domain. *Candida albicans* strains also tend to pair a shorter *CaALS3* allele with a longer *CaALS3* allele suggesting that each allele has a unique function (Oh *et al.*, 2005). The previously described *CaALS8* is an artefact, possibly caused by gene duplication and is now known to be the same gene as *CaALS3* (Zhao *et al.*, 2004). Repeat regions in other portions of the *CaALS* genes, such as the VASES region in the 3' domain of *CaALS7* also contribute to allelic diversity (Hoyer & Hecht, 2000). Examination of two alleles of *CaALS5* from different *C. albicans* strains indicated that sequence polymorphisms can also be found in the generally more conserved 5' domain. Proteins encoded by these alleles have a 2.8% amino acid difference within the N-terminal domain, a figure that was at least double the frequency of sequence differences observed for other *C. albicans* genes for which alleles had been sequenced (Hoyer & Hecht, 2001). Allelic variation of the *CaALS* genes between and within isolates of *Candida* may alter protein function and ignoring allelic variation may lead to incorrect or oversimplified functional conclusions. Strain specific differences in gene regulation have also been observed where the same gene in different strains responded to different regulatory signals (Hoyer *et al.*, 1998a).

6.1.4 *ALS* gene expression

The *CaALS* genes are differentially regulated. Conditions such as components of growth media (*CaALS1*, Hoyer *et al.*, 1998a), morphological form (*CaALS3*, Hoyer *et al.*, 1998a) and growth phase of culture (*CaALS4*, Hoyer *et al.*, 1998b; *CaALS1*, Fu *et al.*, 2002) have an effect on gene expression. Typically in *C. albicans*, one or two genes are expressed under a specific *in vitro* growth condition (Hoyer *et al.*, 2001). Synthesis of *CaALS4* in YEPD began at mid-log phase and increased as the culture reached stationary phase at 30°C. Transcription of *CaALS4* was higher at 30°C than 37°C (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005). Loss of *CaALS2* or *CaALS4* activity decreased *C. albicans* adhesion to vascular endothelial cells but not buccal epithelial cells and analysis of a mutant lacking *CaALS4* showed up-regulation of *CaALS2*, while *CaALS4* expression was up-regulated in a strain lacking *CaALS2* activity suggesting a possible compensatory function between these two very similar genes. Loss of *CaALS4* activity did not affect destruction of RHE however reduction of *CaALS2* expression reduced RHE damage (Zhao *et al.*, 2005). *CaALS2* transcription was strongly up-regulated during germ tube formation in both RPMI-1640 medium and serum-containing media

(Green *et al.*, 2005). *CaALS1* transcription increased when YEPD grown cells were washed in PBS or inoculated into fresh growth medium and transcription of *CaALS3* increased as germ tubes became visible (Green *et al.*, 2005). Deletion of *CaALS3* had the largest effect on *C. albicans* adhesion with a large decrease in adhesion to endothelial and buccal epithelial cells and a marked decrease in RHE damage. In contrast, deletion of *CaALS1* resulted in a slight decrease in RHE damage and a slight but non-significant change in adhesion. There may also be functional redundancy between Als1p and Als3p resulting in a less severe phenotype in the case of the loss of *CaALS1* which is compensated for by *CaALS3* (Zhao *et al.*, 2004). Deletion of *CaALS5*, *CaALS6* or *CaALS7* resulted in a decreased growth rate and increased adhesion to both endothelial and epithelial cells indicating that these Als proteins are not involved in adhesion but have some other function possibly involving maintaining growth rate (Zhao *et al.*, 2007a). These genes are consistently transcribed at lower levels than the other *CaALS* genes suggesting that only a low level of expression is required to produce enough protein to complete the required function (Green *et al.*, 2005; Hoyer *et al.*, 2008). Detection of expression of *CaALS4* from vaginal specimens and models of vaginal candidiasis was more difficult than detection of expression of *CaALS4* from oral specimens and models of oral disease using RT-PCR, suggesting a host-site-specific effect on regulation of the *CaALS* genes (Cheng *et al.*, 2005). *Candida albicans* Als3 is required to induce endocytosis of *C. albicans* by binding to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells and as such is considered a fungal invasin that mimics host cell cadherins and induces endocytosis (Phan *et al.*, 2007). It has recently been shown that Als3 has the capacity to bind ferritin (Almeida *et al.*, 2008) and as iron sequestration by host iron-binding proteins is an important mechanism of resistance to microbial infections this may be another important virulence trait of *C. albicans*. Nobile *et al.* (2008) found that Als1 and Als3 are together required for biofilm formation *in vivo* and that they function by binding to Hwp1 on neighbouring cells suggesting that Als3 in particular has multiple virulence attributes and that the Als family members may have overlapping functions.

6.1.5 Als proteins

Als proteins appear to have specific adhesion profiles with Als1p, Als3p and Als5p being most adherent, Als6p and Als9p showing a decreased adhesion profile and Als7p being non-adherent (Sheppard *et al.*, 2004). The Als proteins appear to be separable into three groups based on surface distributions of hydrophobicity, charge and hydrogen bonding potential. Als1p, Als3p and Als5p constitute Als group A, Als6p and Als7p constitute Als group B and Als2p, Als4p and Als9p constitute Als group C which is more similar to Als group A than Als group B in terms of hydrophobic or electrostatic distribution. These differences in hydrophobicity, charge and hydrogen bonding potential may influence the interaction of these proteins with host cells, and structural differences among the Als proteins may be responsible for differences in function (Sheppard *et al.*, 2004).

6.1.6 The role of the *ALS* genes in biofilm formation

Als proteins and their adhesive properties may be important for biofilm formation. A biofilm can be defined as an association of surface attached bacterial or fungal cells that become encased in ECM material (Costerton *et al.*, 1987; Donlan & Costerton, 2002). Cells in a biofilm exhibit distinct properties, most significant of which is resistance to antimicrobial drugs (Hawser & Douglas, 1995; Ramage *et al.*, 2002). In a study by García-Sánchez *et al.* (2004), *CaALS1* was the most differentially expressed gene in biofilms compared to planktonic cells suggesting its importance in biofilm formation, however *CaALS1* expression is strongly up-regulated when cells are inoculated into fresh media (Green *et al.*, 2005) so it is not surprising that *CaALS1* expression differs between a mature culture such as a biofilm and an earlier growth stage. Nailis *et al.* (2006) also found that *CaALS1* was up-regulated in *C. albicans* biofilm cells compared to planktonic cells. Cells in this study were harvested at stationary phase and as such transcription of *CaALS1* should be in response to biofilm formation as opposed to change in media components as suggested in the study by García-Sánchez *et al.* (2004). O'Connor *et al.* (2005) found up-regulation of *CaALS1* in biofilms grown on silicone elastomer compared to planktonic cells. Nobile *et al.* (2006) suggested that CaAls3p had more of a role in biofilm formation than CaAls1p. A mutant lacking in *CaALS3* was unable to form a mature biofilm *in vitro* or on a silicone

elastomer surface but could form a biofilm *in vivo*. It is likely that this protein is more important in the later stage of biofilm formation when hyphae are present (Hoyer *et al.*, 2008). Loss of *CaALS2* expression resulted in biofilm with a decreased mass (Zhao *et al.*, 2005).

6.1.7 ALS genes in other *Candida* species

ALS genes have been identified in *C. dubliniensis* and *C. tropicalis* using cross hybridisation on southern blots and amplification of genomic DNA using degenerate PCR primers designed from *CaALS* sequences. Although the basic three domain structure is conserved within each species, extensive sequence diversification is present and unique *ALS* genes have arisen. Cross hybridisation on southern blots with probes specific for the tandem repeat and 3' domains of the *CaALS* genes indicate that the *ALS* genes in *C. dubliniensis* (*CdALS*) have similar 5' and tandem repeat domains to *CaALS* but tend to have unique 3' domains largely unrelated to those found in *CaALS*. The *CdALS* genes are found on chromosomes 3 and 6. While the *CaALS* genes are differentially expressed under a variety of conditions including morphological form, growth medium composition, growth phase and strain of *C. albicans*, more *CdALS* genes were expressed under the same growth conditions compared to *CaALS in vitro* and often the same *CdALS* gene profile was seen under many growth conditions suggesting that expression of these genes is constitutive or coordinated (Hoyer *et al.*, 2001).

6.1.8 Real time PCR

Real time RT-PCR is a highly sensitive, easy to perform method which allows accurate quantification of small changes in gene expression (Pfaffl, 2001). This is difficult to achieve with conventional PCR. Real time PCR reactions are characterised by the point in time during cycling when amplification of a target is first detected, rather than the amount of target accumulated after a fixed number of cycles. Newly synthesised PCR products are detected using SYBR Green fluorescent dye, which binds specifically to the minor groove of double strand DNA meaning it detects specific and non-specific products (Morrison *et al.*, 1998). Relative quantification compares transcript abundance of a target gene across multiple samples, using a co-amplified

internal control for sample normalisation for the amount of RNA added to the RT reactions. Standard housekeeping genes usually suffice as internal controls and should have the same copy number in all cells, be expressed in all cells and have a medium copy number. Results are expressed as ratios of the target gene signal to the internal control signal which yields a corrected relative value for the target gene product in each sample. Using the $2^{-\Delta\Delta C_t}$ or comparative C_t method, the data are presented as change in gene expression of the target gene under a test condition relative to some calibrator sample such as an untreated control or a sample at time zero in a time course study and does not require a standard curve to elucidate copy number. Quantifying the relative changes in gene expression using real time PCR requires certain equations, assumptions and testing of these assumptions to properly analyse the data. For these calculations to be valid the amplification efficiencies of the target gene and internal control must be approximately equal and for the calibrator sample, $\Delta\Delta C_t$ equals 0 and 2^0 equals 1, so that the change in gene expression of the calibrator sample equals one by definition. For the treated samples, evaluation of $2^{-\Delta\Delta C_t}$ indicates the change in gene expression relative to the calibrator sample (Livak & Schmittgen, 2001). Fold gene expression is calculated as \log_{10} relative quantification. The comparative C_t method is easy to use for large gene numbers such as this study and a good design process will result in very high assay efficiencies.

Given the importance of the *ALS* gene family in *C. albicans* pathogenesis and the difference in the ability of *C. albicans* and *C. dubliniensis* to adhere to human cells, the aim of this part of the study was to identify and characterise the *CdALS* gene family. The recently sequenced *CdALS* genes were used to design gene-specific conventional and real-time PCR primers for each member of the *CdALS* family and to examine differential *CdALS* and *CaALS* gene expression when cultured under various temperature and media conditions and when adherent to human BECs. Differential expression of the *ALS* genes of *C. dubliniensis* and *C. albicans* may provide some insight into the reduced virulence of *C. dubliniensis* compared to *C. albicans*.

6.2 MATERIALS AND METHODS

6.2.1 Relationship between *C. albicans* and *C. dubliniensis* ALS genes

ALS genes from the *C. dubliniensis* genome were identified using the *Candida dubliniensis* GeneDB website (<http://www.genedb.org/genedb/cdubliniensis/>) and the search term ‘agglutinin’ in the ‘search for’ function. Using *CdALS* nucleotide and amino acid sequences from the *C. dubliniensis* GeneDB website, searches for nucleotide and amino acid similarities were carried out using the BLAST (Altschul *et al.*, 1990 & 1997; Zhang *et al.*, 2000) series of computer programs as detailed in section 2.4. Alignments of *C. dubliniensis* and *C. albicans* ALS nucleotide and amino acid sequences were carried out using the CLUSTAL W (Thompson *et al.*, 1994) sequence alignment computer program. The Artemis Comparison Tool (ACT), Release 6, was used to compare the genome sequences and synteny of genes between the *C. dubliniensis* (*Candida dubliniensis* GeneDB) and *C. albicans* (*Candida* Genome Database; <http://www.candidagenome.org/>) genomes. Artemis is a free genome viewer and annotation tool that allows visualisation of sequence features and the results of analyses within the context of the sequence, and its six-frame translation (Rutherford *et al.*, 2000; Carver *et al.*, 2005). The neighbour joining method (Saitou & Nei, 1987) of constructing phylogenetic trees was used to graphically depict the ALS gene families of *C. dubliniensis* and *C. albicans* using MEGA version 4 (Tamura *et al.*, 2007).

6.2.2 Amplification of the *C. dubliniensis* and *C. albicans* ALS genes using conventional PCR

6.2.2.1 *C. dubliniensis* ALS primer design

Gene-specific primers for the *CaALS* genes were previously described by Green *et al.* (2004; Table 6.1). Gene-specific primers for the *CdALS* genes were designed using the *CdALS* gene sequence and where suitable, were based on the position and length of the *CaALS* primers using Primer3 (<http://frodo.wi.mit.edu/>; Rozen & Skaletsky, 2000) as described in section 2.1.6. The *CdALS* PCR primer pairs amplify PCR products of between 150 and 300 bp and are shown in Table 6.2.

6.2.2.2 CdALS and CaALS PCR conditions

Each 50 µl PCR reaction contained 2 µl template genomic DNA or cDNA, 1× *Taq* reaction buffer (Sigma), 1 mM magnesium chloride (Promega), 400 nM of both CdALS or CaALS forward and reverse primer (Sigma-Genosys) for each *ALS* gene, 100 µM each dATP, dTTP, dCTP and dGTP (Promega) and 0.75 U *Taq* DNA polymerase (Sigma). To validate the specificity of each primer pair for its corresponding *ALS* gene, PCR products were amplified using either *C. dubliniensis* CD36 (Sullivan *et al.*, 1995) genomic DNA for each CdALS primer pair or *C. albicans* SC5314 (Gillum *et al.*, 1984) genomic DNA for each CaALS primer pair. The PCR cycling conditions were initial denaturation for 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and a final extension for 7 min at 72°C. Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in a Thermo Hybaid MBS 0.2G thermal cycler (Thermo Scientific) using either 0.2 ml or 0.5 ml satellite blocks. PCR amplification products were resolved on a 2% (w/v) agarose (Sigma) gel containing 0.013 M ethidium bromide (Sigma) and were visualised on a high performance UV transilluminator (wavelength 345 nm; UVP) and photographed using the Imagestore 7500, version 7.22, gel documentation system (UVP).

6.2.2.3 Actin PCR conditions

PCR amplification of cDNA was carried out to determine whether RNA was correctly reverse transcribed into cDNA using the actin primer pair. PCR amplification using the actin forward (5' TAA TCA TTC AAA ATG GAC GGT 3') and reverse (5' GGA TGG ACC AGA TTC GTC GTA 3') primer pair resulted in a product of 485 bp if genomic DNA or cDNA was amplified and had the RNA not been transcribed into cDNA there would be no product amplified, as RNA is not a valid template for PCR. Each 50 µl PCR reaction contained 2 µl cDNA template, 1× *Taq* reaction buffer, 2 mM magnesium chloride, 400 nM each actin forward and reverse primer (Sigma-Genosys), 100 µM each dATP, dTTP, dCTP and dGTP and 0.75 U *Taq* DNA polymerase. The PCR cycling conditions were initial denaturation for 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 63°C, and 1 min at 72°C, and a final extension for 4 min at 72°C. Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in a Thermo Hybaid MBS 0.2G thermal cycler using either 0.2 ml or 0.5 ml satellite

Table 6.1. *C. albicans* ALS primers for conventional PCR (Green *et al.*, 2004)

| Gene | Primer name | Primer sequence (5' → 3') | Sequence coordinates ^a | Melting temperature ^b | GC% ^c | 2° structure ^d | Primer dimer ^e formation |
|---------------|-------------|---|-----------------------------------|----------------------------------|------------------|---------------------------|-------------------------------------|
| <i>CaALS1</i> | CaALS1F | GAC TAG TGA ACC AAC AAA TAC CAG A | 3024 – 3048 | 62 | 40 | No | No |
| | CaALS1R | CCA GAA GAA ACA GCA GGT GA | 3341 – 3322 | 63 | 50 | No | No |
| <i>CaALS2</i> | CaALS2F | CCA AGT ATT AAC AAA GTT TCA ATC ACT TAT | 571 – 600 | 62.3 | 26 | Weak | No |
| | CaALS2R | TCT CAA TCT TAA ATT GAA CGG CTT AC | 936 – 911 | 64 | 34 | Weak | No |
| <i>CaALS3</i> | CaALS3F | CCA CTT CAC AAT CCC CAT C | 2711 – 2729 | 62.8 | 52 | No | No |
| | CaALS3R | CAG CAG TAG TAG TAA CAG TAG TAG TTT CAT C | 3052 – 3022 | 60.8 | 38 | Very weak | No |
| <i>CaALS4</i> | CaALS4F | CCC AGT CTT TCA CAA GCA GTA AAT | 571 – 594 | 64 | 41 | Very weak | No |
| | CaALS4R | GTA AAT GAG TCA TCA ACA GAA GCC | 926 – 903 | 62 | 41 | Very weak | No |
| <i>CaALS5</i> | CaALS5F | TGA CTA CTT CCA GAT TTA TGC CGA G | 551 – 575 | 66 | 44 | No | No |
| | CaALS5R | ATT GAT ACT GGT TAT TAT CTG AGG GAG AAA | 868 – 839 | 65 | 33 | Weak | No |
| <i>CaALS6</i> | CaALS6F | GAC TCC ACA ATC ATC TAG TAG CTT GGT TT | 528 – 556 | 66.9 | 41 | Weak | No |
| | CaALS6R | CAA TTG TCA CAT CAT CTT TTG TTG C | 679 – 655 | 66 | 36 | Very weak | No |
| <i>CaALS7</i> | CaALS7F | GAA GAG AAC TAG CGT TTG GTC TAG TTG T | 530 – 557 | 68 | 42 | Moderate | No |
| | CaALS7R | TGG CAT ACT CCA ATC ATT TAT TTC A | 735 – 711 | 64.8 | 32 | Weak | No |
| <i>CaALS9</i> | CaALS9F | CCA TAT TCA GAA ACA AAG GGT TC | 1729 – 1751 | 62 | 39 | Very weak | No |
| | CaALS9R | AAC TGA AAC TGC TGG ATT TGG | 1926 – 1906 | 62.8 | 42 | No | No |

See overleaf for legend

^aSequence coordinates are given for each primer where +1 refers to the first 'A' of the ATG start codon for each gene

^bThe temperature in degrees Celsius at which one half of the DNA double strand will dissociate to become single stranded

^cThe GC% is the percentage of Gs and Cs in the primer

^d2° structures are structures which form from interaction of the primer with either itself, such as a hairpin or self dimer, or with the other primer of the pair to form a cross dimer, where the two primers anneal, but do not anneal directly to each other or a primer dimer

^eA small amplicon from the extension of self annealed primers

Table 6.2. *C. dubliniensis* ALS primers for conventional PCR designed using the web based program Primer3

| Gene | Primer name | Primer sequence (5' → 3') | Sequence coordinates^a | Melting temperature^b | GC%^c | 2° structure^d | Primer dimer^e formation |
|----------------|--------------------|-------------------------------------|---|--|------------------------|---------------------------------|---|
| <i>CdALS6</i> | CdALS6F | CCA AAA CAC CGG AAT CAT CT | 524 – 543 | 63.5 | 45 | No | No |
| | CdALS6R | TTG TCA CAT CGT TCT TTG TTG C | 676 – 655 | 64.8 | 40.9 | Very weak | No |
| <i>CdALS1</i> | CdALS1F | CCA GAT CAC CAA CGA TTT CA | 3194 – 3213 | 63.5 | 45 | Very weak | No |
| | CdALS1R | CTA GAA GGA GCG ATA GGT GAT G | 3476 – 3455 | 61.6 | 50 | Very weak | No |
| <i>CdALS4</i> | CdALS4F | CAA TGG GGT TTT CAA CTG CT | 638 – 657 | 63.7 | 45 | Very weak | No |
| | CdALS4R | ATT ATA ACT AAA AGA AGC ATC AAC AGG | 927 – 901 | 60.6 | 29.6 | No | No |
| <i>CdALS2*</i> | CdALS2F | AGC AAT GCT TTG AAC CCA TC | 324 – 343 | 63.8 | 45 | Weak | No |
| | CdALS2Ra | TGG TGC CAT ATA AAG AGT TGT ACC | 601 – 578 | 62.6 | 41 | Weak | No |
| | CdALS2Rb | GTG GTG CCA TAT AAA GAG TTG TAC C | 591 – 567 | 63 | 44 | Weak | No |
| <i>CdALS9</i> | CdALS9F | GGA GAG CCA TAT TCA GAA ACA CA | 4921 – 4943 | 64 | 43 | No | No |
| | CdALS9R | AAT TGG CAC TTC TGG AGT TGA | 5124 – 5104 | 42 | 42 | Very weak | No |
| <i>CdALS7</i> | CdALS7F | CCT CGA AGA GAT CTT GCT TAT GGT | 526 – 549 | 65.4 | 45.8 | Weak | No |
| | CdALS7R | GGG CAT ACT CCA ATC ATT TAT TTC A | 735 – 711 | 65.3 | 36 | No | No |

See overleaf for legend

^aSequence coordinates are given for each primer where +1 refers to the first 'A' of the ATG start codon for each gene

^bThe temperature in degrees Celsius at which one half of the DNA double strand will dissociate to become single stranded

^cThe GC% is the percentage of Gs and Cs in the primer

^d2° structures are structures which form from interaction of the primer with either itself, such as a hairpin or self dimer, or with the other primer of the pair to form a cross dimer, where the two primers anneal, but do not anneal directly to each other or a primer dimer

^eA small amplicon from the extension of self annealed primers

*The primer pair designated CdALS2 amplifies both *CdALS2-1* and *CdALS2-2*

blocks. PCR amplification products were resolved on a 2% (w/v) agarose gel containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm) and photographed using the Imagestore 7500, version 7.22, gel documentation system.

6.2.2.4 PCR product clean-up

The GenElute™ PCR Clean-Up Kit (Sigma) was used to purify PCR products amplified when using each CaALS primer pair and the CdALS6, CdALS1, CdALS4, CdALS9 and CdALS7 primer pairs. The QIAEX II Gel Extraction Kit (Qiagen) was used to purify PCR products amplified when using the CdALS2F/CdALS2Ra and CdALS2F/CdALS2Rb primer pairs. Kits were used according to the manufacturer's instructions. Purified PCR products were resolved on a 2% (w/v) agarose gel containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm).

6.2.2.5 Sequence analysis of *CdALS* and *CaALS* genes amplified using genomic DNA and conventional ALS PCR primers

Individual samples of purified PCR product amplified from genomic DNA, and corresponding forward primers, were sent to Cogenics to be sequenced commercially as described in section 2.4. Sequencing results were received in the form of a text file containing the sequence and a chromatogram. Results were analysed as described in section 2.4. Gene sequences were translated into protein sequences using the Translate tool on the ExPASy (Expert Protein Analysis System) proteomics server (<http://www.expasy.org/>; Gasteiger *et al.*, 2003), which is hosted by the Swiss Institute of Bioinformatics (<http://www.isb-sib.ch/>). Searches for nucleotide and amino acid similarities were carried out using the BLAST series of computer programs as detailed in section 2.4. Alignments of nucleotide sequences were carried out using the CLUSTAL W sequence alignments computer program.

6.2.2.6 Analysis of *ALS* expression in *C. dubliniensis* and *C. albicans* when pre-cultured under different temperature and media conditions

Candida dubliniensis CD36 (genotype 1) and *C. albicans* SC5314 from colonies grown on PDA (Oxoid) plates that were less than 1 week old were pre-cultured in either 50 ml YEPD or 50 ml YEPGal in 250 ml conical flasks (Duran) at either 30°C or 37°C overnight (~16 h) in an orbital incubator (Gallenkamp) at 200 rpm before being diluted into fresh broth and grown to mid-exponential phase (A_{600} : 1). RNA from each sample was isolated and extracted and each RNA sample was DNase-treated and analysed as described in sections 2.5.2 to 2.5.5. cDNA was generated as described in section 2.3.2. The resultant cDNA was used as the template for PCR analysis with each pair of gene-specific CdALS or CaALS primer pairs and the actin primer pair using the reagents and cycling conditions as detailed in sections 6.2.2.2 and 6.2.2.3 above.

6.2.3 Amplification of the *C. dubliniensis* and *C. albicans ALS* genes using real time RT-PCR

6.2.3.1 *C. dubliniensis* real time PCR primer design

Quantitative real time (QRT) PCR primers for the *CaALS* genes were previously described by Green *et al.* (2005; Table 6.3). QRTPCR primers for the *CdALS* genes were designed using Primer Express (Applied Biosystems) as described in section 2.1.6. The QRTCdALS PCR primers are detailed in Table 6.4. As there was only 1 base pair mismatch between the forward and the reverse QRTTEF1 primers and the *CdTEF* gene sequence, the QRTTEF1 primer pair (Green *et al.*, 2005) was used to amplify both *CaTEF* and *CdTEF* genes, which were used as internal controls. As the QRTCaALS2 primer pair did not amplify *C. albicans* SC5314 genomic DNA and the QRTCaALS3 primer pair amplified the non-template control (NTC) indicating the formation of primer dimers by this primer pair, use of these primer pairs was discontinued and new real time primers for *CaALS2* and *CaALS3* were designed using Primer Express as described in section 2.1.6. These primers are detailed in Table 6.5 and are used in this part of the present study instead of the QRTCaALS2 and QRTCaALS3 primer pairs (Green *et al.*, 2005). The QRTALS primers do not cross-react with RNA derived from uninoculated buccal cells (data not shown), demonstrating the utility of these primer

Table 6.3. *C. albicans* ALS primers for real time RT-PCR (Green *et al.*, 2005)

| Gene | Primer name | Primer sequence (5' → 3') | Sequence coordinates^a | PCR product size (bp) |
|---------------|--------------------|---|---|------------------------------|
| <i>CaALS1</i> | QRTCaALS1F | TTC TCA TGA ATC AGC ATC CAC AA | 3213 – 3235 | 53 |
| | QRTCaALS1R | CAG AAT TTT CAC CCA TAC TTG GTT TC | 3265 – 3240 | |
| <i>CaALS2</i> | QRTCaALS2F | TTC CAA GTA TTA ACA AAG TTT CAA TCA CTT A | 569 – 599 | 68 |
| | QRTCaALS2R | ACC AGA TGT GTA GCC ATT TGC AC | 636 – 614 | |
| <i>CaALS3</i> | QRTCaALS3F | AAT GGT CCT TAT GAA TCA CCA TCT ACT A | 2857 – 2884 | 56 |
| | QRTCaALS3R | GAG TTT TCA TCC ATA CTT GAT TTC ACA T | 2912 – 2885 | |
| <i>CaALS4</i> | QRTCaALS4F | TCT GCA ACA CGA GTC AGC TCA | 841 – 861 | 64 |
| | QRTCaALS4R | CCG CAC CAA CAC AAG CAT ATA T | 904 – 883 | |
| <i>CaALS5</i> | QRTCaALS5F | CTG CCG GTT ATC GTC CAT TTA | 806 – 826 | 63 |
| | QRTCaALS5R | ATT GAT ACT GGT TAT TAT CTG AGG GAG AAA | 868 – 838 | |
| <i>CaALS6</i> | QRTCaALS6F | GAC TCC ACA ATC ATC TAG TAG CTT GGT TT | 528 – 556 | 52 |
| | QRTCaALS6R | ACT TGG AAT AAC CCT TGC GAA A | 579 – 558 | |
| <i>CaALS7</i> | QRTCaALS7F | GAA GAG AAC TAG CGT TTG GTC TAG TTG T | 530 – 557 | 51 |
| | QRTCaALS7R | GCG ACA TGG AAA GTC TTT GAC TAA C | 580 – 566 | |
| <i>CaALS9</i> | QRTCaALS9F | AAA TCA ATT ACC ACC CCA GCT G | 4849 – 4870 | 68 |
| | QRTCaALS9R | GAA ACT GAA ACT GCT GGA TTT GG | 4916 – 4894 | |
| <i>TEF1</i> | QRTTEF1F | CCA CTG AAG TCA AGT CCG TTG A | 851 – 872 | 51 |
| | QRTTEF1R | CAC CTT CAG CCA ATT GTT CGT | 901 – 881 | |

^aSequence coordinates are given for each primer where +1 refers to the first 'A' of the ATG start codon for each gene

Table 6.4. *C. dubliniensis* ALS primers for real time PCR designed using the TaqMan® Probe and Primer Design function of Primer Express on a Macintosh platform

| Gene | Primer name | Primer sequence (5' → 3') | Sequence coordinates ^a | Melting temperature ^b | GC% ^c | Length of primer ^d |
|----------------|-------------|--|-----------------------------------|----------------------------------|------------------|-------------------------------|
| <i>CdALS6</i> | QRTCdALS6F | AGT ACG TCA ACC TTT TTG TCT GCT G | 3979 – 4003 | 66.2 | 44 | 25 |
| | QRTCdALS6R | CTG TAA CCT GAA TTG ACA GTT CA | 4123 – 4101 | 60.6 | 36 | 28 |
| <i>CdALS1</i> | QRTCdALS1F | TAC TGT AAT TAT TTA TGA ATC CAT GTC GAG TT | 508 – 539 | 64.9 | 28 | 32 |
| | QRTCdALS1R | ATA TTG ACA GAG GAA GAC TCA ATT GTT GTA | 642 – 613 | 65.4 | 33 | 30 |
| <i>CdALS4</i> | QRTCdALS4F | TCC CAG TCA TCA TCA GTG AAT ATA CTT | 948 – 974 | 64.4 | 37 | 27 |
| | QRTCdALS4R | TTG CCA CAC TTG TTT CAC CAC | 1056 – 1036 | 65.9 | 48 | 21 |
| <i>CdALS2*</i> | QRTCdALS2F | AAT GAT TTT TTA GTT GCC GCC A | 559 – 580 | 68 | 36 | 22 |
| | QRTCdALS2R | GTA TTC GTA AAT CCC ATT GTA CCA GAA | 674 – 648 | 65.3 | 37 | 27 |
| <i>CdALS9</i> | QRTCdALS9F | CTT CAG CTA CTA GGG AAT TAA CCT CTA GAT | 5121 – 5150 | 63.9 | 40 | 30 |
| | QRTCdALS9R | GTA GTC GTG CCA TTG GAT GCT | 5265 – 5245 | 66.1 | 52 | 21 |
| <i>CdALS7</i> | QRTCdALS7F | CCT TTC ACA AGT TTC TTT TCT CAG TCT ATT | 941 – 970 | 65.4 | 33 | 30 |
| | QRTCdALS7R | TCT GCA CTA GGG AAA TGA AGG GTA | 1079 – 1056 | 66.5 | 46 | 24 |

^aSequence coordinates are given for each primer where +1 refers to the first 'A' of the ATG start codon for each gene

^bThe temperature in degrees Celsius at which one half of the DNA double strand will dissociate to become single stranded

^cThe GC% is the percentage of Gs and Cs in the primer

^dLength in bases

*The primer pair designated QRTCdALS2 amplifies both *CdALS2-1* and *CdALS2-2*

Table 6.5. *C. albicans* ALS primers for real time PCR designed using the TaqMan® Probe and Primer Design function of Primer Express on a Macintosh platform

| Gene | Primer name | Primer sequence (5' → 3') | Sequence coordinates ^a | Melting temperature ^b | GC% ^c | Length of primer ^d | 2° structure ^e | Primer dimer ^f formation |
|---------------|--------------|---|-----------------------------------|----------------------------------|------------------|-------------------------------|---------------------------|-------------------------------------|
| <i>CaALS2</i> | MyQRTCaALS2F | AGA TAT ACT AAT GAT TAT GCC TGT GTT GG | 868 – 896 | 63.7 | 34 | 29 | None | No |
| | MyQRTCaALS2R | CAA AAC CGT TAG AAT TAG CTT CAC TAT TAT | 973 – 944 | 63.3 | 30 | 30 | Very weak | No |
| <i>CaALS3</i> | MyQRTCaALS3F | AAC TTG GGT TAT TGA AAC AAA AAC AAT | 2556 – 2582 | 64.1 | 26 | 27 | Weak | No |
| | MyQRTCaALS3R | ACC ATA GGA GTT TCG ATA TTA TTA GGA ATT | 2678 – 2649 | 63.6 | 30 | 30 | Very weak | Yes |

^aSequence coordinates are given for each primer where +1 refers to the first 'A' of the ATG start codon for each gene

^bThe temperature in degrees Celsius at which one half of the DNA double strand will dissociate to become single stranded

^cThe GC% is the percentage of Gs and Cs in the primer

^dLength in bases

^e2° structures are structures which form from interaction of the primer with either itself, such as a hairpin or self dimer, or with the other primer of the pair to form a cross dimer, where the two primers anneal, but do not anneal directly to each other or a primer dimer

^fA small amplicon from the extension of self annealed primers

pairs to monitor *ALS* gene expression in adherent *Candida*. Using the BLAST series of computer programs, a result of “no significant similarity” was obtained when the QRTCdALS and QRTCaALS forward primers were aligned against the human genome indicating that these primers would not amplify any portion of the human genome.

6.2.3.2 Conventional PCR conditions using QRTCaALS and QRTCdALS primers

Each 50 µl PCR reaction contained 2 µl template genomic DNA or cDNA, 1× Green GoTaq® Flexi Buffer (Promega), 2.5 mM magnesium chloride, 400 nM each QRTCaALS (substituting the MyQRTCaALS2 and MyQRTCaALS3 primer pairs for the QRTCaALS2 and QRTCaALS3F primer pairs (Green *et al.*, 2005)), QRTCdALS or QRTTEF1 forward and reverse primer pairs (Sigma-Genosys), 200 µM each dATP, dTTP, dCTP and dGTP and 0.75 U GoTaq® DNA polymerase (Promega). To validate the specificity of each primer pair for its corresponding *ALS* gene, PCR products were amplified using either *C. dubliniensis* CD36 genomic DNA for each QRTCdALS primer pair or *C. albicans* SC5314 genomic DNA for each QRTCaALS primer pair and the MyQRTCaALS2 and MyQRTCaALS3 primer pairs. The QRTTEF1 primer pair was used to amplify both *C. dubliniensis* CD36 and *C. albicans* SC5314 genomic DNA. The PCR cycling conditions were initial denaturation for 5 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C and a final extension for 7 min at 72°C. Amplification reactions were carried out in 0.2 ml or 0.5 ml microfuge tubes in a Thermo Hybaid MBS 0.2G thermal cycler using either 0.2 ml or 0.5 ml satellite blocks and PCR amplification products were resolved on a 2% (w/v) agarose gel containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm) and photographed using the Imagestore 7500, version 7.22, gel documentation system. Where PCR products were to be sent for sequencing, 1× colourless GoTaq® Flexi Buffer (Promega) was used instead of 1× green GoTaq® Flexi Buffer.

6.2.3.3 Elongation factor (EF) exon PCR

PCR amplification of cDNA was carried out to determine whether RNA was correctly reverse transcribed into cDNA and contained no genomic DNA contamination. PCR amplification using the EFB5' (5' ATT GAA CGA ATT CTT GGC

TGA C 3') and EFB3' (5' CAT CTT CTT CAA CAG CAG CTT G 3') primer pair (Maneu *et al.*, 1996; Hube *et al.*, 2000) resulted in a product of 551 bp if cDNA was amplified. If genomic DNA contamination was present, a PCR product of 916 bp would be amplified and had the RNA not been transcribed into cDNA there would be no product amplified, as RNA is not a valid template for PCR. Each 50 µl PCR reaction contained 1× Green GoTaq® Flexi Buffer, 2 mM magnesium chloride, 400 nM each EFB5' and EFB3' primer (Sigma-Genosys), 200 µM each dATP, dTTP, dCTP and dGTP and 0.75 U GoTaq® DNA polymerase. The PCR cycling conditions were initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and a final extension for 7 min at 72°C. Amplification reactions were carried out in a Biometra T Personal DNA thermal cycler (Biometra biomedizinische Analytik) and PCR amplification products were resolved on a 1% (w/v) agarose gel containing 0.013 M ethidium bromide and were visualised on a high performance UV transilluminator (wavelength 345 nm) and photographed using the Imagestore 7500, version 7.22, gel documentation system.

6.2.3.4 PCR product clean-up

The GenElute™ PCR Clean-Up Kit was used to purify PCR products amplified when using the MyQRTCaALS2 and MyQRTCaALS3 primer pairs and each QRTCaALS primer pair. The QIAEX II Gel Extraction Kit was used to purify PCR products amplified when using each QRTCaALS primer pair and the QRTTEF1 primer pair. Kits were used according to the manufacturer's instructions. Concentration of purified PCR product was determined using spectrophotometric analysis.

6.2.3.5 Sequence analysis of the *CdALS* and *CaALS* genes amplified using genomic DNA and real time ALS PCR primers

Individual samples of purified CdALS PCR product amplified from *C. dubliniensis* CD36 genomic DNA and corresponding forward primers, and individual samples of purified CaALS PCR product amplified from *C. albicans* SC5314 genomic DNA and corresponding forward and reverse primers were sent to Cogenics to be sequenced commercially as described in section 2.4. Sequencing results were received in the form of a text file containing the sequence and a chromatogram. Alignments of

nucleotide sequences were carried out using the CLUSTAL W sequence alignments computer program.

6.2.3.6 Determination of real time PCR primer efficiency

Primer efficiency was determined using Ct values recorded with ABI Prism Sequence Detection Systems, version 1.9.1 (Applied Biosciences) on a Macintosh platform and was obtained using 1:4, 1:8, 1:32 and 1:128 dilutions of a 1:100 stock solution of genomic DNA run in an ABI Prism 7700 Sequence Detector (Applied Biosciences). *Candida dubliniensis* CD36 genomic DNA was used for determination of the efficiencies of the QRTCdALS primers and *C. albicans* SC5314 genomic DNA was used for determination of the efficiencies of the QRTCaALS primers. Both *C. dubliniensis* CD36 and *C. albicans* SC5314 genomic DNA was used for determination of the amplification efficiency of the QRTTEF1 primers as TEF was used as the internal control for both *C. dubliniensis* and *C. albicans*. The slope and resultant primer efficiency were elucidated and graphically depicted using Microsoft Excel.

6.2.3.7 Analysis of *ALS* expression in real time in *C. dubliniensis* and *C. albicans* when pre-cultured under different temperature and media conditions

Candida albicans SC5314 and *C. dubliniensis* CD36 (genotype 1; Sullivan *et al.*, 1995), Can6 (genotype 2; Pinjon *et al.*, 1998), CD514 (genotype 3; Gee *et al.*, 2002) and Eg200 (genotype 4; Al Mosaid *et al.*, 2005) from colonies grown on PDA plates that were less than 1 week old were pre-cultured in either 50 ml YEPD or 50 ml YEPGal in 250 ml conical flasks at either 30°C or 37°C overnight (~16 h) in an orbital incubator at 200 rpm before being diluted into fresh broth and grown to mid-exponential phase (A_{600} : 1). *Candida albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 from colonies grown on PDA plates that were no more than 72 h old were pre-cultured in Lee's medium (Lee *et al.*, 1975; Buffo *et al.*, 1984), pH 4.5 at 30°C overnight (~16 h) in an orbital incubator at 200 rpm before being washed once with PBS (Oxoid) and re-suspended in 10% (v/v) filter-sterile new born bovine calf serum (Sigma) in PBS which was freshly made prior to each experiment and pre-warmed to 37°C at a final volume of 2×10^6 cell ml⁻¹. The isolates were individually cultured at 37°C, without shaking, for 3, 3.5, 3, 3.5 and 3.5 h respectively resulting in 100%

hyphal, 87% hyphal, pseudohyphal, pseudohyphal and 90% hyphal cultures respectively. Human BECs were collected and processed as described in the materials and methods section of chapter 3. *Candida albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 from colonies grown on PDA plates that were less than 1 week old were pre-cultured in either 50 ml YEPD or 50 ml YEPGal in 250 ml conical flasks at either 30°C or 37°C overnight (~16 h) in an orbital incubator at 200 rpm and were processed as described in the materials and methods section of chapter 3. Following co-incubation of as much volume of human BECs and *Candida* as was possible while maintaining the ratio of 50:1 yeast cells: human BECs, the human BECs with adherent yeast cells were collected by filtering the sample through a hydrophilic, polycarbonate membrane containing 12 µm pores (Millipore) and washed gently, twice with 10 ml sterile PBS in order to remove any non-adherent yeast cells. The polycarbonate membrane was removed from a Nalgene® polysulfone filter holder with receiver (upper chamber capacity 250 ml, receiver capacity 250 ml; Nalgene® Labware, part of Thermo Fisher Scientific Inc.) and the human BECs with adherent yeast cells were washed from the polycarbonate membrane with 10 ml PBS and collected in a 50 ml screw cap tube with a conical bottom. To ensure reproducibility, each RNA extraction was performed on two separate samples which were prepared on separate days. RNA from each yeast, hyphal and adherent yeast sample was isolated and extracted and each RNA sample was DNase-treated and analysed as described in sections 2.5.2 to 2.5.6. cDNA was generated as described in section 2.3.2 and analysed as described in section 6.2.3.3.

Each 15 µl PCR reaction for each individual QRTALS primer pair contained 1× Power SYBR® green PCR Master Mix (Applied Biosystems) and 500 nM each QRTALS or QRTTEF forward and reverse primer and 1 µl template. Using the ddCt (Relative Qualification) Plate function of the 7500 Fast System Software, version 1.4, from Applied Biosystems Sequence Detection Software on a Windows platform, details of the set up of each real time PCR plate were recorded including the detectors used and the name of each sample. The thermal profile settings (initial denaturation for 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C) for each real time PCR run and the results (recorded at each extension step), including the spectra and amplification plot, were also recorded and graphically depicted using this software. The overall results from each replicate, which were recorded over several ddCt Plates in a 7500 Fast Real-Time PCR system machine (Applied Biosystems), were

compiled using a ddCt Study (7500 Fast System Software, version 1.4) and the results, including amplification plots and fold expression calculated as \log_{10} relative quantification, were graphically depicted.

6.2.3.8 Data analysis

Real time measurement of PCR amplification was achieved by determining the number of PCR cycles necessary to achieve a given level of fluorescence. In this study, the Ct was fixed in the exponential phase of amplification. Data was analysed according to the comparative Ct or $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and graphically depicted using 7500 Fast System Software, version 1.4. Analysis involved comparing the Ct values of the test condition with a calibrator sample; in this part of the present study, the calibrator sample was *Candida* grown to mid-exponential phase in YEPD at 37°C as this is the standard growth temperature and medium for *Candida*. The Ct values of both the calibrator sample and the test conditions were normalised to an appropriate internal control, in this case *TEF1*. For this calculation to be valid, the amplification efficiencies of the target (in this case, the *ALS* genes of *C. dubliniensis* and *C. albicans*) and the internal control must be approximately equal (as determined in section 6.2.3.6).

6.3 RESULTS

6.3.1 Identification of *C. dubliniensis* ALS genes

Seven *ALS* genes were identified in the *C. dubliniensis* genome, compared with eight *ALS* genes in *C. albicans*, and the *ALS* genes in *C. dubliniensis* were re-named based on homology to and synteny with the *CaALS* genes (Table 6.6). *Cd36_86150* was found to be most similar to *CaALS7* (Fig. 6.2; Table 6.6) and *Cd36_86290* was found to be most similar to *CaALS6* (Fig. 6.3; Table 6.6) using ACT and the BLAST series of computer programs. *Cd36_64210* was found to be most similar to *CaALS1* (Fig. 6.4; Table 6.6) and *Cd36_64220* was found to be most similar to *CaALS9* (Fig. 6.5; Table 6.6) using ACT and the BLAST series of computer programs. Using BLAST, *Cd36_64610* was most similar to *CaALS4* and ACT analysis showed that *Cd36_64610* and *CaALS4* were syntenous on the upstream side of both genes (Fig. 6.6; Table 6.6). Using BLAST, the nucleotide sequence of *Cd36_65010* was found to be most similar to *CaALS2*, whereas the amino acid sequence of *Cd36_65010* was found to be most similar to *CaALS4*. ACT analysis showed that *Cd36_65010* was most syntenous to *CaALS2* (Fig. 6.7; Table 6.6). Using BLAST, the nucleotide sequence of *Cd36_64800* was found to have similar nucleotide homology to both *CaALS2* and *CaALS4* (Table 6.6). ACT analysis showed that *Cd36_64800* was also syntenic with *CaALS2* (Fig. 6.8). *Candida dubliniensis* contains no orthologs for *CaALS3* (Fig. 6.9) or *CaALS5*. Schematic diagrams representing the order and position of each *CdALS* gene in relation their *CaALS* orthologs are shown (Figs. 6.10 to 6.13).

The entire CdAls and CaAls protein sequences were used to construct a phylogenetic tree using the neighbour joining method of Saitou & Nei (1987), which depicted the relationship between the *CaALS* and *CdALS* genes (Fig. 6.14). A phylogenetic tree using 461 N terminal amino acids only (data not shown) was constructed and was similar to the phylogenetic tree constructed using the entire protein sequence with the exception that the phylogenetic tree constructed using the 461 N terminal amino acids only grouped *CaALS1*, *CaALS5* and *CaALS3* together, while only *CaALS1* and *CaALS3* were grouped together in the phylogenetic tree constructed using the entire protein sequence.

Table 6.6. *C. dubliniensis* ALS genes identified using the *Candida dubliniensis* GeneDB website

| Syntenic gene name | Chromosomal location | Designated name ^a | Nucleotide homology ^b | Comments |
|--------------------|----------------------|------------------------------|----------------------------------|---|
| <i>Cd36_64210</i> | 6 | <i>CdALS1</i> | 82% to <i>CaALS1</i> | Also 35% nucleotide homology to <i>CaALS5</i> <i>CdALS1</i> = 3888 bp <i>CaALS1</i> = 3783 bp |
| <i>Cd36_64220</i> | 6 | <i>CdALS9</i> | 73% to <i>CaALS9</i> | Most homologous at 5' end. Also 46% homology to <i>CaALS1</i> <i>CdALS9</i> = 5742 bp <i>CaALS9</i> = 5673 bp |
| <i>Cd36_64610</i> | 6 | <i>CdALS4</i> | 79% to <i>CaALS4</i> | Most homologous at 5' end <i>CdALS4</i> = 5970 bp <i>CaALS4</i> = 6303 bp |
| <i>Cd36_64800</i> | 6 | <i>CdALS2-2</i> | 78% to <i>CaALS2</i> | Also 63% nucleotide homology to <i>CaALS4</i> , however better alignment between <i>Cd36_64800</i> and <i>CaALS2</i> <i>CdALS2-2</i> = 8025 bp <i>CaALS2</i> = 7089 bp |
| <i>Cd36_65010</i> | 6 | <i>CdALS2-1</i> | 58% to <i>CaALS2</i> | Also 56% nucleotide homology to <i>CaALS4</i> , however better alignment between <i>Cd36_65010</i> and <i>CaALS2</i> <i>CdALS2-1</i> = 4353 bp <i>CaALS2</i> = 7089 bp |
| <i>Cd36_86150</i> | 3 | <i>CdALS7</i> | 27% to <i>CaALS7</i> | Most homologous at 5' end. <i>CaALS7</i> is shorter than <i>CdALS7</i> <i>CdALS7</i> = 15456 bp <i>CaALS7</i> = 4707 bp |
| <i>Cd36_86290</i> | 3 | <i>CdALS6</i> | 80% to <i>CaALS6</i> | Most homologous at 5' and 3' ends. Low homology in the tandem repeat section between <i>CdALS6</i> and <i>CaALS6</i> <i>CdALS6</i> = 5043 bp <i>CaALS6</i> = 4101 bp |

^aNew name assigned to each *CdALS* gene based on homology to and synteny with the *CaALS* genes

^bPercentage nucleotide homology between the *CdALS* gene and its *CaALS* ortholog determined using CLUSTAL W

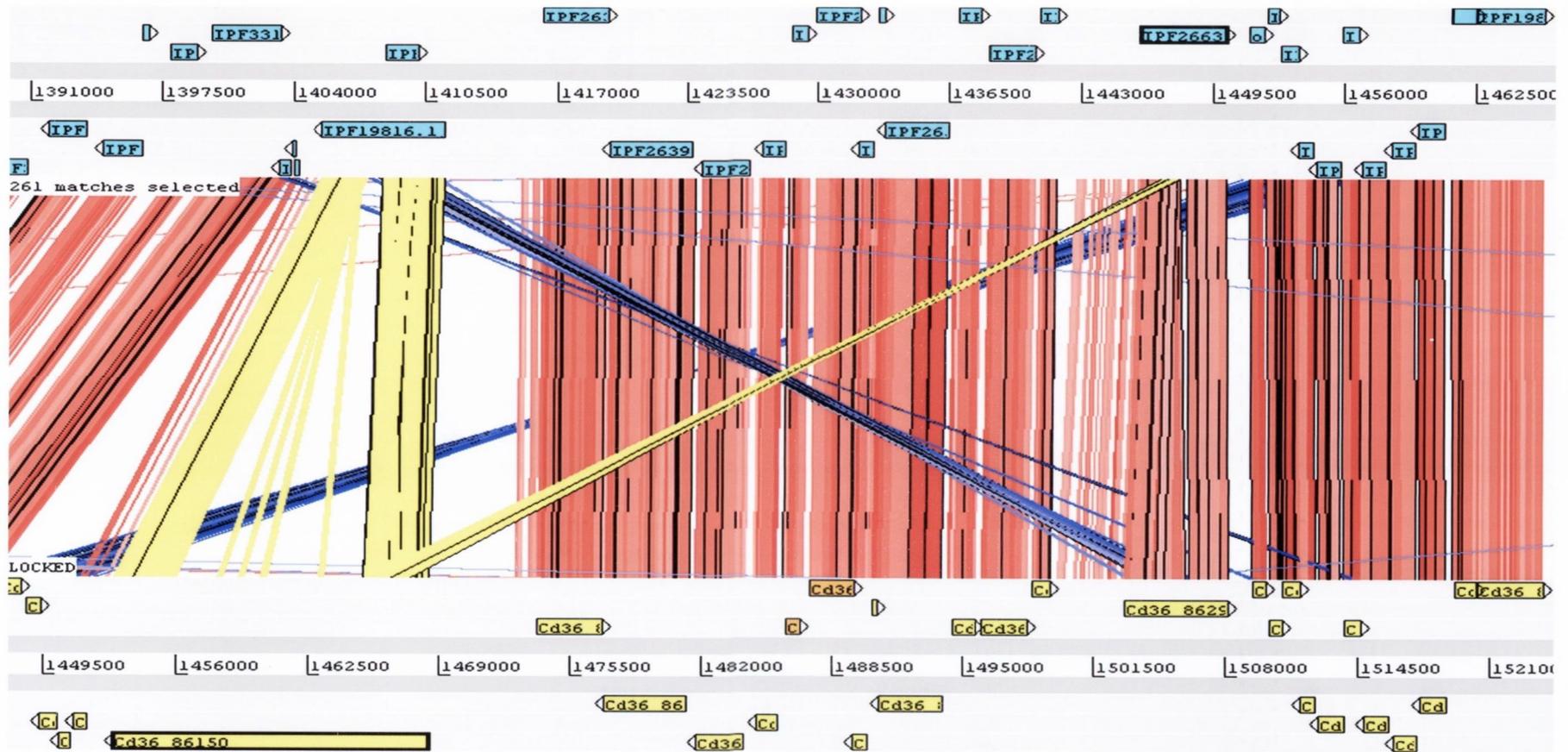


Figure 6.2. Synteny between *Cd36_86150* and *IPF19816.1* (*CaALS7*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_86150* on chromosome 3. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six open reading frames (ORFs), are represented by yellow and orange boxes above (sense) and yellow boxes below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_86150*).

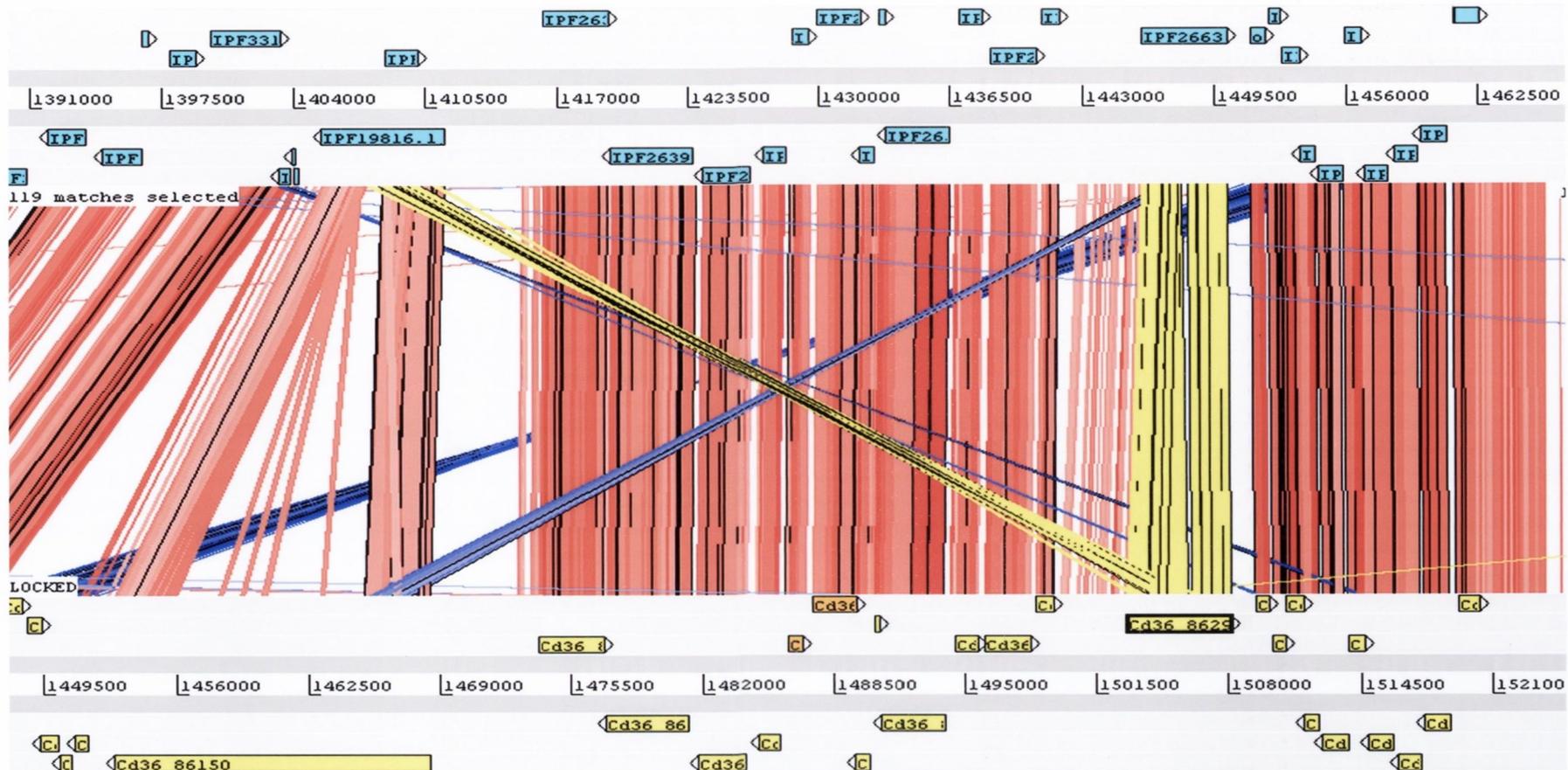


Figure 6.3. Synteny between *Cd36_86290* and *IPF2663.2 (CaALS6)*. This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_86290* on chromosome 3. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six ORFs, are represented by yellow and orange boxes above (sense) and yellow boxes below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_86290*).

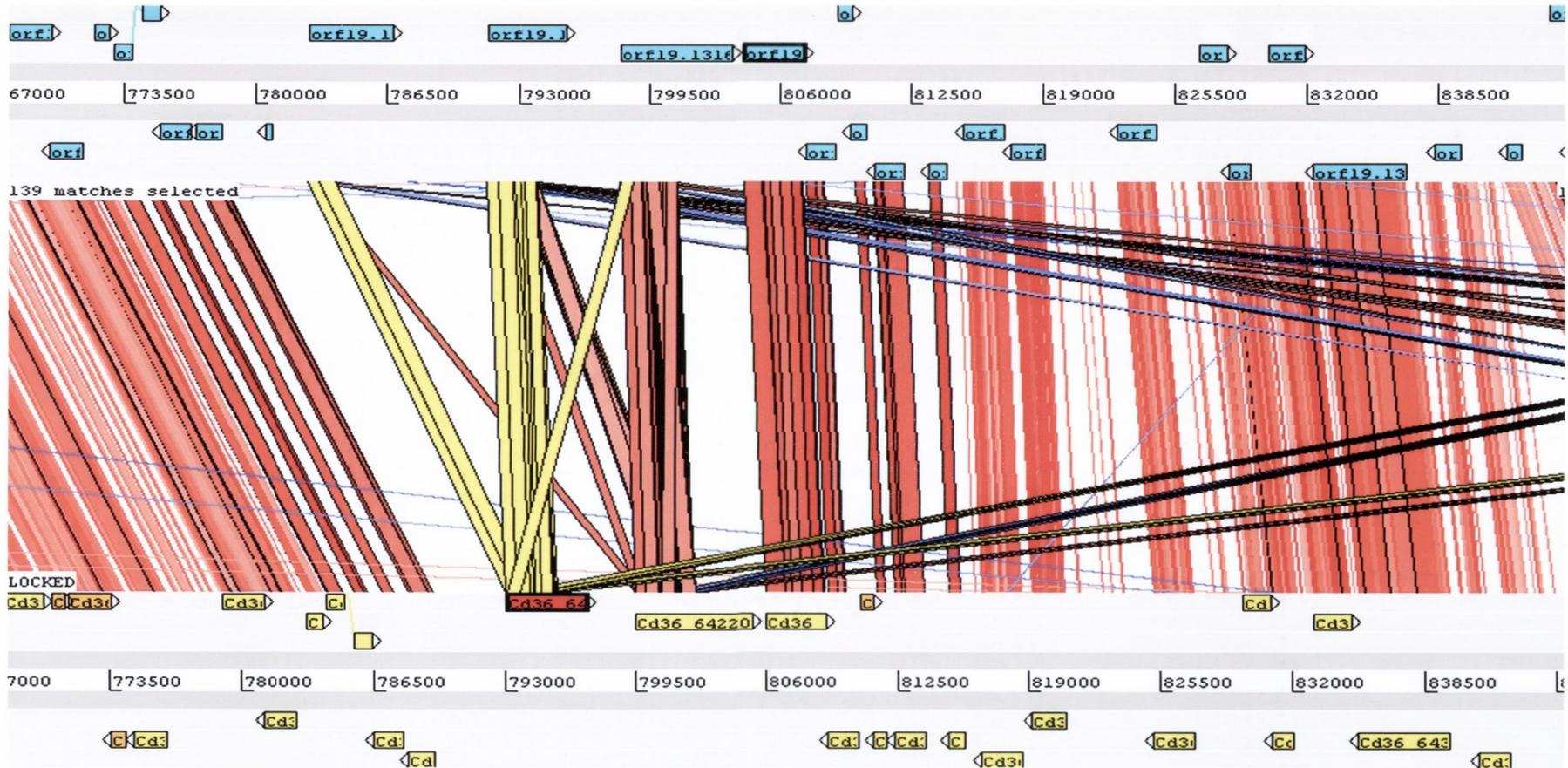


Figure 6.4. Synteny between *Cd36_64210* and *orf19.13163* (*CaALS1*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_64210* on chromosome 6. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six ORFs, are represented by yellow and orange boxes above (sense) and below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. *Cd36_64210* is represented by a red box. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_64210*).

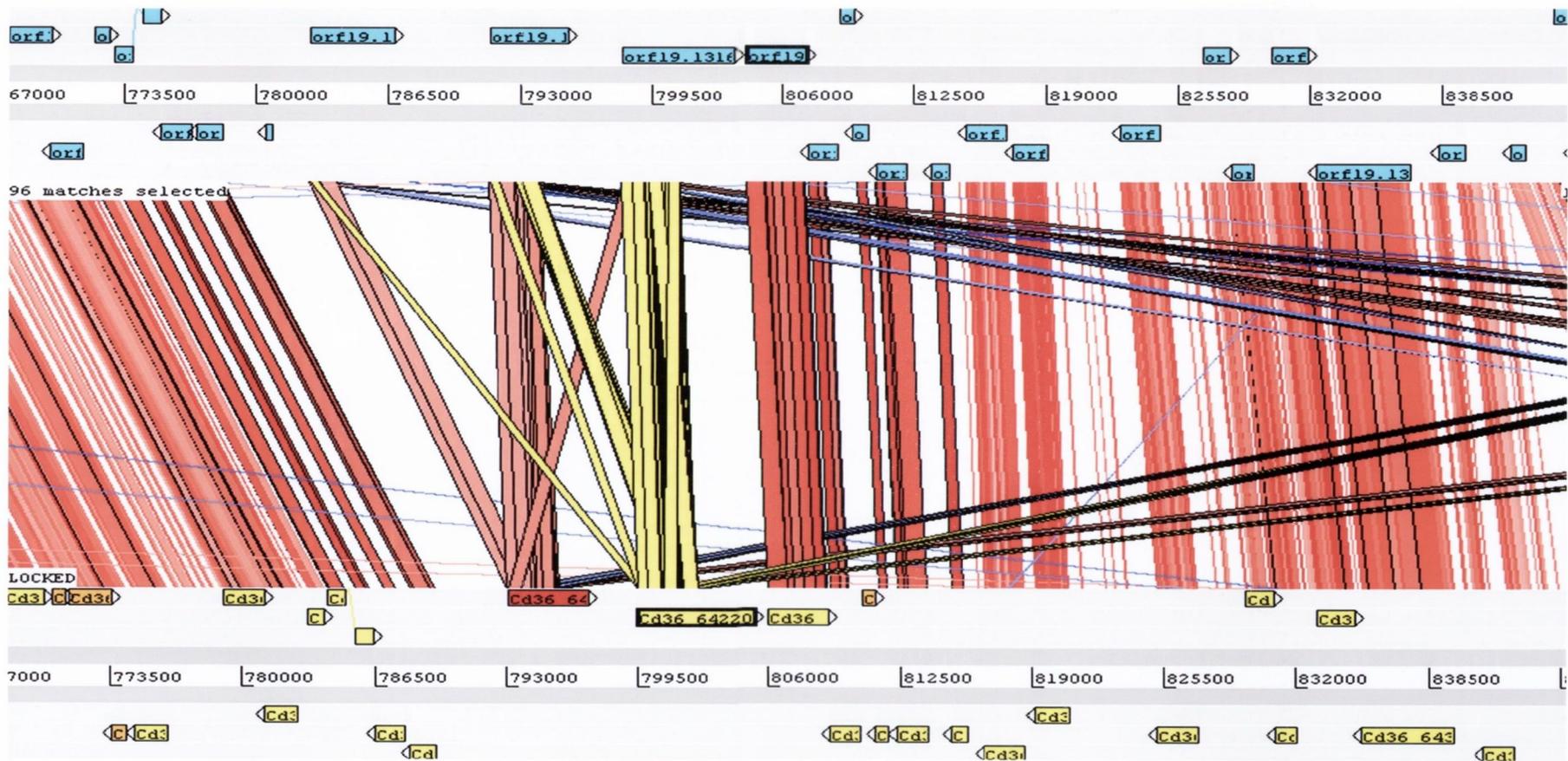


Figure 6.5. Synteny between *Cd36_64220* and *orf19.1316* (*CaALS9*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_64220* on chromosome 6. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six ORFs, are represented by yellow and orange boxes above (sense) and below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_64220*).

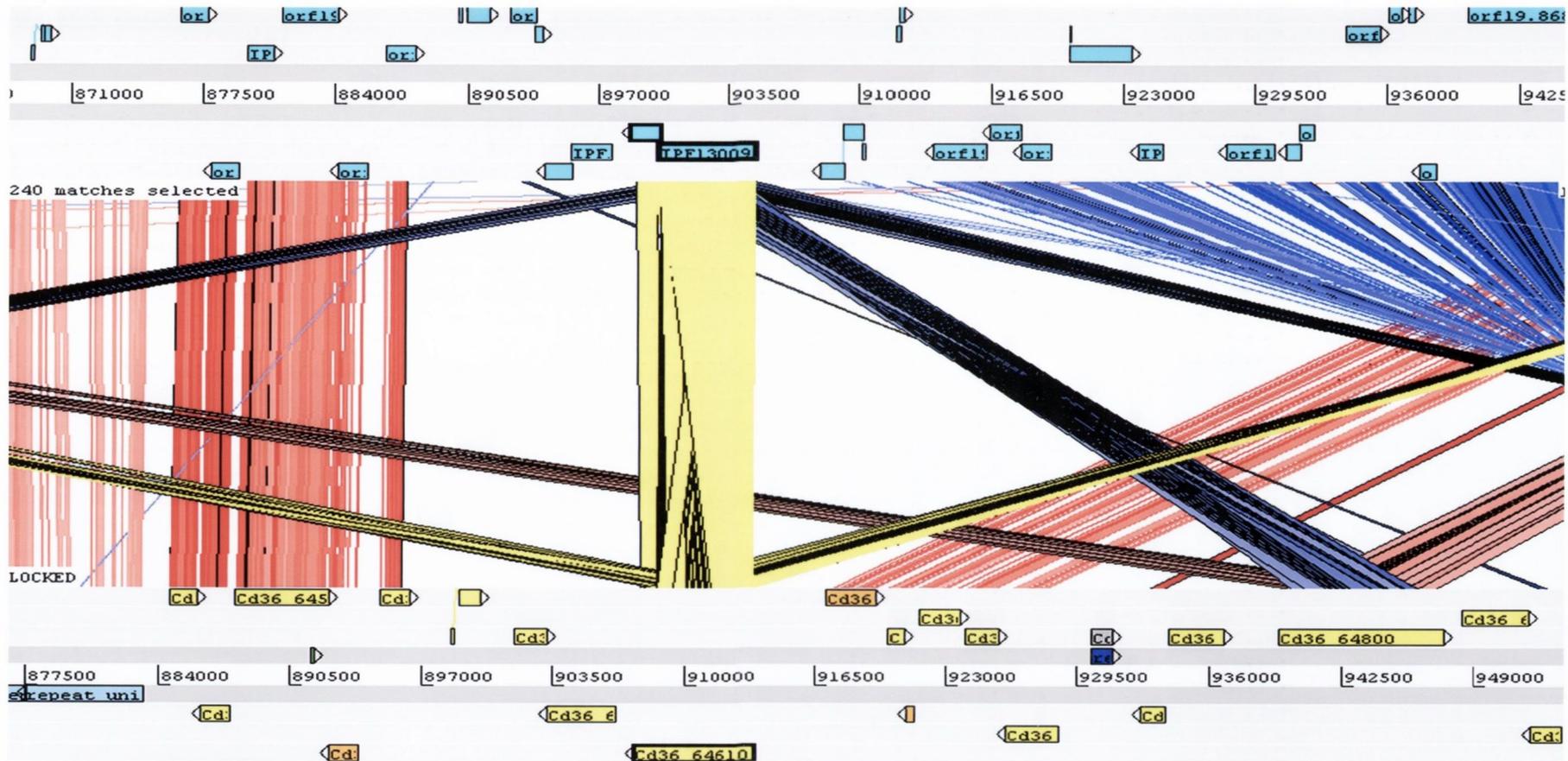


Figure 6.6. Synteny between *Cd36_64610* and *IPF13009.2* (*CaALS4*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_64610* on chromosome 6. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six ORFs, are represented by one grey and several yellow and orange boxes above (sense) and below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_64610*).

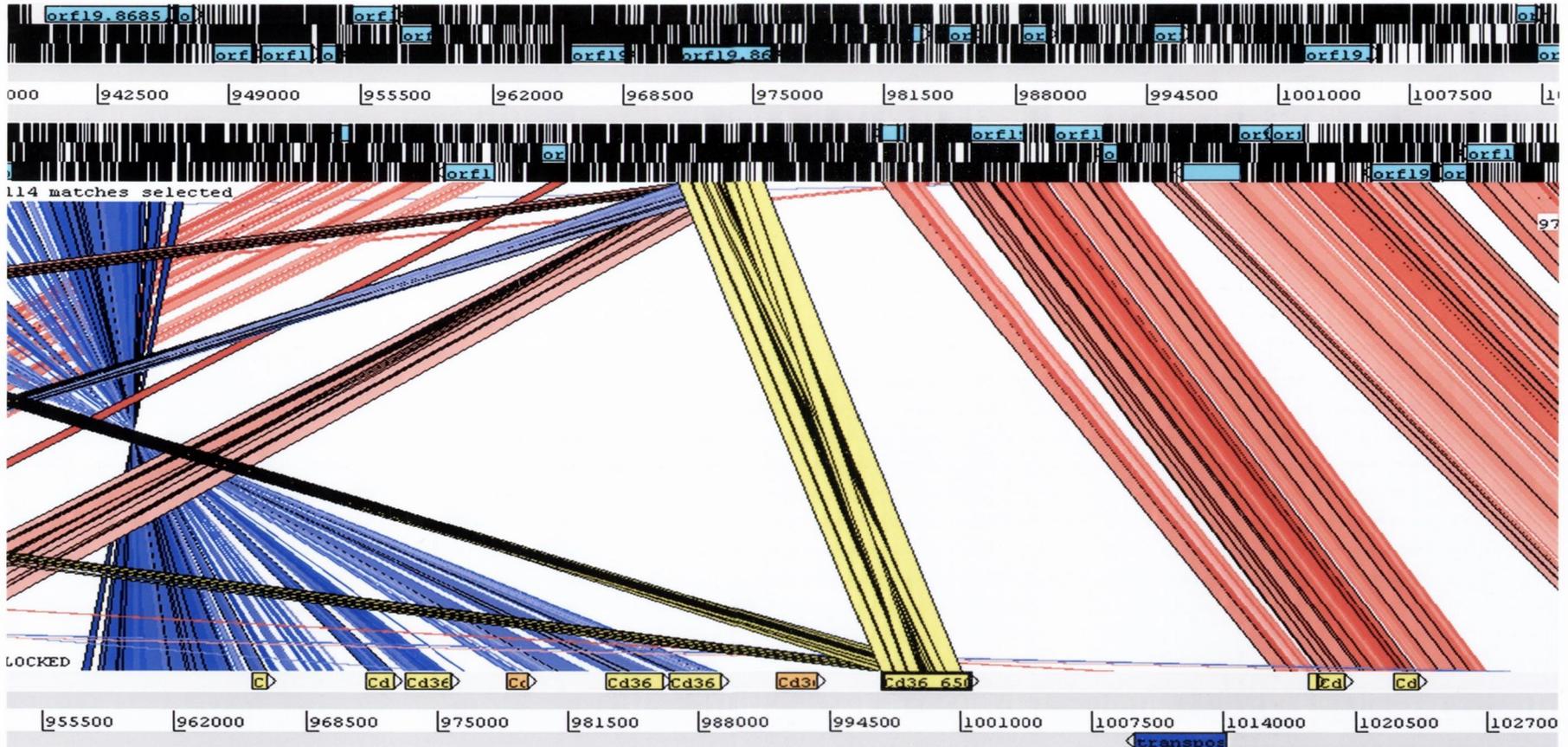


Figure 6.7. Synteny between *Cd36_65010* and *orf19.8699* (*CaALS2*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_65010* on chromosome 6. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis* are represented by yellow and orange boxes above (sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_65010*).

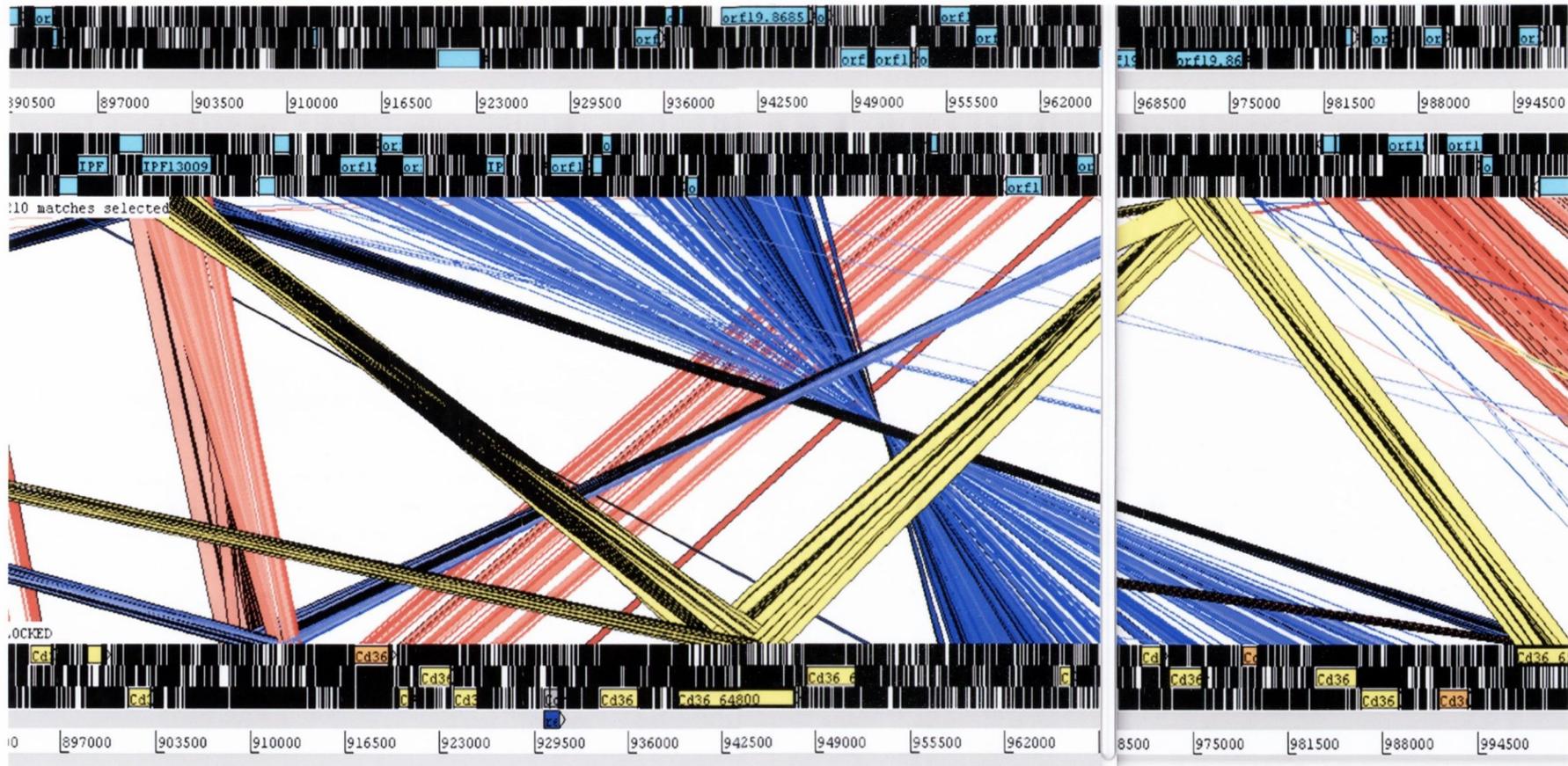


Figure 6.8. Synteny between *Cd36_64800* and *IPF13009.2* (*CaALS4*) and *orf19.8699* (*CaALS2*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *Cd36_64800* on chromosome 6. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of three of the six ORFs, are represented by yellow and orange boxes above (sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches; yellow, *Cd36_64800*).

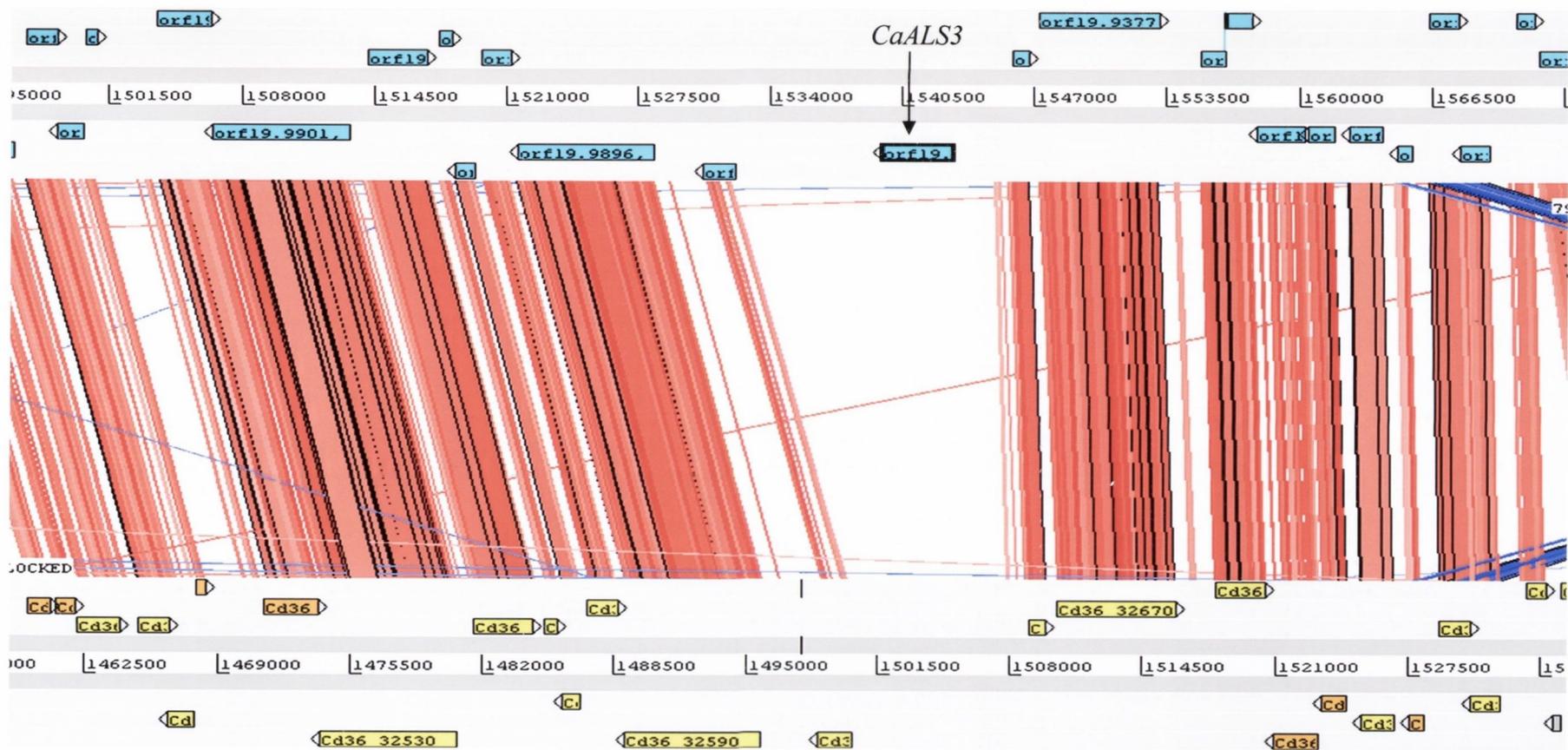
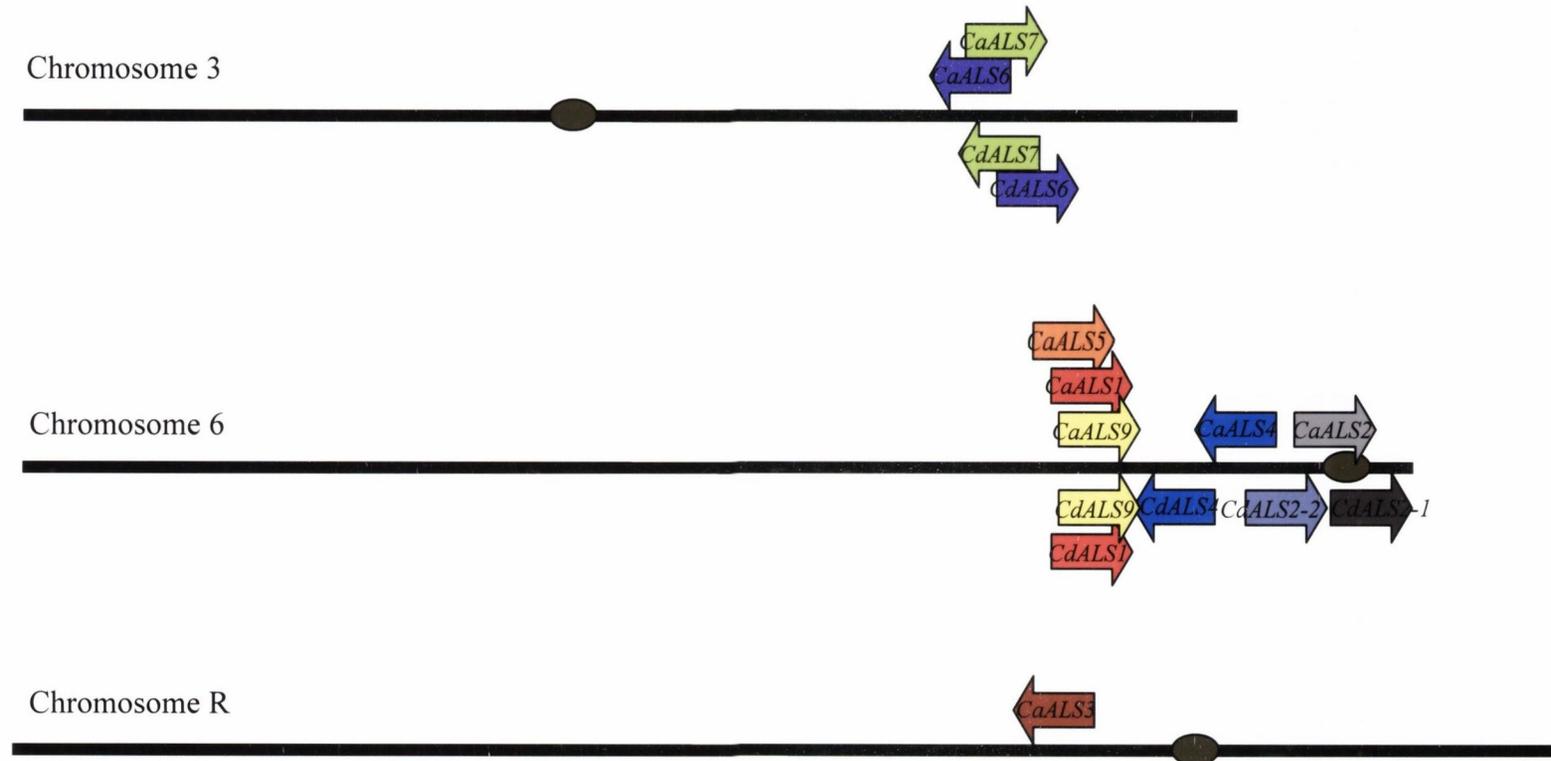
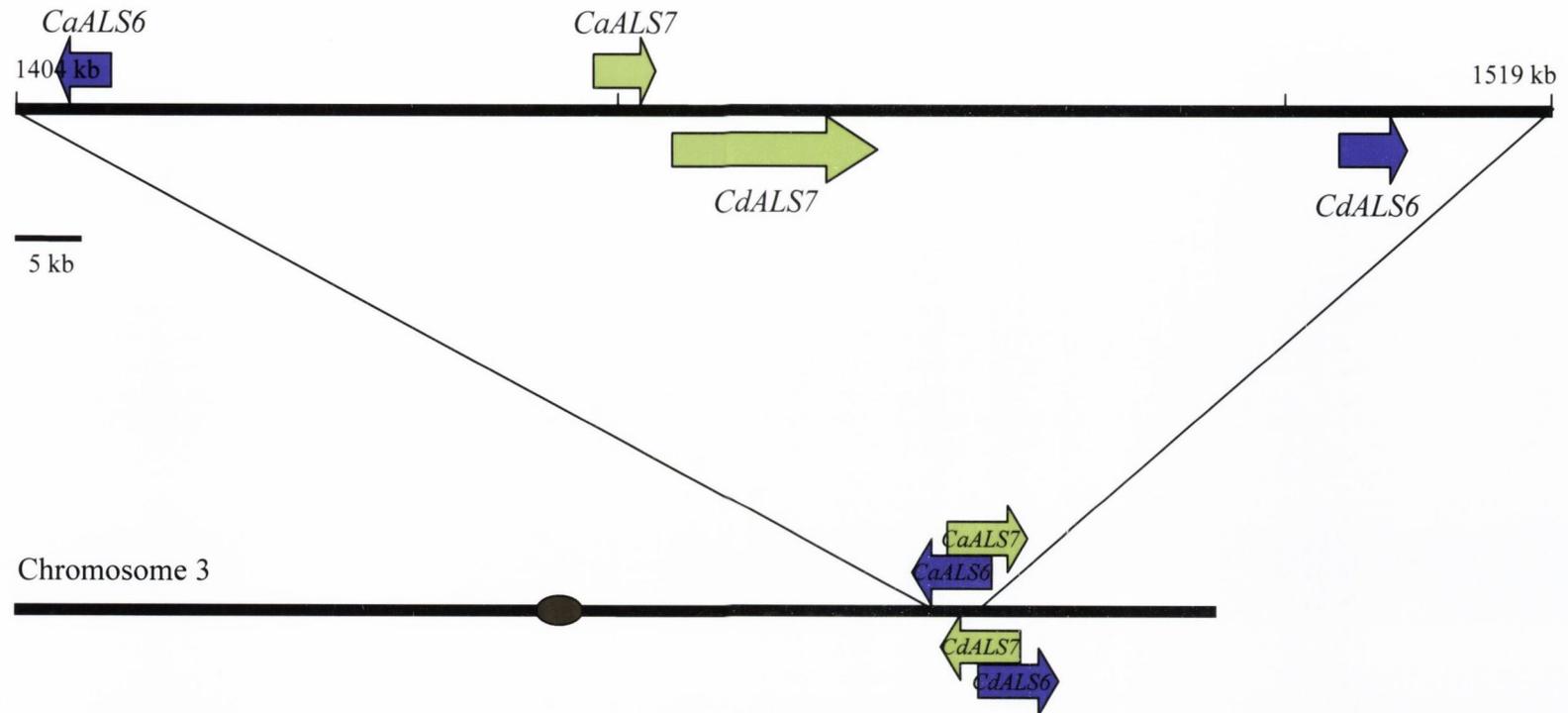


Figure 6.9. *orf19.9379* (*CaALS3*). This annotation was generated from ACT which was used to compare genome sequences and synteny between *C. dubliniensis* (bottom) and *C. albicans* (top) and shows the genomic arrangement of *orf19.9379* on chromosome R. DNA strands are represented by horizontal grey bars above and below a scale bar (in bases). The genes of *C. dubliniensis*, in each of the six ORFs, are represented by yellow and orange boxes above (sense) and below (anti-sense) the *C. dubliniensis* DNA strands and the genes of *C. albicans*, in each of the six ORFs, are represented by light blue boxes above (sense) and below (anti-sense) the *C. albicans* DNA strands. Significant synteny between genes is represented by vertical bars (red, forward matches; blue, reverse matches).



C. albicans centromere ●

Figure 6.10. Schematic diagram representing the position of the *ALS* genes of *C. dubliniensis* and *C. albicans* on each chromosome.



C. albicans centromere ●

Figure 6.11. Schematic diagram representing the position and relative size of the *ALS* genes of *C. dubliniensis* and *C. albicans* located on chromosome 3.

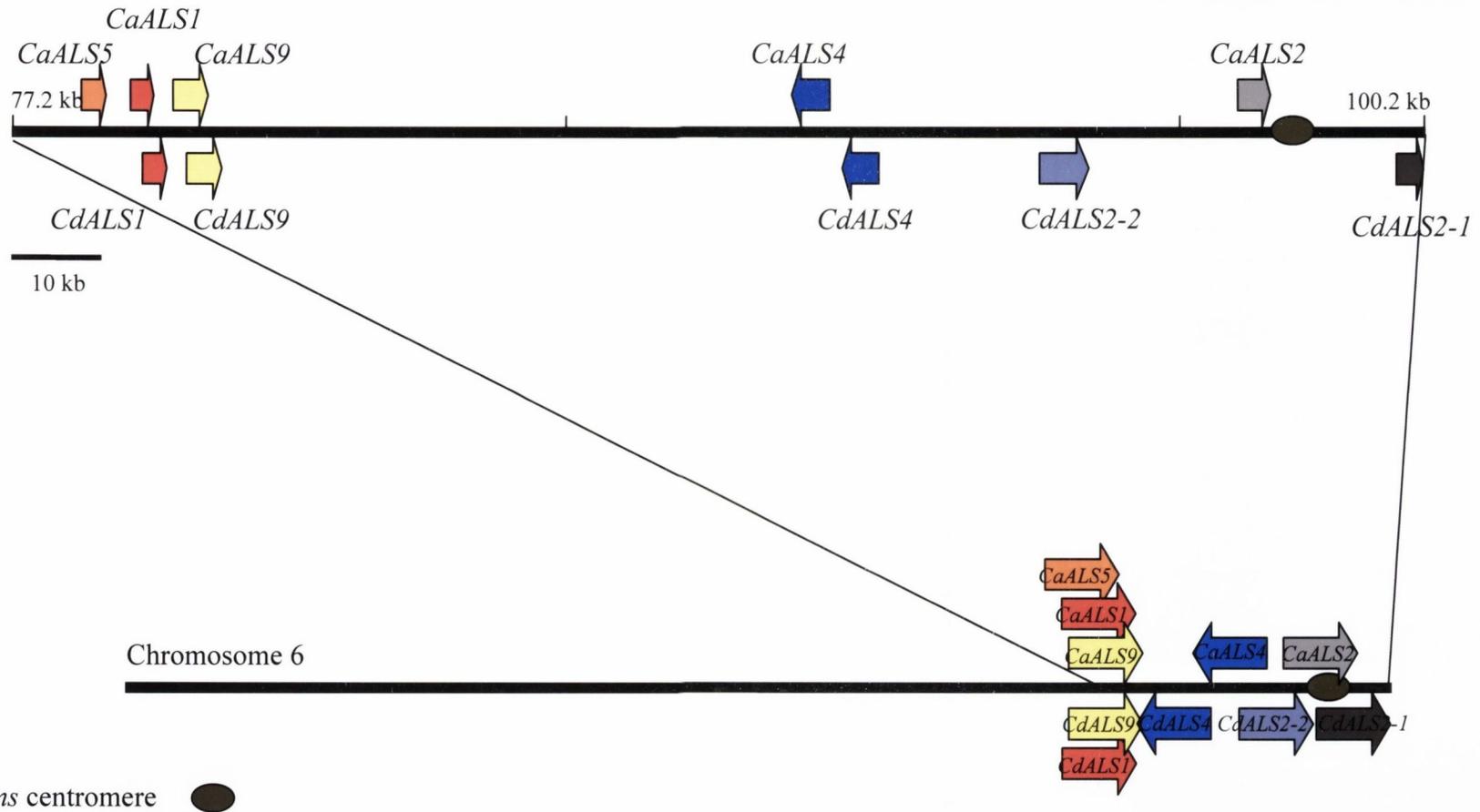
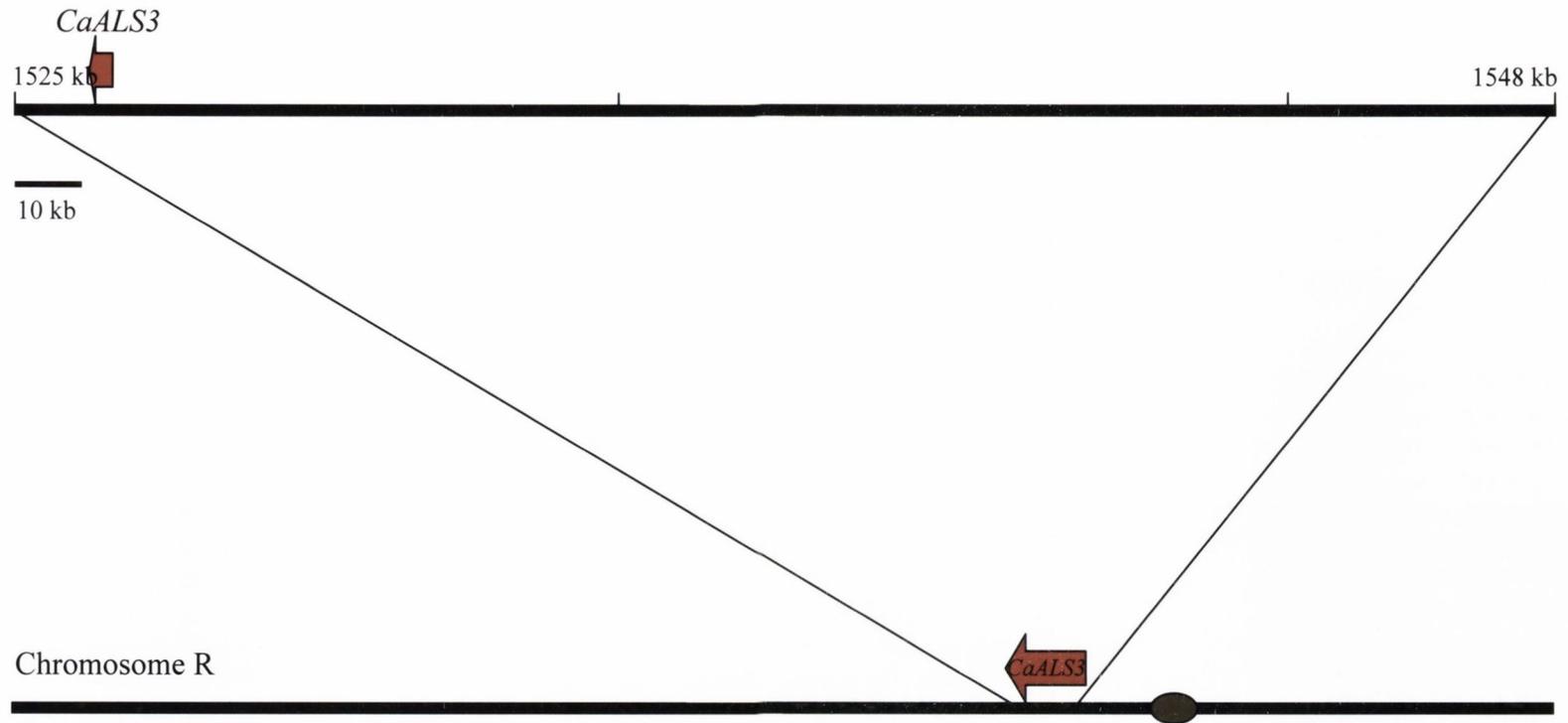


Figure 6.12. Schematic diagram representing the position and relative size of the ALS genes of *C. dubliniensis* and *C. albicans* located on chromosome 6.



C. albicans centromere ●

Figure 6.13. Schematic diagram representing the position and relative size of *CaALS3* which is located on chromosome R.

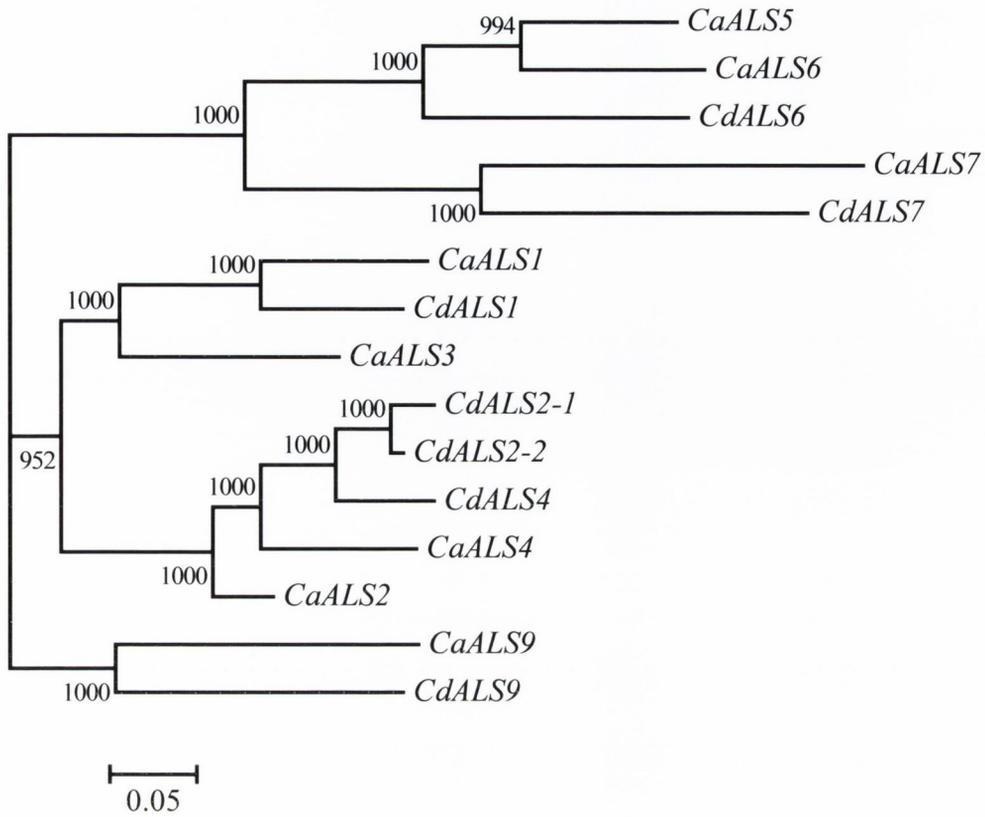


Figure 6.14. Phylogenetic tree depicting the relationship between *CaALS* and *CdALS* genes. The numbers at each node were generated by bootstrap analysis and represent the number of times the topology was generated in 1,000 replicates. The branch lengths are proportional to the similarity between amino acid sequences. The scale bar represents a 0.05% difference in amino acid sequence. This tree was constructed using the neighbour-joining method of Saitou & Nei (1987) and based on the entire protein sequence of each gene.

6.3.2 Amplification of *C. dubliniensis* and *C. albicans* ALS genes using conventional PCR

6.3.2.1 PCR amplification of *CdALS* and *CaALS* genes using genomic DNA and conventional PCR primers

In order to validate the specificity of each primer pair for its corresponding *ALS* gene, each primer pair was validated using genomic DNA. The *CdALS* primer pairs were validated using *C. dubliniensis* CD36 genomic DNA and the *CaALS* primer pairs were validated using *C. albicans* SC5314 genomic DNA. Each primer pair amplified a single product of the expected size.

6.3.2.2 Sequence analysis of PCR products amplified using the *CdALS* and *CaALS* primer pairs and genomic DNA

Nucleotide sequences of all *CdALS* and *CaALS* PCR products were obtained and translated using the Translate tool on the ExpASY proteomics server and a search of the protein database was carried out using the BLAST series of computer programs. Each translated *CaALS* PCR product sequence received a BLAST hit to the correct *CaALS* gene validating the specificity of each *CaALS* primer pair for the *C. albicans* genome. Where no corresponding sequences were found when a search was carried out using the BLAST series of computer programs, such as with the *CdALS* PCR products, the CLUSTAL W sequence alignment computer program was used to compare the nucleotide sequence derived from sequencing of the PCR product with the nucleotide sequence it was designed from. In each case the derived nucleotide sequence for each gene-specific *CdALS* primer pair matched exactly the predicted *CdALS* gene sequence and this validated the specificity of each *CdALS* primer pair for the *C. dubliniensis* genome. With the exception of the *CdALS2F/CdALS2Rb* primer pair, each primer pair differentially amplified a product corresponding to a single *ALS* gene and therefore could be used to assess the expression of individual *ALS* genes in both *C. dubliniensis* and *C. albicans* under various growth conditions. Amplification of both *CdALS2-1* and *CdALS2-2* was carried out using the *CdALS2F/CdALS2Rb* primer pair (Table 6.2).

6.3.2.3 PCR analysis of cDNA using gene-specific CdALS and CaALS primers

cDNA from samples of *C. dubliniensis* CD36 and *C. albicans* SC5314 when grown to mid-exponential phase in either YEPD or YEPGal at either 30°C or 37°C was used as template for PCR using each CdALS and CaALS primer pair. Amplification of *CdALS1*, *CdALS4*, *CdALS2*, *CdALS9*, *CaALS2*, *CaALS3*, *CaALS4* and *CaALS9* occurred under all growth and temperature conditions tested. There was no amplification of *CdALS7*, *CaALS6* or *CaALS7* under all growth and temperature conditions tested. There was no amplification of *CdALS6* and *CaALS5* under the majority of growth and temperature conditions tested; *CdALS6* formed a weak product when cultured in YEPD at 37°C and *CaALS5* formed a weak product when cultured at 30°C in either YEPD or YEPGal (Table 6.7).

6.3.3 Amplification of *C. dubliniensis* and *C. albicans* ALS genes using real time PCR

6.3.3.1 PCR amplification of *CdALS* and *CaALS* genes using genomic DNA and real time PCR primers

In order to validate the specificity of each primer pair for its corresponding *ALS* gene, each primer pair was validated using genomic DNA. The QRTCdALS primer pairs were validated using *C. dubliniensis* CD36 genomic DNA and the QRTCaALS primer pairs used and the MyQRTCaALS2 and MyQRTCaALS3 primer pairs were validated using *C. albicans* SC5314 genomic DNA. The QRTTEF1 primer pair was validated using both *C. dubliniensis* CD36 and *C. albicans* SC5314 genomic DNA. Each primer pair amplified a product of the expected size (Figs. 6.15 and 6.16). The *C. dubliniensis* QRTALS primers are not species specific, i.e. they will also amplify *C. albicans* DNA.

6.3.3.2 Sequence analysis of PCR products amplified using the QRTCdALS and QRTCaALS primer pairs and genomic DNA

Nucleotide sequences for all QRTCdALS PCR products were obtained. As no corresponding sequences were found when using the BLAST series of computer

Table 6.7. Expression of *C. dubliniensis* and *C. albicans* ALS genes under various growth conditions detected by conventional PCR

| Gene | Growth Condition | | | |
|---------------------------|------------------|------------|-------------|-------------|
| | YEPD 30°C | YEPD 37°C | YEPGal 30°C | YEPGal 37°C |
| <i>CdALS6</i> | No product | Weak band | No product | No product |
| <i>CdALS1</i> | Product | Product | Product | Product |
| <i>CdALS4</i> | Product | Product | Product | Product |
| <i>CdALS2^a</i> | Product | Product | Product | Product |
| <i>CdALS9</i> | Product | Product | Product | Product |
| <i>CdALS7</i> | No product | No product | No product | No product |
| <i>CaALS1</i> | Product | Product | Product | No product |
| <i>CaALS2</i> | Product | Product | Product | Product |
| <i>CaALS3</i> | Product | Product | Product | Product |
| <i>CaALS4</i> | Product | Product | Product | Product |
| <i>CaALS5</i> | Weak band | No product | Weak band | No product |
| <i>CaALS6</i> | No product | No product | No product | No product |
| <i>CaALS7</i> | No product | No product | No product | No product |
| <i>CaALS9</i> | Weak band | Product | Product | Product |

^aThe CdALS2 primer pair consisted of CdALS2F and CdALS2Rb and amplified both *CdALS2-1* and *CdALS2-2*

The method used to generate PCR products is detailed in section 6.2.2.2

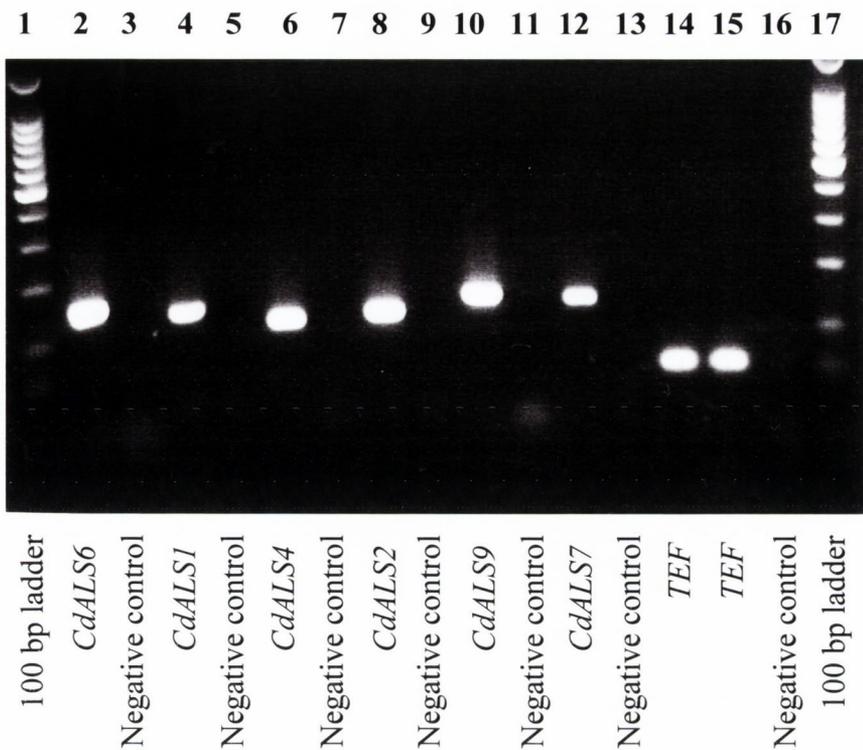


Figure 6.15. PCR products from amplification of *C. dubliniensis* CD36 genomic DNA using QRTCdALS gene-specific primer pairs. PCR reactions were carried out to determine if each primer pair amplified a single gene-specific product. The resulting products were separated on an agarose gel and stained with ethidium bromide. The product size for each primer pair is as follows: QRTCdALS6 primer pair = 145 bp in lane 2, QRTCdALS1 primer pair = 135 bp in lane 4, QRTCdALS4 primer pair = 109 bp in lane 6, QRTCdALS2 primer pair = 116 bp in lane 8, QRTCdALS9 primer pair = 145 bp in lane 10 and QRTCdALS7 primer pair = 139 bp in lane 12. The TEF product was produced using *C. dubliniensis* CD36 genomic DNA (51 bp in lane 14) and *C. albicans* SC5314 genomic DNA (51 bp in lane 15).

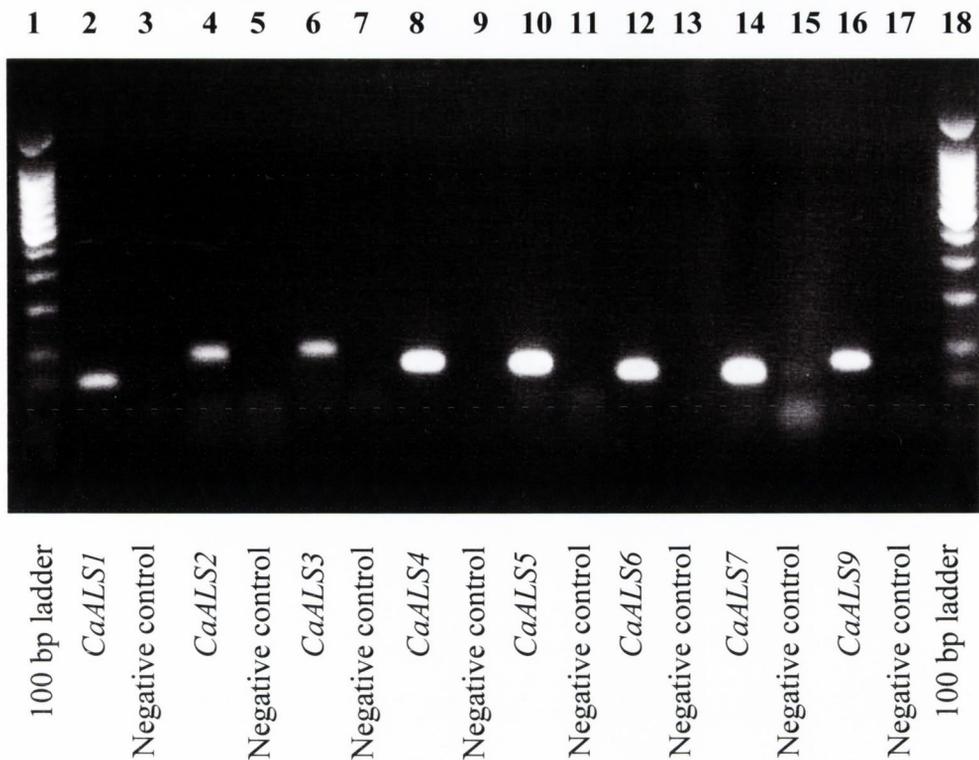


Figure 6.16. PCR products from amplification of *C. albicans* SC5314 genomic DNA using QRTCALS gene-specific primer pairs. QRTCALS gene-specific primers were previously described by Green *et al.* (2005). The PCR products were separated on an agarose gel and stained with ethidium bromide. The product size for each primer pair is as follows: QRTCALS1 primer pair = 53 bp in lane 2, MyQRTCALS2 primer pair = 106 bp in lane 4, MyQRTCALS3 primer pair = 123 bp in lane 6, QRTCALS4 primer pair = 64 bp in lane 8, QRTCALS5 primer pair = 63 bp in lane 10, QRTCALS6 primer pair = 52 bp in lane 12, QRTCALS7 primer pair = 51 bp in lane 14 and QRTCALS9 primer pair = 68 bp in lane 16.

programs due to the very small size of the PCR product formed when using the QRTCdALS PCR primers, the CLUSTAL W sequence alignment computer program was used to compare the nucleotide sequence derived from sequencing of the PCR product with the nucleotide sequence it was designed from. In each case the derived nucleotide sequence for each QRTCdALS primer pair matched exactly the predicted *CdALS* gene sequence and this validated the specificity of each QRTCdALS primer pair for the *C. dubliniensis* genome. With the exception of the QRTCdALS2 primer pair, each primer pair differentially amplified a product corresponding to a single *CdALS* gene. Amplification of both *CdALS2-1* and *CdALS2-2* were carried out using the QRTCdALS2 primer pair (Table 6.4).

Sequencing reactions for all QRTCaALS PCR products failed when using either the corresponding forward or reverse primer for each product due to the extremely small size of these PCR products (51 – 68 bp). As these primer pairs were taken from a published article where the primer specificity had been verified by sequencing of each PCR product which had been amplified using genomic DNA (Green *et al.*, 2005), it was assumed that the primers differentially amplified a single *CaALS* gene and so, use of these primer pairs continued. As the QRTCaALS2 primer pair did not amplify *C. albicans* SC5314 genomic DNA and the QRTCaALS3 primer pair amplified the NTC under conventional PCR conditions, use of these primer pairs was discontinued and new real time primers for *CaALS2* and *CaALS3* were designed as detailed in section 6.2.3.1. Nucleotide sequences for the MyQRTCaALS2 and MyQRTCaALS3 PCR products were obtained and translated using the Translate tool on the ExpASY proteomics server. Both translated PCR product sequences received a BLAST hit to the correct *CaALS* gene validating the specificity of both primer pairs for the *C. albicans* genome. The CLUSTAL W sequence alignment computer program was also used to compare the nucleotide sequence derived from sequencing of the PCR product with the nucleotide sequence it was designed from. In each case the derived nucleotide sequence for each MyQRTCaALS primer pair matched exactly the predicted *CaALS* gene sequence and this validated the specificity of both primer pairs for the *C. albicans* genome. The MyQRTCaALS2 and MyQRTCaALS3 primer pairs replaced the QRTCaALS2 and QRTCaALS3 primer pairs in this part of the present study.

6.3.3.3 Amplification efficiencies of real time ALS PCR primers

Amplification efficiencies were calculated by subtracting the Ct value of the gene of interest (in this case, the *ALS* genes) from the Ct value of the internal control (in this case, the gene, *TEF*) calculated from the same sample and plotting the differences against the log of the template genomic DNA amount. If the slope of the resultant line is less than 0.1, then amplification efficiencies are comparable. Values for each QRTALS primer pair were less than 0.1 (data not shown) and as such it was determined that the amplification efficiencies were comparable and that these primer pairs were suitable for use in real time PCR amplification of the *ALS* genes of *C. dubliniensis* and *C. albicans*.

6.3.3.4 *C. dubliniensis* and *C. albicans* ALS gene expression

Figs. 6.17 to 6.22 and Table 6.8 show the fold expression of the *ALS* genes in adherent and non-adherent *Candida* when pre-cultured under various growth and temperature conditions. In *C. albicans* SC5314, *CaALS2* was down-regulated under all conditions tested. *CaALS1*, *CaALS4* and *CaALS9* were up-regulated to a greater extent in adherent *C. albicans* SC5314 compared to non-adherent *C. albicans* SC5314. *CaALS7* was up-regulated to a greater extent in adherent *C. albicans* SC5314 when grown in YEPD compared to adherent *C. albicans* SC5314 when grown in YEPGal and there was greater expression of *CaALS7* in adherent *C. albicans* SC5314 when grown at 30°C compared to 37°C in the same growth media. *CaALS9* (1.389-fold) was up-regulated to the same extent in hyphal *C. albicans* SC5314 as in adherent *C. albicans* SC5314 while *CaALS5* (-0.258-fold) and *CaALS6* (-0.351-fold) were down-regulated in hyphal *C. albicans* SC5314 compared to up-regulation of these genes in all other conditions tested. *CaALS3* in hyphal *C. albicans* SC5314 was the most up-regulated of all genes and conditions tested (3.854-fold) and was up-regulated to a greater extent than in adherent *C. albicans* SC5314 (Figs. 6.17 and 6.22; Table 6.8).

In *C. dubliniensis* CD36, *CdALS1* and *CdALS6* were up-regulated in adherent and hyphal *C. dubliniensis* CD36 while both genes were down-regulated in non-adherent *C. dubliniensis* CD36. *CaALS2* was down-regulated in adherent and hyphal *C. dubliniensis* CD36 while it was up-regulated in non-adherent *C. dubliniensis* CD36. With the exception of *C. dubliniensis* CD36 grown in YEPGal at 37°C (0.241-fold), *CdALS7* was down-regulated under all conditions tested. When *C. dubliniensis* CD36

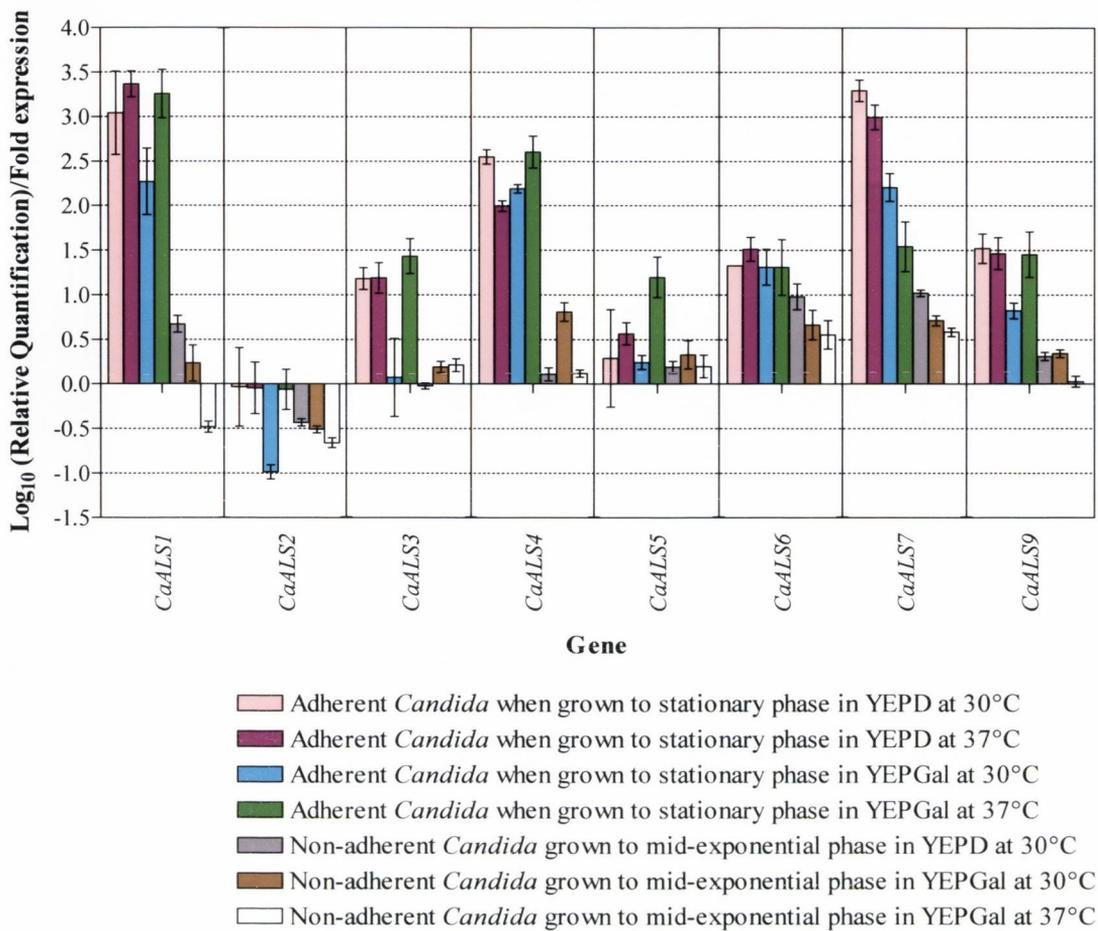


Figure 6.17. Expression of each *CaALS* gene of adherent and non-adherent *C. albicans* SC5314 under various growth and temperature conditions. The expression of each *CaALS* gene of adherent and non-adherent *C. albicans* SC5314 under various growth and temperature conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. albicans* SC5314 grown in YEPD at 37°C.

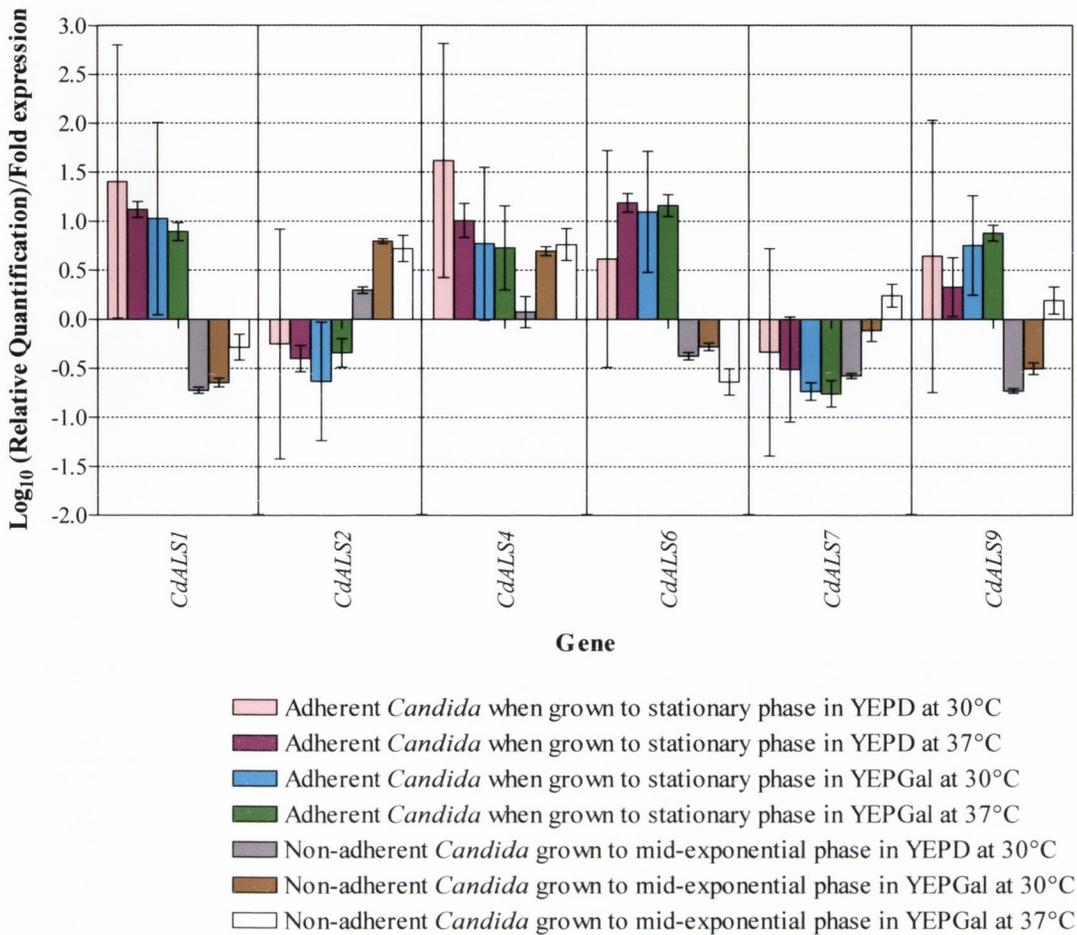


Figure 6.18. Expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* CD36 under various growth and temperature conditions. The expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* CD36 under various growth and temperature conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. dubliniensis* CD36 grown in YEPD at 37°C.

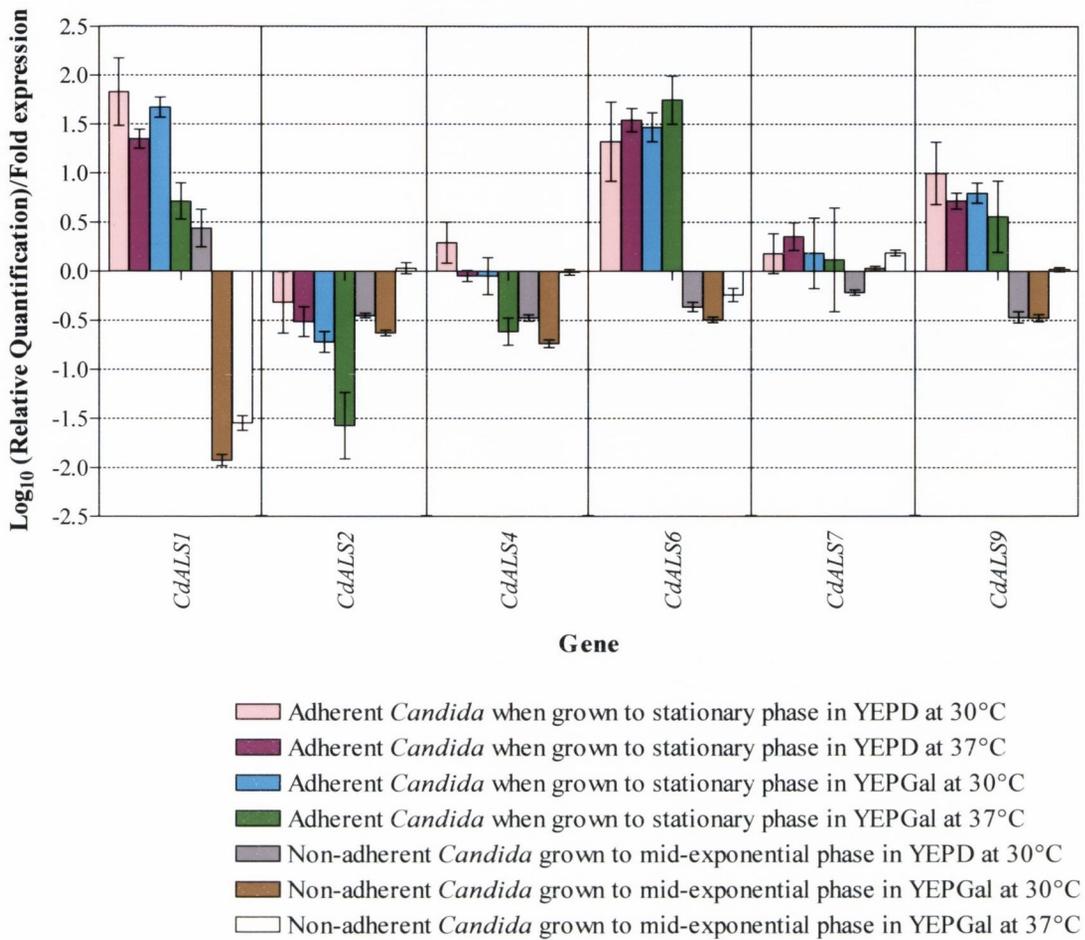


Figure 6.19. Expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* Can6 under various growth and temperature conditions. The expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* Can6 under various growth and temperature conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. dubliniensis* Can6 grown in YEPD at 37°C.

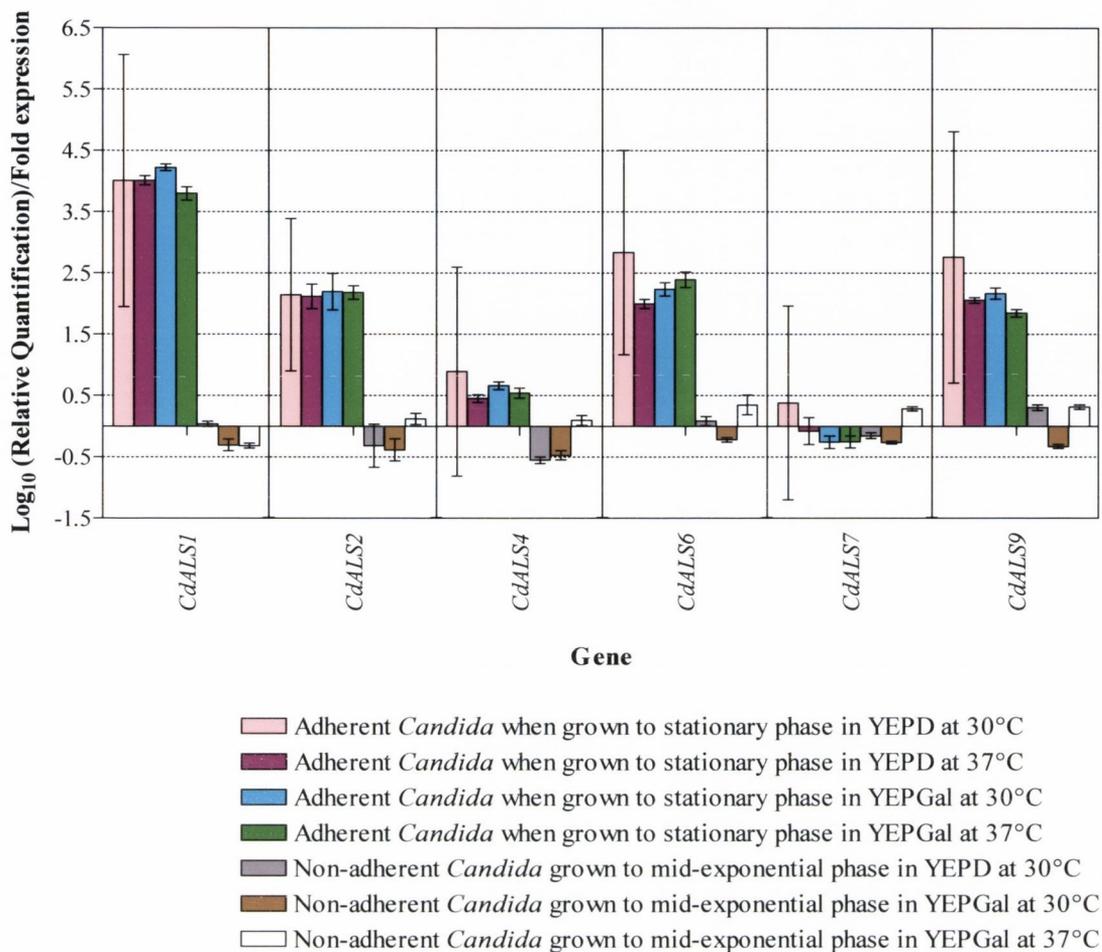


Figure 6.20. Expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* CD514 under various growth and temperature conditions. The expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* CD514 under various growth and temperature conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. dubliniensis* CD514 grown in YEPD at 37°C.

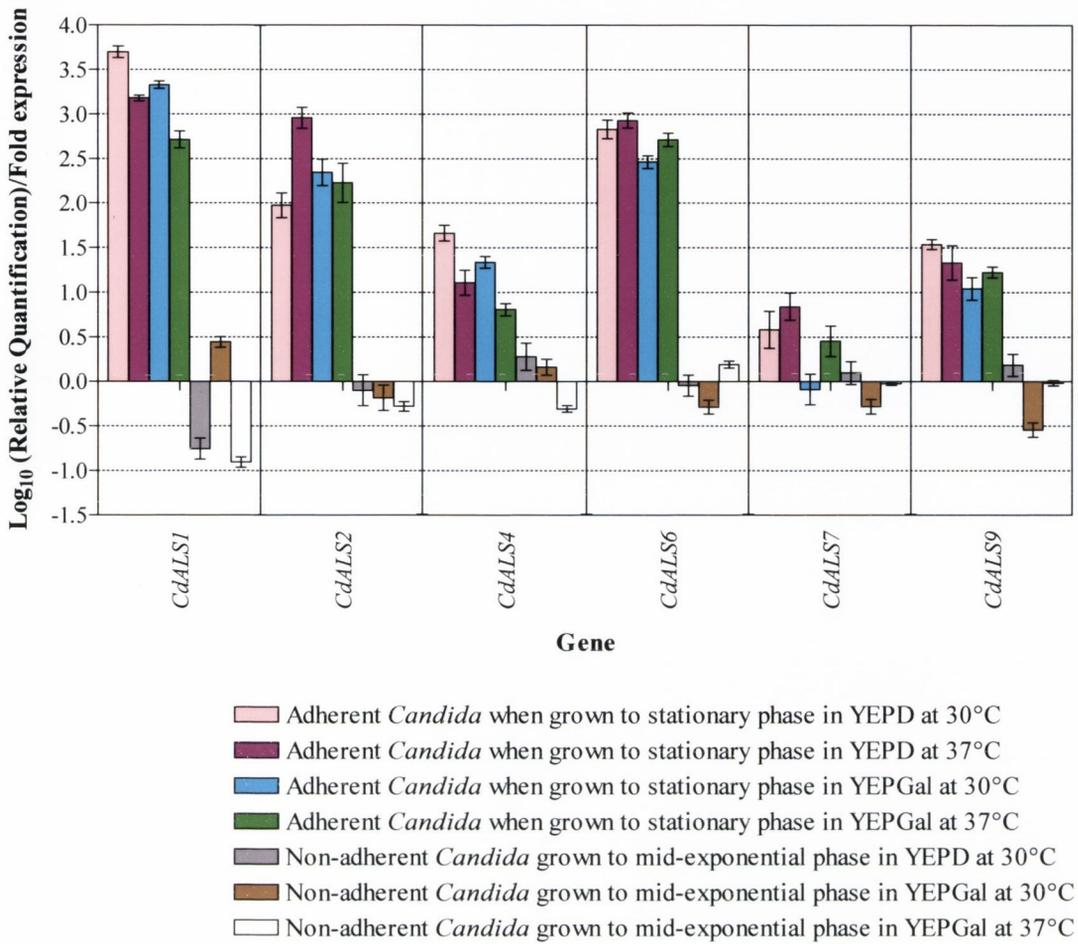


Figure 6.21. Expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* Eg200 under various growth and temperature conditions. The expression of each *CdALS* gene of adherent and non-adherent *C. dubliniensis* Eg200 under various growth and temperature conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. dubliniensis* Eg200 grown in YEPD at 37°C.

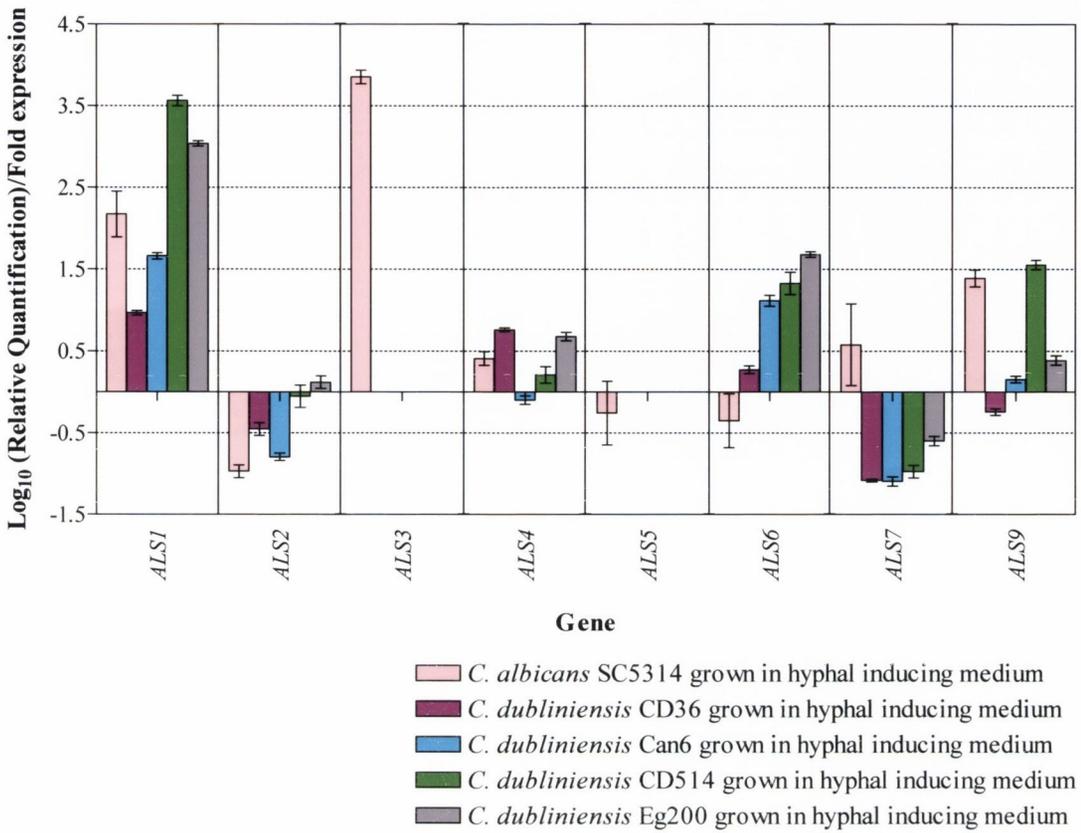


Figure 6.22. Expression of each *CaALS* and *CdALS* gene of *C. albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 under hyphal inducing conditions.

The expression of each *CaALS* and *CdALS* gene of *C. albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 under hyphal inducing conditions are represented by fold expression compared to the calibrator sample of RNA extracted from mid-exponential phase *C. albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 grown in YEPD at 37°C respectively.

was grown in hyphal inducing medium, *CdALS1* was the most up-regulated gene (0.965-fold) and *CdALS7* (-1.082-fold), and *CdALS2* (-0.455-fold) and *CdALS9* (-0.245-fold) to a lesser extent, were the most down-regulated (Figs. 6.18 and 6.22; Table 6.8).

In *C. dubliniensis* Can6, *CdALS2* was down-regulated under all conditions tested with the exception of non-adherent *C. dubliniensis* Can6 when grown in YEPGal at 37°C (0.03-fold). With the exception of adherent *C. dubliniensis* Can6 when grown in YEPD at 30°C (0.291-fold), *CdALS4* was down-regulated under all conditions tested. *CdALS6* was up-regulated in adherent and pseudohyphal *C. dubliniensis* Can6 however, this gene was down-regulated in non-adherent *C. dubliniensis* Can6. *CdALS6* (1.115-fold) was up-regulated to a similar extent in pseudohyphal *C. dubliniensis* Can6 as in adherent *C. dubliniensis* Can6. *CdALS1* (1.661-fold) and *CdALS6* (1.115-fold) were the most up-regulated genes in pseudohyphal *C. dubliniensis* Can6 and *CdALS7* (-1.096-fold) and *CdALS2* (-0.796-fold) were the most down-regulated (Figs. 6.19 and 6.22; Table 6.8).

All *CdALS* genes were down-regulated in *C. dubliniensis* CD514 when grown in YEPGal at 30°C whereas all *CdALS* genes were up-regulated in *C. dubliniensis* CD514 when grown in YEPGal at 37°C and adherent *C. dubliniensis* CD514 when grown in YEPD at 30°C. *CdALS1*, *CdALS2*, *CdALS6* and *CdALS9* were up-regulated to a greater extent in adherent *C. dubliniensis* CD514 than in non-adherent *C. dubliniensis* CD514 and *CdALS1* (3.564-fold) and *CdALS9* (1.553-fold) were expressed at similar levels in pseudohyphal *C. dubliniensis* CD514 as in adherent *C. dubliniensis* CD514. *CdALS1* (3.564-fold) was the most up-regulated of the *CdALS* genes in pseudohyphal *C. dubliniensis* CD514 and *CdALS7* (-0.974-fold) was down-regulated in pseudohyphal *C. dubliniensis* CD514 (Figs. 6.20 and 6.22; Table 6.8).

CdALS1, *CdALS2*, *CdALS4*, *CdALS6* and *CdALS9* were up-regulated to a greater extent in adherent *C. dubliniensis* Eg200 than non-adherent *C. dubliniensis* Eg200. *CdALS1* (3.037-fold) was expressed at similar levels in hyphal *C. dubliniensis* Eg200 as in adherent *C. dubliniensis* Eg200. With the exception of *CdALS6* (0.191-fold), all *CdALS* genes were down-regulated in *C. dubliniensis* Eg200 when grown in YEPGal at 37°C. *CdALS1* was the most up-regulated gene of the *CdALS* genes of *C. dubliniensis* Eg200 when grown in hyphal inducing medium (3.037-fold) while *CdALS7* (-0.599-fold) was down-regulated in this growth medium (Figs. 6.21 and 6.22; Table 6.8).

6.4 DISCUSSION

6.4.1 Re-naming the *ALS* genes of *C. dubliniensis*

The *CdALS* genes were re-named based on their nucleotide and amino acid homology to and synteny with the *CaALS* genes. While most of the *CdALS* genes had obvious orthologs in *C. albicans* (*Cd36_64210/CaALS1* (Fig. 6.4), *Cd36_64610/CaALS4* (Fig. 6.6), *Cd36_86290/CaALS6* (Fig. 6.3), *Cd36_86150/CaALS7* (Fig. 6.2) and *Cd36_64220/CaALS9* (Fig. 6.5)), other *CdALS* genes showed similar homology to more than one *CaALS* gene. This is not surprising considering the *ALS* gene family share a highly conserved 5' domain (Hoyer, 2001; Hoyer *et al.*, 2001). *Cd36_86150* although syntenous with *CaALS7* shares only 27% nucleotide homology with *CaALS7* and is longer than *CaALS7*. This is due the presence of an extra 13 repeats of a sequence of 36 amino acids in *CdALS7*. As these repeat sequences were within *CdALS7*, their presence did not affect the synteny between *CdALS7* with *CaALS7* (Fig. 6.2).

Cd36_65010 was most similar to *CaALS2* based on nucleotide sequence, whereas it was most similar to *CaALS4* based on amino acid sequence. ACT analysis showed that *Cd36_65010* was most syntenous to *CaALS2* (Fig. 6.7). Along with the highly conserved 5' domain across the *ALS* family, the 3' domain is also highly conserved between certain pairs of genes such as *CaALS2* and *CaALS4* (Hoyer *et al.*, 1998b) making them difficult to distinguish from each other and hence the high homology of *Cd36_65010* to both *CaALS2* and *CaALS4*. As *Cd36_65010* showed nucleotide homology to and synteny with *CaALS2* it was re-named *CdALS2*. *Cd36_64800* had 78% nucleotide homology to *CaALS2* and based on the position of the *CaALS* and flanking genes *Cd36_64800* was more syntenous with *CaALS2* than *CaALS4* (Fig. 6.8). *Cd36_64800* appears to be an extra gene which has arisen in *C. dubliniensis* due to inversion, splicing and recombination of the *C. dubliniensis* genome as opposed to the ortholog of a *CaALS* gene which has been deleted from the *C. albicans* genome. *Cd36_65010* is located at the edge of an inversion in sequence between the genomes of *C. dubliniensis* and *C. albicans*. Inversion of this sequence could have caused a duplication of *Cd36_65010* giving rise to *Cd36_64800*. Because of this, it appears that *C. dubliniensis* has two *CdALS2* genes. As *Cd36_65010* was more

similar in length to *CaALS2*, it was re-named *CdALS2-1*. *Cd36_64800* has two large insertions of 432 and 612 amino acids respectively and although *Cd36_64800* is nearer in length to *CaALS4*, it is more syntenous with *CaALS2*, and so was re-named *CdALS2-2*.

There are no orthologs in *C. dubliniensis* for *CaALS3* (Fig. 6.9) or *CaALS5*. In *C. dubliniensis*, there is no corresponding nucleotide sequence to *CaALS3*, suggesting that either the corresponding *CaALS3* ortholog was deleted from *C. dubliniensis* or that it arose in *C. albicans* by insertion or gene duplication. Whereas *CaALS5* shows 35% nucleotide homology to *Cd36_64210*, this latter gene shows more homology to and synteny with *CaALS1*.

6.4.2 Amplification of the ALS genes of *C. dubliniensis* and *C. albicans* using conventional PCR

The aim of this part of the present study was to examine the *ALS* gene expression of *C. dubliniensis* CD36 and *C. albicans* SC5314 yeast cells when grown under different temperature and media conditions. Although the primers and amplification conditions for the *CaALS* genes had been validated and optimised by Green *et al.* (2004), the *CaALS* primer pairs were validated again in this part of the present study using *C. albicans* SC5314 genomic DNA to confirm each primer pairs' specificity for a single *CaALS* gene. While each primer pair was found to be gene-specific, the melting temperature for each *CaALS* primer was found to be higher in the present study than previously stated by Green *et al.* (2004). Sequence analysis of each *CdALS* and *CaALS* PCR product determined the gene-specific discriminatory nature of each primer pair with the exception of the *CdALS2F/CdALS2Ra* and *CdALS2F/CdALS2Rb* primers pairs which both amplified *CdALS2-1* and *CdALS2-2*. Analysis of the PCR product formed from these primer pairs showed that the sequence amplified when using the *CdALS2F/CdALS2Rb* primer pair was more homologous to both *CdALS2-1* and *CdALS2-2* and as such use of *CdALS2Ra* was discontinued.

cDNA from mid-exponential phase cultures of *C. dubliniensis* CD36 and *C. albicans* SC5314 grown in either YEPD or YEPGal at 30°C or 37°C was used as the template for PCR using the *CdALS*, *CaALS* and actin primers. The results showed that not all *ALS* genes were expressed and that *CdALS1*, *CdALS4*, *CdALS2*, *CdALS9*, *CaALS2*, *CaALS3*, *CaALS4* and *CaALS9* were expressed under all growth and

temperature conditions (Table 6.7). These results contradict the published results of Green *et al.* (2004) who found that all *CaALS* genes were expressed in an inoculum culture of *C. albicans* SC5314. The gene expression analysis by Green *et al.* (2004) was carried out on stationary phase cultures of *C. albicans* SC5314, whereas cultures in the present study were examined at mid-exponential phase. The difference in growth phase of the *Candida* cells is likely to account for the difference observed in *ALS* gene expression. As synthesis of *CaALS4* begins at mid-log phase and increases as cultures reach exponential phase (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005), it is not surprising that *CaALS4* was detected in both this study where mid-exponential phase *Candida* cultures were used and in a study by Green *et al.* (2004), where stationary phase *Candida* cultures were used.

In this part of the present study, *CaALS2* and its orthologs in *C. dubliniensis*, *CdALS2-1* and *CdALS2-2*, *CaALS4* and its ortholog in *C. dubliniensis*, *CdALS4* and *CaALS9* and its ortholog in *C. dubliniensis*, *CdALS9* were expressed under all growth conditions indicating that these genes are constitutively expressed in both *C. albicans* SC5314 and *C. dubliniensis* CD36. These results concur with the results of Green *et al.* (2004). While *C. dubliniensis* has no ortholog of *CaALS3*, which was expressed under all growth conditions tested, *CdALS1* which is the mostly closely related *CdALS* gene to *CaALS3* (Fig. 6.14), was also expressed under all growth conditions tested, despite its direct ortholog, *CaALS1*, only being expressed in three of the four conditions tested (Table 6.7). *CaALS7* and its ortholog in *C. dubliniensis*, *CdALS7*, were not expressed under any of the growth conditions tested, while *CaALS6* and its ortholog in *C. dubliniensis*, *CdALS6* showed similar expression profiles (Table 6.7). As *CaALS6* and *CaALS7* are transcribed at lower levels than the other *CaALS* genes, it is possible that these genes are expressed below the limit of detection.

Due to the highly conserved 5' and 3' domains in *CaALS2* and *CaALS4* (Hoyer *et al.*, 1998b) it is not surprising that both *CaALS2* and *CaALS4* are expressed under the same growth conditions. As *CdALS2-1*, *CdALS2-2* and *CdALS4* are also very closely related (Fig. 6.14; 97% amino acid homology between *CdALS2-1* and *CdALS2-2*, 87% amino acid homology between *CdALS2-1* and *CdALS4* and 92% amino acid homology between *CdALS2-2* and *CdALS4*) it is also not surprising that these genes are expressed under the same growth conditions. The similar expression profiles of these genes across the two species is indicative of how closely related they are (71% amino acid homology between *CaALS2* and *CdALS2-1*, 85% amino acid homology between *CaALS2* and

CdALS2-2 and 83% amino acid homology between *CaALS4* and *CdALS4*) as similar growth conditions induced expression.

6.4.3 Amplification of the *ALS* genes of *C. dubliniensis* and *C. albicans* using real time RT-PCR

The main aim of this part of the study was to examine the comparative expression of the *ALS* genes in representative strains of the 4 genotypes of *C. dubliniensis* and of *C. albicans* SC5314 under various growth and temperature conditions using quantitative real time RT-PCR.

cDNA from mid-exponential phase, non-adherent and stationary phase, adherent *C. albicans* SC5314 and *C. dubliniensis* CD36, Can6, CD514 and Eg200 when individually cultured in YEPD or YEPGal at 30°C or 37°C and cDNA from hyphal *C. albicans* SC5314 and hyphal *C. dubliniensis* CD36 and Eg200 and pseudohyphal *C. dubliniensis* Can6 and CD514 was used as a template for PCR using the QRTCdALS, QRTCaALS and EF exon primers. *CaALS2* was down regulated under all growth conditions (Figs. 6.17 and 6.22). Similar results were observed in *C. dubliniensis* where *CdALS2* was down-regulated in *C. dubliniensis* CD36 in adherent and hyphal conditions (Figs. 6.18 and 6.22), in *C. dubliniensis* Can6 under all growth conditions (Figs. 6.19 and 6.22), in *C. dubliniensis* CD514 in non-adherent and hyphal conditions (Figs. 6.20 and 6.22) and in *C. dubliniensis* Eg200 in non-adherent conditions (Fig. 6.21). However, the QRTCdALS2 primer pair amplifies both *CdALS2-1* and *CdALS2-2* and this may be the reason for the difference in expression between *CaALS2* and *CdALS2*. *CdALS2-1* and *CdALS2-2* are practically identical (97% amino acid homology between *CdALS2-1* and *CdALS2-2*) and were initially thought to be one gene so it is possible that they have a similar function. Further work including determination of the protein structure and formation of mutant strains for each *CdALS2* gene could be carried out to further understand the difference(s) between these two practically identical genes. Despite the fact that *CaALS2* was down-regulated under all growth conditions, *CaALS4* was up-regulated under all growth conditions tested (Figs. 6.17 and 6.22). The highly conserved 5' and 3' domains shared by *CaALS2* and *CaALS4* mean that these genes are very similar (85% similar at the amino acid level) so it is interesting that the same condition caused differential regulation and expression of these two genes. It is possible that, despite the similarity in amino acid sequence, a

compensatory function (Zhao *et al.*, 2005) between *CaALS2* and *CaALS4* is being observed. This would explain the different responses of *CaALS2* and *CaALS4* to the same condition. Generally there is more expression of the *CaALS* genes in adherent than non-adherent *C. albicans* SC5314 confirming the role of the *CaALS* genes in adhesion (Fig. 6.17; Table 6.8). *CaALS3* is up-regulated 3.854-fold in hyphal *C. albicans* SC5314, as expected (Fig. 6.22; Table 6.8; Hoyer *et al.*, 1998a; Green *et al.*, 2005).

In *C. dubliniensis* CD36, *CdALS4* was up-regulated under all growth conditions (Figs. 6.18 and 6.22; Table 6.8), as was its ortholog in *C. albicans* SC5314 (Figs. 6.17 and 6.22; Table 6.8). However, in *C. dubliniensis* Can6, *CdALS4* was down-regulated under all growth conditions except one (Figs. 6.19 and 6.22; Table 6.8), showing no clear trend in the regulation of this gene either between or within the species tested. In all *C. dubliniensis* isolates tested, *CdALS1* was the most up-regulated gene under hyphal inducing conditions while *CdALS7* was the most down regulated (Fig. 6.22; Table 6.8). *CdALS2* was also down-regulated under hyphal conditions in all *C. dubliniensis* isolates tested, except Eg200 (Fig. 6.22; Table 6.8). While *C. dubliniensis* does not have a direct ortholog of *CaALS3*, which is transcribed when germ tubes are visible, *CdALS1* is the most closely related *CdALS* gene to *CaALS3* (Fig. 6.14). It is possible then, that *CdALS1* is involved in the production of hyphal cells. In *C. albicans*, Zhao *et al.* (2004) suggested functional redundancy between *CaALS1* and *CaALS3* so it is possible that the ortholog of *CaALS1* (i.e. *CdALS1*) plays a role in hyphal formation in *C. dubliniensis*. The lack of an ortholog for *CaALS3* in *C. dubliniensis* may contribute to the lower induction of hyphal cells in *C. dubliniensis* compared to *C. albicans* and may also contribute to its poor ability relative to *C. albicans* to invade human cells and cause infection (Phan *et al.*, 2007). Interestingly *CaALS1* is the second most up-regulated gene in *C. albicans* SC5314 under hyphal inducing conditions (Fig. 6.22; Table 6.8) suggesting that *CaALS1* plays some role in the hyphal formation. Despite the fact that *CdALS7* was down-regulated under hyphal inducing conditions in all *C. dubliniensis* isolates tested, *CaALS7* was up-regulated (0.577-fold) in *C. albicans* SC5314 (Fig. 6.22; Table 6.8). Deletion of *CaALS7* resulted in decreased growth rate of *C. albicans* suggesting that *CaALS7* has some function in maintaining growth rate. If *CdALS7* also functions in maintaining growth rate, the down-regulation of *CdALS7* may reduce the growth rate of *C. dubliniensis* under hyphal inducing conditions, which may further decrease the ability of *C. dubliniensis* to filament. In this part of the present study, *CdALS2* (which includes *CdALS2-1* and *CdALS2-2*) was down-regulated in all hyphal

and pseudohyphal *C. dubliniensis* isolates tested (with the exception of Eg200), as was *CaALS2* in hyphal *C. albicans* SC5314 (Fig. 6.22; Table 6.8). However, Green *et al.* (2005) observed that *CaALS2* was strongly up-regulated during germ-tube formation in both serum-containing media and RPMI-1640 medium. The method used by Green *et al.* (2005) to induce germ tubes differed significantly from the method used in this study (section 2.1.1) and this probably accounts for the difference in results.

CdALS6 was up-regulated in all adherent *C. dubliniensis* isolates tested (Figs. 6.18 to 6.21; Table 6.8). It was either up-regulated compared to its down-regulation under non-adherent conditions or more up-regulated compared to its up-regulation under non-adherent conditions. *CdALS1* (with the exception of Can6; Figs 6.18, 6.20 and 6.21; Table 6.8) and *CdALS9* (with the exception of CD36; Figs. 6.19 to 6.21; Table 6.8) were up-regulated in all adherent *C. dubliniensis* isolates tested compared to non-adherent conditions.

In this study, mid-exponential phase *Candida* cells which were pre-cultured in YEPD at 37°C were used as the calibrator sample for QRT RT-PCR. This condition was chosen as more RNA could be recovered from mid-exponential phase cells than from stationary phase cells and growth in YEPD at 37°C is the standard growth condition for *Candida*. However, as stationary phase *Candida* cells were used in the BEC adhesion assay to determine adherent candidal numbers, stationary phase adherent *Candida* cells were also used for real time PCR quantification of the *ALS* genes so that the results between the two assays could be compared. In hindsight, using stationary phase *Candida* cells which had been washed in PBS may have been a more appropriate calibrator sample to use in the QRT RT-PCR assay, as stationary phase *Candida* cells which had been washed in PBS were used in both the BEC adhesion assay and in the QRT RT-PCR assay.

6.4.4 Comparative results from conventional and real time PCR

While conventional PCR indicates whether a gene is expressed or not, real time PCR indicates by how much a gene is up- or down-regulated. While the results can not be directly compared due to the quantitative nature of real time PCR, up-regulation of a gene in real time PCR and expression of a product from conventional PCR will be discussed. When grown to mid-exponential phase in YEPD at 30°C, *CaALS1* and *CaALS4* were detected by both real time and conventional PCR. When grown to mid-exponential phase in YEPGal at 30°C, *CaALS1*, *CaALS3*, *CaALS4* and *CaALS9* were detected by both real time and conventional PCR and when grown to mid-exponential phase in YEPGal at 37°C, *CaALS3*, *CaALS4* and *CaALS9* were detected by both real time and conventional PCR. Expression of *CaALS4* was detected in all samples as is expected (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005). *CaALS1* may have some temperature related function as it was detected in samples which were grown at 30°C but not at 37°C. *CaALS3* and *CaALS9* may have some media related function as both genes are expressed in samples grown in YEPGal. It is possible that when galactose is the sugar source, *Candida* form hyphae more easily.

When grown to mid-exponential phase in YEPD at 30°C, *CdALS2* and *CdALS4* were detected by both real time and conventional PCR. When grown to mid-exponential phase in YEPGal at 30°C, *CdALS2* and *CdALS4* were also detected by both real time and conventional PCR and when grown to mid-exponential phase in YEPGal at 37°C, *CdALS2*, *CaALS4* and *CdALS9* were detected by both real time and conventional PCR. As the ortholog of *CaALS4*, *CdALS4* is probably transcribed from mid-log phase onwards (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005). *CdALS2* and *CdALS4* were detected in all samples indicating that there may not be functional redundancy between these genes as there is between *CaALS2* and *CaALS4* in *C. albicans* (Zhao *et al.*, 2005). It should be noted however that the *CdALS2* primer pair amplifies both *CdALS2-1* and *CdALS2-2* and while there may be functional redundancy between *CdALS4* and one of the *CdALS2* genes, it will be difficult to detect until primer pairs are available which differentially amplify *CdALS2-1* and *CdALS2-2*. *CaALS4* and *CdALS4* are detected from all samples using both conventional and real time PCR.

A study by Cheng *et al.* (2005) found that the overall results from expression of *CaALS* genes from clinical vaginal specimens, from a murine model of vaginitis and from reconstituted human vaginal epithelial cells showed that *CaALS1*, *CaALS2*,

CaALS3 and *CaALS9* were more commonly observed. Detection of expression of *CaALS6* and *CaALS7* was next most difficult and *CaALS4* and *CaALS5* were the least readily detectable in clinical vaginal specimens. Transcription of *CaALS6* and *CaALS7* is also low in oral models of candidiasis indicating that a low level of transcription of these genes may be adequate for function. *CaALS4* and *CaALS5* are easy to detect in oral models and *CaALS4* expression was abundant in *Candida* culture used to infect the vaginal models, suggesting that these results are unique and there are host-site specific influences on *CaALS* gene expression (Cheng *et al.*, 2005). A study by Green *et al.* (2006) found that there were a greater number of *CaALS* transcripts in rats 5 days post infection compared to 3 days post infection. Five days post infection *CaALS1*, *CaALS2*, *CaALS3*, *CaALS4*, *CaALS5* and *CaALS9* were detected using RT-PCR. *CaALS6* was only detected from one specimen and *CaALS7* appeared not to be transcribed. Similar results were obtained from oral scrapings from HIV positive human patients with signs of oral candidiasis. *CaALS1*, *CaALS2* and *CaALS3* were detected in all samples, *CaALS4* and *CaALS5* were detected in two samples and *CaALS9* was only detected in one sample. *CaALS6* and *CaALS7* appeared not to be transcribed. Green *et al.* (2006) found these two models showed similar results indicating that the rat infection model may be useful for studying oral *Candida* infection. The results from the studies of Cheng *et al.* (2005) and Green *et al.* (2006) are comparable to the real time RT-PCR results from this part of the present study. Green *et al.* (2006) found that at five days post infection in a hyposalivatory rat model, transcripts of *CaALS1*, *CaALS2*, *CaALS3*, *CaALS4*, *CaALS5* and *CaALS9* were detected. Results of the present study indicate that *CaALS1*, *CaALS3*, *CaALS4*, *CaALS7* and *CaALS9* are up-regulated when adherent to human BECs however, contrary to the study of Green *et al.* (2006) *CaALS2* was down-regulated under adherent conditions and *CaALS5* is regulated to the same amount as non-adherent *Candida* (Fig. 6.17; Table 6.8). *CaALS7* was also found to be up-regulated in this part of the present study while Green *et al.* (2006) found detection of *CaALS7* difficult. While results from this part of the present study can not be directly compared with results from Cheng *et al.* (2005) due to the different human cell types used, *CaALS1*, *CaALS3* and *CaALS9* were found to be up-regulated in this part of the present study and these genes are also transcribed in cases of vaginal infection. Cheng *et al.* (2006) showed that while *CaALS2* was expressed in models of vaginal infection, the detection of *CaALS4* proved to be more difficult despite abundant *CaALS4* expression in the inoculum culture of *Candida*. Contradictory results were found in this part of the

present study however, showing down-regulation of *CaALS2* and up-regulation of *CaALS4* (Fig. 6.17; Table 6.8). This is most probably another example of the functional redundancy between these two closely related *CaALS* genes; *CaALS2* is expressed to compensate for the lack of expression of *CaALS4* in adhesion to vaginal cells, while *CaALS4* is expressed to compensate for the lack of expression in *CaALS2* in adhesion to human BECs (Fig. 6.17; Table 6.8).

Chapter 7

General Discussion

7.1 GENERAL DISCUSSION

It is widely accepted that adhesion of a microorganism to its hosts' cells and tissue is an essential step in the establishment of disease with the more adherent species being generally more pathogenic (Calderone & Braun, 1991; Cannon & Chaffin, 1999; Yang, 2003). However although *C. dubliniensis* genotype 1 isolates show greater adhesion to human BECs than *C. albicans* under two of three conditions tested in this study, *C. dubliniensis* is generally regarded as being less pathogenic than *C. albicans* (Sullivan *et al.*, 2004). The increased ability of *C. dubliniensis* genotype 1 isolates to adhere to human BECs when grown at 30°C in YEPD and at 37°C in YEPGal could be due to the oral origin of the human BECs and *C. dubliniensis* isolates used in this study (Sullivan *et al.*, 1995; Sullivan & Coleman, 1997; Sullivan *et al.*, 1999).

As suggested in chapter 3, it is possible that an effect of the host cells on adherence of *Candida* isolates to human BECs was being observed in the *Candida* adherence assays. Blignaut *et al.* (2002) found a novel *C. albicans* clade present in South Africa which was absent from a collection of isolates from the United States and a further study by this group found that *C. dubliniensis* colonisation is influenced more by race than HIV status (Blignaut *et al.*, 2003). In order to test the theory that a host effect influences the adhesion of *Candida* cells to human BECs a study could be carried out where for example, Arab human BECs only are collected and pooled and tested in the same manner as the BEC assay in this study. Adhesion studies using human cells from different ethnic groups may show different adhesion results and where, for example, only Arab human BECs are used, the adhesion of *C. dubliniensis* genotype 4 isolates may be greater than the other *C. dubliniensis* genotypes. It would also be interesting to test if the adhesion of *C. dubliniensis* to human BECs collected from Arabs living in Ireland would differ from adhesion of *C. dubliniensis* to cells from Irish Caucasian individuals. The diet in Libya and Egypt consists of more natural, fresh food than in Ireland. Less canned foods and preserves are consumed and the diet is high in carbohydrate, rice, pasta and couscous (personal communication, Amilah Ali (Dip. Appl. Biol., B. Sc. (Hons), M. B. D.)). The nature of these differences may contribute to the prevalence of the different genotypes in different parts of the world and a change in these factors, such as a change in diet, may alter the prevalent genotype present.

Despite the fact that the isolates used in this study had been stored and repeatedly sub-cultured they continue to show an ability to adhere to human cells. It is not known what effect, if any, storage and repeated sub-culture had on these isolates. Storage and repeated sub-culture of *C. dubliniensis* causes the loss of colour phenotype when grown on CHROMagar™ Candida medium (Schoofs *et al.*, 1997), a phenomenon that is not seen in *C. albicans*. To investigate whether storage and repeated sub-culture has an effect on the ability of *Candida* to adhere to human cells, a further study could be undertaken whereby fresh clinical isolates are obtained and their ability to adhere to human BECs examined before being stored and repeatedly sub-cultured. After prolonged storage and/or repeated subculture, these isolates could be re-tested and their ability to adhere to BECs compared to that observed upon initial isolation.

Due to the heterogeneous nature of human cells used in the BEC assay in the present study, which may contribute to variation in results, an assay using more homogeneous human cells was used to examine candidal adhesion. The results of the TR146 adhesion assay differed from the results of the human BEC adhesion assay when *Candida* cells were cultured under the same conditions (i.e. when *Candida* was pre-cultured at 37°C in YEPD), with *C. dubliniensis* genotype 4 isolates adhering to the TR146 monolayer in significantly greater numbers than the other genotypes of *C. dubliniensis*. There may be many reasons for this difference as discussed in chapter 4, however the differences in adhesion are most likely due to the differences between the human cell types used rather than differences in the experimental conditions. When *Candida* were pre-cultured in YEPD at 37°C there was no significant difference between the adhesion of *C. dubliniensis* genotype 1 and *C. albicans* to human BECs and to TR146 monolayers.

The use of monolayers of epithelial cells in adhesion assays has some drawbacks in that the monolayers neither mimic the differentiation of cells during maturation, nor the interactions encountered *in vivo*. Perhaps a more realistic model of human oral epithelium is the RHE method. It has been used as a satisfactory model for experimental candidiasis and some of the advantages of this model include a multilayer structure closely resembling the oral epithelium and the ability to artificially reproduce the internal milieu of the oral cavity (Jayatilake *et al.*, 2005). Schaller *et al.* (1998) showed that RHE maintained as multilayer cell cultures, histologically resemble the normal human oral epithelium and the pathological changes that accompany candidal invasion which are akin to human disease. This model of human epithelium is available

commercially, however it is expensive to purchase and if cultured in the laboratory, it takes longer to grow than a monolayer of the same cell line. As such, the TR146 adhesion assay may not be a practical model by which to examine the adhesion of a large number of isolates of *Candida* or another pathogen.

In my opinion, neither the BEC adhesion assay nor the TR146 adhesion assay is better than the other as it is likely that different kinds of adhesion are being examined in both assays. While both assays are useful, both have advantages and disadvantages as discussed in chapters 3 and 4. These advantages and disadvantages must be taken into account when considering which assay to use to investigate adhesion.

There are many factors involved in microbial adhesion to host cells and tissue. The BIAcore™ 3000 biosensor was used to assess the molecular adhesion of *Candida* to ECM proteins in an effort to elucidate some of the ligands to which *Candida* binds. The results of the BIAcore assay reflect the results of the human BEC adhesion assay when *Candida* cells were cultured under similar conditions (i.e. when pre-cultured at 30°C in YEPD), lending credence to the human BEC adhesion assay and indicating that the results of the human BEC adhesion assay may be a more realistic reflection of adhesion *in vivo* than the TR146 adhesion assay. Both the human BEC adhesion assay and the BIAcore assay show that when *Candida* is pre-cultured at 30°C in YEPD *C. dubliniensis* genotype 1 isolates were more adherent to the ligands tested than the other genotypes of *C. dubliniensis* and of *C. albicans*. This fits with the theory that *C. dubliniensis* has adapted to colonise the oral cavity, which is at a lower temperature than body temperature (McFadden *et al.*, 1985).

While previous studies have examined the interaction between whole bacterial cells and various ligands (Clyne *et al.*, 2004; Kinoshita *et al.*, 2007; Nobbs *et al.*, 2007), this is to the best of our knowledge, the first attempt to elucidate the interaction between whole *Candida* cells and various ligands, in this case ECM proteins. In previous studies, the BIAcore biosensor was used to examine a small range of variables (Clyne *et al.*, 2004; Kinoshita *et al.*, 2007; Nobbs *et al.*, 2007), whereas in this study the BIAcore biosensor was used to examine the adhesion of a range of *Candida* isolates to a range of ECM protein ligands. While this method of using the BIAcore determined the rank of the most adherent group of *Candida* to the ECM proteins tested (Figs. 5.9 to 5.12) and the ECM protein to which *Candida* cells adhered most (Fig. 5.15), it could not elucidate specific adhesins involved in the adhesion of *Candida* to the ECM proteins. Using a similar method to Nobbs *et al.* (2007) whereby mutants lacking an adhesin are created,

the BIAcore biosensor could be used to elucidate the adhesion profiles of wild type *Candida* and *Candida* mutants to various ligands, which may help elucidate the targets of candidal adhesins.

The *ALS* genes of *C. albicans* have been implicated in the adhesion of this organism to host cells. The most significant difference between the *ALS* gene families of *C. albicans* and *C. dubliniensis* is the lack of orthologs for *CaALS3* (Fig. 6.9) and *CaALS5* in *C. dubliniensis* and the presence of two *CdALS2* genes (termed *CdALS2-1* and *CdALS2-2*) in *C. dubliniensis* compared to one *CaALS2* gene in *C. albicans*. The lack of an ortholog for *CaALS3* in *C. dubliniensis* is probably a major contributory factor why *C. dubliniensis* is less virulent than *C. albicans*. *CaALS3* has recently been shown to be an invasin (Phan *et al.*, 2007) and has the ability to bind ferritin. The ability of host cells to sequester iron is known to be an important mechanism of resistance to microbial infections (Almeida *et al.*, 2008). Lack of these invasive abilities would confer a less pathogenic phenotype upon *C. dubliniensis*. The lack of an ortholog for *CaALS3* in *C. dubliniensis* goes some way to explaining why *C. dubliniensis* is less virulent than *C. albicans*.

As the results of the BEC adhesion assay and the BIAcore assay concurred, the relationship between the adhesion of *Candida* to human BECs and *ALS* gene expression was examined. While all *ALS* genes were up-regulated in adherent *C. albicans* SC5314 when pre-cultured at 30°C in YEPD (Fig. 6.17), only *CdALS1*, *CdALS4*, *CdALS6* and *CdALS9* were up-regulated in adherent *C. dubliniensis* CD36 (genotype 1; Fig. 6.18) when pre-cultured under the same conditions. However, *ALS1*, *ALS4*, *ALS6* and *ALS9* in adherent *C. dubliniensis* CD36 were not up-regulated to the same extent as in adherent *C. albicans* SC5314 despite the fact that *C. dubliniensis* genotype 1 isolates adhered to human BECs in significantly greater amounts than *C. albicans* when pre-cultured at 30°C in YEPD (Fig. 3.3). *Candida dubliniensis* genotype 1 isolates were also significantly more adherent to human BECs than the other genotypes of *C. dubliniensis* yet *CdALS1*, *CdALS6*, *CdALS7* and *CaALS9* were up-regulated to a greater extent in adherent *C. dubliniensis* Can6 (genotype 2; Fig. 6.9), *C. dubliniensis* CD514 (genotype 3; Fig. 6.20) and *C. dubliniensis* Eg200 (genotype 4; Fig. 6.21) than in adherent *C. dubliniensis* CD36 (genotype 1; Fig. 6.18) when pre-cultured at 30°C in YEPD. These results indicate that a lower level of expression of the *CdALS* genes in *C. dubliniensis* may correlate with adhesion.

Despite the fact that *C. dubliniensis* genotype 1 isolates and *C. albicans* adhere in similar amounts to human BECs when *Candida* cells are pre-cultured at 37°C in YEPD (Fig. 3.5), only *ALS2* and *ALS6* are expressed at similar levels in adherent *C. albicans* SC5314 (Fig. 6.17) and adherent *C. dubliniensis* CD36 (Fig. 6.18). All other *ALS* genes are expressed at greater levels in adherent *C. albicans* SC5314 (Fig. 6.17) than adherent *C. dubliniensis* CD36 (Fig. 6.18). While *ALS2* was down regulated in both species, *ALS4* was up-regulated possibly showing functional redundancy between these two closely related genes (Zhao *et al.*, 2005). *ALS6* may have some function in maintaining growth rate (Zhao *et al.*, 2007a) and the fact that *ALS6* is expressed at similar levels between adherent *C. albicans* and *C. dubliniensis* genotype 1 isolates when they adhere to human BECs in similar amounts may indicate that growth rate plays a role in adhesion. The expression of *ALS1*, *ALS4*, *ALS7* and *ALS9* was greater in adherent *C. albicans* SC5314 (Fig. 6.17) than in adherent *C. dubliniensis* CD36 (Fig. 6.17). This indicates that a lower expression of the majority of *ALS* genes in adherent *C. dubliniensis* compared to adherent *C. albicans* is not necessarily correlated with an adherent phenotype in *C. dubliniensis*.

When pre-cultured at 37°C in YEPGal, *C. dubliniensis* genotype 1 isolates adhered in greater amounts to human BECs than *C. albicans* (Fig. 3.7) however *CaALS1*, *CaALS4*, *CaALS7* and *CaALS9* were up-regulated to a greater extent in adherent *C. albicans* SC5314 (Fig. 6.17) than in adherent *C. dubliniensis* CD36 (Fig. 6.18) when pre-cultured under the same conditions.

Where *C. dubliniensis* genotype 1 isolates adhered to human BECs in significantly greater amounts than the other genotypes of *C. dubliniensis* and of *C. albicans* (i.e. when *Candida* cells were pre-cultured in YEPD at 30°C (Fig. 3.3) and YEPGal at 37°C (Fig. 3.7)) a similar pattern of *ALS* gene expression was detected in *C. dubliniensis* CD36 (Fig. 6.18); all *CdALS* genes were up-regulated with the exception of *CdALS2* and *CdALS6*. *CdALS2* may be down-regulated due to the up-regulation of *CdALS4* and the potential for functional redundancy between these two closely related genes as in *C. albicans* (Zhao *et al.*, 2005). Deletion of *CaALS6* resulted in increased adhesion of *C. albicans* to endothelial and epithelial cells (Zhao *et al.*, 2007a); if *CdALS6* has a similar function to *CaALS6*, down-regulation of *CdALS6* could be partly responsible for the increase in adhesion to human BECs. In contrast, *CdALS6* was up-regulated and *CdALS7* was down-regulated in adherent *C. dubliniensis* CD36 when pre-cultured in YEPD at 37°C (Fig. 6.18) and this coincided with no significant difference

in the adhesion of *C. albicans* and *C. dubliniensis* genotype 1 isolates to human BECs (Fig. 3.5). Deletion of *CaALS7* also resulted in increased adhesion of *C. albicans* to endothelial and epithelial cells (Zhao *et al.*, 2007a); if *CdALS7* has a similar function to *CaALS7* down-regulation of *CdALS7* may result in increased adhesion to human BECs. However, as *CdALS6* is up-regulated, this increase in adhesion may be negated if *CdALS6* has a similar function to *CaALS6*.

Candida dubliniensis genotypes 1, 2 and 4 adhere in significantly greater amounts to human BECs when pre-cultured in YEPD at 30°C than at 37°C (Fig. 3.8) however, there appears to be no significant difference between the *ALS* gene expression of adherent *C. dubliniensis* CD36 (Fig. 6.18), *C. dubliniensis* Can6 (Fig. 6.19) and *C. dubliniensis* Eg200 (Fig. 6.21) when pre-cultured in YEPD at 30°C compared to in YEPD at 37°C. *Candida dubliniensis* genotype 4 and 4v adhered to human BECs in significantly greater numbers when grown in YEPGal than YEPD at 37°C (Fig. 3.9) however, *CdALS1* and *CdALS2* are up-regulated in *C. dubliniensis* Eg200 when pre-cultured in YEPD at 37°C compared to when pre-cultured in YEPGal at 37°C (Fig. 6.21). *Candida albicans* adhere to human BECs in significantly greater numbers when grown in YEPD than in YEPGal at 37°C (Fig. 3.9) and although *CaALS7* is up-regulated in adherent *C. albicans* SC5314 when pre-cultured in YEPD compared to YEPGal at 37°C, *CaALS4* and *CaALS5* are down-regulated when pre-cultured in YEPD compared to YEPGal at 37°C (Fig. 6.17).

7.2 CONCLUSIONS

Data from this study indicate that the different results obtained using the different adhesion assays are as a result of differences in the human cell types used and that *C. dubliniensis* genotype 1 isolates have an increased ability to adhere to human cells than *C. albicans* and the other genotypes of *C. dubliniensis* while being less pathogenic than *C. albicans*.

The data from this study also indicate that the majority of *ALS* genes are involved in the adhesion of *Candida* to human cells as there was greater expression of the majority of *ALS* genes in adherent *Candida* compared to non-adherent *Candida*. In hyphal *C. dubliniensis*, *CdALS7* was down-regulated while *CdALS1* was up-regulated. The down-regulation of *CdALS7* may function in increasing the adhesion of

C. dubliniensis to human cells while *CdALS1*, which is the closest *CdALS* gene to *CaALS3*, may have some function in hyphal formation. *CaALS1* is up-regulated, along with *CaALS3*, in hyphal *C. albicans* cells.

The results indicate that despite the lower virulence of *C. dubliniensis* in relation to *C. albicans*, *C. dubliniensis* genotype 1 isolates have a greater overall ability to adhere to human BECs than *C. albicans* and the other genotypes of *C. dubliniensis* and in conclusion the ability of these *Candida* species to adhere to human cells is not the sole method involved in pathogenicity.

In order to further understand the molecular methods of adhesion of *C. dubliniensis* to human cells comparative microarray analysis of the entire genomes of *C. albicans* and *C. dubliniensis* should be investigated to identify other virulence genes and their differential expression between the two species. Differential primers for *CdALS2-1* and *CdALS2-2* could also be designed to fully elucidate the individual expression of these genes in *C. dubliniensis*, and once this is complete, construction of mutant strains of *C. dubliniensis* lacking *ALS* genes should be carried out to further understand the individual function of each *ALS* gene. Comparative analysis of Als protein structure in *C. dubliniensis* and *C. albicans* may also give further insights into possible differences in function between the orthologs in these two closely related species. If the methods of adhesion of *Candida* to human cells can be elucidated it is possible that novel antifungal treatments or vaccines could be developed to reduce the increasing numbers of infections caused by this organism.



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Appendix I

Table 6.8. Fold expression of *C. dubliniensis* and *C. albicans* ALS genes of adherent and non-adherent *Candida* under various growth conditions detected by real time PCR

| Condition | ALS gene | | | | | | | | |
|-----------------------------|----------------------|----------------------------|-----------------------------|------------------|---------------|----------------|----------------|----------------|---------------|
| | 1 | 2* | 3 | 4 | 5 | 6 | 7 | 9 | |
| <i>C. albicans</i> SC5314 | Adherent 30°C YEPD | 3.041 ^a ± 0.468 | -0.035 ^b ± 0.442 | 1.18 ± 0.123 | 2.549 ± 0.081 | 0.289 ± 0.546 | 1.329 | 3.293 ± 0.12 | 1.52 ± 0.166 |
| | Adherent 37°C YEPD | 3.366 ± 0.145 | -0.045 ± 0.29 | 1.189 ± 0.172 | 1.996 ± 0.06 | 0.564 ± 0.123 | 1.512 ± 0.135 | 2.994 ± 0.139 | 1.463 ± 0.179 |
| | Adherent 30°C YEPGal | 2.27 ± 0.374 | -0.986 ± 0.079 | 0.073 ± 0.438 | 2.19 ± 0.046 | 0.243 ± 0.078 | 1.311 ± 0.201 | 2.206 ± 0.155 | 0.823 ± 0.087 |
| | Adherent 37°C YEPGal | 3.255 ± 0.271 | -0.061 ± 0.226 | 1.433 ± 0.196 | 2.602 ± 0.18 | 1.199 ± 0.227 | 1.309 ± 0.312 | 1.542 ± 0.279 | 1.455 ± 0.255 |
| | Hyphal | 2.172 ± 0.282 | -0.972 ± 0.077 | 3.854 ± 0.083 | 0.407 ± 0.082 | -0.258 ± 0.388 | -0.351 ± 0.329 | 0.577 ± 0.5 | 1.389 ± 0.103 |
| | 30°C YEPD | 0.672 ± 0.096 | -0.433 ± 0.04 | -0.021 ± 0.035 | 0.109 ± 0.072 | 0.191 ± 0.067 | 0.98 ± 0.145 | 1.019 ± 0.037 | 0.311 ± 0.046 |
| | 30°C YEPGal | 0.233 ± 0.203 | -0.51 ± 0.04 | 0.191 ± 0.062 | 0.808 ± 0.104 | 0.328 ± 0.158 | 0.663 ± 0.165 | 0.711 ± 0.057 | 0.342 ± 0.044 |
| | 37°C YEPGal | -0.481 ± 0.063 | -0.66 ± 0.054 | 0.213 ± 0.071 | 0.117 ± 0.04 | 0.197 ± 0.127 | 0.552 ± 0.159 | 0.582 ± 0.047 | 0.028 ± 0.061 |
| <i>C. dubliniensis</i> CD36 | Adherent 30°C YEPD | 1.405 ± 1.396 | -0.252 ± 1.17 | N/A ^c | 1.618 ± 1.195 | N/A | 0.615 ± 1.105 | -0.336 ± 1.057 | 0.642 ± 1.388 |
| | Adherent 37°C YEPD | 1.119 ± 0.083 | -0.401 ± 0.133 | N/A | 1.006 ± 0.173 | N/A | 1.187 ± 0.093 | -0.511 ± 0.536 | 0.328 ± 0.3 |
| | Adherent 30°C YEPGal | 1.026 ± 0.98 | -0.633 ± 0.603 | N/A | 0.772 ± 0.78 | N/A | 1.096 ± 0.616 | -0.736 ± 0.09 | 0.752 ± 0.507 |
| | Adherent 37°C YEPGal | 0.894 ± 0.091 | -0.341 ± 0.145 | N/A | 0.728 ± 0.429 | N/A | 1.159 ± 0.111 | -0.76 ± 0.135 | 0.878 ± 0.08 |
| | Hyphal | 0.965 ± 0.028 | -0.455 ± 0.078 | N/A | 0.761 ± 0.024 | N/A | 0.272 ± 0.048 | -1.082 ± 0.018 | -0.245 ± 0.04 |
| | 30°C YEPD | -0.725 ± 0.03 | 0.297 ± 0.034 | N/A | 0.073 ± 0.159 | N/A | -0.376 ± 0.038 | -0.577 ± 0.026 | -0.73 ± 0.022 |
| | 30°C YEPGal | -0.646 ± 0.044 | 0.796 ± 0.023 | N/A | 0.697 ± 0.045 | N/A | -0.28 ± 0.037 | -0.113 ± 0.115 | -0.503 ± 0.06 |
| | 37°C YEPGal | -0.285 ± 0.131 | 0.721 ± 0.134 | N/A | 0.763 ± 0.162 | N/A | -0.638 ± 0.134 | 0.241 ± 0.116 | 0.191 ± 0.139 |

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|------------------------------|----------------------|----------------|----------------|-----|----------------|-----|----------------|----------------|----------------|
| <i>C. dubliniensis</i> Can6 | Adherent 30°C YEPD | 1.829 ± 0.345 | -0.318 ± 0.314 | N/A | 0.291 ± 0.208 | N/A | 1.322 ± 0.403 | 0.18 ± 0.204 | 0.999 ± 0.32 |
| | Adherent 37°C YEPD | 1.394 ± 0.096 | -0.515 ± 0.153 | N/A | -0.048 ± 0.057 | N/A | 1.54 ± 0.119 | 0.353 ± 0.141 | 0.717 ± 0.082 |
| | Adherent 30°C YEPGal | 1.673 ± 0.103 | -0.72 ± 0.107 | N/A | -0.048 ± 0.189 | N/A | 1.468 ± 0.148 | 0.184 ± 0.359 | 0.797 ± 0.102 |
| | Adherent 37°C YEPGal | 0.713 ± 0.184 | -1.573 ± 0.338 | N/A | -0.615 ± 0.139 | N/A | 1.747 ± 0.245 | 0.116 ± 0.53 | 0.557 ± 0.363 |
| | Hyphal | 1.661 ± 0.039 | -0.796 ± 0.046 | N/A | -0.1 ± 0.051 | N/A | 1.115 ± 0.066 | -1.096 ± 0.057 | 0.154 ± 0.039 |
| | 30°C YEPD | 0.438 ± 0.191 | -0.452 ± 0.023 | N/A | -0.477 ± 0.031 | N/A | -0.365 ± 0.047 | -0.216 ± 0.027 | -0.47 ± 0.057 |
| | 30°C YEPGal | -1.926 ± 0.057 | -0.63 ± 0.03 | N/A | -0.738 ± 0.039 | N/A | -0.496 ± 0.029 | 0.029 ± 0.024 | -0.477 ± 0.036 |
| | 37°C YEPGal | -1.547 ± 0.076 | 0.03 ± 0.057 | N/A | -0.011 ± 0.029 | N/A | -0.242 ± 0.069 | 0.189 ± 0.029 | 0.018 ± 0.022 |
| <i>C. dubliniensis</i> CD514 | Adherent 30°C YEPD | 4.008 ± 2.06 | 2.144 ± 1.24 | N/A | 0.894 ± 1.704 | N/A | 2.834 ± 1.67 | 0.383 ± 1.58 | 2.76 ± 2.05 |
| | Adherent 37°C YEPD | 4.009 ± 0.076 | 2.12 ± 0.2 | N/A | 0.453 ± 0.063 | N/A | 1.994 ± 0.072 | -0.078 ± 0.219 | 2.055 ± 0.047 |
| | Adherent 30°C YEPGal | 4.22 ± 0.053 | 2.193 ± 0.3 | N/A | 0.662 ± 0.064 | N/A | 2.235 ± 0.107 | -0.259 ± 0.103 | 2.167 ± 0.091 |
| | Adherent 37°C YEPGal | 3.794 ± 0.107 | 2.181 ± 0.11 | N/A | 0.542 ± 0.083 | N/A | 2.39 ± 0.125 | -0.253 ± 0.098 | 1.846 ± 0.06 |
| | Hyphal | 3.564 ± 0.063 | -0.057 ± 0.137 | N/A | 0.208 ± 0.1 | N/A | 1.328 ± 0.136 | -0.974 ± 0.075 | 1.553 ± 0.057 |
| | 30°C YEPD | 0.038 ± 0.045 | -0.315 ± 0.351 | N/A | -0.554 ± 0.055 | N/A | 0.085 ± 0.073 | -0.154 ± 0.05 | 0.305 ± 0.046 |
| | 30°C YEPGal | -0.307 ± 0.095 | -0.388 ± 0.18 | N/A | -0.473 ± 0.076 | N/A | -0.221 ± 0.038 | -0.265 ± 0.021 | -0.33 ± 0.034 |
| | 37°C YEPGal | -0.315 ± 0.043 | 0.122 ± 0.093 | N/A | 0.099 ± 0.08 | N/A | 0.35 ± 0.158 | 0.286 ± 0.035 | 0.314 ± 0.038 |
| <i>C. dubliniensis</i> Eg200 | Adherent 30°C YEPD | 3.698 ± 0.066 | 1.974 ± 0.139 | N/A | 1.663 ± 0.087 | N/A | 2.828 ± 0.104 | 0.581 ± 0.206 | 1.539 ± 0.056 |
| | Adherent 37°C YEPD | 3.179 ± 0.033 | 2.955 ± 0.117 | N/A | 1.107 ± 0.14 | N/A | 2.928 ± 0.085 | 0.84 ± 0.153 | 1.332 ± 0.193 |
| | Adherent 30°C YEPGal | 3.328 ± 0.042 | 2.345 ± 0.148 | N/A | 1.336 ± 0.065 | N/A | 2.463 ± 0.072 | -0.089 ± 0.171 | 1.039 ± 0.126 |
| | Adherent 37°C YEPGal | 2.713 ± 0.096 | 2.227 ± 0.221 | N/A | 0.806 ± 0.067 | N/A | 2.713 ± 0.073 | 0.451 ± 0.172 | 1.224 ± 0.062 |
| | Hyphal | 3.037 ± 0.032 | 0.118 ± 0.077 | N/A | 0.678 ± 0.051 | N/A | 1.681 ± 0.034 | -0.599 ± 0.058 | 0.385 ± 0.056 |
| | 30°C YEPD | -0.755 ± 0.118 | -0.1 ± 0.173 | N/A | 0.277 ± 0.152 | N/A | -0.046 ± 0.118 | 0.096 ± 0.129 | 0.183 ± 0.123 |
| | 30°C YEPGal | 0.443 ± 0.06 | -0.185 ± 0.141 | N/A | 0.162 ± 0.09 | N/A | -0.287 ± 0.077 | -0.281 ± 0.082 | -0.543 ± 0.081 |

| | | | | | | | | |
|-------------|----------------|---------------|-----|----------------|-----|--------------|----------------|----------------|
| 37°C YEPGal | -0.906 ± 0.056 | -0.28 ± 0.053 | N/A | -0.307 ± 0.037 | N/A | 0.191 ± 0.04 | -0.024 ± 0.019 | -0.019 ± 0.031 |
|-------------|----------------|---------------|-----|----------------|-----|--------------|----------------|----------------|

^aFold expression shown in black font indicates up-regulation

^bFold expression shown in red font indicates down-regulation

^cNot applicable

*In the case of *CaALS2*, the QRTC*aALS2* primer pair amplifies *CaALS2* only however, in the case of *CdALS2* the QRTC*dALS2* primer pair amplifies both *CdALS2-1* and *CdALS2-2*