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**Analysis of morphogenesis and chlamydospore
formation by *C. albicans* and *C. dubliniensis***

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

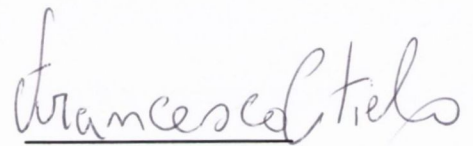
Francesco Citiulo

March 2010

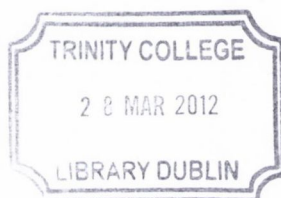
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Francesco Citiulo



Thesis 9337

To my mother, to my father



Summary

Candida dubliniensis is the species that is most closely related to *Candida albicans*. Despite their close phylogenetic relatedness, epidemiological and infection model data suggest that *C. albicans* is a far more successful pathogen. The reasons for this disparity in virulence are still unclear, however, it has been shown that *C. dubliniensis* is less able than *C. albicans* to produce hyphae under a wide range of *in vitro* and *in vivo* conditions. The purpose of this study was to investigate the comparative effects of glucose/galactose and methionine on morphogenesis in the two species.

We studied the effect of altering the concentration of glucose/galactose and methionine in growth media where these were the sole source of carbohydrate and amino acids. We found evidence for a role of the sulphur-containing amino acids, in particular methionine, in combination with low carbohydrate concentrations, in the morphogenesis of *C. albicans* and *C. dubliniensis*, with some significant differences evident between the two species. We found that while *C. albicans* was able to produce hyphae under a broad range of carbohydrate concentrations (e.g. 1% to 0.025% (w/v)), most *C. dubliniensis* strains produced hyphae only in media containing low carbohydrate concentrations (i.e. 0.025% (w/v)). The differential effect of methionine and glucose/galactose led to the formulation of a defined medium (i.e. YNB containing 0.025% (w/v) glucose or galactose and 20 mg/L methionine pH 3.7) in which *C. albicans* grows exclusively in the yeast form while *C. dubliniensis* grows as hyphae and pseudohyphae with copious chlamydospores. We have demonstrated that these phenotypic differences are at least in part due to species-specific downregulation of the transcriptional repressor-encoding gene *NRG1* in *C. dubliniensis*. Our data suggest that there may be differences in the methionine biosynthetic and metabolic pathways in the two species. To further study this we carried out an analysis of the regulators of methionine biosynthesis. The most divergent genes in this pathway in *C. albicans* and *C. dubliniensis* were found to be *CBF1* and *MET4*, homologues of genes encoding the transcriptional activators that in *S. cerevisiae* regulate the whole sulphur amino acid biosynthesis pathway and also pathways involved in general metabolism. Heterologous expression of the *C. albicans* *CBF1* gene in *C. dubliniensis* did not have any detectable effect on the phenotype of this species, suggesting that there is no defect

in this protein in *C. dubliniensis*. We investigated the phenotype of the *C. albicans* $\Delta cbf1$ mutant (obtained from Joachim Morschhauser in the University of Wurzburg) and found that deletion of this gene in *C. albicans* resulted in a reduced capacity to produce hyphae, form biofilm and cause tissue damage in a reconstituted human epithelial infection model. Interestingly, deleting the putative *MET4* gene in *C. albicans* resulted in no detectable phenotype, suggesting that unlike in *S. cerevisiae*, this gene may not be involved in the control of methionine biosynthesis in this species.

In this study we also identified conditions that lead to the hyperproduction and purification of chlamydospores in both *C. albicans* and *C. dubliniensis*. The function of these large thick-walled cells is unknown and they have been very poorly studied to date. Staining these cells with the molecular probe FUN-1 revealed that chlamydospores are metabolically active structures but, in contrast to blastospores, this activity is significantly reduced after 10-15 days when the chlamydospores, after maturation, appear to enter a dormant state, possibly due to mitochondrial inactivation. The addition of nutrients and serum to the chlamydospore suspensions results in a gradual resumption of the metabolic activity of chlamydospores that are not older than 30 days. After incubation in these conditions, some (10-20%) chlamydospores were observed to produce new yeast cells by budding, or to produce pseudohyphae or hyphae depending on the precise environmental conditions. In order to further investigate the biology of chlamydospores we compared global gene expression in *C. dubliniensis* yeast cells and chlamydospores. This revealed that the 11% of the genes greater than 2-fold upregulated in chlamydospores were genes regulated by the transcriptional repressor Nrg1. These mainly included genes involved in glucose metabolism and glucose transport. Other upregulated genes included genes involved in stress response, cell cycle control, chromatin regulation, chromosome rearrangements, DNA repair-recombination and pheromone response. Genes related to mitochondrial respiratory functions and biogenesis were found to be downregulated in chlamydospores. Our data confirm that chlamydospores represent a specialised growth form of *C. albicans* and *C. dubliniensis*. Since both species have maintained the capacity to produce these complex structures it is likely that they play some as yet unidentified role in the biology of these two pathogenic species.

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Acknowledgements

It is a pleasure to thank the many people who made this thesis possible.

Firstly I would like to thank my supervisor Prof. Derek Sullivan for his constant support, guidance and patience throughout the course of this project. I would like also to thank Prof. David Coleman and Dr. Gary Moran for their constant supervision and support. I am also thankful for the opportunities given to me to present my research on both national and international conferences. I would like also to acknowledge all the members of the Can Train project for their hospitality during workshops, meetings and practical experiences in their laboratories. I would like to acknowledge the financial support of the European program FP6, Can Train, Marie Curie actions and of the Microbiology Research Laboratory in the Dental School and Hospital.

I wish to thank the friendship and help of everyone in the lab, Ray, Peter, Anna, Leanne, Emanuelle, Mohammed, Brendan, Maria, Phillipa and Orla, in particular I wish to thank Judy, Brenda and Emma-Louse for proof reading, helping me with the formatting and give me advises on the thesis. I also wish to thanks our laboratory manager Mary O'Donnell for her help and for keeping the lab running efficiently.

I would like also to thanks Prof. Joachim Morschhauser for his gift of the *C. albicans* $\Delta cbf1$ strain. I wish to thank all my friends for their friendship and for the fun of the nights out. In particular I would like to thank Peter, Ray, Adam, Giuseppe and my housemates Rosario and Antonio.

I would like to thank Chiara, thank you for your encouragements, support and your constant love.

I would like to thank my whole family in particular my brother Roberto, Laura, my Mum and Dad, thank you for your encouragement, love and for supporting my idea to continue my studies.

Abbreviation

AIDS	acquired immunodeficiency syndrome
A540, A600	absorption at 540 nm or 600 nm, respectively
ATP	adenosine 5' -triphosphate
Azole	synthetic <i>N</i> -substituted azoles, including the imidazole and triazole antifungal antibiotics
BLAST	Basic local alignment tool
bp	base pairs
cfu	colony forming units
Ci	curie
CIM	chlamydospore induction medium
cm	centimetre
CO ₂	carbon dioxide
CPM	counts per minutes
C _t	cycle threshold
Δ	deletion (of a gene)
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylenePyrocarbonate
DMEM	Dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
EDTA	ethylenediamine tetraacetic acid
e.g.	for example
<i>et al.</i>	and others
FUN-1	[2-choro-4-(2,3-dihydro-3-methyl- -(benzo-1,3-thiazol-2-yl)-methylidene)- -1phenylquinoliniumiodide]
g	gram
<i>g</i>	gravitational force
h	hour

HIV	human immunodeficiency virus
IPTG	isopropyl-β-D-thiogalactopyranoside
i.e.	that is
kb	kilobase pair
L agar	Luria agar
L broth	Luria broth
L	Litre
LDH	Lactate dehydrogenase
M	molar
Mb	megabase
mg	milligram
μg	microgram
ml	millilitre
μl	microlitre
min	minute
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
nm	nanometre
no.	number
O.D _x	optical density at x nm
ORF	open reading frame
PAS	Periodic Acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
p.f.u.	plaque forming units
rDNA	ribosomal DNA
RHE	Reconstituted human oral epithelium
RNA	ribonucleic acid
RNase	ribonuclease

rpm.	revolutions per minute
s	second
sarcosyl	<i>N</i> - lauroylsarcosine, sodium salt
S_{AB}	similarity coefficient
SDS	sodium dodecyl sulphate
SSC	salt, sodium citrate
ST	sequence type
TBE	tris-borate EDTA
Tris	tris (hydroxymethyl) aminoethane
UK	United Kingdom
USA	United States of America
U.V.	ultraviolet
v/v	% "volume in volume" expresses the number of millilitres of an active constituent in 100 millilitres of solution
w/v	% "weight in volume" expresses the number of grams of an active constituent in 100 grams of solution or mixture
X-gal	5-bromo-4-chloro-indoyl- β -D-galactoside
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)- -2H-tetrazolium-5-carboxanilide
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPM	Yeast peptone maltose
YPS	Yeast extract peptone maltose
~	approximately
>	greater than
<	less than
$\geq$	greater than or equal to
$\leq$	less than or equal to

Publications:

Purification and germination of *Candida albicans* and *Candida dubliniensis* chlamydospores cultured in liquid media.

Francesco Citiulo, Gary P. Moran, David. C. Coleman and Derek J. Sullivan. *FEMS Yeast Research* (October 9, 2009)

The *Candida dubliniensis* genome reveals evolutionary changes associated with systemic candidiasis.

Andrew P. Jackson, John Gamble, Tim Yeomans, Gary Moran, David Saunders, David Harris, Martin Aslett, Jamie F. Barrell, **Francesco Citiulo**, David Coleman, Piet deGroot, Tim Goodwin, Thomas Keane, Michael Quail, Carol Munro, Arnab Pain, Russell Poulter, Marie-Adèle Rajandream, Hubert Renault, Martin Spiering, Adrian Tivey, Neil Gow, Barclay Barrell, Derek Sullivan, Matthew Berriman.

Genome Res. 2009, 19: 2231-2244, Sep 10

Chapter 1

General Introduction

1.1. The genus *Candida*

The incidence of fungal infection has increased over the last 20 years coinciding with the increase in the number of individuals infected with the human immunodeficiency virus (HIV) and those receiving immunosuppressive therapies or broad-spectrum antibiotics. Infection due to *Candida* species accounts for approximately 80% of all major systemic fungal infection (Coleman *et al.*, 1997b; Odds *et al.*, 1987). The genus *Candida* comprises more than 150 species of yeasts. Only a small number of these species are of clinical relevance in humans including *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei*. *Candida albicans* is the most pathogenic *Candida* species and is responsible for 50-65% of systemic infections (Odds *et al.*, 2007; Kibbler *et al.*, 2003; Coleman *et al.*, 1998; Odds, 1987). However over the last decade there has been an increase in the reported prevalence of other *Candida* species, especially *C. glabrata* and *C. krusei*. (Sullivan *et al.*, 2004; Yang & Lo, 2000). The reasons behind this epidemiological shift are not clear. However, evidence is accumulating that the widespread therapeutic and prophylactic use of antifungal medications may have been a significant factor in the selection of these organisms as they can exhibit reduced susceptibility to commonly used antifungal drugs relative to *C. albicans* (Ponton *et al.*, 2000).

1.1.1. *Candida* species as commensal organisms and opportunistic pathogens

Candida species are a normal part of the oropharyngeal vaginal and gastrointestinal flora (Ruhnke & Maschmeyer, 2002). However, when host defences are compromised *Candida* species behave as opportunistic pathogens and can establish infection. The transition from commensalism to infectious agent requires a susceptible host, but it also involves an active process in which changes in the pathogen's gene expression are regulated by an interplay with host responses. The mechanisms behind this transition are not currently clear. Immunodepression of the host is a significant factor for the development of *Candida* infection. The interaction between the host and the pathogen begins on the epithelial surfaces. A healthy epithelium constitutes an important primary barrier that prevents the pathogen establishing an infection. In the oral cavity, salivary antimicrobial peptides are considered to be an important part of the host innate defence system in the

prevention of microbial colonisation. Histatin-5 specifically exhibits potent activity against *Candida* sp. (Nikawa *et al.*, 2002; Nikawa *et al.*, 2001). Torres *et al.* (2009) have shown histatin-5 levels to be significantly reduced in the saliva of HIV-infected individuals, indicating an important role for this antimicrobial peptide in maintaining *C. albicans* as a commensal, as HIV-infected individuals are particularly vulnerable to oral candidiasis caused by *C. albicans*. Proinflammatory cytokines are also constitutively produced by oral and vaginal epithelial cells. Those molecules play a role in regulating fungal growth and may also serve to maintain *Candida* sp. as a commensal (Lilly *et al.*, 2006; Weissenbacher *et al.*, 2004).

1.1.2. Clinical manifestation of candidal infections

Candida species are the causative agents of a variety of superficial and deep-seated infections in humans. The most common sites of mucosal *Candida* infections are the oropharynx and the vagina. The most common clinical form of candidiasis is pseudomembranous candidiasis (commonly referred to as 'thrush') in which *Candida* cells form a thick layer of yeast and hyphae with the presence of abundant extracellular matrix on the epithelial surfaces. Oral candidiasis is the most common mycosis in AIDS patients and is often the first clinical manifestation of HIV infection (Samaranayake *et al.*, 2002; Coleman *et al.*, 1997a; Sullivan *et al.*, 1997). Vaginal carriage of *Candida* species and other yeasts is reported to range from 10 to 20% in healthy women. Although vulvovaginal candidiasis is more common in HIV-infected women, approximately 10% of women with predisposing factors such as immunosuppression and uncontrolled diabetes suffer recurrent episodes of infection (Fidel, 1998). Chronic mucocutaneous candidiasis (CMC) is a syndrome in which patients have chronic and/or recurrent infections of the skin, nails, and mucous membranes with *Candida* species. Individuals at risk of CMC are those unable to develop effective cell-mediated immune responses against *Candida*.

Invasive candidiasis refers to *Candida* infections, other than those of the skin and mucous membranes, when *Candida* cells penetrate through the epithelial barrier. Invasive candidiasis includes candidaemia (colonisation of the bloodstream by *Candida* species), acute or chronic disseminated candidiasis and less frequently infection of a single or multiple deep organs. Invasive candidiasis is generally the result of penetration of *Candida* cells into the bloodstream and the subsequent spread

of the infective organism (i.e. Candidaemia). Invasive candidiasis is associated with high mortality. Risk factors for candidaemia and disseminated candidiasis also include the use of antibiotics prior to infection (Wenzel, 1995), the presence of intravenous catheters, neutropenia or malignant disease of the haematopoietic system (i.e. leukaemia) (Kullberg & Oude Lashof, 2002). It has been shown that broad-spectrum antibiotics eliminate the endogenous bacterial flora and allow the overgrowth of *Candida* and other yeast species (Samonis *et al.*, 1994; Wenzel, 1995). For this reason it has been hypothesised that the vast majority of invasive infections are caused by endogenous *Candida* strains that belong to the patient's normal flora. However several studies have highlighted the correlation between previous gastrointestinal colonisation by *Candida* species and candidaemia (Kullberg & Oude Lashof, 2002).

1.1.3. Epidemiology of candidiasis

Reports on the prevalence of *Candida* carriage in healthy individuals vary widely, but indicate that *Candida* yeast may be present in the oral flora of up to 60% of the healthy population (Odds, 1987). However, carriage of the organism does not necessarily predispose the host to infection. While *C. albicans* is still the most common cause of *Candida* infections, the recovery of non-*C. albicans* *Candida* species from sites of infection, thought to be less pathogenic than *C. albicans*, such as *C. parapsilosis*, *C. glabrata* and *C. tropicalis* has increased (Coleman *et al.*, 1998). The reasons for this shift in the epidemiology of candidiasis may be linked to the increased longevity of severely immunocompromised, HIV-positive individuals. It has also been suggested that the widespread therapeutic and prophylactic use of antifungal drugs such as fluconazole might also have contributed to the emergence of less susceptible non-*C. albicans* *Candida* species. It is generally accepted that *C. krusei* is inherently resistant to fluconazole (Rex *et al.*, 2000). Furthermore, several studies have reported that a significant proportion of *C. glabrata* isolates are resistant to fluconazole and itraconazole (Fidel *et al.*, 1999). Resistance to azole drugs has also been described in other non-*C. albicans* *Candida* species, including isolates of *C. dubliniensis*, *C. tropicalis* and *C. guilliermondii*, which are frequently recovered from HIV-infected patients following protracted azole therapy. Consequently, it has been suggested that these species have been selected by the

pressure of antifungal drugs (Moran, 1998; Pfaller *et al.*, 1999; Pinjon *et al.*, 2005b) because they are less susceptible to azole drugs than *C. albicans*.

1.2. *Candida dubliniensis*

1.2.1. *C. dubliniensis*, a distinct taxon within the genus *Candida*

The increased incidence of infections caused by non-*C. albicans* *Candida* species in the 1980s and 1990s was also accompanied by a number of reports of “atypical isolates” from laboratories around the world, the majority of which were isolated from the oral cavity of HIV-infected individuals and AIDS patients (Coleman *et al.*, 1993; Sullivan *et al.*, 1993; McCullough *et al.*, 1994; Boerlin *et al.*, 1995; Le Guennec *et al.*, 1995). Phenotypically, these organisms were similar to *C. albicans* in that they produced germ tubes and chlamydospores. However, many of these isolates were found to yield atypical carbohydrate and nitrogen-source substrate assimilation profiles when analysed with commercially available yeast identification kits and unusual DNA fingerprinting patterns with *C. albicans*-specific complex fingerprinting probes (Sullivan *et al.*, 1993). Sullivan *et al.* carried out an extensive study of the phenotypic and genotypic characteristics of atypical isolates recovered from the oral cavities of HIV-infected patients in Ireland and Australia. The data from these experiments, in particular the phylogenetic data, lead these authors to conclude that these atypical isolates constituted a novel, distinct taxon within the genus *Candida* which they named *C. dubliniensis*. *Candida dubliniensis* is the species now known to be the species phylogenetically closest to *C. albicans*. Subsequent studies demonstrated that *C. dubliniensis* had a widespread geographic distribution especially in HIV-infected patient groups (Sullivan & Coleman, 1998; Sullivan & Coleman, 1997; Sullivan *et al.*, 1995).

1.2.2. Phenotypic characteristics of *C. dubliniensis*

Candida dubliniensis shares many phenotypic and genotypic traits with *C. albicans*, this can lead to misidentification and (in some studies) its prevalence may have been underestimated. It grows well at 30°C and 37°C on media used routinely for the isolation and culture of yeast such as Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Yeast Peptone Dextrose broth (YPD). On solid media its

colonial morphology is almost identical to that of *C. albicans* with both species producing colonies of similar size, shape and colour. Furthermore, both species are capable of the phenomenon of phenotypic switching (see section 1.3.5.).

Candida dubliniensis is not as capable as *C. albicans* of growth at higher temperatures. Isolates of *C. dubliniensis* grow poorly or not at all at 42°C, and fail to grow at 45°C (Pinjon *et al.*, 1998; Sullivan *et al.*, 1995). In contrast, the majority of *C. albicans* isolates grow well at both temperatures. Growth at a temperature of 45°C was described as the basis of a simple test to distinguish between the two species (Pinjon *et al.*, 1998). Generally, *C. albicans* appears to have a growth advantage over *C. dubliniensis*. In rich media such as YPD broth, the doubling times of *C. dubliniensis* strains are longer than those of *C. albicans*. Furthermore, in mixed cultures *C. albicans* shows a competitive advantage over *C. dubliniensis* (Kirkpatrick *et al.*, 2000). In order to accurately determine the clinical significance of *C. dubliniensis*, several recent studies have attempted to develop useful phenotypic and genotypic tools for discriminating between it and *C. albicans*. These have included media in which the two species exhibited different colonial morphology (Alves *et al.*, 2006; Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001), direct immunological tests, biochemical and enzymatic tests and PCR-based assays (Somogyvari *et al.*, 2007; Neppelenbroek *et al.*, 2006; Campanha *et al.*, 2005; Mirhendi *et al.*, 2005; Bujdakova *et al.*, 2004; Kurzai *et al.*, 1999).

A commonly used medium for the identification of medically important *Candida* species is the commercially available CHROMagar Candida medium (Odds & Bernaerts, 1994). On this media *C. albicans* colonies are a light blue-green colour, whereas *C. dubliniensis* colonies are a dark green colour on primary isolation (Sullivan & Coleman, 1998; Schoofs *et al.*, 1997). However, this medium is only useful for the preliminary isolation of *C. dubliniensis* following primary culture from clinical specimens as its distinctive colony colouration may be lost following storage and subculture (Schoofs *et al.*, 1997). *Candida dubliniensis* is also less tolerant of environmental stresses such as high concentrations of NaCl and H₂O₂ in the growth media (Tosello *et al.*, 2007).

1.2.3. Epidemiology of *C. dubliniensis*

Candida dubliniensis isolates were initially recovered from the oral cavities of HIV-infected and AIDS patients from Ireland, Australia and the United Kingdom (Coleman *et al.*, 1997a; Sullivan *et al.*, 1995). Reports of the recovery of *C. dubliniensis* from patients in other geographic locations grew rapidly following the first description of the species in 1995. Retrospective studies of culture collections showed that between 1.2 and 2% of isolates originally identified as *C. albicans* were in fact *C. dubliniensis* isolates with some isolates originally recovered in the 1970's (Jabra-Rizk *et al.*, 2000; Jabra-Rizk *et al.*, 1999; Odds *et al.*, 1998). *Candida dubliniensis* is now known to have a worldwide distribution. *Candida dubliniensis* isolates have mainly been recovered from the oral cavities of HIV-infected and AIDS patients (Ponton *et al.*, 2000; Meiller *et al.*, 1999; Kirkpatrick *et al.*, 1998; Coleman *et al.*, 1997b; Sullivan & Coleman, 1997). Isolates of *C. dubliniensis* have also been recovered from the oral cavities of diabetic patients and healthy individuals (Jabra-Rizk *et al.*, 2000; Jabra-Rizk *et al.*, 1999; Odds *et al.*, 1998). Coleman, Sullivan and other authors (Ponton *et al.*, 2000; Sullivan *et al.*, 1999; Coleman *et al.*, 1997a) reported that *C. dubliniensis* was found in 3.5% of Irish asymptomatic HIV-negative healthy individuals, indicating that *C. dubliniensis* can form part of the normal oral flora. In the same study *C. dubliniensis* was recovered from 32% of Irish AIDS patients exhibiting clinical symptoms of oral candidiasis and from 25% of asymptomatic Irish AIDS patients. *Candida dubliniensis* isolates from the oral cavity are most commonly recovered in mixed culture with other *Candida* species, especially *C. albicans* (Coleman *et al.*, 1997b). However, pure cultures of *C. dubliniensis* have been recovered from individuals exhibiting clinical symptoms of oral candidiasis, suggesting that this organism can be responsible for oral infections. However, unlike *C. albicans*, *C. dubliniensis* is rarely found in the oral microflora or in other anatomic sites of normal healthy individuals and has only rarely (approx. 2%) been associated with systemic infection (Carr *et al.*, 2005; Meis *et al.*, 1999). The vast majority of *C. dubliniensis* isolates identified to date are susceptible to all of the commonly used antifungal agents, however, reduced susceptibility to azole drugs has been observed in clinical isolates and can be induced *in vitro* (Pinjon *et al.*, 2005a; Pinjon *et al.*, 2005b; Martinez *et al.*, 2002; Perea *et al.*, 2002).

1.2.4. Phylogenetic analysis of *C. dubliniensis* and determination of its genome sequence

In the first study describing *C. dubliniensis*, Sullivan *et al.* (1995) compared the sequence of 500 bp of the V3 variable region of the large rRNA subunit genes from nine isolates of *C. dubliniensis* and from reference strains of *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata* and *C. krusei*. A phylogenetic tree was produced based on these sequences in which *C. dubliniensis* isolates formed a homogeneous cluster which were 100% identical and significantly different from the other *Candida* species analysed (Sullivan *et al.*, 1995). The *C. dubliniensis* sequence was 2.3% divergent from the corresponding *C. albicans* sequence. This study also indicated that *C. albicans* and *C. stellatoidea* were so closely related (0-0.02 % sequence divergence) as to be considered a single species (Sullivan *et al.*, 1995). These results were later confirmed by the analysis of the V3 region from a further five *C. dubliniensis* isolates from Ireland, the UK, Argentina and Switzerland (Sullivan *et al.*, 1997). Moran *et al.*, (2004) used *C. albicans* whole genome DNA microarrays to perform comparative genomic hybridization study between *C. albicans* and *C. dubliniensis* in order to identify genomic differences that might explain the difference in virulence between both species. The results indicated that *C. dubliniensis* genomic DNA demonstrated a significant degree of nucleotide sequence homology (> 60 %) with the *C. albicans* gene-specific sequences. However, 4.4 % of the sequences (representing 247 genes) exhibited significant sequence divergence (< 60 % homology) or absence in *C. dubliniensis* (Moran *et al.*, 2004).

In order to investigate the molecular biology of *C. dubliniensis* its genome has been sequenced by the The Wellcome Trust Sanger Institute Pathogen Genomics group and annotated in collaboration with Prof. Derek Sullivan *et al.* of the School of Dental Science, Trinity College, Dublin and Prof. Neil Gow *et al.* of the Department of Molecular and Cell Biology, University of Aberdeen.

Phylogenetic distance between *C. albicans* and *C. dubliniensis* and their relative pathogenicity do not correlate. In addition, comparison of *C. albicans* and *C. dubliniensis* genomes by comparative genomic hybridization using *C. albicans* microarrays revealed that there was a majority of shared genes but there is also a small set of highly diverged genes. These diverged genes seemed to be related to the yeast-hypha transition. In order to determine what specific genes confer to *C.*

albicans an increased capacity to cause disease, the sequence of *C. dubliniensis* genome was compared with the *C. albicans* one. The *C. dubliniensis* genome was sequenced using a whole genome shotgun approach. Gene detection was conducted in comparison with the known gene order in *C. albicans* using Artemis Comparison Tool. Using this method it was found that *C. albicans* has 169 species-specific genes that include known hypha-specific virulence factors, (i.e. the aspartyl proteinases *SAP4* and *SAP5* and the invasin *ALS3*), (Jackson *et al.*, 2009). It was also found that *C. dubliniensis* possessed 115 pseudogenes that in *C. albicans* were orthologs of filamentous growth regulator genes, that also have a possible role in pathogenesis. The principal differences between the two genomes regarded the *TLO* gene family of putative transcription factors and the *IFA* family of putative transmembrane proteins, that also are supposed to play a role in pathogenesis. The study provided useful information to better understand the reasons for the different epidemiology of the two species. *Candida albicans* has increased its parasitic capacity with expansions of specific gene families whereas *C. dubliniensis* has had widespread deletions in those genes. Jackson *et al.* (2009) referred this phenomenon as reductive evolution of loci made redundant after ecological restriction of *C. dubliniensis* to its specialised host niches.

1.2.5. Antifungal susceptibility and resistance in *C. dubliniensis*

The relationship between fluconazole usage and the emergence of *C. dubliniensis* still remains controversial. Borg-Von Zepelin, *et al.* (2002) showed that in the presence of fluconazole, the adherence of *C. dubliniensis* to epithelial cells increased, while the adherence of *C. albicans* decreased under the same conditions (Borg-von Zepelin *et al.*, 2002). However, the majority of *C. dubliniensis* isolates are susceptible to the most commonly used antifungals (Jabra-Rizk *et al.*, 2000; Polacheck *et al.*, 2000; Jabra-Rizk *et al.*, 1999; Meiller *et al.*, 1999; Pfaller *et al.*, 1999; Odds *et al.*, 1998; Moran *et al.*, 1997). Pfaller *et al.* (1999) showed that 97% of the *C. dubliniensis* isolates that they tested were susceptible to fluconazole. Compared to *C. albicans*, *C. dubliniensis* MICs for fluconazole, itraconazole and ketoconazole tend to be significantly higher than those of *C. albicans* isolates (Odds, 1998). Furthermore, sequential exposure of fluconazole-susceptible clinical isolates of *C. dubliniensis* to increasing concentrations of fluconazole in solid media resulted

in the recovery of fluconazole-resistant derivatives (Moran *et al.*, 1998 & 1997). Moran *et al.* (1998) analysed the mechanism of resistance to fluconazole in both *in vitro* -generated fluconazole resistant derivatives and in fluconazole-resistant clinical isolates of *C. dubliniensis*. It was found that overexpression of the *CdMDR1*, the *C. dubliniensis* homologue of the *C. albicans* *MDR1* gene, in *C. dubliniensis* was involved in mediating reduced accumulation of drug in fluconazole-resistant clinical isolates and *in vitro*-generated derivatives (Moran *et al.*, 1998). The importance of *CdMDR1* in fluconazole resistance was confirmed by deletion of both alleles of the gene *CdMDR1* in a *C. dubliniensis* strain resistant to fluconazole. The deletion rendered the derivative strain susceptible to fluconazole (Wirsching *et al.*, 2001). Upregulation of *CdCDR1*, the *CDR1* homologue from *C. dubliniensis* has been observed in fluconazole-resistant clinical isolates and *in vitro*-generated derivatives (Moran *et al.*, 1998). However, in a subsequent study, Moran *et al.* (2002) demonstrated that while CdCdr1p is important for mediating reduced susceptibility to itraconazole and ketoconazole, it is not required for fluconazole resistance (Moran *et al.*, 2002). In contrast, in *C. albicans*, resistance to fluconazole is mainly associated with overexpression of *CDR1* (Moran *et al.*, 1998). Furthermore, Moran *et al.* (2002) investigated the reasons for the differential regulation of *CDR1* expression in *C. albicans* and *C. dubliniensis*. The investigators reported the high prevalence (58%) of a nonsense mutation in the *CdCDR1* gene encoding a non-functional CdCdr1p protein amongst *C. dubliniensis* isolates (Moran *et al.*, 2002). Resistance to other antifungal drugs such as ketoconazole, amphotericin B has not yet been reported in *C. dubliniensis* (Pfaller *et al.*, 1999; Ryder *et al.*, 1998).

1.3. *C. albicans* and *C. dubliniensis* virulence traits and virulence determinants

1.3.1. The yeast-to-hypha transition in *C. albicans* and *dubliniensis*

C. albicans and *C. dubliniensis* are polymorphic fungi and they can undergo, *in vivo* and *in vitro*, a yeast-to-hypha transition responding to several environmental conditions such as a shift in pH, temperature, the presence of serum and nutrient starvation (Meis *et al.*, 1999; Land *et al.*, 1975a; Land *et al.*, 1975b; Taschdjian *et al.*, 1960). Other species such as *C. tropicalis* are also capable of this dimorphic switch (Odds, 1987). However, the exact role of hyphae in the pathogenesis of infection is a controversial subject (Ryley & Ryley, 1990). Although it was believed that hyphae could constitute the virulent or invasive form of *Candida*, evidence from animal models of infection has demonstrated that blastospores are also capable of penetrating deep into tissue (Odds, 1994). The recent finding that some *C. albicans* knock-out mutants that are unable to produce hyphae are not completely avirulent in *in vivo* models, and that mutants blocked in the hyphae form are less virulent than the co-respective wild type, support the hypothesis that hyphae are not the only virulent form of *C. albicans*. Although perhaps not essential for pathogenesis, *Candida* hyphae possess two important properties which may enhance virulence: their ability to adhere to host tissues and thigmotropism. Studies have shown that the induction of germ-tube formation induces an increase in the binding ability of *C. albicans* to oral epithelial cells (Sandin *et al.*, 1982; Fukazawa & Kagaya, 1997). Thigmotropism is the ability to sense and interact with a surface (Sherwood *et al.*, 1992). *In vitro*, hyphae have been demonstrated to possess the ability to detect the presence of gaps and pores in membrane filters (Sherwood *et al.*, 1992). Scanning electron microscopy has shown that hyphae will penetrate pores and follow the course of grooves in membrane filters in a directed fashion. *In vivo*, this property may allow the fungus to penetrate intercellular spaces or lesions in the epithelial surface, enhancing invasiveness.

The filamentous growth of *C. albicans* and *C. dubliniensis* species includes two distinct morphologies, pseudohyphae and hyphae. Pseudohyphal cells have an elliptical shape and are attached end-to-end, budding in a unipolar pattern and remaining attached after cytokinesis, forming branched chains of elongated buds and

colonies comprised of pseudohyphae appearing fibrous or rough. Hyphae are narrower than pseudohyphal cells (~2 µm) and have parallel walls with no obvious constriction at the site of septation (Berman & Sudbery, 2002). Many studies relate *C. albicans* pathogenesis to its ability to produce true hyphae which are more able to invade tissue than pseudohyphae (Liu, 2002).

1.3.2. Comparison of virulence in *C. albicans* and *C. dubliniensis*

Since *C. dubliniensis* has been characterised as a different species to *C. albicans*, few studies have been carried out to clarify the characteristics of this fungus in terms of pathogenesis and virulence and their correlation with the host immune response. Sullivan *et al.* in 2004 reported that *C. dubliniensis* is rarely identified as a cause of systemic infection and the site of infection is mostly limited to the oral cavity of HIV-infected patients (Sullivan *et al.*, 2004). Only in rare cases *C. dubliniensis* has been found present in different site of the human body than the oral cavity (Sullivan *et al.*, 2005). Stokes *et al.* (2007) compared the ability of the two species to colonise and infect tissue in animals using the oral intragastric mouse model. When immunocompromised mice were infected with the two species it was found that in the majority of cases *C. dubliniensis* failed to colonise the animal or to cause infection when the animal was rendered immunocompromised. Histological analysis of infected animals revealed that in the various organs, *C. albicans* was present in both the yeast and hyphal form. In contrast, in the small number of animals in which *C. dubliniensis* was detected it was only present in the yeast form (Stokes *et al.*, 2007). Vilela *et al.* also previously reported the higher rate of survival of mice injected with *C. dubliniensis* compared to mice injected with *C. albicans* (Vilela *et al.*, 2002). When mice were inoculated with 2×10^6 yeast cells of each of the two species, those inoculated with *C. albicans* died fast after the injection, while only few mice inoculated with some strains of *C. dubliniensis* died. They also demonstrated that the host mice inflammatory reaction was aggressive with *C. dubliniensis* and mild with *C. albicans*.

Other factors like different adherence to buccal epithelial cells, the production of extracellular enzymes and the phenotypic switching are also considered to contribute to the diverse virulence of the two species.

1.3.3. Comparison of hyphal induction in *Candida albicans* and *Candida dubliniensis*. A distinguishing virulence trait

Despite the fact that *C. albicans* and *C. dubliniensis* are genotypically and phenotypically very similar a clear difference between them is their different capacity to cause disease in humans as *C. dubliniensis* is rarely identified as a cause of systemic infection (Sullivan *et al.* 2004). To understand if this difference in pathogenesis is related to the ability of the two species to produce hyphae, Stokes *et al.* (2007) investigated the ability of strains of *C. albicans* and *C. dubliniensis*, to produce hyphae under a wide range of *in vitro* hypha-induction conditions and also to invade tissue in the Reconstituted Human Epithelial model (RHE). They found that hyphal production *in vitro* is less efficient in *C. dubliniensis* compared to *C. albicans*. Under the conditions normally used to induce hyphae, *C. albicans* was far more able to produce hyphae than *C. dubliniensis*. From all the media tested, the only medium that consistently induced hyphae in *C. dubliniensis* was water to which 10% (v/v) calf serum was added, however, under these conditions the efficiency of induction was still significantly less than that of *C. albicans*. They also compared the growth of *C. albicans* and *C. dubliniensis* in the RHE infection model; the latter grew exclusively in the yeast form, forming a non-invasive layer along the surface of the epithelial tissue. In contrast *C. albicans* produced abundant hyphae that invaded the tissue. In contrast, there are some *in vitro* conditions, some media with added plant extracts (e.g: Staib agar, Tobacco agar, Tomato juice (V8) agar, Pal's agar) on which *C. albicans* grows as yeast and *C. dubliniensis* produces hyphae and pseudohyphae (Khan *et al.*, 2004; Al Mosaid *et al.*, 2003; Al Mosaid *et al.*, 2001; Staib & Morschhauser, 1999). Al Mosaid *et al.* (2003) studied the production of a hyphal fringe around *C. dubliniensis* colonies grown on Pal's agar (sunflower seed agar) at 30°C for 48 to 72 h as a method to discriminate between isolates of this species and *C. albicans* with 100% accuracy. Of 128 *C. dubliniensis* isolates tested on this medium, all produced a hyphal fringe. In contrast, none of the 124 *C. albicans* isolates tested produced a hyphal fringe.

Candida dubliniensis exhibits slower kinetics of hyphal formation compared to *C. albicans*. It was suggested that this may contribute to *C. dubliniensis* lower virulence (Gilfillan *et al.*, 1998). In some *C. dubliniensis* isolates production of hyphae

appeared to be slower in *C. dubliniensis* than in a reference strain of *C. albicans* (Gilfillan *et al.*, 1998).

1.3.4. *C. albicans* and *C. dubliniensis* capability to adhere to and interact with surfaces: proteolytic enzymes and lipases

Adherence is considered in most models of microbial infection to be an important prerequisite for colonisation and tissue penetration. Adherence in *C. albicans* is believed to be multifactorial, involving multiple surface localised molecules. Evidence suggests that the surface mannoproteins of *Candida* species are important ligands involved in the adherence of *Candida* cells to host epithelia. Receptors for these adhesins include surface exposed sugars on the host cell such as L-fucose and N-acetylglucosamine and proteins such as fibronectin (Bailey *et al.*, 1995; Pendrak & Klotz, 1995; Calderone & Braun, 1991). A gene called *INT1*, encoding an adhesin, has been isolated from *C. albicans*. The protein encoded by this gene is structurally related to the α -subunit of leukocyte β 2-integrins and facilitates binding of *C. albicans* cells to fibrinogen and to other proteins found on the surface of epithelial cells (Gale *et al.*, 1996). Roles for this protein in intracellular signalling and the induction of germ tube formation have also been proposed (Hostetter, 1996).

Candida albicans and *C. dubliniensis* have the ability to adapt to changing environmental conditions through the regulation of virulence determinants, in the different morphological forms. Among the most important of these are proteolytic enzymes (Saps) and lipases. These enzymes have been hypothesised to be involved in tissue breakdown and invasion (Sweet, 1997). The SAPs in *C. albicans* are a large family of proteins. Their association with virulence is based on the ability of these enzymes to degrade a wide range of host proteins, including those in the squamous states of the epithelial surfaces, which may facilitate tissue invasion (Schaller *et al.*, 2000). *In vitro* studies suggest that in *C. albicans* the expression of different *SAP* genes is controlled by environmental signals (i.e. glucose, galactose concentrations and particular amino acids concentrations) and by the yeast to hypha transition (Hube *et al.*, 1994). *SAP2* appears to be the most abundantly expressed protease gene in the yeast phase, whereas *SAP4*, *SAP5* and *SAP6* seem to be preferentially expressed when cells undergo the yeast to hypha transition. It seems that individual SAPs may have specific roles at different stages of infection (Hube *et al.*, 1994). Hube *et al.*

created a series of knock-out derivatives of *C. albicans* for the SAPs enzymes ($\Delta sap1$, $\Delta sap2$ and $\Delta sap3$) and reported that when each of these mutants was injected intravenously in mice, the animals exhibited increased survival rates compared to control animals injected with the wild-type strain. This evidence suggests that SAPs contribute to the overall virulence of *C. albicans* in disseminated infections.

1.3.5. Phenotypic switching and mating

Candida albicans and *C. dubliniensis* are capable of rapid and reversible phenotypic switching. This transition influences virulence, mating behaviour, and biofilm formation. Switching can be observed *in vitro* using specific culture conditions, especially stress-inducing conditions (Alby & Bennett, 2009), in which isolates can yield colonies with variant morphologies; smooth, rough, wrinkled or opaque colonies. The most commonly studied switching system is the white to opaque transition. When examined by microscopy, the cells of the smooth white colonies were found to be typical budding yeast cells, however, the cells of the opaque colonies were found to be elongated, rod shaped cells (Anderson & Soll, 1987). In high frequency switching strains these variants appeared at a frequency 1.4×10^{-4} , which is a much higher frequency than the natural occurrence of point mutations. Slutsky *et al.* and Anderson & Soll first hypothesised that phenotypic switching probably involved either a reversible DNA reorganisation event or a heritable change in heterochromatin structure which affects gene expression (Anderson & Soll, 1987; Slutsky *et al.*, 1985). Alby & Bennett showed that a wide range of factors can induce high rates of switching from white to opaque. These included different forms of environmental stimuli, genotoxic and oxidative stress, as well as intrinsic factors such as mutations in DNA repair genes (Alby & Bennett, 2009). A number of genes which are differentially expressed between the white and opaque phases and also control the yeast-to-hypha transition have been identified (see section 1.4). Anderson *et al.* (1987) have proposed that the switch from the opaque phase to the white phase involves the activation of white-phase-specific *trans*-acting factors that activate white-phase-specific genes, and the repression of opaque-phase-specific *trans*-acting factors, leading to the deactivation of opaque-phase-specific genes.

Some evidence also indicates that switching may affect the expression of virulence factors (i.e SAPs). Kvaal *et al.* (1997) have demonstrated that white-phase cells are more virulent than opaque cells in a mouse model of systemic infection but opaque-cells may have some advantages in specific host niches. Miller & Johnson (2002) described a connection between white-opaque switching and mating. These authors found that the ability of a *C. albicans* strain to undergo white-opaque switching was regulated by the *MTL* locus. They also found that opaque-phase α and a cells mated approximately 10^6 times more efficiently than the same cells in the white phase. These findings explained why only a subset of clinical isolates of *C. albicans* underwent white-opaque switching; a and α cells, but not a/a cells, were competent for switching. It also explained the low level of mating previously reported, as the previous studies used *C. albicans* white cells. With the deletion of one *MTL* locus, or the forced loss of one homolog of chromosome 5, which carries the *MTL* allele, different laboratories generated hemizygous a -type and α -type mating strains and studied the mating mechanism of these strains (Miller & Johnson, 2002; Magee & Magee, 2000). The mating products were mononuclear and tetraploid in DNA content. The efficiency of *C. albicans* mating, however, both *in vitro* and *in vivo* was relatively low, in some cases as low as one successful mating event per ten million potential mating partners. The reduction in ploidy for returning to a diploid state occurred in *C. albicans* through chromosome loss during rounds of mitotic division, thereby forming a parasexual cycle but no meiosis was identified. Examination of a number of clinical *C. albicans* isolates has revealed that between 3% and 7% can undergo white-opaque switching; these are naturally occurring homozygous a/a or α/α strains. The residue of the clinical isolates were heterozygous (a/α) at the *MTL* and did not undergo white-opaque switching or undergo with very low frequency. One possibility as to why *C. albicans* incorporated white-opaque switching into its mating behaviour is that white-opaque switching has evolved to allow *Candida* to direct mating to a specific environmental niche in the host. Perhaps certain steps in the mating process, such as pheromone signalling and membrane fusion, are incompatible with *C. albicans* survival in certain environments of a mammalian host. However the ability of *C. albicans* to alter its phenotype may allow the organism to rapidly adapt to different host microenvironments during the course of infection. The role of switching and mating in *C. albicans* biology and virulence remains to be established, since population analysis suggests that there is little

chance for sexual reproduction. However *C. albicans* could, at least in principle, use mating and the parasexual cycle for increasing genetic variation during infection. Genetic variation is known in *C. albicans* to be very low but there are cases of pathogens that have mechanisms that introduce bursts of genetic variation during infection and this could also be one possibility for existence of mating in this species.

1.4. *C. albicans* environmental sensors and morphogenetic signalling pathways

1.4.1. Sensing environmental factors and signalling, an overview

The ability of *C. albicans* to switch from the yeast to hyphal forms of growth in response to various environmental factors represents an excellent model system for the study of how signalling pathways can coordinate cellular growth and development. The various hyphal inducers trigger a wide range of signal transduction pathways involved in morphogenesis. The two best studied pathways are the mitogen-activated protein kinase (MAPK) pathway, that transduces environmental signal through a phosphorylation cascade and the cAMP-protein kinase (PKA) that transduces signals through an increase in the intracellular level of cyclic adenosine monophosphate (cAMP).

Besides the MAPK and cAMP pathways, additional pathways influence the morphogenetic transition. For example the Tup1-Nrg1-mediated pathway represses filamentation independently from the MAPK and cAMP pathways. Despite extensive studies and the growing number of identified genes involved in the process of morphogenesis, the early stages of inducer sensing are not well understood.

An open question that remains to be answered is how external signals are sensed and how the sensors activate the various signal transduction pathways. Very few upstream sensors have been characterised: those that have been identified include the general amino acid sensor Ssy1p, the carbohydrate sensor CaGpr1p, the high affinity glucose sensor-transporter Hgt4p and the ammonium sensor-transporter Mep2p (Brown *et al.*, 2006; Maidan *et al.*, 2005a; Poulsen *et al.*, 2005).

1.4.2. The MAPK, cAMP and *TUP-NRG1* mediated pathways and their role in filamentation

The pathways involved in filamentation that are mediated by MAP kinases include the Cek1-mediated pathway and the HOG pathway. The Cek1 pathway involves the Cst20 PAK-like protein, the Hst7 MAP kinase kinase, the Cek1 MAP kinase, and the Cph1 transcription factor, a homolog of *S. cerevisiae* Ste12 (Liu *et al.*, 1994). Mutants in these genes exhibit defects in hyphal development to a different degree on certain media and have reduced virulence in animal models. The HOG pathway participates in the sensitivity of and the response to osmotic stress. The *C. albicans* *HOG1* gene was cloned by functional complementation of the osmosensitive phenotype associated with *S. cerevisiae* $\Delta hog1$ mutants (Alonso-Monge *et al.*, 2003). *HOG1* is also responsive to other stress conditions and participates in two morphogenetic programmes: filamentation and chlamydospore formation (see section 1.6.4). Eiseman *et al.* showed that Hog1 is a repressor of the filamentation, this repression occurred both under serum limitation and under other partially inducing conditions, such as low temperature, low pH, or nitrogen starvation (Eiseman *et al.*, 2006). The $\Delta hog1$ mutant exhibited a filamentous phenotype characterised by the presence of pseudohyphae under these conditions.

The cAMP pathway, which leads to an increase in the level of the intracellular cAMP is comprised of Cdc35, Pde2, Cap1, Tpk1, Tpk2 and Bcy1 which ultimately control the activity of the transcription factor Efg1 (Staib *et al.*, 2002; Braun & Johnson, 2000; Stoldt *et al.*, 1997) (Fig. 1.1). The Efg1 protein is a strong regulator of morphogenesis in *C. albicans*, since it influences the yeast-to-hypha interconversion and also regulates white-opaque switching and chlamydospore formation (Sonneborn *et al.*, 1999). Over-expression of Efg1p leads to enhanced filamentation in the form of pseudohyphae in liquid medium and mutants lacking this transcriptional factor are not able to form chlamydospores (Sonneborn *et al.*, 1999). The Efg1p pathway appears to be more important than the Cph1p pathway for hyphal morphogenesis, because $\Delta efg1$ mutants are unable to form hyphae in most inducing conditions, e.g., in the presence of serum or GlcNAc as well as during growth on Spider medium (Ernst, 2000; Stoldt *et al.*, 1997), while *cph1* mutants show only a morphogenetic block on Spider medium (Kohler & Fink, 1996). $\Delta efg1$ mutants have a strongly reduced virulence in the mouse model of infection (Lo *et al.*,

1997), while *cph1* mutants essentially retain virulence (Lo *et al.*, 1997). Nevertheless, *efg1 cph1* double mutants show even a further attenuation in virulence than the Δ *efg1* single mutant, suggesting that both transcriptional regulators are required for virulence. Both the MAPK and cAMP pathway are activated by a common upstream factor, CaRas1 (Feng *et al.*, 1999), which is thought to be activated by CaCdc25 as in *S. cerevisiae* (Goldberg *et al.*, 1993). There are other pathways than the MAPK and cAMP pathways involved in morphogenesis. The Tup1-Nrg1-mediated pathway represses filamentation independently from Efg1 and Cph1 (Braun & Johnson, 2000). Disruption of the *TUP1* gene in *C. albicans* results in a mutant that grows exclusively in the hyphal form. Braun and Johnson (2000) have hypothesised that *C. albicans* possesses a mechanism that can sense hyphal inducing environmental stimuli, which can relieve Tup1 mediated repression of hyphal promoting genes. One year later, Murad *et al.* (2001) characterised Nrg1 a transcriptional regulator that represses filamentous growth in *Candida* by recruiting the co-repressor Tup1 to target genes (Fig. 1.1). *C. albicans* Nrg1 mutant cells were predominantly filamentous under non-filament-inducing conditions and their colony morphology resembled that of Δ *tup1* mutants (Murad *et al.*, 2001). *C. albicans* Δ *nrg1* mutants formed chains of relatively short pseudohyphal cells in YPD at 25°C, and more extended pseudohyphae in synthetic complete medium. They also formed wrinkly colonies on YPD agar (Braun *et al.*, 2001). The Δ *tup1* and Δ *nrg1* mutants displayed phenotypic differences. Unlike the Δ *tup1* strain, the Δ *nrg1* mutant grew at 42°C, grew on low glucose media and formed true hyphae on glycerol. Chlamydospore formation that was delayed in the Δ *tup1* mutant, was instead rapid and abundant in the Δ *nrg1* strain (Braun *et al.*, 2001). Therefore, compared with CaNrg1, CaTup1 appears to play additional roles in the growth and development of *C. albicans*. Braun *et al.* (2001) showed that growing *C. albicans* in serum at 37°C, a potent inducer of filamentous growth, caused a reduction of *Nrg1* mRNA, suggesting that filamentous growth is induced by the down-regulation of *Nrg1*. They showed that the expression of *Nrg1* in *C. albicans* under a non-regulated promoter partially blocked the induction of filamentous growth. They also identify two filament-specific genes, *Ece1* and *Hwp1*, whose transcription is repressed by *Nrg1* under non-inducing conditions. These genes constitute a subset of those under Tup1 control (Braun & Johnson, 2000).

1.4.3. The upstream environmental sensors

The *C. albicans* homologue of the *S. cerevisiae* Ssy1p amino acids sensor has been recently characterised. CaSsy1 is important for the induction of genes encoding amino acid permeases. Deletion of this gene results in a defect in filamentation in serum and amino acid based media (Poulsen *et al.*, 2005). Maidan and Miwa have investigated the function of CaGpr1, a putative sensor protein that activates the cAMP-PKA pathway, as occurs in *S. cerevisiae* (Maidan *et al.*, 2005a; Maidan *et al.*, 2005b; Miwa *et al.*, 2004). It was shown that the *C. albicans* Gpr1 homologue is important for cell and colony morphology on solid media and provided evidence that it functions upstream in the cAMP pathway. Maidan and Miwa also showed an important role for micromolar concentrations of specific amino acids such as methionine and proline as triggers for hyphal morphogenesis through Gpr1. In the absence of methionine both wild type and $\Delta gpr1/\Delta gpr1$ strains form smooth colonies. Addition of methionine (at various concentrations) induced filamentation in the wild type strain but not in the $\Delta Cagpr1/\Delta Cagpr1$ mutant (Maidan *et al.*, 2005b). Brown *et al.* (2006) demonstrated that Hgt4 is implicated in the sensing of low glucose levels and appears also to sense fructose and mannose and is required for optimal growth in presence of these carbohydrates. Growth in galactose was not affected by $\Delta hgt4$ mutation. They also attributed to this sensor an important role in the fermentation pathway. Biswas & Morschhauser (2005), showed that in response to nitrogen starvation, *C. albicans* expresses the *MEP1* and *MEP2* genes, which encode two ammonium permeases that enable growth when limiting concentrations of ammonium are the only available nitrogen source. In addition to its role as an ammonium transporter Mep2p also has a central function in the induction of filamentous growth on a solid surface under limiting nitrogen conditions. When ammonium is absent or present at low concentrations, Mep2p activates both the Cph1p-dependent mitogen-activated protein (MAP) kinase pathway and the cAMP dependent signalling pathway via the interactions of its C-terminal cytoplasmic tail with Ras1p. The tail is essential for signalling but dispensable for ammonium transport. In contrast, under ammonium-replete conditions that require transporter-mediated uptake, Mep2p is engaged in ammonium transport and signalling is blocked such that *C. albicans* continues to grow in the budding yeast form.

Fig. 1.1

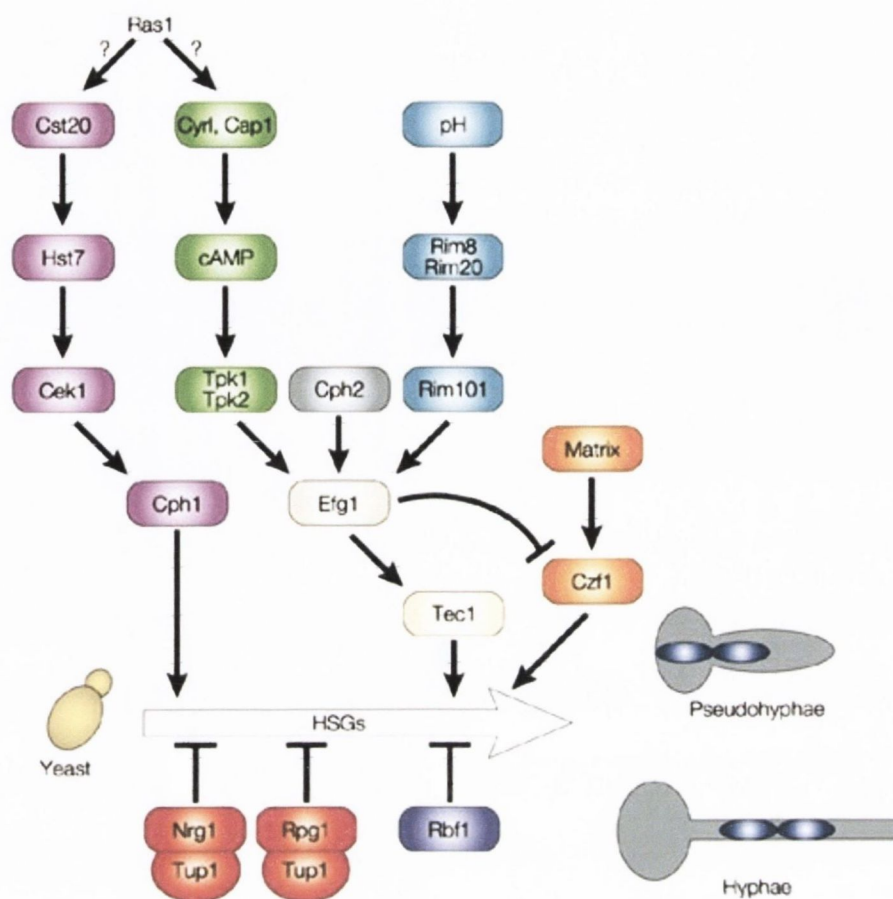


Fig. 1.1 Schematic diagram of the pathways involved in the yeast-to-hypha transition in *C. albicans*.

(taken from *Candida albicans*: A molecular revolution built on lessons from budding yeast, Judith Berman & Peter E. Sudbery, Nature Reviews Genetics 3, 918-932 (December 2002)).

It is not known currently if sensor/transporters upstream of the methionine uptake/biosynthesis pathway can activate the signalling pathways implicated in the yeast-to-hypha transition. Neither is it known if transcriptional activators involved in the regulation of the genes in the methionine pathway can also activate the cAMP-PKA pathway or the MAPK pathway.

1.5. Biosynthesis of sulphur-containing compounds and their importance in fungi

1.5.1. Role of sulphur, sulphur-containing amino acids and sulphur containing compounds in fungi

Sulphur-containing compounds, especially cysteine, methionine, and S-adenosylmethionine are essential for the growth of all cells. Methionine initiates the synthesis of nearly all proteins in all organisms, whereas cysteine plays a critical role in the structure, stability, and catalytic function of many proteins. S-adenosylmethionine plays a pivotal role in methyl group transfer and in polyamine biosynthesis. Furthermore, sulphur is found as an essential component in many other biologically important molecules such as glutathione.

Glutathione (γ -glutamyl-cysteinyl-glycine, GSH) is a ubiquitous tripeptide, synthesised from inorganic sulphate, methionine or cysteine, in which the thiol/thiolate group of its cysteine residue is reversibly oxidised to generate oxidised glutathione. It is the most abundant intracellular redox-active sulfhydryl compound, and it acts as a major cellular redox buffer. Maintenance of the cellular redox state is the most important role of glutathione, protecting cells from the effect of reactive oxygen species (ROS) which due to their powerful oxidant reactivity, cause oxidative damage to nucleotides, proteins, and lipids if they are not properly removed from cells. Glutathione functions as an electron donor for glutathione peroxidase that reduces hydrogen peroxide (H_2O_2) to water and contributes to ROS degradation (Sheehan *et al.*, 2001). ROS are by-products of normal aerobic metabolism (Sumimoto, 2008) but pathogenic fungi also have to tolerate these compounds during the course of host colonisation and infection (Swindle & Metcalfe, 2007). Phagocytic cells (i.e. macrophages, dendritic cells) are the first line of defence against fungal infections. These cells generate ROS, including superoxide, hydrogen peroxide

(H₂O₂), and hydroxyl radicals, that can destroy phagocytosed pathogens (Swindle & Metcalfe, 2007). In addition, to glutathione several well-characterised enzymes, such as the superoxide dismutases, catalases, peroxidases, and glutathione peroxidases, are produced to eliminate ROS (Herrero *et al.*, 2008). Pathogens have a strong co-regulation of these well-conserved antioxidation mechanisms to evade phagocyte defences (Hassett & Cohen, 1989); thus, the production of these enzymes may be related to virulence.

It is believed that pathogenic fungi acquire methionine, cysteine and glutathione from the host, but in some host niches the biosynthesis of those compounds from inorganic sulphate may also be important. An auxotrophy for those compounds may reduce the ability of the pathogen to colonise the host.

Scully *et al.* (2006) demonstrated that cysteine and methionine auxotrophy affected host-range restriction in *A. flavus*. An *A. flavus* strain that was a cysteine/methionine auxotroph due to an inability to reduce sulphate to sulphite showed auxotrophy for those compounds and exhibited a severe diminution or a complete lack of conidial production on a variety of standard agar media and on various plant species. However, it retained its ability to infect insects and to re-emerge from and adequately conidiate on the insect cadavers as a culmination of the pathogenic life cycle (Scully & Bidochka, 2006). Similar studies with other pathogenic fungal species (e.g. *P. brasiliensis*, (Paris *et al.*, 1985)) emphasised the role of nutrition and auxotrophy for sulphur containing metabolites in the host–pathogen relationship with respect to host-niche restrictions.

1.5.2. The biosynthesis of methionine and cysteine from sulphate in *S. cerevisiae*.

For all microorganisms the biosynthesis of sulphur-containing amino acids first requires the capacity to accumulate sulphur atoms from the growth medium and then the transformation of the transported ligands in the reduced form of the sulphur atom sulphide (S²⁻) (Marzluf, 1997). *Saccharomyces cerevisiae* expresses a large number of both transport and biochemical activities that permit it to use a variety of inorganic and organic sulphur sources. The element sulphur occurs in a variety of compounds in which its oxidative state can change from -2 (sulphide), the most reduced form present in sulphur amino acids, to +6 (sulphate), its most oxidised form present in minerals in the earth such as CaSO₄ and FeS₂. In *S. cerevisiae* sulphate assimilation

is carried out in sequential reactions (Fig. 1.2); in which extracellular sulphate (SO_4^{2-}) is transformed into sulphide (S^{2-}). For cysteine and methionine biosynthesis sulphate is sequentially reduced to sulphite which is sequentially reduced to sulphide by sulphite reductase. The transsulphuration pathway is used in *S. cerevisiae* to synthesise cysteine and methionine by the formation of homocysteine and cysteine via formation of cystathionine (Marzluf, 1997).

1.5.3. Transcriptional regulation of the sulphur amino acid pathway

Cbf1p, (centromere binding factor 1) a protein containing a basic helix-turn-helix motif domain, common to DNA binding factors (Yudkin, 1987), has been demonstrated to be involved in the regulation of the Met pathway in *S. cerevisiae*, in addition this protein has also been implicated in the control of chromosome segregation (Cai & Davis, 1990). *S. cerevisiae* $\Delta cbf1$ mutants exhibit auxotrophy for methionine and cysteine, a 10 fold increase in meiotic and mitotic chromosome loss and sensitivity to drugs that disrupt microtubule function. Cbf1p has been shown to be essential for viability in the yeasts *Kluyveromyces lactis* and *C. glabrata* (Stoyan *et al.*, 2001). Numerous studies have been performed to investigate the mechanism of action of Cbf1p in *S. cerevisiae* and it is believed that it exerts its activity by interacting with Met4p, a leucine zipper DNA binding factor, which is the main transcriptional activator of the sulphate-assimilating pathway (Thomas *et al.*, 1992). Enzyme assays on $\Delta met4$ cell extracts revealed undetectable levels of all the enzymes required for sulphate assimilation and $\Delta met4$ mutants are auxotrophic for methionine (Mountain *et al.*, 1993). It has been shown that Cbf1p and Met4p fully activate the Met transcription together by forming heterodimers (Thomas *et al.*, 1992).

1.6. *C. albicans* and *C. dubliniensis* chlamydospores

1.6.1. Chlamydospore cell structure

Chlamydospores are large, spherical, thick-walled cells formed only by *C. albicans* and *C. dubliniensis* in the *Candida* genus, which are usually produced at the end of pseudohyphal tips under certain conditions. Chlamydospores have rarely been observed during infection and their role in the *Candida* life cycle and pathogenesis is

not fully known. Chlamydospores develop at the tip of suspensor cells in a budding fashion and grow until they reach their maximum diameter of approximately 8-10 μm . Subsequently a septum is formed between the suspensor cell and the chlamydospore (Staib & Morschhauser, 2007). Electron micrographs of chlamydospores have shown that chlamydospore cell walls are double-layered, consisting of a thin, electron transparent outer layer surrounding a thick electron dense inner layer. The outer layer of the chlamydospore is continuous with the wall of the suspensor cells (Shannon, 1981). The cytoplasmic organisation of a chlamydospore is similar to the organisation of a yeast cell although there is a greater content of ribonucleic acids and lipids. Vidotto *et al.* first identified the presence of a nucleus in chlamydospores using the stain DAPI (4,6-diamidino-2-phenylindole) (Vidotto *et al.*, 1996). Martin *et al.* (2005) found that nuclear division during chlamydospore formation from the suspensor cell occurred within the suspensor cell, followed by the migration of one nucleus into the immature chlamydospore, the process of nuclear migration and maturation takes around 5 days. Nuclear division across the suspensor-chlamydospore junction was never detected, whereas this is typical at the necks of budding yeast cells.

1.6.2. Chlamydospores *in vitro* culturing conditions

The *in vitro* culturing conditions that maximise production of chlamydospores include nutrient-poor media supplemented with detergents and incubation at room temperature preferentially under oxygen limitation and in the dark. Corn meal and rice extract agar, Tween 80-oxgall-caffeic acid (TOC) are the best known chlamydospore-inducing media in both species (Sullivan *et al.*, 1995) (Koehler *et al.*, 1999) (Jabra-Rizk *et al.*, 1999). The production of chlamydospores has also been used as a tool to distinguish *C. albicans* and *C. dubliniensis* from other species of the *Candida* genus and to distinguish between these two closely related species as *C. dubliniensis* was first characterised as being able to produce greater numbers of chlamydospores than *C. albicans*. In contrast to *C. albicans* isolates, which, in the traditional media, tend to produce single chlamydospores attached terminally to pseudohyphae by a single suspensor cell, isolates of *C. dubliniensis* tend to produce abundant chlamydospores, frequently arranged in contiguous pairs or triplets attached to a single suspensor cell (Sullivan *et al.*, 1995).

Fig .1.2

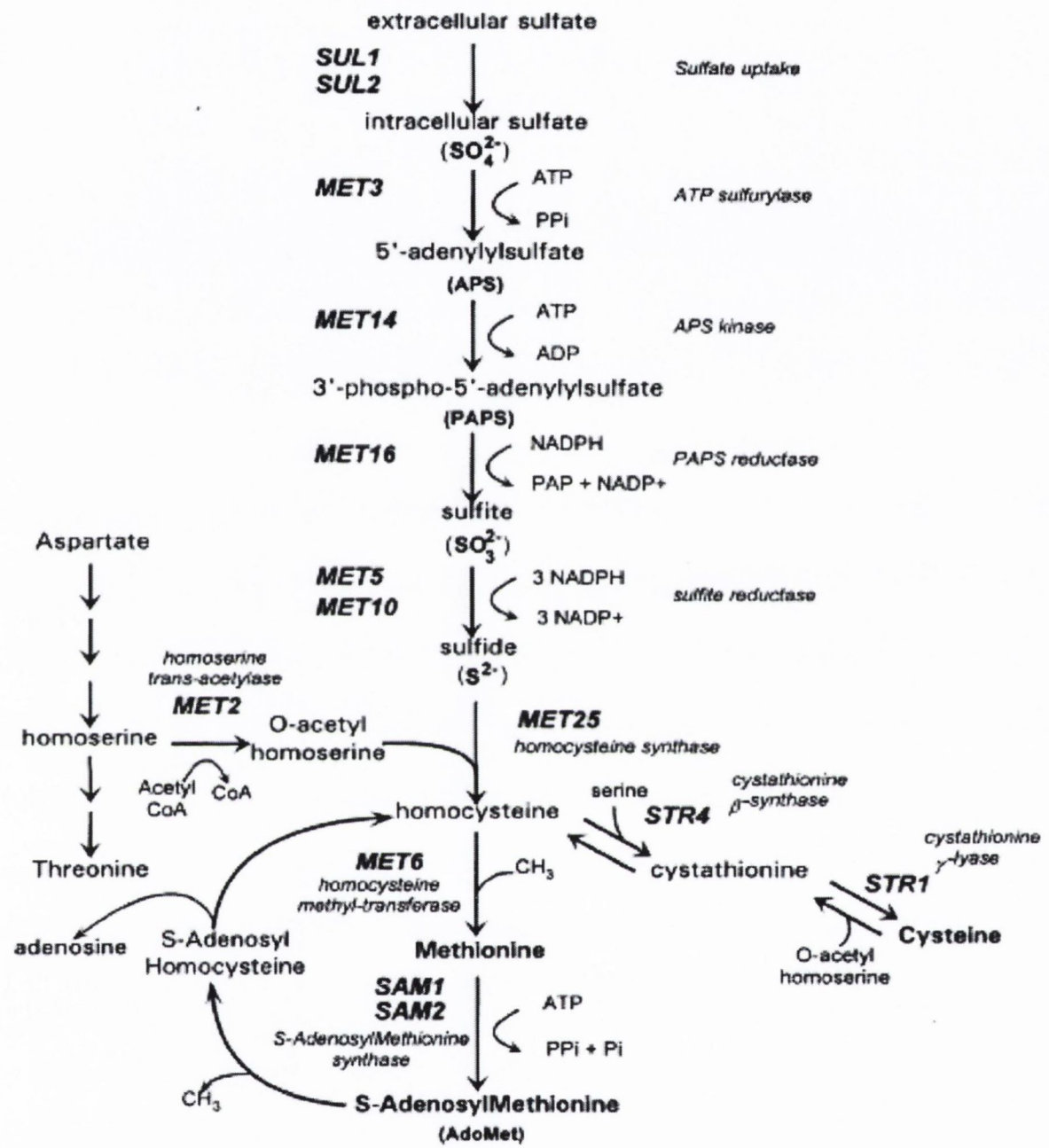


Fig. 1.2 Methionine biosynthesis from sulphate in *S. cerevisiae*.

The genes in the first steps of the pathway are regulated by the transcriptional activator *CBF1* (and its cofactor *MET4*). *MET4* is instead the main transcriptional activator in the second part of the pathway promoting the glutathione biosynthesis. Almost all the *S. cerevisiae* genes in the pathway show a good degree of homology with uncharacterised *Candida* genes possibly involved in the methionine pathway. (from Marzluf, G. A. (1997). Molecular genetics of sulfur assimilation in filamentous fungi and yeast. *Annu Rev Microbiol* 51, 73-96).

Staib and Morschhauser first discovered in 1999 that when *C. dubliniensis* is grown on Staib agar it forms rough colonies composed mainly of pseudohyphae and chlamydospores while under the same conditions *C. albicans* produces smooth colonies composed solely of yeasts (Staib & Morschhauser, 1999). Other solid media have been described in which *C. dubliniensis* produces chlamydospores while *C. albicans* does not, these include Pal's agar, sunflower agar, caffeic acid-ferric agar, citrate agar, tobacco agar and tomato-juice agar (Alves *et al.*, 2006; Kumar & Menon, 2005; Al Mosaid *et al.*, 2003). The environmental signals responsible for species-specific production of chlamydospores on these media are unclear.

In order to fully investigate the molecular biology of chlamydospores it would be essential to be able to purify them by separating them from their suspensor cells and associated hyphae and pseudohyphae. There are two obstacles to the purification of chlamydospores: (i) the invasion of solid agar media by the mycelium and (ii) the tight association of chlamydospores with their parental cells. Gunasekaran and Hughes separated chlamydospores from the mycelial component by sonication, Simonetti *et al.* used a sulphuric acid treatment which caused lysis of the mycelium, Fabry *et al.* used β -glucuronidase which allowed reasonable levels of purity to be achieved, however, the yield of chlamydospores was low (Fabry *et al.*, 2003; Vidotto *et al.*, 1988; Gunasekaran & Hughes, 1978; Simonetti & Strippoli, 1971). The ability to produce chlamydospores in liquid culture would greatly facilitate the purification and analysis of these intriguing structures. To date only one study has observed this, when Staib & Morschhauser (2005b) recently described a rapid method to produce chlamydospores in undefined liquid medium.

1.6.3. Presence of chlamydospore in *in vivo* samples.

There are few reports of the presence of chlamydospores in *in vivo* samples. Chlamydospores have been observed *in vivo* in patients affected with very severe forms of candidiasis (i.e. *Candida* endocarditis or other systemic candidiasis in HIV-infected patients (Chabasse *et al.*, 1988; Heineman *et al.*, 1961), in more than one report the presence of these structures was revealed only by a scrupulous post-mortem autopsy of the patient. There are also reports of the presence of chlamydospores in the histological samples from patients with milder *Candida* infection coinciding with the administration of a putative inducing agent (i.e. therapy

for other bacterial or fungi infections (Wilborn & Montes, 1980)). This paucity of reports of the *in vivo* presence of chlamydospores could also be related to the low number of specific studies done so far for their *in vivo* identification.

Heineman *et al.* (1961) observed the presence of chlamydospores in histology samples in a case of fatal superinfection of *C. albicans* endocarditis during treatment of staphylococcal endocarditis. Chlamydospores were observed in the cardiac vegetation, as well as in kidney abscesses. The authors commented that this finding was related to the pathogenicity of *C. albicans*, hypothesising that the structures could enable the species survival “in the adverse environment of the bodily fluids”. Wilborn & Montes (1980) analyzed the oral surfaces of 12 patients with chronic mucocutaneous candidiasis using scanning electron microscopy. They found that high density of *C. albicans* populated the surface of the infected mouth of each patient. Although pseudohyphae were the predominant forms that they found, blastospores and chlamydospores were also found.

Reports also exist of chlamydospores associated with cases of candidal endophthalmitis and infection of costal cartilages. Schonborn & Schmidt (1971), demonstrated the presence of chlamydospores in dermatological specimens taken from skin and nails of patient affected from onychomycosis. Cole *et al.* (1991) detected the production of chlamydospores *in vivo* in a mouse model of systemic infection. The group noted the presence of chlamydospores in histological sections of the gastric mucosa of orally infected mice after the administration of cyclophosphamide plus cortisone acetate and the antifungal agent cilofungin.

1.6.4. Genetic control of chlamydospore formation in *C. albicans*

Chlamydospores are not produced by the model yeasts *S. cerevisiae* and *S. pombe*, so there is limited understanding of the pathways that govern their development. To determine if specific signal transduction pathways leading to hyphal development also had a function in the formation of chlamydospores Sonneborn *et al.* tested various mutants in the cAMP and MAPK pathways for their ability to form chlamydospores (Sonneborn *et al.*, 1999). The *cph1* mutant, lacking the Cph1p transcription factor activated by the Cek1p MAP kinase and a *tpk2* strain which lacks an isoform of PKA, were competent in the formation of chlamydospores. On the other hand, the *efg1/efg1* mutant was completely deficient for chlamydospore

formation (Sonneborn *et al.*, 1999). More recently Eisman *et al.* (2006) showed that the Hog1 mitogen-activated protein (MAP) kinase that is mainly implicated in an adaptive response to both osmotic and oxidative stress and in the yeast-to-hypha transition (as a repressor), also plays a role in chlamydospore formation, as an inducer. They also showed that the inability of the $\Delta hog1$ mutants to form chlamydospores can be suppressed when additional elements of the CEK1 pathway (i.e. *CST20* or *HST7*) are altered. Both the *EFG1* and *HOG1* genes are essential in the formation of chlamydospores, involving a MAPK signal transduction pathway and the cAMP pathway in this process. Eisman *et al.* demonstrated that both Hog1 and Efg1 proteins from the two signalling pathway, act independently, since overexpression of the *EFG1* gene did not restore the ability to form chlamydospores in the *hog1* mutant, and similarly, overexpression of *HOG1* does not restore the formation of chlamydospores in the *efg1* mutant (Eisman *et al.*, 2006). A different approach to identify genes controlling chlamydospore development in *C. albicans* was used by Staib & Morschhauser (2005a). Based on the observation that only *C. dubliniensis*, but not *C. albicans*, produced chlamydospores under certain environmental conditions, (e.g. during growth on Staib agar (Staib & Morschhauser, 1999)) a *C. albicans* genomic library was integrated into the *C. dubliniensis* genome and transformants were screened for clones in which filamentation and/or chlamydospore production on Staib agar was suppressed. This screen identified the *NRG1* gene. Expression of *CaNRG1* in *C. dubliniensis* repressed pseudohyphae and chlamydospore formation (Staib & Morschhauser, 2005a).

Nobile *et al.* using a forward genetic approach defined the basis of the genetic regulation that governs chlamydospore formation. Based on the phenotypes of homozygous insertion mutants and reconstituted strains they identified 6 genes (i.e. *ISW2*, *MDS3*, *RIM13*, *RIM101*, *SCH9* and *SUV3*) that are required for efficient chlamydospore formation. *SUV3*, *SCH9*, and *ISW2*, are involved in mitochondrial function, glycogen accumulation, and chromatin remodelling, respectively. Rim101 is a proteolytically activated regulator of alkaline-induced filamentation, a zinc finger protein similar to *S. cerevisiae* Rim101 (YHL027W) transcriptional activator needed for entry into meiosis. Mutations in *ISW2*, *SCH9* and *SUV3* completely abolish chlamydospore formation. Mutations in *RIM13*, *RIM101* and *MDS3* delay normal chlamydospore formation. For these taken all together, it is clear that chlamydospore production involves a complex genetic program and it is also

possible that chlamydospore-inducing conditions might govern not yet defined signalling pathways that regulate filamentation.

Although some genes which are involved in chlamydospore formation in *C. albicans* have been identified, no clue about the biological role of this structure has been obtained so far. As mentioned previously, chlamydospore development is a specific characteristic of *C. albicans* and *C. dubliniensis* whose natural habitats are humans and other warm-blooded animals and which are normally not found in the environment. This may indicate a yet undiscovered role of chlamydospores in the pathogenesis of these two species.

Aims and objectives of the project

- To investigate the differential responses of *C. albicans* and *C. dubliniensis* to varying concentrations of methionine and glucose.
- To investigate the role of the methionine biosynthetic pathway (and its regulators Cbf1 and Met4) in morphogenesis in *C. albicans* and *C. dubliniensis*.
- To investigate the role of chlamydospores in *C. albicans* and *C. dubliniensis* life cycle

Chapter 2

General materials and methods

2.1. General Materials and Methods

2.1.1. Strains and culture conditions

The *Candida* strains used in this study are listed in Table 2.1. All yeast isolates were from the culture collection of the Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School and Hospital, University of Dublin Trinity College Dublin, Ireland. The double disruptant of the *CBF1* gene was kindly obtained from J. Morchhauser, University of Wurzburg, Germany. *Candida* strains were routinely cultured on yeast extract-peptone-dextrose agar (YPD) at 30°C. For liquid culture, isolates were grown in yeast extract-peptone-dextrose (YPD) broth at both 30°C and 37°C in a Gallencamp orbital incubator (New Brunswick Scientific Company Incorporated, Edison, New Jersey, USA) set at 200 rpm. Phosphate buffered saline pH 7 (PBS) (Oxoid) was used to resuspend and wash *Candida* cells.

2.1.2. Bacterial strains

Escherichia coli strains were routinely cultured on Luria-Bertani agar (LA), pH 7.4 at 37°C in an orbital incubator (Gallencamp) at 200 rpm. The *E. coli* strain DH5 α [F- ϕ 80d, *lacZ* Δ m15, *endA1*, *recA1*, *hsdR17*, (rK-mK+), *supE44*, *thi-1*, *gyrA96*, Δ 9*lacZYA*-F), U169] was used as a host strain for plasmid pBluescript II Ks (-) (Stratagene, La Jolla, California, USA), (Hanahan *et al.* 1991).

2.1.3. Chemicals, enzymes and oligonucleotides

Analytical-grade or molecular biology-grade chemicals were purchased from Sigma-Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland), or Roche Diagnostics Ltd. (Lewes, East Sussex, United Kingdom). Zymolyase 20T (21,600 U/g) was purchased from the Seikagaku Corporation (Tokyo, Japan). Enzymes were purchased from the Promega Corporation (Madison, Wisconsin, USA) and from New England Biolabs (Beverly, Massachusetts, USA) and used according to the manufacturer's instructions. DNA molecular weight markers were purchased from Promega Corporation. XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) was purchased from Sigma-Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland). FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-

methyldene)-1phenylquinoliniumiodide] was purchased from Molecular Probes, Europe BV (Leiden, The Netherlands).

2.1.4. Buffers and Solutions

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

TBE buffer was prepared at 20X concentration and consisted of 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA. This was diluted in ultra-purified water to a final concentration of 0.5 X and was used as the buffer for agarose gel electrophoresis. SSC buffer was prepared at 20 X concentration and consisted of 3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7.0.

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

2.2. Recombinant DNA techniques

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

Small scale preparations of plasmid DNA from *E. coli* were made using the GenElute™ (Sigma-Aldrich) plasmid miniprep kit according to the manufacturer's instructions.

2.2.2. Purification of restriction endonuclease-generated DNA fragments from agarose gels

Fragments were electrophoresed in agarose gels and viewed on a UV transilluminator which emits UV light at 345 nm (Ultra Violet Products Ltd., Cambridge, United Kingdom). The fragment of interest was extracted from the gel and purification was carried out using the Gel purification kit Qiaex II (Qiagen, Crawley, West Sussex UK) kit according to the manufacturer's instructions.

Table 2.1.
***Candida dubliniensis* isolates**

Human isolates	Origin ^a	Yr of isolation	Underlying patient condition	Sample	ITS Genotype	Source/Reference
CD36	Ireland	1988	HIV Pos	Oral	1	(Sullivan <i>et al.</i> , 1995; Gee <i>et al.</i> , 2002)
CD06033	Ireland	2006	CF	Sputum	1	This study
CD0603	Ireland	2006	CF	Sputum	1	This study
CD060213	Ireland	2006	CF	Sputum	1	This study
CD0604	Ireland	2006	CF	Sputum	1	This study
CD06038	Ireland	2006	CF	Sputum	1	This study
CD06045	Ireland	2006	CF	Sputum	1	This study
CD06037	Ireland	2006	CF	Sputum	2	This study
CD06036	Ireland	2006	CF	Sputum	2	This study
CD060215	Ireland	2006	CF	Sputum	3	This study
CD604	France	2000	HIV Pos	Throat	1	This study
CD603	France	2000	HIV Pos	Throat	1	This study
CM1a ^b	Australia	1991	HIV Pos	Oral	1	(Sullivan <i>et al.</i> , 1995; Gee <i>et al.</i> , 2002)
SA105	S. Arabia	2002	Diabetes	Oral	1	(Al Mosaïd <i>et al.</i> , 2005)
SA115	S. Arabia	2002	Diabetes	Oral	1	(Al Mosaïd <i>et al.</i> , 2005)
SA116	S. Arabia	2002	Diabetes	Oral	1	(Al Mosaïd <i>et al.</i> , 2005)
SA108	S. Arabia	2002	Diabetes	Oral	3	(Al Mosaïd <i>et al.</i> , 2005)
SA100	S. Arabia	2002	Leukaemia	Oral	3	(Al Mosaïd <i>et al.</i> , 2005)
SA121	S. Arabia	2002	S/P Renal Tx	Oral	4	(Al Mosaïd <i>et al.</i> , 2005)
Eg202	Egypt	2002	Cancer	Oral	4	(Al Mosaïd <i>et al.</i> , 2005)
Eg203	Egypt	2002	Cancer	Oral	1	(Al Mosaïd <i>et al.</i> , 2005)
Eg204	Egypt	2002	Cancer	Oral	1	(Al Mosaïd <i>et al.</i> , 2005)
Eg207	Egypt	2002	Diabetes	Oral	4	(Al Mosaïd <i>et al.</i> , 2005)
p6785	Israel	1999	HIV Neg	Urine	3	(Al Mosaïd <i>et al.</i> , 2005; Polacheck <i>et al.</i> , 2000)
p7276	Israel	1999	HIV Neg	RT	3	(Al Mosaïd <i>et al.</i> , 2005)
p7718	Israel	1999	HIV Neg	Wound	4	(Gee <i>et al.</i> , 2002; Al Mosaïd <i>et al.</i> , 2005)
CD71	Argentina	1994	HIV Pos	Oral	1	(Sullivan <i>et al.</i> , 1997; Gee <i>et al.</i> , 2002;)
CD98923	India	1998	HIV Pos	Oral	1	(Al Mosaïd <i>et al.</i> , 2001)
B1324	USA	1998	N/A	Tongue	1	This study
B341	USA	1998	N/A	Throat	1	This study
PM6-2	Chile	2006	N/A	N/A	1	This study
P2	Switzerland	1993	HIV Pos	Oral	1	(Gee <i>et al.</i> , 2002; Al Mosaïd <i>et al.</i> , 2005)
1504	Slovakia	2005	N/A	Tonsular swab	1	(Melkusova <i>et al.</i> , 2005)
8882	Slovakia	2005	CH	Tonsular swab	1	(Melkusova <i>et al.</i> , 2005)
9097	Slovakia	2005	Leukaemia	Tonsular swab	1	(Melkusova <i>et al.</i> , 2005)
966/3(1)	Slovakia	2005	N/A	Sputum	1	(Melkusova <i>et al.</i> , 2005)
966/3(2)	Slovakia	2005	N/A	Sputum	1	(Melkusova <i>et al.</i> , 2005)
CCY29-177-1	Slovakia	2005	Pelvic organ inflammation	Cervial swab	1	(Melkusova <i>et al.</i> , 2005)
MLNIH0479	Thailand	2005	Leukaemia	Blood	1	(Bujdakova <i>et al.</i> , 2004)
MLNIH0720	Thailand	2005	TB, Anaemia, Diabetes	Oral	1	(Bujdakova <i>et al.</i> , 2004)
49831	Japan	2005	Anaemia	Sputum	2	(Bujdakova <i>et al.</i> , 2004)
IFM49883	Japan	2005	N/A	N/A	2	(Bujdakova <i>et al.</i> , 2004)
IFM0492	Thailand	2005	Cancer	Blood	1	(Bujdakova <i>et al.</i> , 2004)
IFM49832	Japan	2005	Diabetes	Sputum	1	(Bujdakova <i>et al.</i> , 2004)
Can4	Canada	1996	HIV Pos	Oral	2	(Pinjon <i>et al.</i> , 1998; Gee <i>et al.</i> , 2002;)
CD539	U.K	1994	AIDS	Oral	2	(Pinjon <i>et al.</i> , 1998; Gee <i>et al.</i> , 2002;)
CBS2747	Netherlands	1952	HIV Neg	Sputum	2	(Meis <i>et al.</i> , 1999; Gee <i>et al.</i> , 2002)

Continued overleaf

Table 2.1. Continued

C. dubliniensis isolates

Human isolates	Origin ^c	Yr of isolation	Underlying patient condition	Sample	ITS Genotype	Source/Reference
CD519	Ireland	1997	AIDS	Oral	3	(Gee et al., 2002)
CD75004	UK	1975	Diabetes	Oral	2	(Pinjon et al., 1998; Gee et al., 2002)
49831	Japan	2005	Anaemia	Sputum	2	(Bujdakova et al., 2004)
IFM49883	Japan	2005	N/A	N/A	2	(Bujdakova et al., 2004)
IFM0492	Thailand	2005	Cancer	Blood	1	(Bujdakova et al., 2004)
IFM49832	Japan	2005	Diabetes	Sputum	1	(Bujdakova et al., 2004)
98-277	Spain	1998	N/A	Oral	1	(Brena et al., 2004)
95-677	Spain	1995	HIV Pos	Oral	1	This study
94-234	Spain	1994	HIV Pos	Oral	1	(Brena et al., 2004)
CD505	Ireland	1989	HIV Pos	Oral	1	(Gee et al., 2002)
CD06032	Ireland	2006	Healthy	Oral	1	This study
CD06027	Ireland	2006	Healthy	Oral	1	This study
CD0512	Ireland	2005	Systemic steroids	Oral	1	This study
CD524	Ireland	1997	HIV Neg	Oral	1	(Gee et al., 2002)
17P	Argentina	N/A	Gingivitis	Oral	2	This study
18P	Argentina	N/A	Periodontitis	Oral	2	This study
22BP	Argentina	N/A	Periodontitis	Oral	2	This study
25P	Argentina	N/A	Periodontitis	Oral	2	This study
110P	Argentina	N/A	Periodontitis	Oral	2	This study
140P	Argentina	N/A	Gingivitis	Oral	2	This study

C. albicans isolates

Human isolates	Origin ^c	Yr of isolation	Underlying patient condition	Sample	ITS Genotype	Source/Reference
SC5314						(Gillum et al., 1984)
CA4153A						(Slutsky et al. 1985)
CY	Ireland	N/A	N/A	N/A	N/A	This study
Δcbf1				SC5314 derivative		(Biswas et al., 2002)
Met4/Δmet4				SC5314 derivative		This study
Δmet4/Δmet4				SC5314 derivative		This study
MET4::SAT1				SC5314 derivative		This study
KJ	Ireland	N/A	N/A	N/A	N/A	This study
JM1	Ireland	N/A	N/A	N/A	N/A	This study
M182CA	Ireland	N/A	N/A	N/A	N/A	This study
8.1	Ireland	N/A	N/A	N/A	N/A	This study
15.1	Ireland	N/A	N/A	N/A	N/A	This study
17.1	Ireland	N/A	N/A	N/A	N/A	This study
19.1	Ireland	N/A	N/A	N/A	N/A	This study
20.2	Ireland	N/A	N/A	N/A	N/A	This study
21.1	Ireland	N/A	N/A	N/A	N/A	This study
28.1	Ireland	N/A	N/A	N/A	N/A	This study

Continued overleaf

Table 2.1. Continued

C. albicans isolates

31.1	Ireland	N/A	N/A	N/A	N/A	This study
32.1	Ireland	N/A	N/A	N/A	N/A	This study
36.1	Ireland	N/A	N/A	N/A	N/A	This study
39.1	Ireland	N/A	N/A	N/A	N/A	This study
40.1	Ireland	N/A	N/A	N/A	N/A	This study
45.1	Ireland	N/A	N/A	N/A	N/A	This study

Abbreviations: HIV Pos; Human immunodeficiency virus-infected; HIV Neg; Human immunodeficiency virus negative; CF; Cystic Fibrosis; S. Arabia; Saudi Arabia; S/P Renal Tx; stage-post renal transplantation; N/A; not available; CH; Congenital Hydrocephalus; RT; Respiratory tract; TB; Tuberculosis; AIDS; Acquired immunodeficiency syndrome patient.

2.2.3. Ligation of DNA fragments

Purified DNA fragments were ligated to pBluescript II KS (-) phagemid digested with an appropriate restriction endonuclease. Ligation reactions were carried out in a 20 µl volume, with a 3:1 ratio of insert to vector DNA in 1x ligase buffer (300 mM Tris-HCL (pH 7.8 at 25°C), 100 mM DTT and 10 mM ATP), with 1 U of T4 DNA ligase (Promega Corporation, Madison, Wisconsin USA.).

2.2.4. Transformation

Transformation of competent *Escherichia coli* DH5α by CaCl₂ and identification of recombinants was carried out according to the method of Sambrook *et al.* (1989).

2.2.5. Extraction of *Candida* genomic DNA

Total genomic DNA of *Candida* isolates was prepared from cells grown overnight in YPD broth as described by Gallagher *et al.* (1992).

2.2.6. Polymerase chain reaction

Specific sequences of *C. dubliniensis* genomic DNA were amplified by PCR and cloned into pGEM® T Easy Vector System I (Promega). Oligonucleotide primers were synthesised by Sigma-Aldrich. Amplification reactions were carried out in 50 µl volumes containing 2.5 U *Taq* DNA polymerase (Promega), 1x *Taq* reaction buffer, 2 mM MgCl₂, 250 µM (each) dATP, dTTP, dGTP, dCTP (Promega) 10 pm of both a forward and reverse primer and 10 ng of genomic DNA template. Amplification products were separated by electrophoresis through 0.8% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide. DNA molecular weight marker was loaded on each gel in an adjacent lane. The products were visualised on a UV transilluminator following electrophoresis at 80 V with constant current for 1 hour.

2.2.7. DNA sequence analysis

Nucleotide sequence analysis was performed commercially by Cogenics (Saffron Walden, Essex, United Kingdom) using the dideoxy chain terminating method of Sanger *et al.*, using an automated Applied Biosystems 377 DNA sequencer and dye-labeled terminators (Applied Biosystems, Foster City, Calif.) (Sanger *et al.*, 1977).

Chromatograms were analysed using the 373A Data Analysis program version 1.2.0 (Applied Biosystems). DNA and protein analysis was carried out using the DNA StriderTM software for DNA and protein analysis (CEA/Saclay, Gif-sur-Yvette, France). Sequence alignments were carried out using the CLUSTAL W sequence analysis computer software package (Higgins & Sharp, 1988). Searches of the EMBL and Genbank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programmes (Altschul *et al.*, 1990).

2.3. Heterologous expression of the *C. albicans* *CBF1* gene in *C. dubliniensis* genome

2.3.1. Cloning of the *C. albicans* *CBF1* gene and upstream region in the vector pCDRI

To amplify the *C. albicans* *CBF1* gene and upstream promoter region a forward primer containing the restriction site *NotI* and a reverse primer containing *SacII* were designed based on the sequence of the open reading frame (ORF) 19.2876 ± 1000 bp (Table 2.2). A PCR reaction was carried out as outlined in 2. 2. 6. The PCR amplimer was then cloned into pGEM by standard procedures (2.2.3). The fragment was excised by a digestion with *NotI* and *SacII* enzymes and cloned into the integrating vector pCDRI (Moran *et al.*, 2007) previously digested with the same enzymes.

2.3.2. Integration of the pCDRI plasmid in *C. dubliniensis* genome

pCDRI integrates into the truncated non functional gene *CDR1* (the CDR region of pCDR1 consists of + 1800 to + 2400 of the *C. dubliniensis* *CDR1* gene (accession n AJ439073; (Moran *et al.*, 2002)). To allow integration, the plasmid was linearised with the *NcoI* enzyme within the CDR region.

Candida dubliniensis strains were transformed by electroporation (Kohler *et al.*, 1997). Cells from YPD medium preculture were diluted 10⁴ in 50 ml of fresh YPD medium and grown overnight at 30°C to an optical density at 600 nm (OD 600) of 1.6 to 2.2, which was found empirically to yield the best transformation efficiency. Transformation was carried out as outlined by Staib *et al.* (2001). Nourseothricin

resistant transformants were selected on agar plates containing 100 µg/ml nourseothricin (cloNAT, Werner BioAgents, Jena, Germany). Single colonies were picked after 3 days of growth at 30°C, and subcultured.

2.3.3. Identification of the expression of *CaCBF1* gene in *C. dubliniensis* with reverse transcriptase PCR.

2.3.3.1. Total RNA extraction and cDNA preparation from Candida isolates.

Candida cells were harvested in mid exponential phase (O.D. 600 0.8-1.0) from 50 ml YPD or minimal medium (SD) (2% glucose, 0.67 yeast nitrogen base without amino acids) at 2,500x g for 5 min. Pellets were frozen immediately in liquid nitrogen. For each sample a 2 ml screw capped Sarstedt tube containing 0.3 g RNase-free glass beads was prepared. The cell pellets were resuspended in 1 ml Tri-Reagent (Sigma) and quickly transferred to the tube containing the beads. The cells were then homogenised in a Fastprep FP120 Bead Beater (Savant Instruments, Inc. Holbrook, NY) for 30 s at speed 6. The homogenate was transferred to a fresh eppendorf and incubated for 5-10 min at 15-20°C. After the addition of 200 µl chloroform the sample was vortexed for 15 s and then centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was carefully removed to a fresh 1.5 ml Eppendorf tube and 500 µl of isopropyl alcohol was added to the sample and left to incubate at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 10 min at 4°C. The pellet formed was resuspended in DEPC H₂O and to remove DNA and polysaccharides two volumes of 6 M LiCl was added and incubated at -20°C for 2h. Following a centrifugation at 12,000 x g for 15 min, the resulting pellet was carefully washed in 1 ml of 70% (v/v) ethanol. The ethanol was removed and the pellet was air dried briefly. The pellet was resuspended in a final volume of 30 µl of DEPC H₂O and stored at -80°C. The concentration of each RNA sample was assessed by measuring the A260 (1unit of A260 = 42 µg RNA) and 15 µg were loaded on 1% (w/v) agarose gel to assess the integrity of each samples.

First strand cDNA synthesis was performed using the Superscript II kit (Invitrogen, Bio Science Ltd., Dun Laoghaire, Dublin, Ireland). One µg of total RNA and 1 µg of oligo dT12-18 (0.025mM) primer was added to each tube to obtain a total volume of 11 µl. RNA was denatured at 70°C for 10 min and cooled to 42°C to allow primer annealing. Subsequently 5X first standard buffer, 2 µl 0.1M DTT and 1µl RNase inhibitor (40 U/ µl)

(Ambion, Applied Biosystem, Warrington, U.K.), were added to each tube. The mixture was incubated at 42°C for 2 min, and then 1 µl of Reverse Transcriptase (200 U/ µl), was added to each sample. The reaction was further incubated at 42°C for 60 min, followed by 70°C for 15 min.

2.3.3.2. Discriminative PCR on the *CaCBF1* gene.

To study the expression of the *CaCBF1* in *C. dubliniensis* a PCR on a region of low identity between the *CBF1* homologues in the two species was designed. For the specific amplification of the *C. albicans* *CBF1* gene two specific primers were designed (Table 2.3). A PCR reaction was carried out as outlined previously on the cDNA synthesised from the *C. dubliniensis* CD36 and the strain carrying the *C. albicans* *CBF1* gene (CD36CaCBF1).

2.4. Distruption of the *CaMET4* gene

2.4.1. PCR amplification of *C. albicans* *MET4*

To amplify the 5' and 3' regions of *CaMET4* ORF 19.5312 the primer sets MetKF/MetKR and MetSIIF/MetSIR (with *KpnI/XhoI* and *SacII/SacI* restriction sites included at the respective 5' and 3' ends, Table 2.4) were designed using the nucleotide sequence of the *CaMET4* gene. Reactions were carried out as outlined previously except 2.5 mM MgCl₂ was used with 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 56°C, and extension for 4 min at 72°C; this was followed by a final incubation at 72°C for 10 min. The 5' and 3' PCR amplimers of *CaMET4* were then cloned separately into pGEM by standard procedures (2.3.3) to generate the plasmids pFC002 and pFC003.

2.4.2. Construction of a disruption cassette

The *SAT1*-flipper cassette in the plasmid pSFS2A was used to characterize the function of the *MET4* gene in *C. albicans*. The *SAT1* flipping method relies on the use of a cassette that contains a dominant nourseothricin resistance marker (*caSAT1*) for the selection of integrative transformants and a *Candida* adapted *FLP* gene that allows the subsequent excision of the cassette, which is flanked by *FLP* target

Table 2.2

Primer	Sequence*	coordinates
CBF1F	ATGCGCGGCCGCTTCAATTC ^{CGATCTTCTGG}	-919 to -899
CBF1R	ATGCCCCGCGGTCTGCTTGTTTCGCCTCTTT	+1952to +1972

*restriction enzyme sites are underlined in the primer sequence

Table 2.3

Primer	Sequence	coordinates
CaRCbf1F	TTCTGATTCTGCCCACACTG	+399 to +419
CaRCbf1R	TTGTCCAATGCAGATGTTTGA	+515 to +536

Table 2.4

Primer	Sequence*	coordinates
5' gene end	MetKF (<i>KpnI</i>)	GGCCCGGTACCGACATTTCGGAGAAGTCACG -479 to -460
	MetXR (<i>XhoI</i>)	GGCCCTCGAGTATACTTTGTAATAATCTCTGTTTG -25 to -2
3' gene end	MetSIIF (<i>SacII</i>)	GCCGCCGCGGTTAGAACTGTCATATAAGGAAAC +1158 to +1181
	MetSIR (<i>SacI</i>)	GGCCGAGCTCTGTTGAAAAATGTCTTTACTGG +1699 to +1702
	Metup	CTATGGCTGCAATATAACTATAG -873 to -851
	FLP1	TTCCGTTATGTGTAATCATCC

*restriction enzyme sites are underlined in the primer sequence



sequences, from the genome. Two rounds of integration and excision generate homozygous mutants that differ from the wild-type parent strain only by the absence of the target gene, and reintegration of an intact gene copy for complementation of mutant phenotypes is performed in the same way, as described by (Reuss *et al.*, 2004).

To make a *MET4* specific *SAT1*-flipper cassette the cloned upstream product, from pFC002 was released by digestion with *KpnI* and *XhoI* from the 'TA' plasmid vector pBluescript II KS [-] (Norrander *et al.*, 1983), gel purified and ligated to *KpnI/XhoI* digested pSFS2A plasmid to generate the plasmid pMET4KX.

Transformation into *E. coli* was done with this construct. Subsequent screening for recombinant colonies containing the pSFS2A plasmids with the upstream fragment was done by extracting the plasmid and digesting it with *XhoI* and *KpnI* enzymes.

The positive clones containing the upstream fragment in pSFS2A were then used, in the next step of cassette construction, for incorporation of the downstream *SacI/SacII* fragment. The 'TA' cloned downstream fragment was released from pFC003 by digestion of the plasmid with *SacI* and *SacII*. The fragment was cloned in the pSFS2A-upstream recombinant plasmid to generate the plasmid pMet4KXS. A screening for recombinants containing both upstream and downstream fragments was done by PCR using the previous positive clones for the upstream region. An additive control was done by digesting the same clones in two different reactions with the enzymes *XhoI/KpnI* and *SacI/SacII* verifying the presence of released fragments in the two cases and sequencing the plasmid containing the complete *SAT1*-flipper cassette.

Candida albicans strains were transformed by electroporation with the standard procedure (2.3.2). Nourseothricin resistant transformants were selected on agar plates containing 200 µg/ml nourseothricin (cloNAT, Werner BioAgents, Jena, Germany). Single colonies were picked after 3 days of growth at 30°C, and subcultured. Positive transformants were first identified by PCR in order to verify the integration of the cassette in the targeted gene and the construction of heterozygous mutant. In order to flip the pMet4KXS cassette back out, heterozygous mutant were cultivated in maltose broth overnight at 37°C and then streaked onto plates with a drug concentration of 25 µg/ml and 10 µg/ml, which allowed for sensitive revertants to be

identified as small colonies. A second transformation was carried out on the nourseothricin sensitive heterozygous mutant and resistant colonies were screened for the right position of the cassette on the targeted wild type allele by PCR.

2.4.3. Verification of the deletion of both alleles of the *CaMET4* gene by PCR and southern hybridisation.

2.4.3.1. Digestion of the genomic DNA

Digestions of genomic DNA were carried out with 10 µg of genomic DNA in a 50 µl volume containing 15 U in total of the restriction enzymes *Bam*HI and *Hind*III (Promega) in the appropriate restriction enzyme buffer according to the manufacturer's instructions. Electrophoresis was performed in 0.8-1% (w/v) agarose gels in 0.5x TBE buffer containing 0.5 µg/ml ethidium bromide, in horizontal gel trays. Restriction endonuclease-generated DNA fragments in 1x final sample buffer were applied to the gel wells. The appropriate DNA size standards were loaded in the first well of each gel. Electrophoresis was performed at 50-100 volts (with constant current) until the bromophenol blue tracking dye had reached the end of the gel. Following electrophoresis, gels were visualised on a U.V. transilluminator (wavelength 345 nm) and photographed using an Imagestore 7500 Version 7.2.2 Gel documentation system.

2.4.3.2. Transfer of DNA fragments from agarose gels

Following the separation of restriction endonuclease digest-generated DNA fragments by agarose gel electrophoresis, DNA was transferred to nylon membranes by capillary action using the method of Southern (1975). The positions of DNA reference size markers were marked on the gel with a sterile Pasteur pipette. The DNA was then depurinated by soaking the gels in 0.02 M HCl with gentle shaking. Following depurination, the DNA was denatured by soaking the gel in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation, after which the gels were placed in a neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for a further 45 min with shaking.

DNA fragments were transferred to Biobond Plus nylon membranes (Sigma-Aldrich) using 20 x SSC as the transfer buffer. Following transfer, the positions of

DNA reference size standards were manually marked on the membrane using a pencil. The membrane was then rinsed in 2 x SSC and the DNA was fixed using a UV crosslinker (CL-508, UVI tec., Cambridge, UK) set at 0.120 J/cm².

2.4.3.3. Random primer labelling of DNA fragments with [α -³²P]dATP

The DNA fragment amplified by PCR using the primers MetKF and MetXR (Table 2.4) was used as a probe in Southern hybridization experiments. The probe was labelled with [α -³²P]dATP by random primer labelling using the Prime-a-gene kit (Promega). DNA was denatured for 2 min at 95°C. Denatured DNA was added to a reaction mixture containing 1 x labelling buffer, supplied by the kit manufacturer, contained a random mixture of dNTPs (dTTP, dCTP and dGTP) and bovine serum albumin (BSA). To the reaction mixture were added 3 μ l of [α -³²P]dATP (6,000 Ci/mmol; 220 TBq/mmol) (Amersham) and 5 U of Klenow DNA polymerase, and the reaction was incubated at room temperature for 1-2 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction mixture through a size exclusion matrix in columns (Sigma-Aldrich) as specified by the manufacturer.

2.4.3.4. Southern hybridisation

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

Radiolabelled probe ($\sim 2 \times 10^6$ d.p.m.) was denatured by boiling for five minutes followed by incubation on ice. The denatured probe was then added to the prehybridisation solution and incubated with the membrane at 65°C for 18 h. Unbound probe was removed from the membranes following hybridisation by washing the membrane in the bottle with a solution of 2x SSC, 0.1% (w/v) SDS at room temperature for 15 min, followed by a wash at 37°C in 0.1x SSC, 0.5% (w/v) SDS for 30 min and finally a high stringency wash at 65°C in 0.1x SSC, 0.5% (w/v)

SDS for 30 min. After washing, the membranes were wrapped in Saran wrap (Dow Chemical Co., Germany) and placed in an autoradiography cassette with an intensifying screen (Kodak BioMax MS-1) and exposed to Kodak Biomax X-ray film overnight at -70°C. Autoradiograms were developed with Kodak GBX developer and fixed in Kodak GBX fixer using the dilutions recommended by the manufacturer.

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

2.5. Phenotypic Analysis of *C. albicans* SC5314, *C. dubliniensis* CD36 and derivatives

2.5.1. Induction of hyphae

To determine the ability of *C. albicans* to undergo the yeast-to-hypha transition, budding cells were grown overnight at 30°C with vigorous shaking in YPD medium. To examine colony morphology on solid media, 10 to 200 cells were incubated per plate for 4 to 6 days at 30°C or 37°C on different media. These included YPD medium (1% (w/v) yeast extract, 2% (w/v) Bacto Peptone, and 2% w/v glucose) or synthetic dextrose (SD) medium (2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids [Difco]). Hyphal production was also tested on Yeast Nitrogen Base without amino acids (6.7 g/L, YNB w/o a.a.) containing different concentrations of the carbohydrates glucose, galactose and sucrose at the concentrations 2%, 1%, 0.5%, 0.25%, 0.1% 0.05% and 0.025% (w/v) and methionine, cysteine or proline at the concentrations 0, 5, 10, 20, 25, 30, 40, 60, 100 mg/l with buffering the media at neutral pH or at pH 3.7. Media were solidified with 2% (w/v) bacto agar (Difco) or 1.35% (w/v) agarose type 1 from Sigma. Synthetic low dextrose (SLD) medium is composed of 0.17% YNB (w/v) medium containing 0.5% (w/v) (NH₄)₂ SO₄ and 0.1% (w/v) glucose.

For serum plates, 10% (v/v) fetal calf serum (FCS) was added to YPD at 50°C after autoclaving. Cultivation under embedded growth conditions was performed as described in (Brown *et al.*, 1999). For BSA plates 1.17% (w/v) Yeast Carbon base, 0.01% (w/v) Yeast Extract, 0.2% (w/v) BSA was solidified with 2% (w/v) bacto agar (Difco). Exponentially-growing *Candida* cells, grown on YPD overnight at 37°C in

shaking condition, were firstly washed twice in PBS then diluted to a concentration of 1×10^8 cells/ml. Serial 1:10 dilution of the two strains were made and spotted on the plate. Plates were incubated at 37°C for 3 to 5 days.

Liquid formulations of YNB containing varying concentrations of glucose, galactose, methionine, cysteine and proline, as described for solid media were used to examine hypha production in liquid media. Exponentially growing *Candida* cells were inoculated in the liquid formulation of these media and cultured for 6-7 days at 30°C or 37°C with shaking in a Gallencamp orbital incubator (New Brunswick Scientific Company Incorporated, Edison, New Jersey, USA) set at 200 rpm. For Lee's temperature and pH experiments (Lee *et al.*, 1975) cultures were started from an overnight culture grown in Lee's medium at 25°C (at pH 4.5 for pH shift experiments). A suspension of 1×10^6 cells of this overnight culture was used to inoculate 100 ml of temperature-adjusted (37°C) medium or pH adjusted medium (pH 7.5). Cultures were grown for ~3h at either 37°C or pH 7.5 to induce hyphae or 25°C or pH 4.5 to maintain yeast growth. A Nikon Eclipse 600 microscope (Nikon) fitted with a super high power mercury lamp (Nikon) was used to measure the percentage of the population undergoing the transition. One hundred cells were counted and the percentage of hyphal cells was determined from this.

2.5.2. Methionine uptake assay

C. albicans SC5314 and *C. dubliniensis* CD36 were grown to an OD₆₀₀ of 0.8 in YNB w/o amino acids supplemented with ammonium sulphate, containing 1% (w/v) glucose and 20 mg/l methionine. Briefly, 1.6 ml of culture was harvest by centrifugation in a previously weighed eppendorf 2.0 ml tube and washed once with 1 ml of a solution containing 10 mM Mes-tris pH 6.4, 2 mM MgCl₂, 25 mM KCl (buffer A), then pelleted, weighed and subsequently resuspended in 400 µl (assay volume) of buffer A supplemented with 2% glucose.

The rate of methionine uptake was determined at two different concentration of substrate: 1 mM and 0.01 mM methionine containing S³⁵ methionine (1000 Ci/mmol, Amersham) at a final concentration of 1.25 µCi/µmol. The assay was conducted at 30°C and three different times of incubation in the presence of the substrate were used: 15, 45 and 75 sec. A 100 µl aliquot of the incubated cells was taken at every time point, spotted on glass fibre filters (Whatman GF/C) and

immediately washed with buffer A containing glucose, to remove unbound methionine. Filters were then transferred to scintillation vials with 10 ml of scintillation fluid (Beckman) and radioactivity was measured with a Beckman liquid scintillation counter. The controls blank in each experiment consisted of one filter in which cells were spotted after incubation with the same concentration of unlabeled methionine and one filter in which labelled methionine was added to the filter, and then washed away, in the absence of the cellular component. Eight replicates for each condition were made.

2.5.3. Auxotrophy test and Biolog phenotype arrays

In order to assess the ability of the Δ *Camet4* to synthesise methionine from sulphate, its growth was compared to that of the wild-type strain SC5314 on solid and liquid media with ammonium sulphate as the sole source of sulphate (i.e. YNB w/o a.a. (pH 7) containing 5 g/L (w/v) ammonium sulphate and 2% (w/v) glucose). Cells of the two strains were grown on YPD overnight at 37°C then washed twice in PBS and either streaked on plate or inoculated in liquid media as described above.

The growth on various carbon, nitrogen and phosphorus sources of the strains CaSC5314 and the Δ *Camet4* was tested using the Biolog phenotype microarray (PM) technology (Hayward, CA, USA). The basis for these assays is a culture medium that contains all the nutrients needed for cell growth (e.g. C, N, P, S, K, Na, Mg, Ca, Fe, amino acids, purines, pyrimidines, and vitamins). To measure which C, N, S, P metabolic pathways are active in fungi (e.g. *C. albicans* w.t. strains and in the Δ *Camet4* mutant) the wells in the different plates (PM1 and 2 for carbon sources, PM 3B for nitrogen sources, PM4A for phosphorus and sulphur Sources) contain this medium with all ingredients provided at sufficient levels except the C-source, or the N-, S-, P-sources which are omitted. The various wells in the different metabolic pathway assays provide 190 alternative C-sources, 400 N-sources, 100 P-sources and 100 S-sources.

For any of these compounds, if the cell has a transport system and a catabolic pathway for that chemical, it will metabolise the chemical, producing NADH in the process. The electron transport pathway in the cell will then take electrons from NADH and pass some of them on to the tetrazolium dye. Reduction of the tetrazolium dye due to increased cell respiration results in formation of a purple

colour in the well. Sources that are strongly metabolised rapidly form a dark purple colour, whereas sources that are weakly metabolised slowly form a light purple colour. The rates and extent of colour formation in each well of the PM can be monitored by measuring the absorbance at 595 nm. The assays were performed in 100 μ L cultures in 96-well culture plates. Exponentially growing cells of the wild type and the $\Delta CaMet4$ mutant from an overnight culture in YPD were washed in PBS, diluted to O.D.₆₀₀ = 0.1 and resuspended respectively in PM2 medium for the carbon assimilation test and in PM 4 for sulphur assimilation tests, 0.25 ml of this solution were inoculated in every well of the Array plate. The absorbance at 595 nm was measured at regular time points (6-12-18-24 and 48h) with a microtiter plate reader (Genosys 2 spectrophotometer, Thermo Spectronic, Dublin, Ireland).

2.5.4. Resistance to heavy metals and oxidative stress resistance

Overnight broth cultures of *C. albicans* and *C. dubliniensis* strains were grown in YPD broth at 37°C. To examine oxidative stress resistance, resistance to divalent cations, heavy metals and to the compounds Calcofluor white, caffeine, congo red, 10-fold dilutions of a 10^7 cfu/ml suspension of each strain was spotted on YPD plates containing the indicated concentration of the different compounds: 1, 3, 6 mM H₂O₂, 20 μ M CdCl₂, 0.3M LiCl, 0.5M MgCl₂, 0.7M NaCl, 80 μ g ml⁻¹ Calcofluor white, 200 μ g ml⁻¹ Congo red, 10 mM caffeine. Experiments were performed on three separate occasions.

2.5.5. Biofilm formation on plastic surface assay

Isolates tested for the ability to form biofilm were first cultured at 37°C for 18 h in YPD. A loopful of this culture was used to inoculate 50 ml of yeast nitrogen base (YNB) medium supplemented with 100 mM glucose. After overnight broth culture at 37°C, the yeasts were harvested in the late exponential growth phase. Before use in adhesion experiments, the yeasts were washed twice with 5 ml of phosphate buffered saline (PBS; pH 7.2). The washed cells were then resuspended in growth medium (YNB) and adjusted to an optical density of 0.38 at 520 nm (10^7 cells/ml). One hundred μ l of cell suspension (10^6 cells) in YNB containing glucose (100 mM) was added to each well of a pre-sterilised, polystyrene, 96-well plate and incubated for 90 min at 37°C in static conditions. Afterwards, the cell suspensions were aspirated,

and each well washed twice with 150 µl of PBS to remove loosely adherent cells. A total of 100 µl of yeast nitrogen base medium was then transferred into each of the washed wells and the plates were incubated at 37°C. The biofilms were allowed to develop for 66 or 80 h, and then the biofilm growth was quantified by an XTT reduction assay. All assays were carried out on three different occasions in triplicate. As controls, three wells of each microtiter plate were handled in an identical fashion, except that no *Candida* suspensions were added.

2.5.5.1. XTT reduction assay

XTT (Sigma, St. Louis, Mo.) solution (1 mg/ml in PBS) was prepared, filter sterilised through a 0.22-µm-pore-size filter, and stored at -70°C. Menadione (Sigma) solution (0.4 mM) was prepared and filter sterilised immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume. The biofilms were first washed three times with 200 µl of PBS, and then 200 µl of PBS and 12 µl of the XTT-menadione solution were added to each of the prewashed wells and the control wells. The microtiter plate was then incubated in the dark for 2 h at 37°C. Following incubation, 100 µl of solution was transferred to new wells and the colour change in the solution was measured with a microtiter plate reader (Genosys 2 spectrophotometer, Thermo Spectronic, Dublin, Ireland) at 490 nm. The absorbance values for all new wells were preread at 490 nm, since their levels of absorbance may display deviations. The absorbance values for the controls were then subtracted from the values for the test wells to eliminate spurious results due to background interference.

2.5.6. Reconstituted Human Epithelium (RHE) infection

For RHE infection two different types of tissue were used: a commercially available tissue, TR146 cutaneous carcinoma derived cell line from Skinethic Laboratories (Nice, France) and A431 vaginal epithelial cells (an infection model developed in Steffen Rupp's laboratory Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany) grown on inert polycarbonate 0.5 cm² discs.

For the infection experiment *C. albicans* SC5314 and $\Delta CaCBF1$, *C. dubliniensis* CD36, Egf200 and Ansa28 were grown on PDA for 48h at room temperature, and several colonies were resuspended in PBS and washed twice. Subsequently 10 ml of YPD were inoculated with 2×10^5 cfu/ml of exponentially growing-cells of each strain and incubated overnight at 25°C with shaking at 200 rpm. Ten ml of YPD were then inoculated with 4×10^6 cells of the strains from the culture at 25°C and incubated overnight at 37°C with shaking. A 3 ml volume of each culture was washed twice in PBS and resuspended in 1ml of PBS. The density was then adjusted to 4×10^6 cells/ml. The 0.5 cm² discs containing the tissue were inoculated with 50 µl of this suspension and controls with 50 µl of PBS. Inoculated cultures were incubated at 37°C, 5% (v/v) CO₂ at 100% humidity for 6, 12, 24, 48 h. The tissues were then cut from the insert and stored for sectioning in Karnovsky's solution (4% (v/v) formaldehyde in PBS) at 4°C. Then tissue were dehydrated in ethanol and embedded in paraffin wax using an automated tissue processor (Shandon Citadel 1000) on an overnight cycle comprising two water steps followed by 70% ethanol, 90% ethanol 96% ethanol, isopropanol, isopropanol:xylol (1:1), xylol I, xylol II, paraffin I, paraffin II. Tissues were sliced from the paraffin bloc in 5 µm sections using a rotary microtome (Leica RM2145), slices were then mounted on microscope slides. Sections were stained with Periodic acid Schiff (PAS), (McManus, 1946) and Papanicolaou reagent (Papanicolaou, 1942) to visualise fungal and cellular element.

2.5.6.1. Lactate dehydrogenase assay for epithelial damage

The release of lactate dehydrogenase (LDH) from epithelial cells into the cell-culture medium was measured to quantify the extent of epithelial cell damage. The CytoTox96® non radioactive cytotoxicity assay (Promega, Madison, WI) was used

to measure the amount of LDH in each sample. The CytoTox96® assay quantitatively measures LDH that is a stable cytosolic enzyme released into the medium upon cell lysis. Released LDH in the culture supernatant is measured with a 30 min coupled enzyme assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. The reaction was assayed, for the different *Candida* strains and at the time points specified above, at 480 nm using a Genios plate reader (Tecan UK). One unit of LDH is equivalent to 1 mM formazan formed per reaction (LDH U/L was calculated as: absorbance at 480 nm/ 15600 (extinction coefficient) x 1000 x1000).

2.5.7. Co-culture with murine macrophages

The ability of *C. albicans* SC5314 and $\Delta CaMet4$ mutant, to proliferate in co-culture with Murine RAW264.7 macrophages was determined. The endpoint dilution assay (EDA) was used to quantify the survival of the wild type and mutant strain in presence of murine macrophages (method adapted from Epstein Cynthia *et al.* 2001). Murine RAW264.7 macrophages were seeded at a density of 1.5×10^6 cells/ml in the wells of a 96 well plate in 100 μ l of Dulbecco's modified Eagle's medium DMEM (Invitrogen, Carlsbad, California) supplemented with 10% fetal calf serum. Macrophages were cultured over night at 37°C in the presence of 5% (v/v) CO₂. Five serial 10-fold dilutions of a suspension of 2×10^6 cells/mL of the *C. albicans* wild type and the knock out strain were resuspended in 50 μ l and added to the monolayer. Plates were then incubated at 37°C in presence of 5% (v/v) CO₂ for 48h. After incubation, plates were stained with crystal violet and the colonies formed were counted.

2.6. Studies on Chlamydospores

2.6.1. Culture of *C. albicans* and *C. dubliniensis* chlamydospores on solid and liquid media

A defined solid medium comprising 6.7 g/l Yeast Nitrogen Base (without amino acids; with ammonium sulphate) (YNB w/o a.a.), 0.025% (w/v) galactose, 25 mgL⁻¹ methionine and 2% (w/v) Bacto agar (Difco labs), was found to induce high levels of

chlamydospore production by *C. dubliniensis* when incubated in the dark at room temperature for 3-4 days. A liquid chlamydospore-inducing medium (LCIM) comprising 6.7 gL⁻¹ YNB w/o a.a., 0.5% (w/v) galactose, 20 mg/l methionine and 5% (v/v) new born calf serum was used to induce high levels of chlamydospore production in *C. dubliniensis*. Inocula were made from *C. dubliniensis* blastospores incubated on YPD agar for 24 – 48 h at 37°C. Plates were left at room temperature for 6 to 12 h and a single colony then inoculated into 10 ml of liquid YPD and incubated with shaking at 37°C for 2 h. One microliter (approx. 10² cells) of this culture was then inoculated into 50 ml of the chlamydospore-induction medium contained in a parafilm-sealed 100 ml conical flask or petri dish. Cultures were then incubated statically at room temperature in the dark. *C. albicans* chlamydospores were induced by incubation in liquid and on solid media containing corn meal broth, 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg/l methionine. Addition of 0.025% YNB w/o a.a. (v/v) and 0.05% Tomato juice (Oxoid) was used to increase chlamydospore production in some *C. albicans* clinical isolates. The starting inocula were prepared as for *C. dubliniensis*. Chlamydospores were identified by their size, refractile cell wall and staining with lactophenol cotton blue.

Liquid YPD or Dulbecco's modified Eagle's medium (DMEM) supplemented with 5-10% (v/v) human serum was used for the reactivation of dormant chlamydospores in both species. Mycelium-attached or purified chlamydospores of both species were centrifuged at 25,000 g for 2 min. The resulting pellet was then resuspended in YPD or DMEM and incubated at 37°C for between 3 to 12 h to allow resumption of metabolic activity. After reactivation, incubation in YPD with or without serum or DMEM with serum was carried out at room temperature or at 37°C with or without 5% (v/v) CO₂ to induce germination.

Bacto agar [2% (w/v)] was used as solidifying agent for experiments on solid cultures. When incubated in solid media, to avoid agar invasion of hyphae and to create low oxygen conditions *C. albicans* and *C. dubliniensis* were cultured between two cellophane baking filter sheets (BioRad). A first filter sheet was placed at the top of the agar then blastospores of the two species, treated as above, at a concentration of 1x10³ cells in a volume of 100-200 µl, were inoculated on the filter. A second filter was placed on top of the first after a short drying period to decrease the moisture content between the two filters.

2.6.2. Purification of chlamydospores

Mycelia grown in liquid chlamydospore induction medium were centrifuged at 14,000 rpm for 2 min. Chlamydospore rich mycelia grown in the solid formulation of the medium between the two filter sheets were washed out from the filters using PBS, collected and centrifuged at 12,000 g for 2 min. The resulting pellets from both culturing methods were then resuspended in 1 M sorbitol in citrate phosphate buffer. Purification of chlamydospores from the mycelial component was obtained by repetitive sonication of the suspensions for 10 min in a Vitasonic II sonicating bath at 25 KHz. In order to achieve a higher level of purity of chlamydospores from hyphal and yeast cells, sonicated samples were incubated with 700 U/ml zymolyase 20T (Seikagaku Corp., Tokyo, Japan) for 2 h at room temperature. Samples were then sonicated again as described above and the chlamydospores pelleted by centrifugation (3000 g for 5 min). Purified chlamydospores were resuspended in phosphate buffered saline (PBS). For molecular biology studies on the structures, 100% pure suspensions of chlamydospores were obtained after incubation for 3-4 h in a solution containing 2% (v/v) Triton X-400, 1 mM EDTA, 1% (w/v) SDS, 100 mM NaCl 10 mM Tris pH 8 and 700 U/ml zymolyase in phosphate citrate buffer and 1M sorbitol. Chlamydospores were pelleted as above. The purity of suspensions prepared with the different method was assessed by light microscopy.

2.6.3. Identification of metabolic activity

Cells were stained with the fluorescent probe 2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1phenylquinoliniumiodide (FUN-1 (Molecular Probes, OR, USA)) to determine the level of metabolic activity in isolated chlamydospores and in germinated cells. Chlamydospores at different stages of exposure to nutrient rich media and serum (0 h, 3 h, 6 h, 12 h, 24 h time points) were stained with 0.5 μ M FUN-1 in 10 mM Na-HEPES solution (pH 7.2) supplemented with 2% (w/v) glucose (GH solution) in the dark at room temperature for 30 min. Fluorescence was measured with a fluorescence microscope (Nikon Eclipse 600) equipped with filter with excitation at 470-590 nm.

2.6.4. DAPI and calcofluor staining

To stain the cell wall and the nucleus of chlamydospores, 100 μ l samples of mycelia-attached chlamydospores or purified chlamydospores were either washed twice in PBS or fixed in 5% paraformaldehyde and then resuspended in 100 μ l calcofluor white solution (1 mg/ml) and 10 μ l of 4',6-diamidino-2-phenylindole (DAPI) solution 10 mg/ml. Five microlitre volumes of the samples were then mounted on glass slides and cells were analysed by fluorescence microscopy with a DAPI filter.

2.6.5. Phagocytosis assay

Murine RAW264.7 macrophages were seeded at a concentration of 1.5×10^6 cells in the wells of a 6 well plate containing sterile coverslips, on the top of the coverslip. Macrophages were cultured overnight at 37°C in the presence of 5% (v/v) CO₂. The monolayer was then washed with warm DMEM (Sigma) and 1 ml of the medium was added to each well. Purified dormant or activated chlamydospores of *C. albicans* and *C. dubliniensis* (1×10^3 or 1×10^4) were then added to the monolayer. Dormant chlamydospores were incubated in PBS or water following purification whereas re-activated chlamydospores were incubated in YPD supplemented with 5% serum. Chlamydospores were co-cultured with macrophages for 3, 5, 10, 15, 20 h. At each time point the medium was removed and acridine orange (Sigma) at 0.1% (v/v) in DPBS was added to the wells. After 10 minutes staining the dye was removed and the coverslips washed twice with PBS. Coverslips were examined by fluorescence microscopy with a DAPI filter.

2.6.6. Electron microscopy

Chlamydospore rich mycelia or chlamydospore suspensions were fixed overnight in PBS containing 2.5% (v/v) glutaraldehyde and 2% (w/v) formaldehyde. The cells were washed briefly in PBS, dehydrated in graded concentrations of ethanol and critical point-dried in 100% CO₂. Specimens were mounted on aluminium stubs, coated with gold and viewed using a Zeiss Supra 35 variable pressure field emission scanning electron microscope.

For transmission microscopy chlamydospores were prepared as for SEM, then fixed for 1 h in 3% (v/v) glutaraldehyde in cacodylate buffer, washed once in

cacodylate buffer, resuspended in 1% (v/v) osmium tetroxide and 1% (w/v) potassium ferricyanide in cacodylate buffer, and incubated for 30 min at room temperature. Cells were then washed four times in dH₂O, resuspended in 1% (w/v) thiocarbohydrazide in water, and incubated for 5 min at room temperature. Cells were again washed in dH₂O, incubated in 1% (v/v) osmium tetroxide and 1% (w/v) potassium ferricyanide in cacodylate buffer for an additional 5 min, and washed again in dH₂O. The cells were then incubated in saturated uranyl acetate for 2 h and dehydrated through a graded series of ethanol washes as for the SEM samples. The dehydrated samples were embedded in Epon 812, sectioned, and images were collected on a JEOL 2100 microscope at 80 kV.

2.7. Comparative whole genomic transcript profiling of yeast and chlamydospores using the microarray technique

2.7.1. Media and conditions used for comparison of the gene expression

C. dubliniensis was used in this study because of its enhanced ability to produce chlamydospores under simple defined conditions (2.6.1.). To induce chlamydospores *C. dubliniensis* was cultured in the CIM as described above for 4 days. As the number of the chlamydospores produced is strongly dependent on the galactose concentration of the incubation medium and from the starting inoculum we set up parallel cultures in CIM for gene expression studies; one control culture containing a 20-fold higher galactose concentration (0.5% w/v) than standard CIM and a starting inoculum of 1×10^7 cells, incubating at room temperature with shaking at 80 rpm, condition that did not induce chlamydospore formation and a second culture with standard galactose poor CIM (0.025% w/v) and a starting inoculum 1×10^2 cells in static condition which induced abundant chlamydospores production.

2.7.2. Microarray procedure

In order to compare the transcript profile of chlamydospore and yeast cells, Two Colour Microarray-based gene expression Analysis (Agilent Technologies) was employed.

RNA was extracted from yeast inducing culture and chlamydospore inducing cultures using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Samples were treated with TURBO DNase (Ambion) to remove residual genomic DNA. Around 300 ng of total RNA in a volume of 4.25 μ l was used as a starting material for the array technique. For the synthesis of cDNA, a reaction containing 5 U MMLV-RT, 0.5 μ l of 10mM dNTPs mix, 0.25 μ l RNaseOut, 2 μ l 5x standard buffer, 1 μ l 0.1 M DTT was assembled for each sample. Samples were incubated at 40°C for 2 h, then at 65°C for 15 min., after the reactions were kept on ice. The resulting cDNA was transcribed in labelled Cyanine 3 or Cyanine 5 RNA (cRNA) using the transcription master mix provided in the kit (Fig. 2.1). Samples were then incubated at 40°C for 2h. The cRNA was purified using the RNeasy Mini Kit (Qiagen) according the manufacturer instructions. The purified cRNA was resuspended in 30 μ l of RNase-free water. The concentration of the cRNA was measured at an optical density of 260 nm.

2.7.2.1. Slide design and hybridization

A *C. dubliniensis* oligonucleotide microarray was designed by Dr. Gary Moran based on the *C. dubliniensis* genome sequence generated at the Sanger Centre in Cambridge. A set of 5,999 predicted ORFs from the CD36 genome were used to design a *C. dubliniensis* expression microarray. Two unique 60mer oligonucleotides were designed specific for each ORF using the Agilent eArray probe design tool. Whole genome microarrays were printed on glass 4x44k slides by Agilent technologies. Each 60mer was printed in quadruplicate.

For the hybridization of 4x44k slides 825 ng of the two dye labeled cRNA was used in a reaction containing 25X Fragmentation buffer 2.2 μ l, 10x blocking agent (prepared as specified from the manufacturer) and RNase free water to 55 μ l. Samples were compared as specified on Fig. 2.2.

The microarray slide was left to hybridize over night at 65°C in an hybridization oven (Hybaid, Teddington, Middlesex, U.K.). After hybridization the slides were washed in glass slides-staining dish. A first wash was done in a dish completely filled with gene expression (GE) wash buffer 1 at room temperature for less than a minute, second wash in a second dish filled with GE 2 at room temperature for 1 min, third was done with GE 2 prewarmed (provided in the kit) at 37°C for 1 min.

2.7.2.2. Data analysis

Slides were scanned using the GenePix personal 4100A scanner (Axon) and data was extracted with GenePix Pro 6.1 (Axon). Spots were flagged absent and excluded from analysis if the fluorescent signal was less than background +1 standard deviation. Raw data was imported into GeneSpring GX10 (Agilent Technologies) and normalised using Lowess normalization. A ratio of the fluorescent signal in chlamydospores relative to yeast cell cultures was determined at each spot and the replicate spot values averaged to determine a relative expression value for each ORF represented on the array. Genes exhibiting a 2-fold difference in expression were selected for further analysis. A *t* test was performed between the two data sets using the variance derived from replicate spots. Those genes with a *p* value <0.025 or <0.05 (exact values quoted in text) that passed the Benjamini-Hochberg multiple correction test to remove false differential gene expression were further analyzed with the programs GO Slim Mapper (<http://www.candidagenome.org/cgi-bin/GO/goTermMapper>) and GO gene Ontology Term Finder (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>) to find significant gene ontology terms shared between the two differentially regulated category of genes and discover what process they may be involved in.

2.7.3. Real time analysis

The Real time approach was used to validate the array results. In the Real Time experiment the housekeeping gene *ACT1* (Cd36_12690) was used as internal control. The analysis was carried out using the $2^{-\Delta\Delta C_t}$ model (Schmittgen & Livak, 2008). The genes examined were *HHD2* (Cd36_32340), *RBT5* (Cd36_40190), *MSH6* (Cd36_12230) and *CBF1* (Cd36_46060). The amplification efficiency of the targeted genes and the reference gene was assessed by comparing the changes in C_T observed over several template dilutions. According the Livak and Schmittgen method several dilutions (1, 1/2, 1/4, 1/8,) of the *C. dubliniensis* genomic DNA (starting from a concentration of 0.5 μ g) were made. For each dilution sample a quantitative PCR was performed using the primers directed to the targeted genes and the internal control. The average C_T for the sets of primers was determined and it was plotted versus the log of DNA dilutions. The slope for each different amplification was calculated using linear regression. PCR efficiency was calculated using the equation

Fig. 2.1

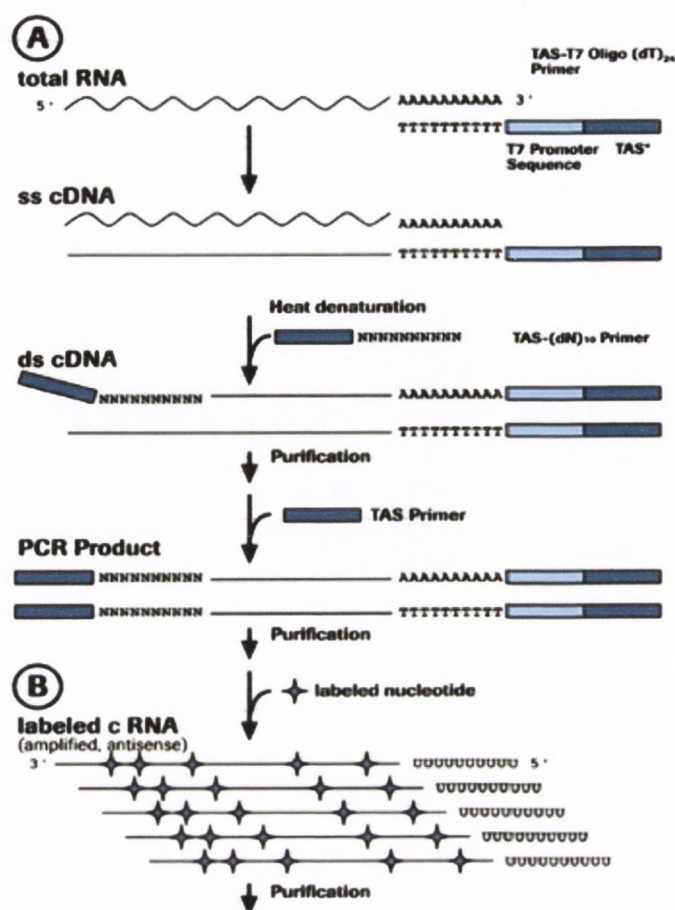


Figure 5.1 c-RNA synthesis

RNA target is prepared in sufficient quantities including a linear amplification step after first-strand cDNA synthesis. This requires the use of a special primer, containing a random sequence with no significant homology to any known sequences in public databases (Target Amplification Sequence) in addition to T7 promoter and oligo (dT) sequences, for first-strand cDNA synthesis. The TAS - region generates a 3' anchor on the cDNA for subsequent PCR amplification with a TAS-PCR primer. The resulting PCR-amplified cDNA is then transcribed into labeled cRNA by a linear amplification step.



Journal of the
Royal Society of
Medicine

Volume 81, No. 12, December 1988

ISSN 0959-6288

Printed in Great Britain

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Fig. 2.2

Array1_1	Array 1_2	Array 1_3	Array 1_4
cRNA from chlamydospore sample 1 labelled with cyanine3 V.S. cRNA from yeast sample1 labelled with cyanine5	cRNA from chlamydospore sample 2 labelled with cyanine5 V.S. cRNA from yeast sample 2 labelled with cyanine3	cRNA from chlamydospore sample 3 labelled with cyanine3 V.S. cRNA from yeast sample 3 labelled with cyanine5	cRNA from chlamydospore sample 3 labelled with cyanine5 V.S. cRNA from yeast sample 3 labelled with cyanine3

Fig. 2.2 Diagram showing the disposition of the samples on the array slide

The mRNA was extracted from chlamydospore rich cultures and from yeast cell rich cultures on 3 separate occasions and labelled either with cy3 and cy5. Differently labelled c-RNA generated from chlamydospore and yeast cells were co-hybridised in the 4 chambers of the 4x44k *C. dubliniensis* genome array slide.

Table 2.5

Primer	sequence	coordinates
HHT2F	TGGCTAGAACAAAACAAACAGC	+3 to +25
HHT2R	TCTTGACACCACCAGTAGATGG	+112 to +135
MSH6F	AATTGCCGAATCAGTTTTGC	+3201 to +3221
MSH6R	CGTTGTTGCTTGATTTGTGG	+3320 to +3340
GIN1F	GAAGAAGAAGGGGGTTTTGG	+885 to +905
GIN1R	CTTCACGGGAGATGTAAATGG	+998 to +1019
CBF1F	ACCATGAGCCTGAAGTTTCC	+296 to +316
CBF1R	GGTTTGGCAGGATTAGTTGC	+422 to +444
ACTF	AGCTCCAGAAGCTTTGTTCAGACC	+768 to +791
ACTR	TGCATACGTTTCAGCAATACCTGGG	+918 to +941
RBT5F	TGCTGGTGTCACTGCTATCC	+45 to +65
RBT5R	TTGGTCGTAGATTCTGTCAGC	+150 to +171

$m = - (1/\log E)$ where m is the slope and E is the efficiency. Real-time measurement of PCR amplification can be achieved by determining the numbers of PCR cycle necessary to achieve a given level of fluorescence. In this study, the C_t was fixed in the exponential phase of the PCR. Expression analysis of the genes relative to *ACT1* in chlamydospores and yeast cells was investigated using 0.5 μg of cDNA retro-transcribed as previously described from RNA derived from the above mentioned growth conditions. Primers used in the experiment are listed in Table 2.5. Analysis of transcript was carried out in 15 μl reactions, using SYBR Green PCR Master Mix (Applied Biosystems), in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles (thermal cycling conditions: initial step of 94°C for 10 min, followed by 40 cycles of 94°C, 15 sec and 59°C, 30 sec and 72°C, 30 sec). The relative changes in gene expression were quantified using the $2^{-\Delta C_t}$ equation (Schmittgen & Livak, 2008).

Chapter 3

The effects of glucose and methionine on *C. albicans* and *C. dubliniensis* morphology

3.1. Introduction

3.1.1. Amino acid sensing and morphogenesis

Although the production of hyphae can be induced in *C. albicans* and *C. dubliniensis* by a wide variety of environmental stimuli (e.g. temperature shift, serum, *N*-acetyl glucosamine), the sensing and signalling pathways involved in this morphological transition are poorly understood. Similarly, the reasons why *C. dubliniensis* is less efficient at producing hyphae than *C. albicans* are also unknown. In order to further investigate how hyphae are induced by specific nutrients in these two species it was decided to investigate the effects of altering the concentration of glucose/galactose and methionine in growth media on morphology, where these are the sole source of carbohydrate and amino acids respectively. The reason for investigating the role of methionine was due to the study of Maidan *et al.* (2005b), which demonstrated that this amino acid interacts with the *C. albicans* glucose receptor Gpr1, which has been shown to play a role in controlling hypha formation. A strong relationship between filamentation and the general amino acid sensing mechanism has already been described. Brega *et al.* (2004) demonstrated that deletion of *CSY1* (which encodes an amino acid sensor) results in a lack of amino acid-mediated activation of amino acid transport, a lack of induction of transcription of specific amino acid permease genes and altered filamentation. No direct relationship between the sensing of the specific amino acids methionine and cysteine and filamentation has been identified yet. However, Kaur & Mishra (1991) studied the transport of four amino acids (L-methionine, L-phenylalanine, L-lysine and L-alanine) during pH-regulated dimorphism in *C. albicans*. The permeases responsible for amino acid uptake responded differently to differentiation and the transport activities varied during the course of morphogenesis. An increase in uptake around the time of evagination was observed with all four amino acids in both the strains studied. The uptake rates of L-methionine and L-phenylalanine were greater in fully differentiated hyphae, while the rate of L-lysine uptake was higher in fully-differentiated buds. Uptake rates of L-alanine did not show any morphotypic related variation. No other genes have been characterised in this biosynthetic pathway in *C. albicans*; however most of the characterised *S. cerevisiae* genes involved in the methionine biosynthesis share a good degree of similarity with *C. albicans*.

3.2. Results

3.2.1. The effects of varying carbohydrate concentration on the growth morphology of *C. albicans* and *C. dubliniensis* in the presence of limited methionine

Candida albicans strains SC5314 and 3153A and *C. dubliniensis* strains CD36 and Wu284 were grown on YNB solid medium (pH 7.0) w/o a.a. supplemented with methionine at a concentration of 20 mg/L and varied concentrations of glucose, galactose or sucrose (0.025% to 2% (w/v)). *Candida albicans* was observed to produce hyphae over a wide range of carbohydrate concentrations (i.e. 1% to 0.025% (w/v)). However, under the same conditions *C. dubliniensis* produced hyphae only at very low sugar concentrations (i.e. 0.1-0.025% (w/v)). At higher concentrations the *C. dubliniensis* colonies were uniformly smooth (Fig. 3.1). Identical results were obtained when glucose was replaced with galactose and cysteine for methionine (data not shown). The results obtained were similar for 18/18 strains of *C. albicans* and 15/18 *C. dubliniensis* strains tested.

Three *C. dubliniensis* strains (Eg200, P7718, P2) were found to produce hyphae under similar conditions to the *C. albicans* strains (i.e. 0.025% to 1% (w/v) glucose or galactose and 20 mg/L methionine (Fig. 3.2, A)). Subsequent investigation revealed that these strains were homozygous α/α at the mating type locus. Homozygote a/a *C. dubliniensis* strains (i.e. Ansa 28, P7276, J11) produced hyphae under similar conditions as a/ α strains tested. The length of hyphae was significantly greater in the α/α strain tested under conditions for maximal hyphae production by all *C. dubliniensis* strains (Fig. 3.2, B).

3.2.2. The effects of varying methionine concentration on the growth morphology of *C. albicans* and *C. dubliniensis* in the presence of limited carbohydrate

To compare the effect of altering the levels of methionine on the production of hyphae by the two species, *C. albicans* SC5314 and *C. dubliniensis* CD36 were incubated on YNB agar medium w/o amino acids (pH 7.0) containing glucose at a concentration of 0.025% (w/v) (i.e. a glucose concentration at which the two species produced hyphae when methionine was present at a concentration of 20 mg/L) and

Figure 3.1

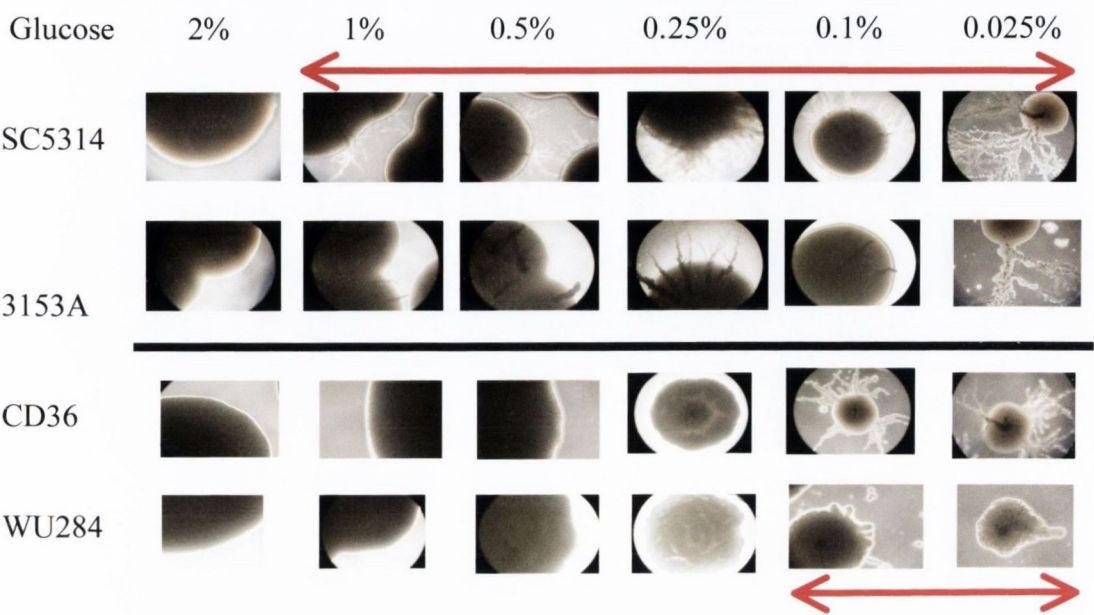


Figure 3.1 Effect of glucose on hyphae induction in *C. albicans* SC5314, 3153A and *C. dubliniensis* CD 36,WU284 in the presence of low level of methionine.

Glucose in a concentration range of 0.025% to 1% (w/v) induces hyphae formation in both *C. albicans* strains. In the concentration range of 0.025% to 0.1% *C. dubliniensis* colonies form hyphae. The media contained methionine at a concentration of 20 mg/L, a concentration that has been found to be optimal for hyphal induction at low glucose concentration. (The red arrows indicate the glucose concentration range in which the two species expressed hyphae). Plates were incubated for 5 days at 30°C.

Fig. 3.2

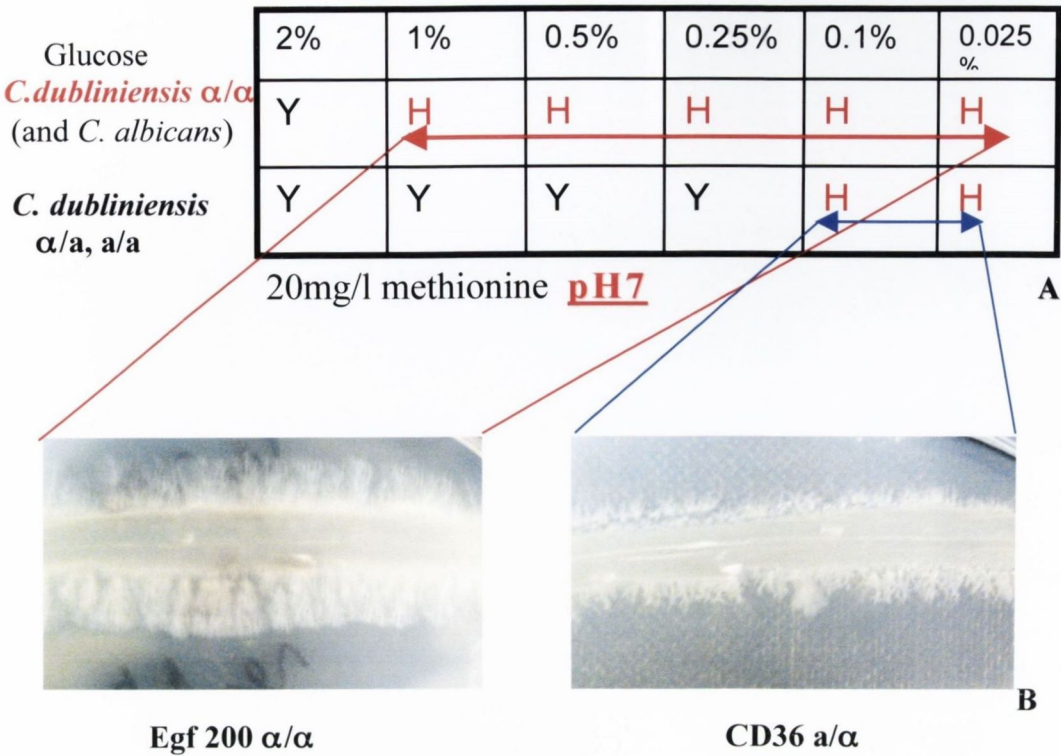


Fig. 3.2 Effect of glucose on hyphal induction and hyphal fringe appearance in the different *C. dubliniensis* mating type at pH 7.

A, *C. albicans* produced hyphae when incubated on medium containing 2% to 0.025% (w/v) glucose. The majority of *C. dubliniensis* strains (a/α , a/a) produced hyphae only at very low glucose concentrations (0.1 to 0.025%). *C. dubliniensis* strains that were found to be α/α homozygous at the mating type locus expressed hyphae in the same glucose range as *C. albicans* (Y=yeast, H=hyphae).

B, Under conditions specific for hyphae formation in *C. dubliniensis* the level of hyphae was significantly greater for α/α strains (e.g. Egf200) compared to a/α strains (e.g. CD36). The strains shown were incubated for 5 days at 30°C in presence of 0.1% (w/v) glucose.

levels of methionine ranging between 10 mg/L to 60 mg/L. High concentrations of methionine (i.e. 60 mg/L) were found to inhibit the production of hyphae by *C. albicans*, in contrast, an increased concentration of methionine appears to have little effect on the levels of hyphal induction observed in *C. dubliniensis* (Fig. 3.3).

The effect of methionine concentration on the morphogenesis of *C. albicans* was also investigated at a concentration of glucose that permitted hyphae production in *C. albicans* but not in *C. dubliniensis* (i.e. 0.25% (w/v)). It was found that without methionine *C. albicans* did not produce hyphae, whereas with methionine concentrations ranging from 10 to 25 mg/L there was strong hyphal induction. A methionine concentration greater than 60 mg/L had an inhibitory effect on hyphae expression in the same glucose range (Fig. 3.4). Similar results were also obtained when glucose concentrations of 1% to 0.25% (w/v) were used (data not shown). Interestingly, when similar experiments were performed in liquid media both species grew exclusively in the yeast form, regardless of the glucose or methionine concentrations.

3.2.3. A simple synthetic medium to distinguish between *C. albicans* and *C. dubliniensis*

It was found that decreasing the pH of the YNB medium containing glucose at concentrations between 0.1 - 0.025% (w/v) and 20 mg/L (w/v) methionine to pH 3-4 enhanced hypha formation in *C. dubliniensis*, increasing the percentage of colonies with hyphae in the media. In contrast, the pH decrease had the opposite effect on *C. albicans*, strongly reducing the number of colonies producing hyphae when glucose was ranging from 1% to 0.025% (w/v). This finding provided the possibility to develop the first synthetic medium for the discrimination between the two species based on their colony morphology. When incubated on solid medium containing glucose or galactose at a concentration of 0.025% (w/v) and 20 mg/L methionine at pH 3.7, *C. albicans* grew as smooth colonies without hyphae while *C. dubliniensis* grew as rough colonies producing copious hyphae and chlamydospores. Interestingly, the liquid formulation of the media also induced growth in the mycelial form in *C. dubliniensis* (pseudohyphae and chlamydospores) when incubated at room temperature without shaking for 4-5 days. In contrast *C. albicans* grew in the yeast form. The use of galactose instead of glucose in the solid and liquid formulation of

the medium induced a higher level of production of chlamydospores by *C. dubliniensis* compared to that obtained with glucose. For this reason a medium comprising of YNB with 0.025% (w/v) galactose and 20 mg/L methionine at pH 3.7 was used to produce large amounts of chlamydospores by *C. dubliniensis* and was named chlamydospore induction medium (see chapter 5). In this medium, 39 of 40 *C. dubliniensis* isolates produced true hyphae or pseudohyphae and chlamydospores while none of 18 *C. albicans* strains tested produced hyphae or chlamydospores (Fig. 3.5).

3.2.4. Expression of the *C. albicans* and *C. dubliniensis* transcription factor Nrg1 in the defined chlamydospore induction medium

It has previously been reported that differential expression of the transcription repressor Nrg1 is responsible for the differential ability of *C. albicans* and *C. dubliniensis* to produce chlamydospores on Staib agar (Staib & Morschhauser, 2005a). We therefore decided to investigate how this gene is regulated in each species when incubated in the defined chlamydospore induction medium (CIM). *Candida albicans* and *C. dubliniensis* strains expressing the fusion protein *pNRG1-GFP* (Moran *et al.*, 2007) were incubated for 5 days in a medium comprised of 6.7 g/L YNB, 20 mg/L methionine containing varying concentrations of galactose (e. g. 2%, 1%, 0.5%, 0.25%, 0.01%, 0.025% (w/v)). No significant differences in fluorescence were observed in cultures containing >0.5% (w/v) galactose. However, when the medium contained a concentration ranging from 0.5% to 0.025% (w/v) galactose there was a strong drop in the fluorescence of *C. dubliniensis* cultures which was absent in *C. albicans*, indicating a down-regulation of *NRG1* expression (Fig. 3.6). Therefore, it is possible that the reason for the differences in morphology of *C. albicans* and *C. dubliniensis* grown on the defined chlamydospore inducing medium is at least partially due to differential expression of *NRG1*.

3.2.5. The effect of the low glucose low methionine medium on *C. dubliniensis* virulence

Moran *et al.* (2007) have previously shown that $\Delta nrg1$ mutants of *C. dubliniensis* are better able to produce hyphae, are more virulent in the RHE infection model and are able to escape macrophage killing. Therefore, since incubation in the

Fig. 3.3

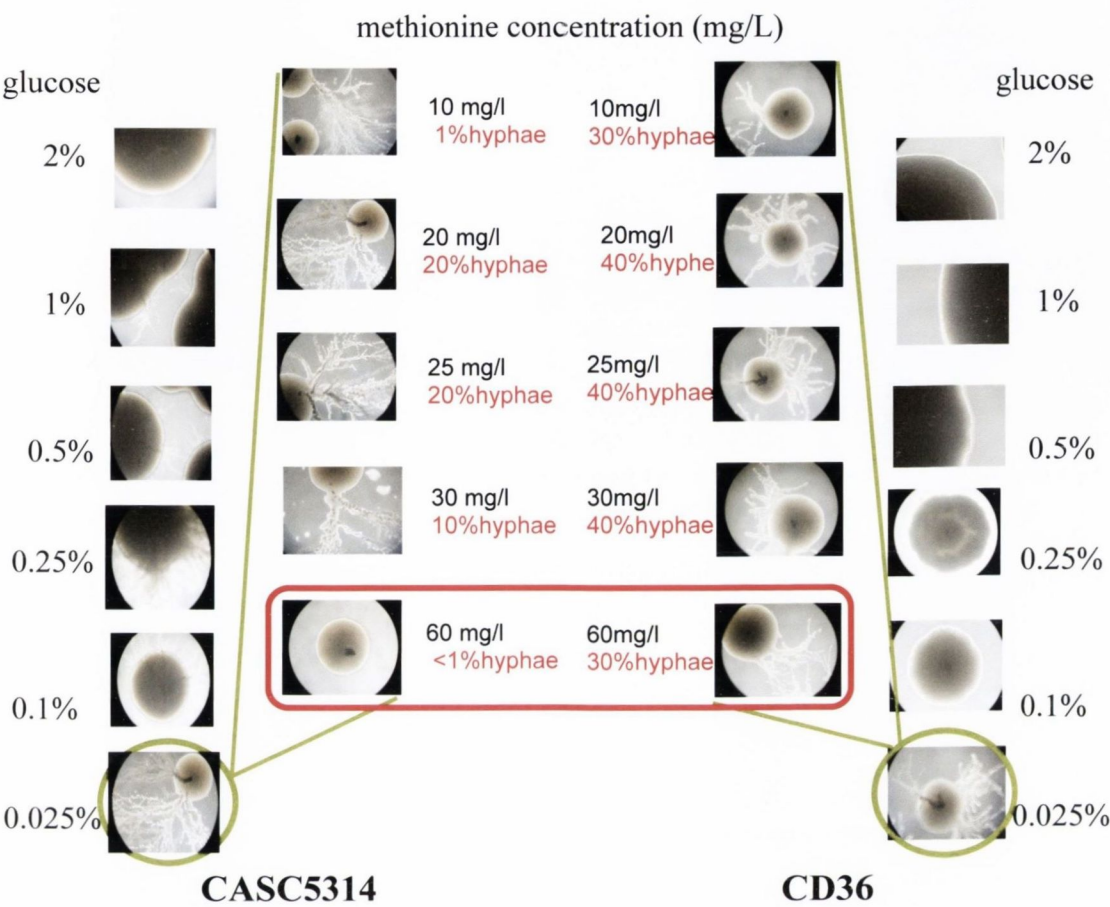


Figure 3.3 High concentrations of methionine inhibit hypha formation in *C. albicans* but not in *C. dubliniensis*. When methionine was added at the concentration of 60 mg/L instead of 25 mg/L into media containing 0.025% (w/v) glucose, an inhibition of hyphae in *C. albicans* but not in *C. dubliniensis* was observed. Plates were incubated for 5 days at 30°C.

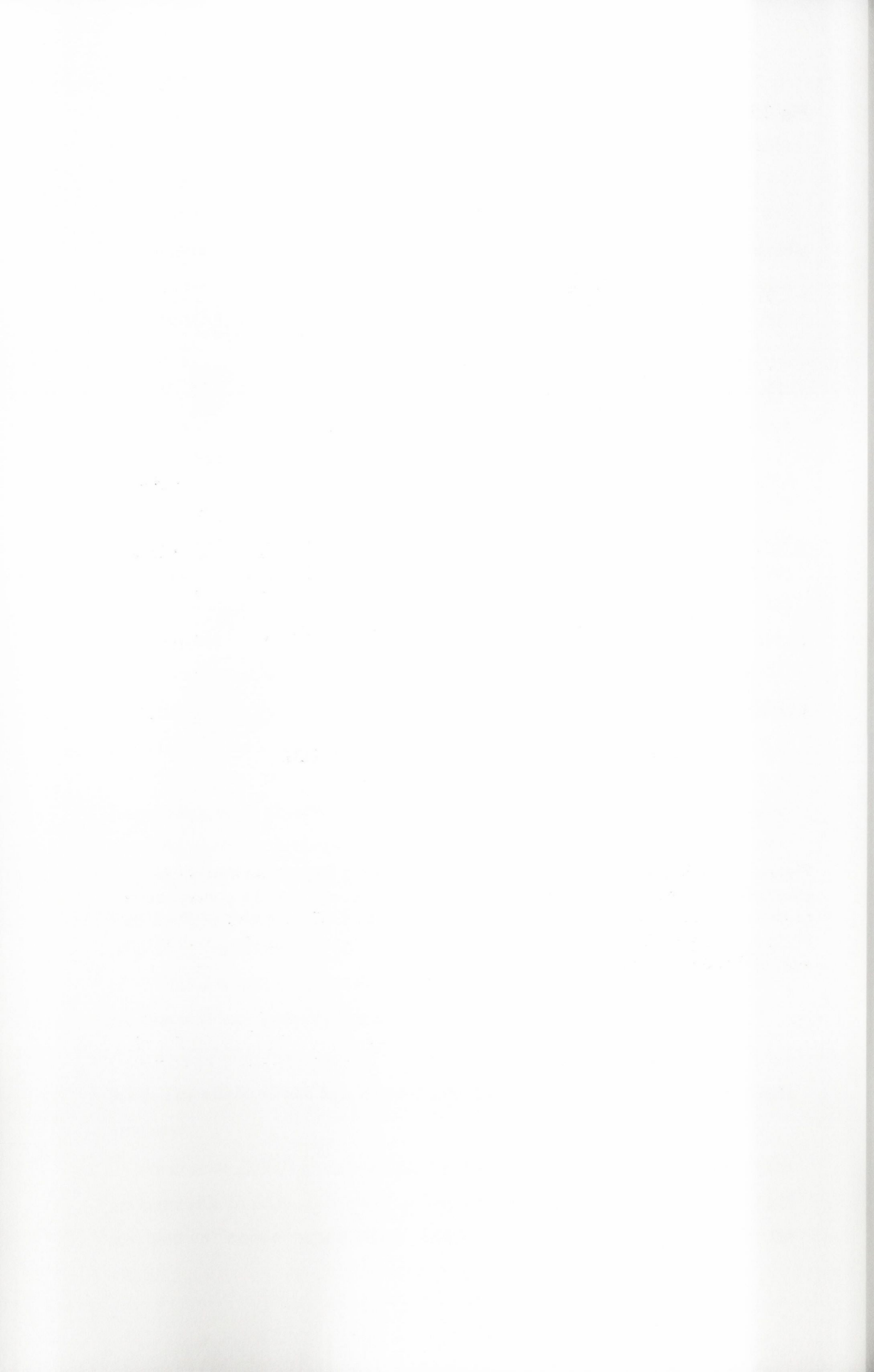


Fig. 3.4

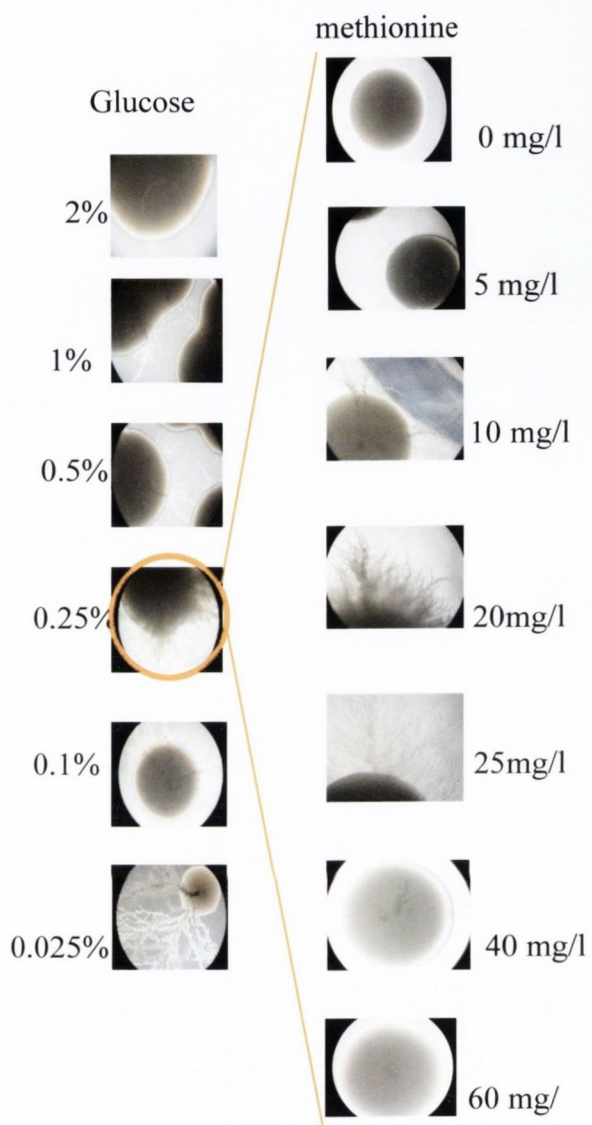


Fig. 3.4 The effect of methionine on hyphal inhibition in *C. albicans*.
Without methionine *C. albicans* did not produce hyphae, however methionine ranging from 10 to 25 mg/L induced strong hyphal formation, while high levels of methionine (≥ 40 mg/L) had an inhibitory effect on hyphae induction.

Fig. 3.5

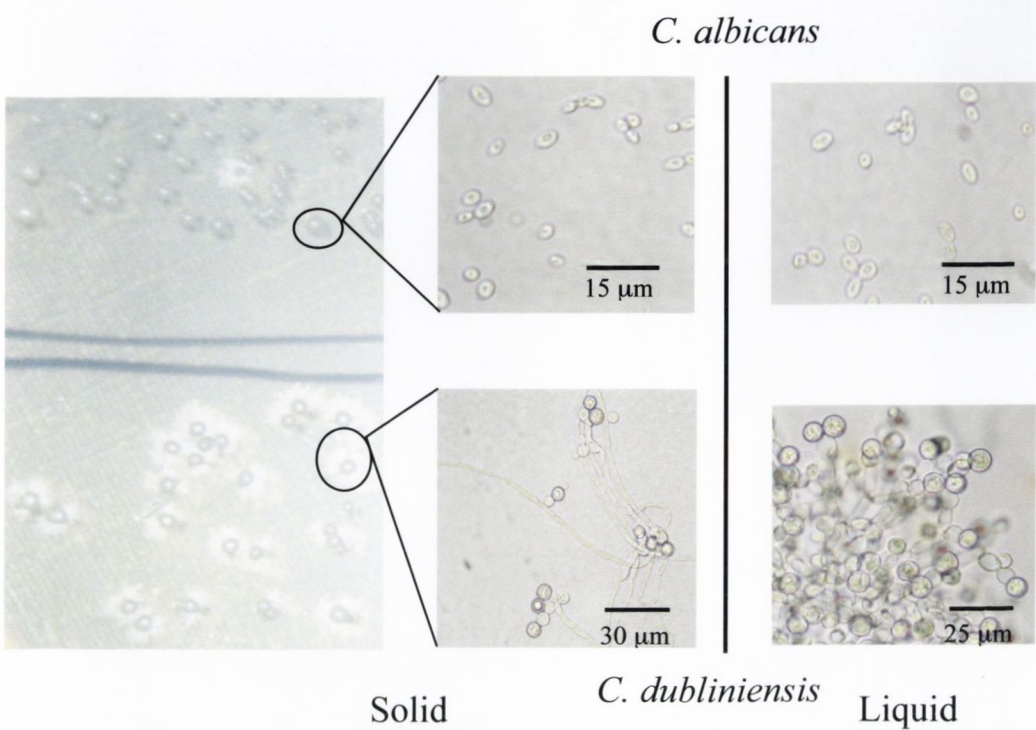


Fig. 3.5 *C.albicans* and *C. dubliniensis* morphology in defined solid and liquid medium.

When the two species were incubated in liquid or solid YNB with 0.025% glucose (w/v) and 20 mg/L methionine at 30°C, pH 3.7, *C. albicans* grew in the yeast form, whereas *C. dubliniensis* grew as hyphae, pseudohyphae and produced copious chlamydospores.

Fig. 3.6

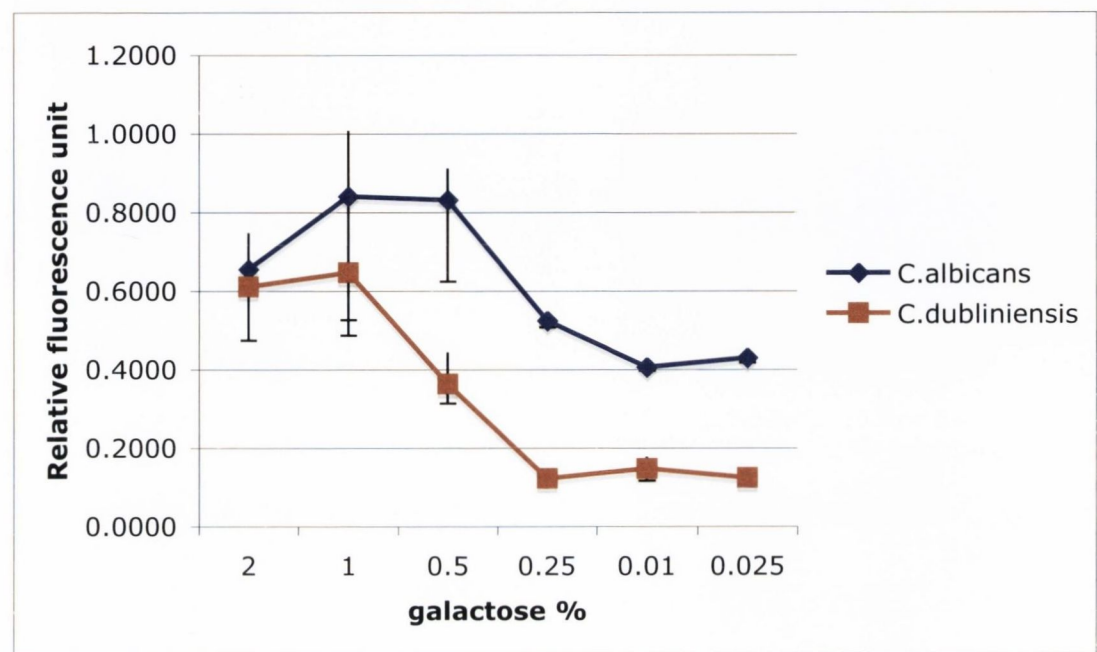


Fig. 3.6 Fluorescence of the *C. albicans* and *C. dubliniensis* strains expressing the pNrg1-GFP gene fusion.

The *C. albicans* and *C. dubliniensis* pNrg1-GFP strains were incubated in a medium comprising of 6.7 g/L YNB, 20 mg/L methionine containing various concentrations of galactose (2%, 1%, 0.5%, 0.25%, 0.01%, 0.025% (w/v)). Fluorescence of the two strains was measured after 5 days incubations.

chlamydospore induction medium results in a downregulation of *NRG1* expression we investigated whether pre-incubation in this medium had an effect on *C. dubliniensis* virulence in the RHE and murine macrophage infection model. For RHE experiments three *C. dubliniensis* strains belonging to different genotypes and mating types (i.e. Eg200, CD36, Ansa28) were cultured for 2-3 days at room temperature on solid CIM. Inocula made from these cells were found to produce more hyphae and cause more damage to RHE than conventionally prepared inocula (YNB at 30°C for 2 days). Increased evidence of tissue damage in the RHE model was shown by the LDH cytotoxicity assay and histological examination of the infected tissue (Fig. 3.7). The ability of the three different mating type strains to filament in co-culture with RAW 264.7 macrophages was also tested using the two different pre-incubation conditions. When pre-cultured under normal conditions the *C. dubliniensis* strains tested were unable to escape from macrophages and were killed quickly, unlike the *C. albicans* strains tested which escaped from the macrophages and survived. When the strains were incubated in the CIM all the *C. dubliniensis* strains tested produced more hyphae and were better able to survive exposure to the macrophages. Strains that were α/α homozygotes at the mating type locus produced the most hyphae and escaped from the macrophages most efficiently (Fig. 3.8).

3.3. Discussion

3.3.1. Low glucose or galactose in combination with methionine differentially affect morphogenesis in *C. albicans* and *C. dubliniensis*

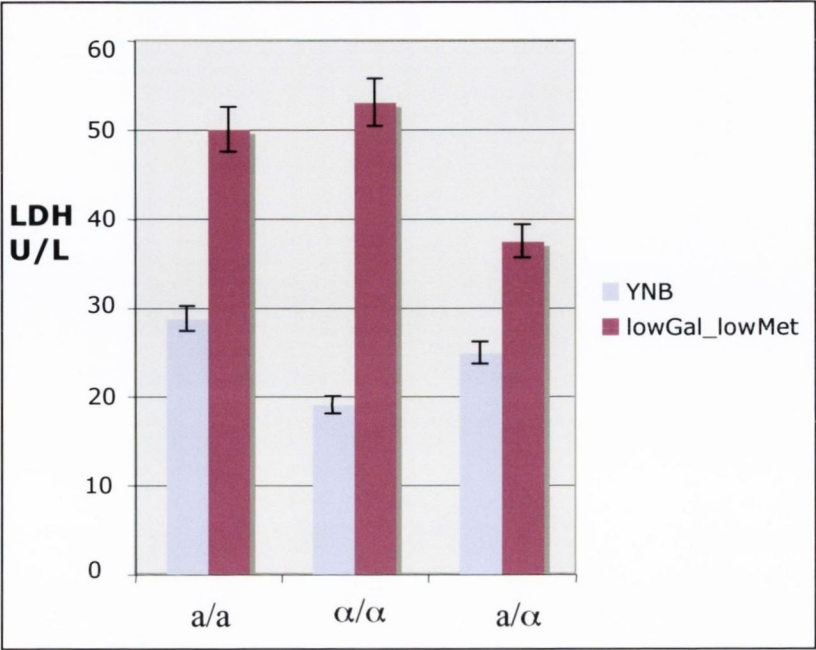
The biochemical events regulating the dimorphic transition in *C. albicans* and *C. dubliniensis* are not completely defined. Similarly, the reasons why *C. dubliniensis* is less efficient at producing hyphae than *C. albicans* are also poorly understood. Amino acids, jointly with glucose and other carbohydrates, are important morphogens in *C. albicans* but to date there is a paucity of studies describing their effects on the ability to trigger the yeast-to-hypha transition in this pathogen, and in *C. dubliniensis*. Of the amino acids, we concentrated on the effect on morphogenesis of sulphur-containing amino acids (in particular cysteine and methionine) for their important role in regulating numerous cellular processes. The methionine/cysteine biosynthetic pathway is of considerable interest in pathogenic fungi. For example, the saprobic mycelial form of the human pathogen *Histoplasma capsulatum* is prototrophic while the pathogenic yeast form requires cysteine or methionine (Maresca & Kobayashi, 1989; Boguslawski & Stetler, 1979). Similar results have been observed in two other dimorphic pathogenic fungi infecting humans, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* (Medoff *et al.*, 1987).

The ATP sulphurylase (Met3) and the methionine synthase (Met6) involved in the initial stages of the methionine biosynthetic pathway, are implicated in pathogenesis, survival *in vivo* and fluconazole resistance in *Cryptococcus neoformans* (Pascon *et al.*, 2004; Yang *et al.*, 2002). In addition, the methionine/cysteine biosynthetic pathway is of interest from the perspective of antifungal drug development. In fact, the antifungal agent azoxybacilin inhibits growth by repressing transcription of fungal sulphate-assimilation enzymes of the methionine/cysteine biosynthetic pathway (Aoki *et al.*, 1996; Ohwada *et al.*, 1994).

For our initial focus on the methionine/cysteine biosynthetic pathway we decided to examine the morphology of *C. albicans* and *C. dubliniensis* when grown under conditions of low glucose (or galactose) and methionine concentrations. In addition, we evaluated the effect of medium pH on colony morphology. While *C. albicans* was found to produce hyphae under a broad range of carbohydrate concentrations (e.g. 1% to 0.025% (w/v)), most *C. dubliniensis* strains produced hyphae only in low

Fig. 3.7

A



B

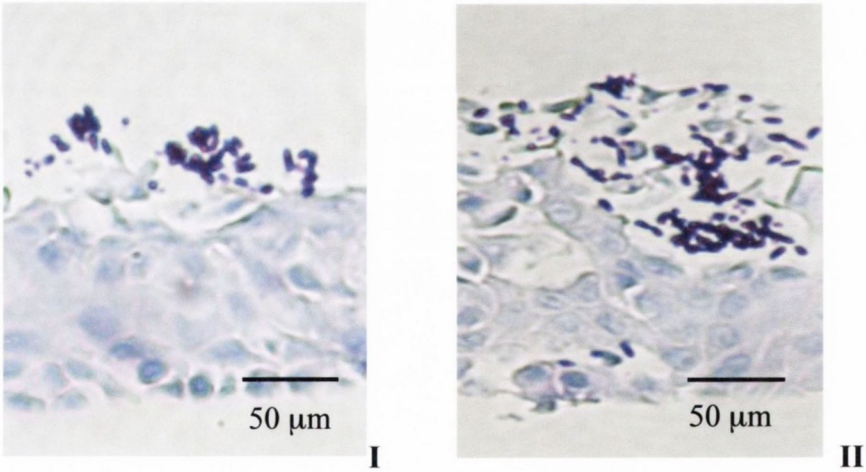


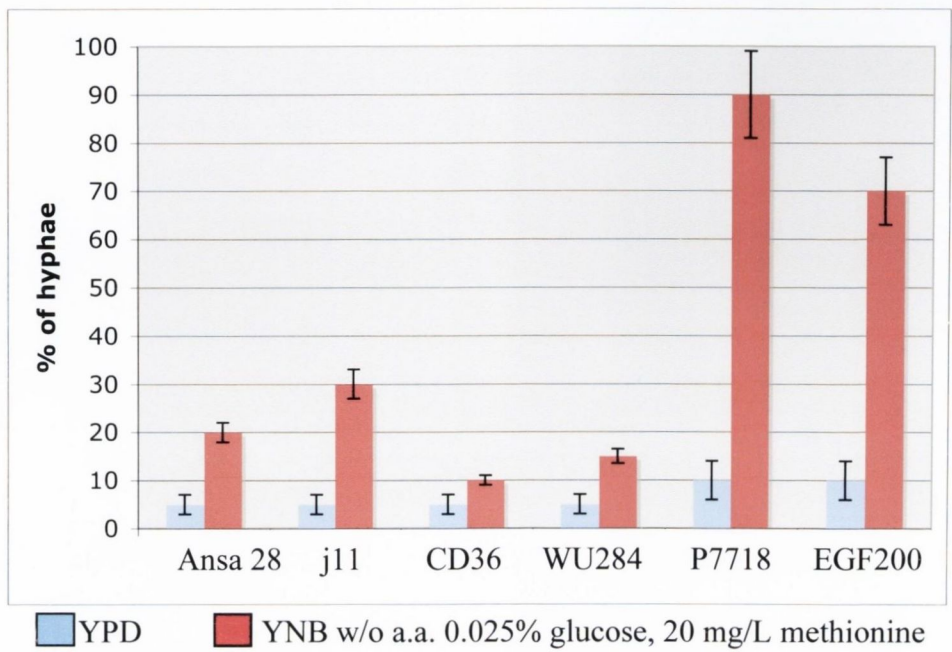
Figure 3.7 *C. dubliniensis* strains grown on low galactose low methionine medium cause more tissue damage in the RHE infection model.

A, Measurement of LDH activity released into the supernatant by *C. dubliniensis* strains Ansa 28 (a/a), Egf200 (α/α), CD36 (α/a) 48 h post inoculation. Inocula were prepared by culturing strains in YPD (blue) and YNB w/o a.a. 0.025% glucose, 20 mg/l methionine pH 3.7 (purple).

B, Histological analysis of RHE infected with the *C. dubliniensis* strain Egf200 (α/α) grown in YPD (I) and YNB w/o a.a. 0.025% glucose, 20 mg/l methionine pH 3.7 (II).

Fig. 3.8

A



B

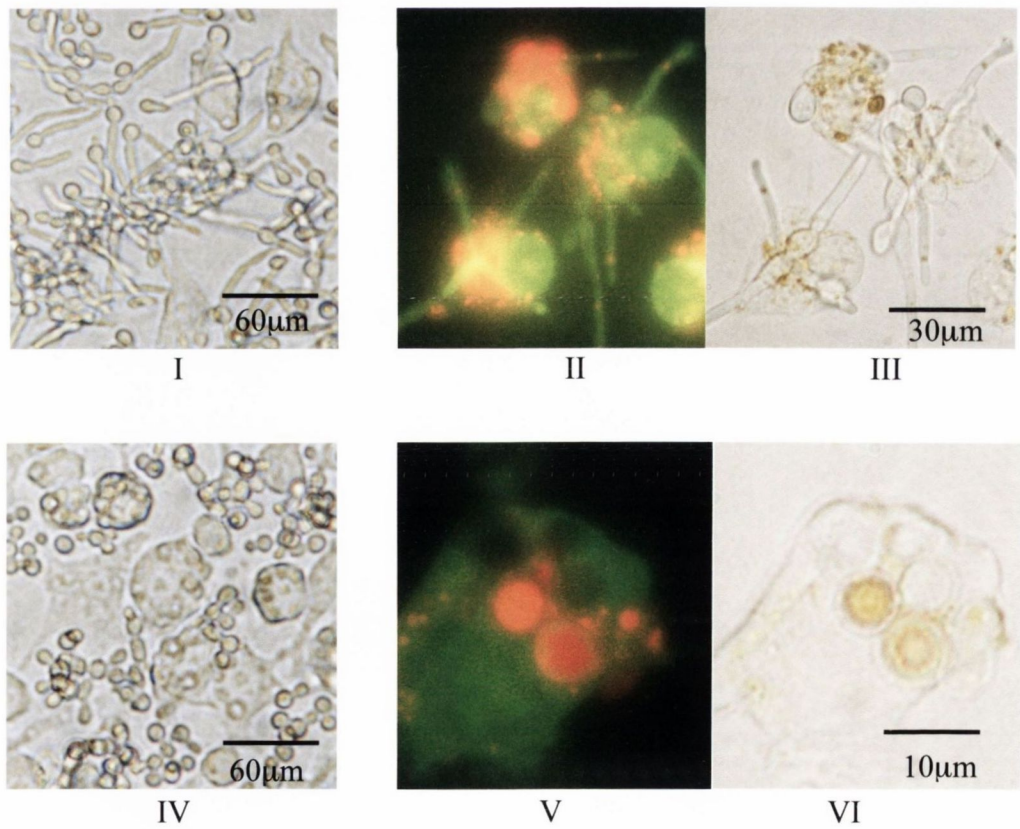


Fig. 3.8 Hypha formation by *C. dubliniensis* α/α α/a and a/a mating types strains co-incubated with murine macrophages.

A, Hypha formation by *C. dubliniensis* strains 1, Ansa 28 (a/a); 2, j11 (a/a); 3, CD36 (α/a); 4, WU284 (α/a); 5, P7718 (α/α); 6, EGF200 (α/α) when co-incubated with macrophages. Inocula were prepared using cells cultured in YPD (blue) and in YNB w/o a.a. 0.025% glucose, 20 mg/L methionine pH 3.7 (Red), previous co-culture with macrophages. Cell producing hyphae were expressed in percentage of the total population. **B**, Viability of Egf200 (α/α) (panel I-III) and Ansa28 (a/a) (panel IV-VI) pre-incubated in YNB w/o a.a. 0.025% glucose, 20 mg/L methionine pH 3.7 previous exposure to macrophages, was assessed by staining with acridine orange staining (panels II, V). Panel I, IV 200x magnification. Panels II, III, V, VI 400X magnification.

carbohydrate concentrations (i.e. 0.025% (w/v)). Three *C. dubliniensis* strains that were shown to be α/α homozygotes at the mating type locus were found to express hyphae under a wider range of sugar concentrations than the two other mating types. While investigating the effect of methionine in the presence of low carbohydrate concentrations in the induction of hyphae in *C. albicans* and *C. dubliniensis*, we found an inhibitory effect of high concentrations of methionine (i.e. > 60 mg/L) in hypha expression in *C. albicans* that is absent in *C. dubliniensis*. The ability of *C. albicans* to produce hyphae under a broader range of nutrient concentrations could potentially explain the reason for the enhanced ability of *C. albicans* to colonise humans and cause disease. For the first time we have shown that glucose-induced hypha expression can also be influenced by amino acid concentrations; in particular cysteine and methionine at high concentrations had an inhibitory effect on hyphae production in *C. albicans* that was absent in *C. dubliniensis*. This raises the possibility of an interaction between the sensing of glucose and the sensing of these amino acids that was also hypothesised by Maidan *et al.* (2005b). We have also described the identification of a defined condition (i.e. YNB containing 0.025% (w/v) glucose or galactose and 20 mg/L methionine, pH 3.7) which causes *C. albicans* to grow as a yeast while *C. dubliniensis* grows as hyphae, pseudohyphae and chlamydospores. The observation of these differences in the conditions necessary for hypha induction between the two species may be related to differences in the niches in the human body of the two species and some evolutionary advantage of *C. dubliniensis* that allowed it to have differentiated its ability to produce hyphae only in particular body sites that perhaps *C. albicans* is less able to colonise.

We further studied the regulation of the transcriptional regulator *NRG1* in the low glucose low methionine medium. The *NRG1* gene was first described in *S. cerevisiae* by Park *et al.*, the gene in the yeast encodes a 25-kDa zinc-finger DNA binding protein that specifically binds to two regions in the upstream activation sequence of the *STAI* gene (Park *et al.*, 1999). *STAI* encodes a secreted glucoamylase the secretion of which is controlled in the yeast by the mating type locus MAT/ a and α haploid yeast cells secrete high levels of the enzyme, but a/α diploid cells produce undetectable amounts. (Dranginis, 1989; Yamashita & Fukui, 1985). Park *et al.* described that the disruption of *NRG1* caused a five-fold increase in the level of the *STAI* transcript in the presence of glucose. The expression of *NRG1* itself was

inhibited in the absence of glucose. These findings indicate that in *S. cerevisiae* Nrg1 acts as a DNA-binding repressor and mediates glucose repression of *STA1* expression in a circuit of regulation in which mating type and ploidy play an important role. In *C. albicans* Nrg1 was identified by Braun *et al.* and described as a repressor of filamentous growth. Nrg1 mutant cells are predominantly filamentous under non-filament-inducing conditions (Braun *et al.*, 2001). This transcriptional regulator was also found to be involved in the differential morphogenesis of *C. albicans* and *C. dubliniensis* in Staib agar (see general introduction). In this medium the transcriptional regulator was found to be downregulated in *C. dubliniensis* but not in *C. albicans* (Staib & Morschhauser, 1999). Moran *et al.* found that deletion of both copies of *CdNRG1* increased the formation of true hyphae by *C. dubliniensis* in response to serum, exogenous cAMP and CO₂. In addition, deletion of this gene enhanced filamentation and survival of *C. dubliniensis* in co-culture with murine macrophages. In the reconstituted human oral epithelium infection model, the $\Delta nrg1$ mutant caused increased tissue damage relative to the wild-type strain (Moran *et al.*, 2007). We found that when the medium was supplemented with glucose or galactose in the range from 0.5% to 0.025% (w/v) there was a downregulation of *NRG1* expression in *C. dubliniensis* that was absent in *C. albicans* (this down-regulation was identified as a drop in the fluorescence of the *C. dubliniensis* strain expressing *pNRG-GFP* compared to the fluorescence of the *C. albicans* strain expressing *pNRG-GFP*). Therefore, it is likely that the reason for the differences in morphology of *C. albicans* and *C. dubliniensis* grown on the defined chlamyospore inducing medium is due to differential expression of *NRG1*. These observations suggest that galactose and methionine at low pH can downregulate the transcriptional regulator *NRG1* in a species-specific manner. To further verify this, a *C. albicans* genomic library could be integrated into the *C. dubliniensis* genome and transformants screened for clones in which filamentation and/or chlamyospore production on the CIM is suppressed. Such a screen could identify the genes that are responsible in *C. albicans* for the suppression of hyphae and chlamyospores in this medium. Since incubation in the CIM results in a down regulation of *NRG1* expression we investigated whether pre-incubation in this medium had an effect on *C. dubliniensis* virulence. It was found that when pre-incubated in this medium different *C. dubliniensis* strains, especially those that were found to be α/α homozygotes, were more able to produce damage to

the epithelium in reconstituted human epithelia experiments than when they were incubated in the ordinary pre-incubation condition (i.e. YEPD) in which *NRG1* was not downregulated. It has been suggested that in *S. cerevisiae* some genes such as *STA1*, (Dranginis, 1989; Yamashita & Fukui, 1985), that are under the control of the mating type could also be directly regulated by *NRG1*. *NRG1* could also itself be regulated (in a still undefined way) by some factor under the regulation of the mating type locus. To verify this further the *C. dubliniensis* strain expressing *pNRG1-GFP* that is heterozygous at the mating type locus could be manipulated with targeted mutagenesis to generate the strains $\Delta mat \alpha/mat a$ and the opposite $\Delta mat a/mat \alpha$. The deletion could also be complemented in the opposite combination to generate the two homozygous strains $\Delta mat \alpha/mat a::mat a$ and $\Delta mat a/mat \alpha::mat \alpha$. The expression of *NRG1* and the virulence of those strains could then be tested to examine the hypothesis that *NRG1* is regulated by the mating type locus, and the possibility that *C. dubliniensis* strains with different mating types have differences in their virulence.

The data presented here suggest that methionine and glucose sensing pathways, as has been observed in other pathogenic fungi, are likely to be important virulence determinants in both *C. albicans* and *C. dubliniensis in vivo*.

Chapter 4

Investigation of the role of Cbfl and Met4 in *C. albicans*.

4.1. Introduction

4.1.1. Cbfl and Met4, two important transcriptional activators in methionine biosynthesis in *S. cerevisiae*: evaluation of their roles in *C. albicans* and *C. dubliniensis*

The incubation of *C. albicans* and *C. dubliniensis* in media containing low concentrations of glucose (as the sole source of carbohydrate), methionine (as the sole source of amino acid), and sulphate (as the only source of sulphur) can result in the two species exhibiting different growth morphologies (see chapter 3). This suggests that differences in the abilities of the two species to sense and/or transport these compounds in the environment may be involved in the differential phenotype of the two species. In order to investigate the possible reasons for this, the genes involved in glucose and methionine transport in *C. dubliniensis* were compared with their *C. albicans* homologues to determine if there are any significant differences between the two species. Several glucose transporter proteins have been identified in *C. albicans*, however only two, the Gpr1p and Hgt4p have been characterised, and their role in sensing carbohydrates and in the activation of the signalling pathway has been elucidated (see general introduction). In contrast relatively little is known about the proteins related to the sensing and biosynthesis of methionine in *C. albicans*. Biswas *et al.* (2003) studied the function of the main transcriptional activator of methionine biosynthesis from sulphate (Cbf1p) in *C. albicans* by deleting the *CBF1* gene. The authors found that the $\Delta cbf1$ mutants exhibited a slow growth phenotype and were temperature sensitive at 42°C. In addition, the mutants were auxotrophic for sulphur amino acids, and could grow on minimal medium only when it was supplemented with either methionine or cysteine, suggesting that *CaCBF1* is necessary for the expression of genes involved in the assimilation of inorganic sulphate. Deletion of *CaCBF1* also resulted in morphological abnormalities, with many cells being unusually large. The $\Delta cbf1$ mutants showed neither enhanced sensitivity to the microtubule destabilizing agent thiabendazole, nor did they exhibit an increased frequency of chromosome loss suggesting that Cbf1p, in contrast to its function in *S. cerevisiae*, is not necessary for efficient chromosome segregation in *C. albicans* but is only involved in the activation of the methionine biosynthetic genes.

The authors did not test the mutant for its virulence in *ex vivo* or *in vivo* models of infection.

In *S. cerevisiae* Met4 is an important transcriptional activator of methionine biosynthesis. Thomas *et al.* (1992) showed that in this yeast induction of the sulphate assimilation pathway and methionine biogenesis is not achieved solely by Cbf1p. This induction requires another trans-acting factor, encoded by the *MET4* gene, that forms heterodimers with the Cbf1 protein. In *S. cerevisiae* the Met4 transcription factor and its co-factor Cbf1 not only control the expression of sulfur metabolism but also regulate oxidative stress response genes (e.g. glutathione and the methyl donor protein, S-adenosylmethionine; Wheeler *et al.*, 2003). In addition Met4 coordinates the transcriptional program and cell cycle progression in response to cadmium and arsenic stress and other heavy metals (Su *et al.*, 2008). Met4 lacks DNA-binding activity and requires either Cbf1 or one of the two homologous proteins Met31 and Met32 for promoter association. Accordingly, *S. cerevisiae* $\Delta met4$, $\Delta cbf1$, and $\Delta met31 \Delta met32$ double mutants are methionine auxotrophs (Su *et al.*, 2008). It has been found that Met4 activity is regulated by nutrient and oxidative stress conditions in a manner which is dependent on the SCF(Met30) ubiquitin ligase. The mechanism of SCF(Met30)-dependent ubiquitinylation of Met4 that controls Met4 activity is not completely understood. Rouillon *et al.* (2000) found that the SCF(Met30) complex mediates the transcriptional repression of the *MET* gene network by triggering degradation of the transcriptional activator Met4p when intracellular S-adenosylmethionine (AdoMet) increases. This AdoMet-induced Met4p degradation is dependent upon 26S proteasome function. Unlike Met4p, Cbf1p was found not to be regulated at the protein level. The author also demonstrated that Met30p was a short-lived protein, which localises within the nucleus (Rouillon *et al.*, 2000). Menant *et al.* demonstrated that instead intracellular cysteine level was the main trigger of the degradation of Met4 *in vivo* analysing the regulation of the degradation of a fully functional Met4-Cbf1 chimera, in which Met4 was fused to the DNA binding domain of Cbf1. The degradation mechanism that the authors identified was, as shown before, dependent on the SCF(Met30) complex (Menant *et al.*, 2006). These data highlight a fine regulation mechanism on the Met4 protein in *S. cerevisiae*.

The main purpose of this part of the study was to investigate the functions of Cbf1 and Met4 in *C. albicans*.

4.2. Results

4.2.1. Comparison of glucose and methionine sensors/transporters and of genes involved in methionine metabolism in *C. albicans* and *C. dubliniensis*

The homologues of the *C. albicans* high affinity glucose transporter gene *HGT4* and of *GPR1* have been identified in the *C. dubliniensis* genome (<http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/>). When aligned and compared the *C. albicans* and *C. dubliniensis* genes were found to be 90% identical (Fig. 4.1, A). Since this is the typical level of divergence observed between *C. albicans* and *C. dubliniensis* genes it is likely that these two genes function in the same manner in both species and are therefore unlikely to be involved in the differences in growth morphology evident when grown in low glucose-containing media. Attempts were made to compare the dynamics of glucose uptake in both species under these conditions, however, these experiments failed due to the low levels of glucose present in the growth media.

Both species have a homologue of the gene encoding the general amino acid sensor Ssy1p, but since these two genes are 93% identical, it is likely that they function in a similar manner in both species. The two species also possess two genes that encode for two sensors/transporters specific for methionine, Mup1p and Mup3p, low affinity and high affinity transporters, respectively. The two genes share more than 90% nucleotide identity suggesting that it is unlikely that they differ in their ability to transport methionine. To confirm that transport of methionine is similar in the two species and in this way dismiss the possibility that Mup1p and Mup3p play a role in the different morphogenesis of the two species in the low glucose low methionine medium the two species were subjected to assays designed to measure the uptake of methionine using ^{35}S methionine in two different concentrations of the unlabeled substrate, low concentration (0.01 mM) and high concentration (1 mM). No differences were found in the rate of methionine uptake of the two species, suggesting that the differential effect of methionine concentration on the morphology of the two species is not related to a difference in uptake of this compound (Fig. 4.2). In order to investigate if there are any differences in the enzymes responsible for sulphate metabolism and methionine biosynthesis in the two species, the genes encoding these enzymes in both species were compared as it has been suggested

previously that genes involved in the initial stages of the methionine biosynthetic pathway (i.e. methionine synthase) are involved in the pathogenesis of filamentous fungi such as *C. neoformans* (Pascon *et al.*, 2004). As can be seen in Fig. 4.1 all of the genes characterised in *S. cerevisiae* involved in the biosynthesis of methionine were identified in the genome of *C. albicans* and were all found to show homology with their counterparts in *C. dubliniensis*. Interestingly however, the genes encoding the transcriptional regulators *CBF1* and *MET4* of this pathway were found to exhibit a higher level of divergence than normal (i.e. 70% and 80%, respectively at the nucleotide level). The *C. albicans* and *C. dubliniensis* Cbf1 proteins are interestingly only divergent in the oligomerisation domain (60% homology at the protein level). However, the DNA-binding domain is highly homologous in the two species (97% homology at the protein level) (Fig. 4.3).

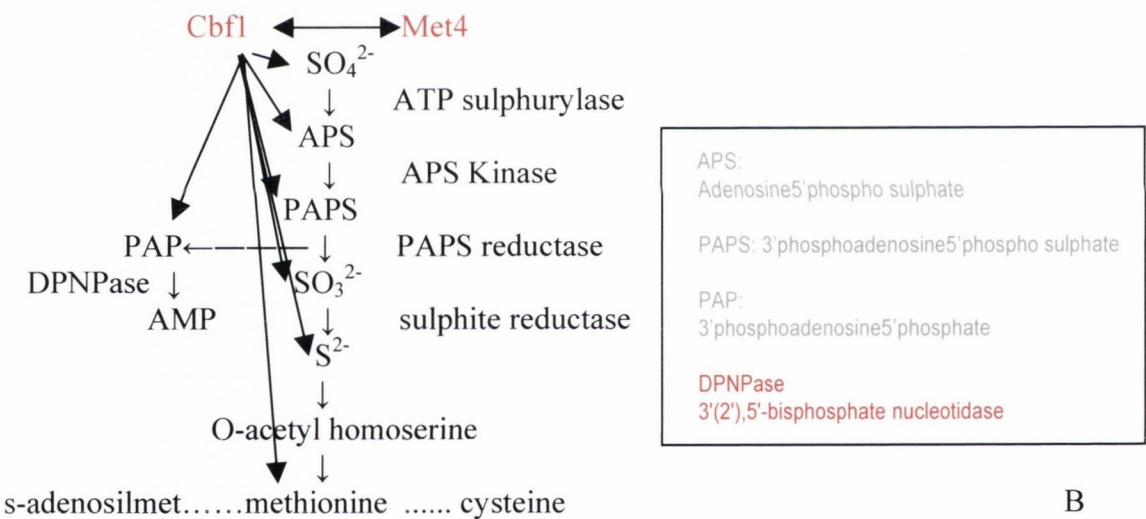
4.2.2. Heterologous expression of the *C. albicans* *CBF1* gene in the *C. dubliniensis* genome

In order to investigate whether this divergence may play a role in the different growth morphology of the two species in media containing low concentrations of methionine the *C. albicans* *CBF1* gene and its promoter was integrated into the truncated, non functional *CDR1* gene of *C. dubliniensis* CD36 (CD36CaCbf1) (Fig. 4.4). Nourseothricin-resistant transformants were selected on agar plates containing 100 µg/ml nourseothricin. A reverse transcriptase PCR using specific primers for the *C. albicans* *CBF1* gene (CaRCbf1F, CaRCbf1R; table 2.3) that amplifies a sequence-specific region of the *C. albicans* gene was carried out to confirm the expression of the *C. albicans* gene in *C. dubliniensis* (Fig. 4.4). The growth of the *C. dubliniensis* wild type and of the CD36CaCbf1 was compared at different temperatures, for its ability to induce hyphae (i.e. Spider medium, SLD medium supplemented with serum, Lee's medium and on BSA agar), in media containing low methionine (i.e. YNB solid medium (pH 7.0) w/o a.a. supplemented with variable concentrations of methionine (5 to 60 mg/L (w/v)) and variable concentrations of glucose, galactose or sucrose (0.025% to 2% (w/v)) (data not shown) and for resistance to oxidative stress (Fig. 4.6). There were no detectable differences between the wild type and the mutant (CD36CaCbf1) strain. It was concluded that that the *CBF1* gene of *C. dubliniensis* is probably fully functional and the integration of the *C. albicans* *CBF1*

Fig. 4.1

<i>C. albicans</i> and <i>C. dubliniensis</i> glucose and methionine sensor/transporter homolog genes	Identity	<i>C. albicans</i> and <i>C. dubliniensis</i> first steps methionine biosynthesis homologues	Identity
<i>GPR1</i>	92%	<i>ATP sulphurylase</i>	94%
<i>HGT4</i>	90%	<i>APS Kinase</i>	92%
<i>SSY1</i>	93%	<i>PAPS reductase</i>	93%
<i>MUP1</i>	93%	<i>Sulphite reductase</i>	92%
<i>MUP3</i>	94%	<i>CBF1</i>	70%
		<i>MET4</i>	80%

A



B

Fig. 4.1 Degree of sequence identity shared by the glucose and methionine sensors/transporters gene homologues in *C. albicans* and *C. dubliniensis*.
A, Percentage sequence identity of the homologues in *C. albicans* and *C. dubliniensis*.
B, Methionine biosynthesis pathway in *S. cerevisiae*. Each step is regulated by Cbfl and Met4. The *S. cerevisiae* enzymes implicated in the methionine biosynthesis have corresponding homologues in *C. albicans*.

Fig. 4.2

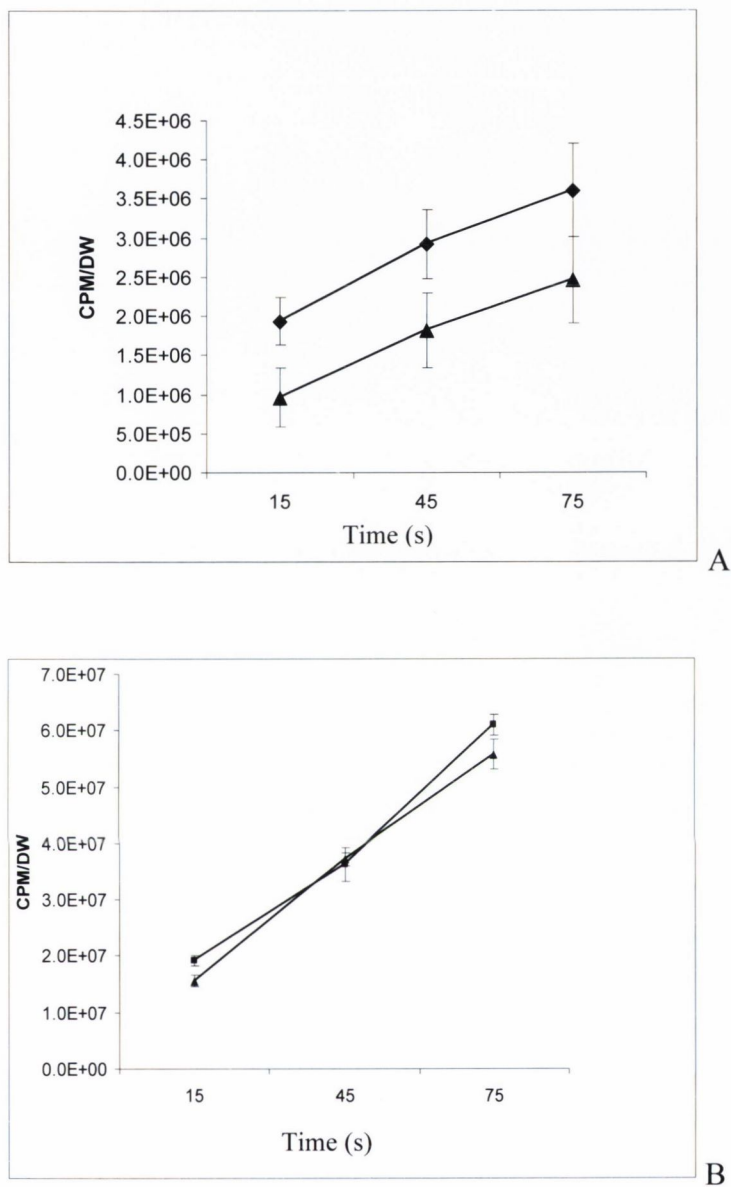


Fig. 4.2 Uptake of methionine by ▲ *C. albicans* and ■ *C. dubliniensis*
Uptake was measured using (A) 1mM or (B) 0.01 mM of unlabelled methionine in the presence of 1.25 $\mu\text{Ci}/\mu\text{mol}$ ^{35}S methionine. The data points represent the average CPM divided by the dry weight plotted as a function of time.

Fig. 4.3

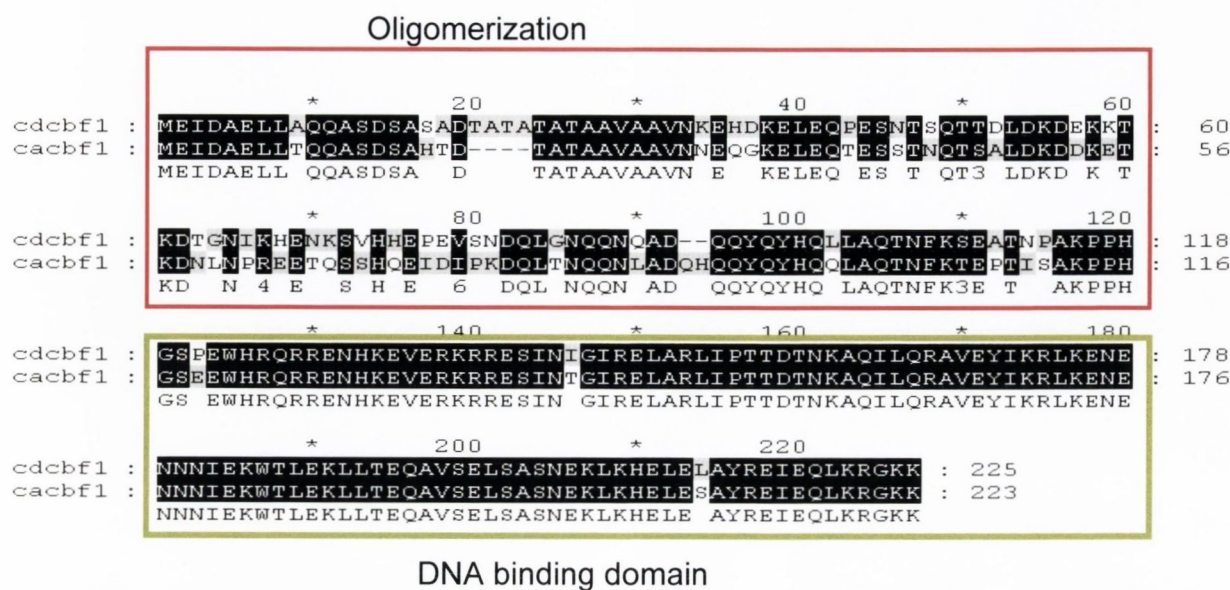


Fig. 4.3 Alignment of the *C. albicans* and *C. dubliniensis* Cbf1 amino acid sequences.
The homologues share 97% sequence identity in the DNA binding domain (in green), and only 60% identity in the oligomerization domain (in red).

Fig. 4.4

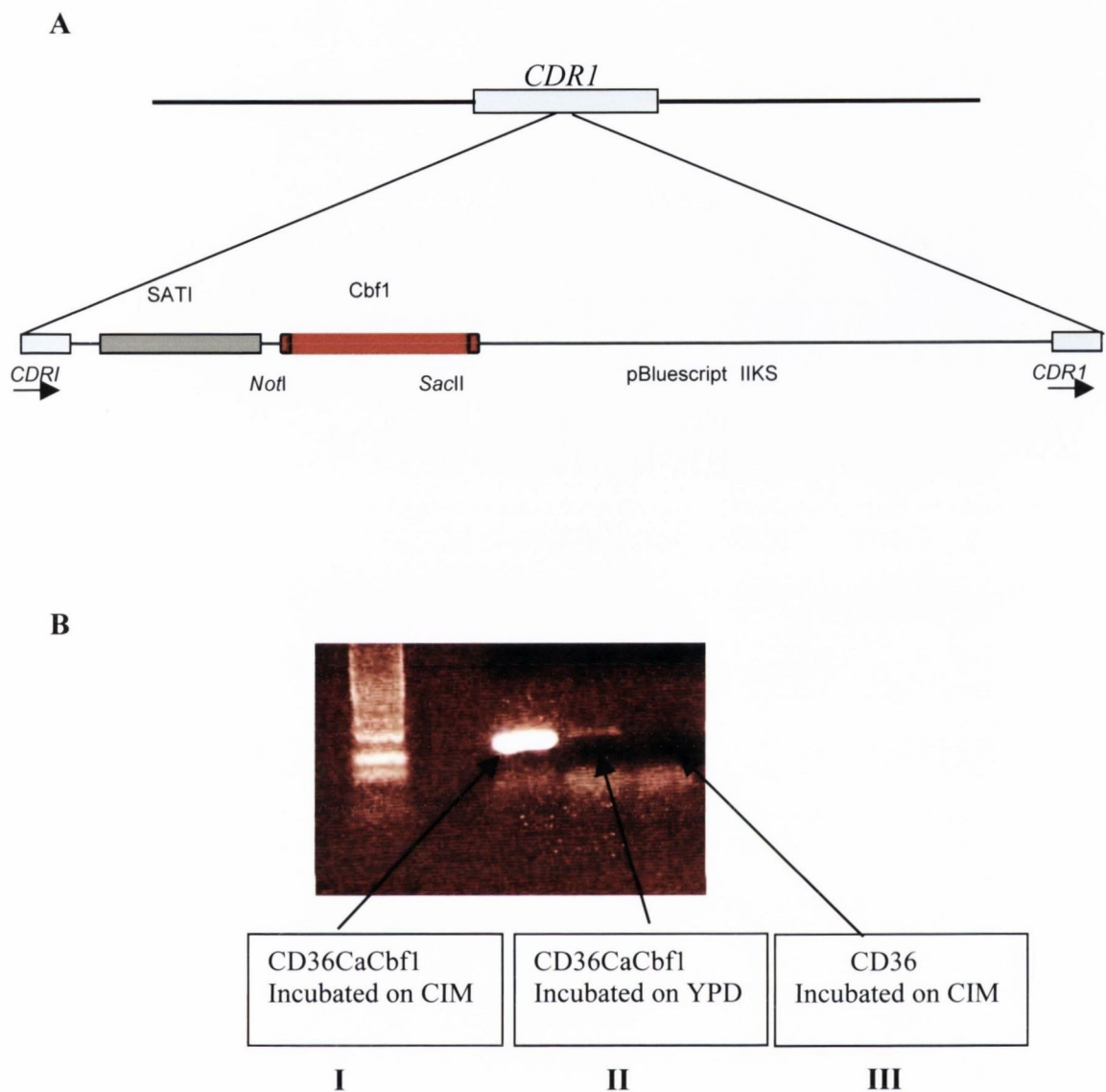


Fig. 4.4 Reverse transcriptase PCR showing the expression of the *C. albicans* Cbf1 in the *C. dubliniensis* genome.

A, The construct was inserted in the *CDR1* gene of *C. dubliniensis* using the integrating vector pCDR1. The *CDR* region consists of + 1800 to + 2400 of the *C. dubliniensis* *CDR1* gene.

B, Reverse transcriptase PCR was conducted on the integrant strain CD36CaCbf1 with primers specific for the *C. albicans* gene. To assess the expression of the *C. albicans* *CBF1* gene, the integrant strain (panel I, II) was incubated in CIM (I) and in YPD (II). A reverse transcriptase PCR conducted on the wild type CD36 strain (III) showed that the primers were specific for the *C. albicans* gene.

gene does not complement any defect, such as poor growth observed at 42°C, or in hyphae induction.

4.2.3. Analysis of the *C. albicans* $\Delta cbf1$ mutant

It was hypothesised that the co-factor transcriptional activator Cbf1p could play an important role in *Candida*, as in *S. cerevisiae* and other filamentous fungi, in regulating S-adenosylmethionine and glutathione synthesis. To investigate this, the growth of the *C. albicans* wild type and the double disruptant of the *CBF1* gene (obtained from Joachim Morschhauser, University of Wurzburg, Germany) were compared for their ability to produce hyphae, for their resistance to oxidative stress, for their ability to form biofilm in an *in vitro* model and for damage to epithelial cells in a reconstituted human epithelium model.

*4.2.3.1. Comparison of the ability of *C. albicans* $\Delta cbf1$ mutant and of the wild type strain to express hyphae in different media (hyphae induction assay)*

The ability of the $\Delta cbf1$ and wild type strain SC5314 to produce hyphae in serum or in response to pH shift and in the presence of proteins in the culture medium was tested on various media (i.e. YPD plus 10% (v/v) serum, Spider medium, SLD medium with serum, Lee's medium and on BSA agar). 1×10^6 cfu/ml of exponentially growing cells of the two strains were added to YPD and liquid SLD medium containing 10% (v/v) serum and incubated at 37°C. Hyphae induction was measured overtime (i.e. 15 min, 30 min, 1 h, 2 h, 5 h after inoculation). For pH and temperature shift experiments 1×10^6 cfu/ml exponentially-growing cells of the two strains were incubated in Lee's medium pH 4.5 at 25°C. After overnight incubation 1×10^6 cells from this culture were subcultured in Lee's medium pH 7.5 and incubated at 25°C or 37°C and the percentage of the population of the two strains that was expressing hyphae was measured after 3 h of incubation. No differences between the $\Delta cbf1$ and the wild type in the ability to produce hyphae in response to pH shift or to the presence of serum in the medium were found (data not shown). But interestingly when exponentially growing cells of the wild type and the mutant strain were spotted on BSA agar plates (1.17% Yeast Carbon base, 0.01% Yeast Extract, 0.2% BSA) and incubated at 37°C for 3-4 days the $\Delta cbf1$ mutant failed to produce hyphae while the wild type produced hyphae efficiently. (Fig. 4.5). No differences however, were

found in the amount of proteinase secreted into the medium by the knock out mutant and the wild type (data not shown).

4.2.3.2. Oxidative stress sensitivity of $\Delta cbf1$ mutant

The growth of the *C. albicans* wild type, the double disruptant for the *CBF1* gene, the wild type *C. dubliniensis* CD36 and CD36 expressing *C. albicans* *CBF1* (CD36CaCbf1) was compared in media containing H₂O₂ as an agent of oxidative stress. Exponentially growing cells of the strains were diluted to the concentration of 1×10^8 cfu/ml and serial 1:10 dilution the strains were made and spotted on YEPD agar supplemented with 1 mM and 6 mM H₂O₂ and incubated at 37°C for 3 days. The *C. albicans* $\Delta cbf1$ mutant was found to be more sensitive to oxidative stress than the wild type. No significant differences in resistance to oxidative stress were found between the *C. dubliniensis* CD36 and the CD36CaCbf1 (Fig. 4.6).

4.2.3.3. Biofilm formation by $\Delta cbf1$ mutant on plastic surface

Global transcriptional analysis experiments have shown up-regulation in *C. albicans* of the genes implicated in the methionine biosynthesis during biofilm formation (Murillo *et al.*, 2005). Such a finding suggested the possibility that the *C. albicans* $\Delta cbf1$ mutant could be defective in biofilm formation.

To test this possibility a simple biofilm test was employed. Biofilm formation was analysed by measuring the ability to adhere and to grow on polystyrene plastic surfaces using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide] reduction assay as a method to quantify the number of viable cells. The $\Delta cbf1$ mutant was found to be less able to form biofilm compared to the wild type and the reconstituted strain (Fig. 4.7, A). Upon analysis of the biofilm by microscopy a lower level of hyphae production was evident in the biofilm formed by the mutant compared to those formed by the wild type (Fig. 4.7, B).

4.2.3.4. Ability of $\Delta cbf1$ mutant to invade a reconstituted human epithelium

The ability of the *C. albicans* wild type and the $\Delta cbf1$ mutant strain to invade a multilayer of TR146 buccal epithelial cells and A431 vaginal epithelial cells (infection model developed in Stefan Rupp's laboratory, Fraunhofer Institute, Stuttgart) was tested.

Fig. 4.5

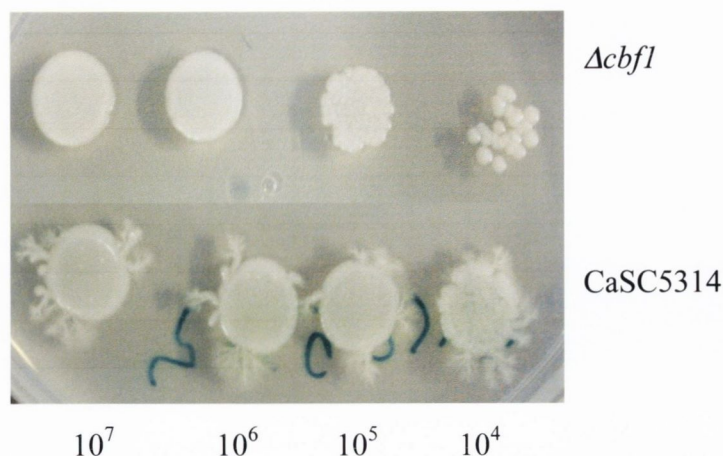


Fig. 4.5 Hypha production of *C. albicans* strains *Δcbf1* and SC5314 on BSA agar. Cells were plated at an initial concentration of 1×10^7 followed by a series of 10 fold dilutions on plates containing medium comprising of 1.17% Yeast Carbon base, 0.01% Yeast Extract, 0.2% BSA.

Fig. 4.6

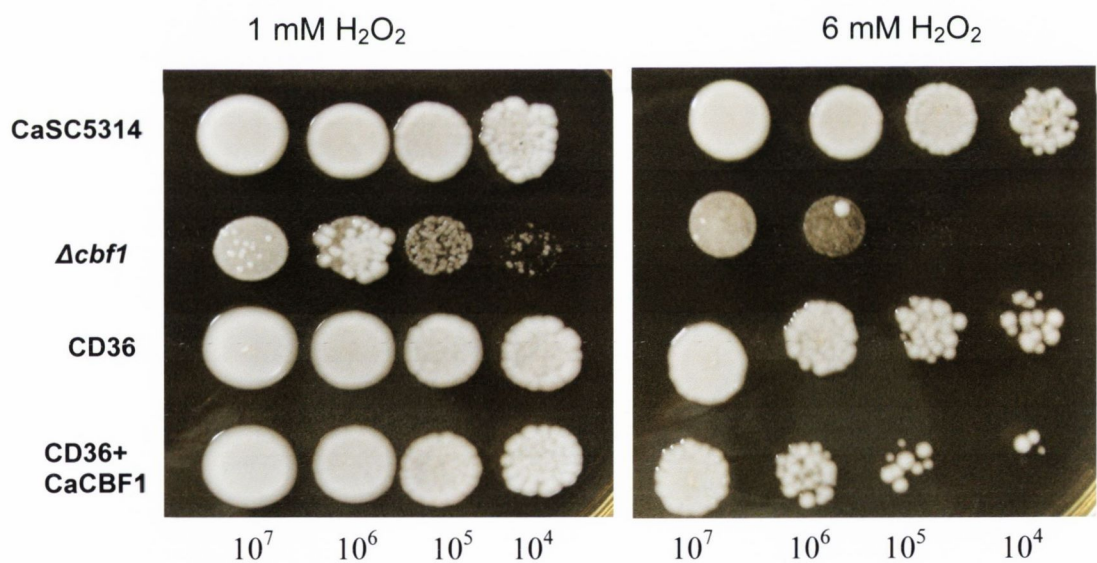
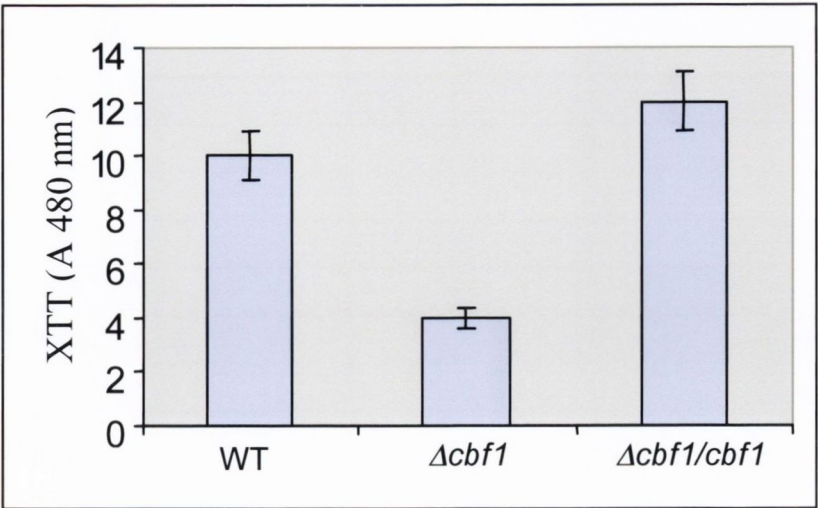


Fig. 4.6 Sensitivity of the *Δcbf1* mutant and the integrated strain CD36CaCBF1 to oxidative stress.

The strains were grown on YEPD agar containing 1 mM or 6 mM H_2O_2 for 3 days at $37^\circ C$. Cells were plated at an initial concentration of 1×10^7 followed by a series of 10 fold dilutions.

Fig. 4.7
A



B

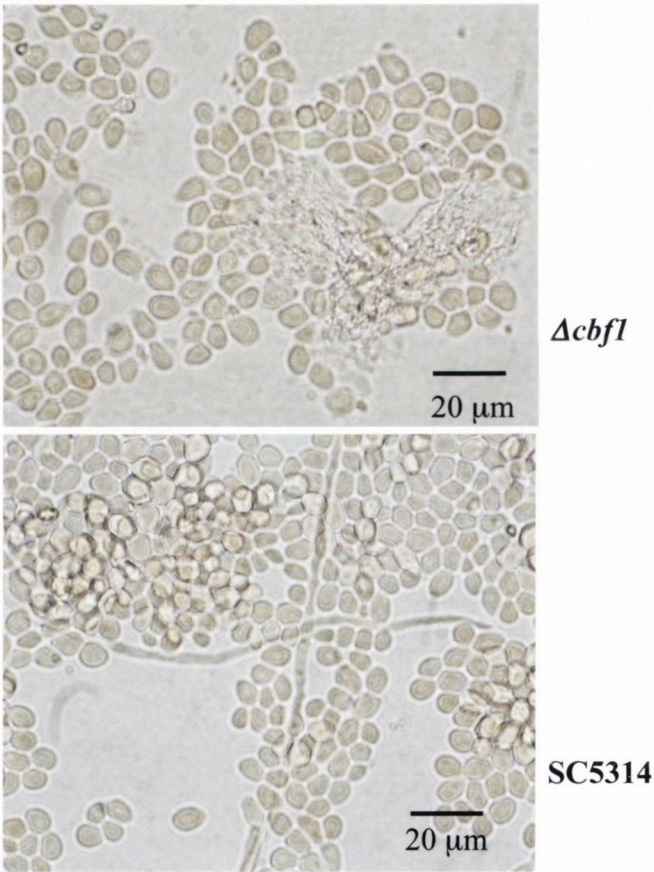


Fig. 4.7 Comparison of biofilm formation by *C. albicans* SC5314 and the mutant $\Delta cbf1$.

A, Biofilm production by the *C. albicans* wild type, the $\Delta cbf1$ mutant and the reconstituted strain was quantified as mean of XTT absorbance at 480 nm.

B, Photographs of biofilm produced by the *C. albicans* $\Delta cbf1$ mutant and the wild type SC5314 (X 400 magnification). Note the absence of hyphae in the $\Delta cbf1$ biofilm.

The damage that the *Δcbf1* and the wild type caused to buccal epithelial cells TR146 (from Skinethic) was measured at 3 different time points (6, 24, 48 h) using the Promega Citotox® colourimetric assay that quantifies the activity of the cytosolic enzyme lactate dehydrogenase from the epithelial cells, released into the medium as a result of cell disruption (Fig. 4.8, A). The results indicate that the *C. albicans* wild type strain was far more able to damage the epithelium than the *Δcbf1*. This was confirmed by histological examination at 48 h (Fig. 4.8, B). Similar results were also obtained using A431 vaginal epithelial cells which showed that the wild type strain invaded the tissue while the *Δcbf1* mutant caused far less tissue invasion and damage (Fig. 4.9).

4.2.4. Deletion of the *CaMET4* with the SAT1 flipper cassette

The *C. albicans* ORF 19.5312 is an uncharacterised ORF that has been identified through homology with Met4 of *S. cerevisiae*. The two proteins share 42% of identity. In order to assess the function of the Met4 transcriptional activator in *C. albicans* the two alleles of the gene were deleted by targeted mutagenesis and the effect of this inactivation on the phenotype of the resulting mutant was evaluated. The technique used was the dominant selection marker *CaSAT1* (Reuss *et al.*, 2004) that confers resistance to nourseothricin combined with the FLP-mediated marker that allows the subsequent excision of the cassette. The two rounds of integration and excision generate homozygous mutants that lack the target gene. In order to create a disruption cassette, a region at the -1000 of the 5' end of the gene and a region at the +1000 3' end of the gene were amplified and respectively cloned into the *KpnI/XhoI* and *SacII/SacI* sites of pFS2A. A 490 bp fragment from the 5' end of the *C. albicans* *MET4* gene was amplified using the primers pair MetKF and MetXR. This fragment was then digested with *KpnI* and *XhoI* and cloned in the vector pBluescript II KS [-] (Norrandar *et al.*, 1983). At the 3' end of the gene a 500 bp fragment was amplified using the primer pair MetSIIF and MetSIR (Table 2.4), (Fig. 4.10) and this was digested with *SacI* *SacII* and cloned into the vector pBluescript II KS [-]. Both fragments were subcloned from pBluescript II KS [-] into the *KpnI/XhoI* and *SacII/SacI* sites flanking the *SAT1* gene in pSFS2A (Reuss *et al.*, 2004) to generate the plasmid pMet4KXS. The *C. albicans* strain SC5314 was transformed with the *KpnI/SacI* fragment from pMet4KXS. In order to detect the correct integration of the

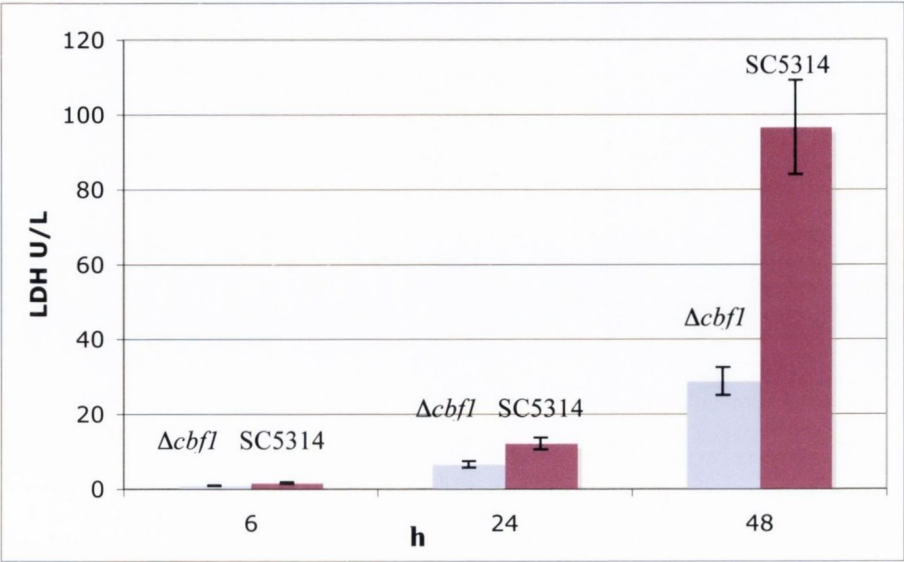
cassette at the *CaMET4* locus, nourseothricin resistant transformants were screened using PCR with specific primers, one on the integrative plasmid (FLP1) the other on the upstream flanking chromosomal DNA region of the *MET4* gene (Metup) (Fig. 4.10, Table 2.4). Fifteen positive transformants were identified. In order to disrupt the second copy of the *MET4* gene excision of the cassette was induced by expressing the FLP recombinase in the presence of maltose. The *FLP* gene allows the excision of the cassette from the genome and confers again to the single-knock out sensitivity to nourseothricin. In order to achieve this positive transformants were grown in yeast peptone maltose YPM overnight at 30°C so the *MAL2* promoter would induce FLP expression. Putative revertants were spread onto plates with a drug concentration of 10 µg/ml which allowed for nourseothricin sensitive revertants to be identified as small colonies. A second round of transformation was carried out to disrupt the second allele of the gene. Five putative revertants were retransformed with the construct and a series of colonies resulting from these transformations were screened by PCR using the primers MetKF and MetSIR to identify mutant lacking the wild type gene. Five independent transformants that were missing both copies of the wild type gene were identified. A second round of excision was carried out to flip out the cassette integrated in the second copy of the gene. A southern blot analysis was carried out on 3 independent transformants to verify the gene disruption (Fig. 4.11).

4.2.5. Phenotypic analysis of the $\Delta CaMet4$

In *S. cerevisiae* the *MET4* transcriptional activator has a role in methionine biosynthesis, glutathione and S-adenosylmethionine biosynthesis and protection against various stresses. To evaluate the role in *C. albicans* virulence of the transcriptional activator CaMet4, the *C. albicans* double disruptant for the transcriptional activator was subjected to a variety of phenotypic tests to evaluate its role in methionine biosynthesis, in the ability to use different carbon sources and in the protection against oxidative stress.

Fig. 4.8

A



B

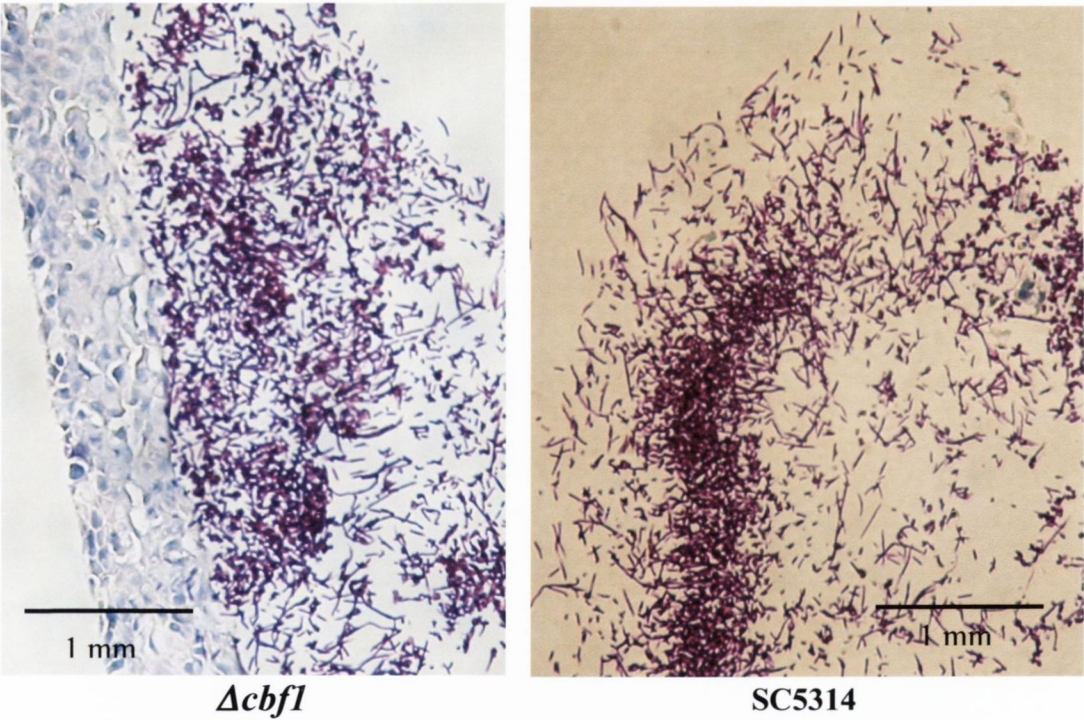
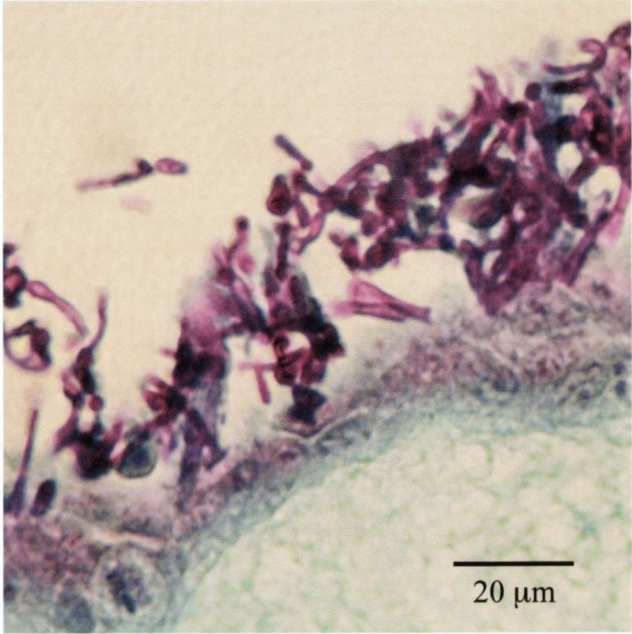


Fig. 4.9 Damage to the buccal epithelial cells TR146 by *C. albicans* SC5314 and the $\Delta cbf1$ mutant.

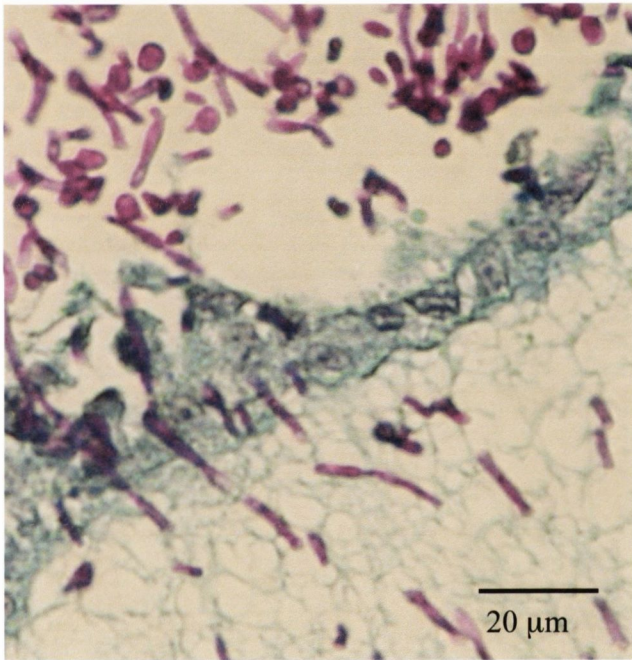
A, The damage that the mutant and the wild type strain conferred to the epithelium was measured using the Promega Citotox® colorimetric assay as a means of measuring the LDH activity at 6, 24 and 48 h time points. The wild type (pink) causes more epithelial damage than the $\Delta cbf1$ mutant (blue).

B, Histological examination of the epithelium following 40 h incubation with the $\Delta cbf1$ mutant or the wild type strain SC5314 (X 200 magnification).

Fig. 4.9



$\Delta cbf1$



SC5314

Fig. 4.9 Histological examination of A 431 vaginal epithelial cells upon infection with *C. albicans* $\Delta cbf1$ and SC5314 strains.

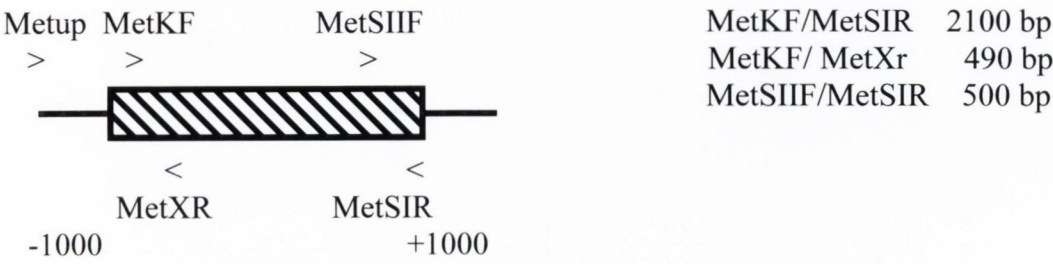
Epithelial cells were inoculated with either the $\Delta cbf1$ mutant or the SC5314 strain, and histological examination was carried out following 40 h incubation (X 400 magnification).

Fig. 4.10

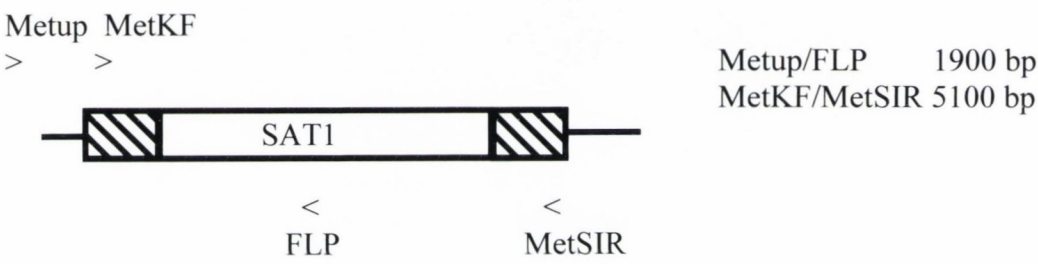
Genotype

Primer pair

WT Met4



MET4::SAT



METΔ::FRT



Fig. 4.10 Schematic diagram of PCR-based strategy to generate allelic genotypes of *MET4* transformants.
Primer pairs used to detect the genotypes of the different transformants and the expected PCR size are shown above.

Fig. 4.11

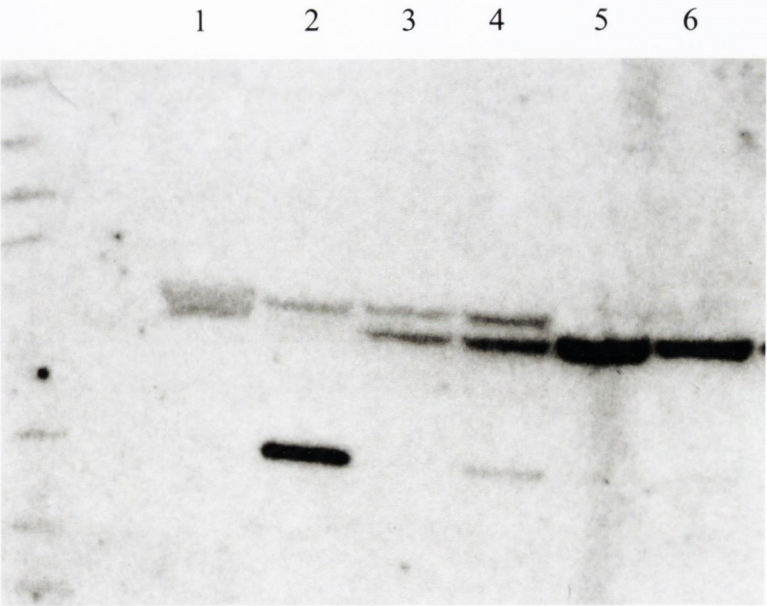


Fig. 4.11 Southern blot analysis showing the disruption of the *MET4* gene

The genomic DNA of the wild type *C. albicans* SC5314 (WT) and derivative strains were digested with the enzymes *Bam*HI and *Hind*III. Digestion products separated by electrophoresis through a 0.8 (w/v) agarose gel, transferred to a nylon membrane and hybridised with a radiolabelled probe. The radiolabelled probe was generated by random primer labelling on a PCR amplified fragment using the primers MetKF and MetXR (Table 2.4).

Lane 1, the *MET4/MET4* wild type strain (WT). Lane 2, Transformant 1; *MET4/met4::SAT1*. Lane 3, Transformant 1; *MET4/met4Δ*. Lane 4, Transformant 2; *MET4/met4Δ*. Lane 5, Transformant 1 *met4Δ/met4Δ*. Lane 6, Transformant 2 *met4Δ/met4Δ*.

4.2.5.1. The effect of deleting *CaMet4* on *C. albicans* growth

Given the role of Met4 in the biosynthesis of sulphur-containing amino acids in *S. cerevisiae* we expected that deletion of the *MET4* gene in *C. albicans* would result in methionine auxotrophy. In order to test this, the Δ *Camet4* was grown on solid and liquid media with ammonium sulphate as the sole source of sulphate (i.e. YNB w/o a.a. (pH 7)) containing ammonium sulphate 5 g/L (w/v) and 2% glucose (w/v)). No difference in growth was observed between the wild type and the Δ *Camet4* on either solid or liquid media. This suggests that the Δ *Camet4* mutant is able to assimilate ammonium sulphate for sulphur containing amino acid synthesis therefore this gene is not required for methionine biosynthesis. In order to test if deletion of the *CaMET4* gene has an effect on the assimilation of carbohydrate or phosphorus and sulphur-containing compounds, we compared the growth of the mutant and the wild type strain in the Biolog phenotype MicroArray system.

Each well of the array in the sulphur and carbon assimilation plate is designed to test a different auxotrophy possessing a different sulphur compound from which sulphur amino acids can be synthesised or a different carbon source or phosphate that can be used for growth. PMs use a redox chemistry that employs cell respiration as a universal reporter. The reduction of a tetrazolium dye results in the change in colour of the medium in the well that can be directly related to growth rate. Exponentially growing cells of the wild type and the Δ *CaMet4* mutant from an overnight culture in YPD were washed in PBS, diluted to O.D.₆₀₀ = 0.1 and resuspended respectively in PM2 medium for the carbon assimilation test and in PM4 for sulphur assimilation tests, 0.25 ml of this solution was inoculated in every well of the array plate. The absorbance at 595 nm was measured. No differences were found in the ability of the wild type and the Δ *met4* mutant to grow in the different carbon sulphur and phosphorus sources.

4.2.5.2. Effect of deleting *CaMET4* on stress tolerance

To investigate the possible role of Met4 in glutathione biosynthesis and its possible role in defence against oxidative stress and heavy metal stresses the $\Delta met4$ mutant was tested, in comparison with the wild type, in a range of stress tolerance tests. Exponentially growing cells of the wild type SC5314 and the $\Delta met4$ mutant strains were diluted to a concentration of 1×10^8 cells/ml and serial 1:10 dilutions of the strains were made and spotted on YEPD plates supplemented with LiCl, NaCl, ZnCl, MgCl₂, CdCl₂, calcofluor, caffeine, congo red, menadione, and the plates incubated at 37°C for 3 days. No difference was observed in the ability of the mutant and wild type strains to tolerate the stresses resulting from these compounds.

The ability of the mutant to survive in macrophages was also tested. The endpoint dilution assay (EDA) was used to quantify the survival of the wild type and the mutant in the presence of mouse macrophages. Murine RAW264.7 macrophages were seeded at a concentration of 1.5×10^6 cells/ml in the wells of a 96 well plate. Macrophages were cultured overnight at 37°C in the presence of 5% (v/v) CO₂. Several 10-fold dilutions of a suspension 2×10^6 cells/ml of the *C. albicans* wild type and the $\Delta met4$ mutant were added to the monolayer. Plates were then incubated at 37°C in the presence of 5% (v/v) CO₂ for 48h. Following incubation plates were stained with crystal violet and the colonies formed were counted. No differences in the survival of the wild type and mutant strains were observed.

4.3. Discussion

4.3.1. The role of the transcriptional activators *CBF1* and *MET4* in *C. albicans* virulence

Upon comparison of the genes that encode the transporters and enzymes involved in the uptake and biosynthesis of methionine in *C. albicans* with those of the well-characterised budding yeast *S. cerevisiae*, it was observed that all yeast genes had a homologue in the *C. albicans* genome. This indicates that the biosynthesis of the amino acids methionine and cysteine may be similar between the two species and that the homologous genes may have conserved functions. All the *C. albicans* genes involved in this pathway were found to share homology with their counterparts in *C.*

dubliniensis. Interestingly however, the genes encoding the homologues of the *S. cerevisiae* transcriptional regulators *CBF1* and *MET4* of this pathway were found to exhibit a higher level of divergence than other genes in the two related species. We decided to study these two genes further because we hypothesised that this divergence could be responsible for differences in their functions (e.g. the degree of conservation in the *CBF1* oligomerisation domain is exceptionally low in the two closely related species suggesting that the two homologues may have species-specific roles (i.e. interaction with different transcriptional activators or formation of specific oligomers) in *C. albicans* and *C. dubliniensis*). However heterologous expression of the *C. albicans* *CBF1* gene in *C. dubliniensis* did not have any observed effect on the phenotype of this species, having no effect on hyphae or chlamydospore production in media containing low methionine in *C. dubliniensis*. This suggests two possibilities, firstly that the *C. dubliniensis* gene is fully-functional and the expression of the *C. albicans* gene in this species does not complement any defect or, secondly, that the degree of divergence in the oligomerisation domain between the two homologues is so high that the *C. albicans* transcriptional activator can not carry out all its original functions in this species (e.g. the inability of the CaCbf1 to form heterodimers with other *C. dubliniensis* proteins).

It was hypothesised that the co-factor-transcriptional activator Cbf1 could play an important role in *Candida*, as it does in *S. cerevisiae*. To investigate this, the phenotype of *C. albicans* wild type and the phenotype of the *CBF1* double disruptant were compared. The ability of the $\Delta cbf1$ and the wild type strain SC5314 to produce hyphae was compared. In the majority of hyphae-inducing media there was no difference in the levels of hyphae produced by the wild type and the knock out strain. However the $\Delta cbf1$ was unable to produce hyphae when grown on BSA agar. The inability of $\Delta cbf1$ to form hyphae in this specific medium may be indicative of a possible relationship between the transcriptional activator and the mechanism involved in the sensing of proteins in the environment. The $\Delta cbf1$ mutant also showed increased sensitivity to oxidative stress indicating that the mutant may have a lower intracellular level of glutathione (that is involved in the first response to reactive oxygen species) due, possibly, to a link between the transcriptional activator and the synthesis of glutathione. The $\Delta cbf1$ mutant was also less able, compared to the wild type, to form biofilm, implicating methionine biosynthesis in this process. In

RHE experiments the $\Delta cbf1$ mutant showed decreased ability to damage epithelial cells. Histological examination showed that when two RHE models (TR146 buccal epithelial cells and A431 vaginal epithelial cells) were infected with the $\Delta cbf1$ mutant it was capable of undergoing the yeast-to-hypha transition upon interaction with the epithelium, although less than the wild type, but was unable to invade the cells and formed a non invasive layer on the surface of the epithelium. These data indicate that *CaCBF1* and sulphur metabolism play a significant role in *C. albicans* virulence.

In order to assess the function of the Met4 transcriptional activator in *C. albicans* we have identified in the *C. albicans* genome an ORF (19.5312) that encodes for a putative protein annotated as Met4. We have found that this *C. albicans* protein shares more than 40% identity with *S. cerevisiae* Met4. Moreover we found that synteny was not conserved around the *S. cerevisiae* *MET4* and the *C. albicans* putative *MET4* loci. We deleted both alleles of this gene in *C. albicans* by targeted mutagenesis and investigated the effect of this inactivation on the phenotype of the resulting mutant. To our surprise the *C. albicans* $\Delta met4$ mutant was not auxotrophic for methionine and was found to be capable of growth in minimal media without methionine. Further analysis of the mutant using the Biolog phenotype MicroArray revealed that there was no difference in the ability of the mutant and wild type strains to grow on a wide range of nitrogen and carbon sources. To investigate the possible role of Met4 in glutathione biosynthesis the $\Delta met4$ mutant was tested, in comparison with the wild type, in a range of stress tolerance tests. However, no difference was observed in the ability of the mutant and wild type strains to tolerate LiCl, NaCl, ZnCl, MgCl₂, CdCl₂, calcofluor, caffeine, congo red or menadione.

In addition the ability of the mutant to survive in co-culture with macrophages was also tested. The Endpoint dilution assay (EDA) was used to quantify the survival of the wild type and the mutant in the presence of mouse macrophages. No difference between the wild type and the $\Delta met4$ mutant were found in their ability to resist the macrophage killing mechanism.

These data suggest two possibilities, the first one is that the role of Met4 in *C. albicans* is very different from its role in *S. cerevisiae* and that transcriptional rewiring has occurred in these pathways. This hypothesis can be supported by the fact that other metabolic pathways also underwent transcriptional rewiring in the

related fungi *S. cerevisiae* and *C. albicans*. One example is the galactose metabolism pathway. The genes encoding these enzymes are syntenically arranged, but the upstream regulatory regions are highly divergent. In *S. cerevisiae*, the Leloir-pathway genes are positively regulated by Gal4p acting through the UAS(G) sequence CGG(N(11))CCG. However, in *C. albicans*, the Gal4p and UAS(G) combination is found to regulate genes unrelated to galactose metabolism (Martchenko *et al.*, 2007; Rokas & Hittinger, 2007). Martchenko *et al.*, (2007) identified a palindromic sequence that controls *GAL10* expression in *C. albicans* in the presence of galactose. This palindrome is found upstream of other Leloir-pathway genes in *C. albicans*, and in the absence of other regulatory sequences, activation of expression through this sequence in the presence of galactose requires Cph1p, the homolog of the Ste12p transcription factor of *S. cerevisiae*. The *S. cerevisiae* transcription factor Ste12 is not related to the galactose pathway but instead controls two distinct developmental programs, mating and filamentation. Although the cellular process of galactose induction of the Leloir pathway is conserved between the two organisms, the regulatory circuits achieving the cellular process are completely distinct (Martchenko *et al.*, 2007). The other possibility is that the *C. albicans* ORF 19.5312 that has been annotated as Met4, and encodes hypothetical protein that has been found to be the most similar protein to the *S. cerevisiae* Met4, is in fact not a homolog of the *S. cerevisiae* *MET4* gene.

Chapter 5

Analysis of *C. albicans* and *C. dubliniensis* chlamydospores

5.1. Introduction

Chlamydospore formation has served for a long time for identification of the human fungal pathogens *C. albicans* and *C. dubliniensis* and also as a distinctive characteristic between the two species, but the biological function of these structures still remains unclear. They have been proposed previously to simply be a dead-end or an *in vitro* growth artefact and not to have any role in pathogenesis because they have only rarely been described in clinical specimens. Previously, attempts have been made to investigate the characteristics of *C. albicans* chlamydospores produced using traditional solid culture media. However, the results of these studies were inconsistent. Jansons and Nickerson described the viability of young (30-40 hours) *C. albicans* chlamydospores based on the observation of budding (Jansons & Nickerson, 1970a). Although for some other authors (Martin *et al.*, 2005) at this stage a chlamydospore can not be considered a structure distinct from a suspensor cell and the true viable cell under these circumstances is the suspensor cell not the chlamydospore. In contrast Bakerspigel & Burke were unable to induce germination of chlamydospores leading them to propose that these structures have a role as storage cells (Bakerspigel & Burke, 1974).

After the discovery of the ability of *C. albicans* and *C. dubliniensis* to undergo mating new interest was shown by researchers in these intriguing structures with the suggestion that they could be involved in a parasexual (or possibly sexual) cycle of the species (Bennett & Johnson, 2005). Rustad *et al.* assayed various *C. albicans* laboratory strains containing disruptions of either the entire *MTL* locus or specific genes within the locus for their ability to form chlamydospores. They also tested clinical strains that were homozygous for one of the two *MTL* loci. They found that no change in chlamydospore formation was seen in these strains compared to the standard laboratory strain, demonstrating that chlamydosporulation was unlikely to be mating-type specific (Rustad *et al.*, 2006).

In order to fully investigate the biology of chlamydospores it is first of all necessary to be able to purify them in sufficient numbers from their associated suspensor cells and hyphae and pseudohyphae. In the past, using chlamydospores cultured on solid media, attempts were made to separate chlamydospores from mycelial cells using sulphuric acid treatment (Vidotto *et al.*, 1988), sonication and enzymatic treatment with β -glucuronidase (Fabry *et al.*, 2003). These methods

allowed reasonable levels of purity to be achieved, however, the density of chlamydospores in the starting cultures was low and the purification process may have lead to damage to the chlamydospore wall (Vidotto *et al.*, 1988; Gunasekaran & Hughes, 1978; Simonetti & Strippoli, 1971). The ability to harvest high yields of chlamydospores from liquid culture would greatly facilitate the purification and analysis of these poorly studied structures. To date only one study has observed this, Staib & Morschhauser (2005b) recently described a rapid method to produce chlamydospores in complex liquid Staib and sunflower medium.

The purpose of the current study was to develop defined culture conditions to generate high yields of chlamydospores to facilitate their purification and subsequent analysis.

5.2. Results

5.2.1. A defined liquid medium for abundant, species-specific chlamydosporulation in *C. dubliniensis*

In a previous chapter (see chapter 3) it was shown that when *C. dubliniensis* was cultured under very specific conditions (i.e. on solid media containing low concentrations of glucose and methionine) large numbers of chlamydospores were produced. In order to investigate these cells further, experiments were designed to improve their production and to attempt to purify them. Due to the nature of the colonial morphology of *C. dubliniensis* when grown on solid media that induce filamentation (e.g. agar invasion by the hyphae and pseudohyphae to which the chlamydospores are attached), agar-containing media are not ideal for these purposes. In order to determine if high levels of chlamydospores could be produced in liquid medium, a defined liquid medium comprising YNB (without amino acids) supplemented with 0.025% (w/v) glucose or galactose and (20 mg/L) methionine at pH 3.7 was tested. When *C. dubliniensis* CD36 was incubated in this medium at room temperature without shaking for 3-4 days in 50 ml of medium in 100 ml conical flasks sealed with parafilm (or Petri dishes), copious chlamydospores were produced. Under these conditions the chlamydospore/ yeast cell ratio was approximately 80-90 chlamydospores per yeast cell, (counted directly on the pseudohyphae). In this medium 39 of 40 *C. dubliniensis* isolates produced

chlamydospores, while none of the *C. albicans* strains tested were able to produce chlamydospores under these conditions. Increasing the concentration of galactose or glucose added to the medium resulted in a reduction in the chlamydospore/ yeast cells ratio due to a general increase in biomass due to increased growth of blastospores and pseudohyphae. Inocula were made from *C. dubliniensis* blastospores incubated on YPD agar for 24–48 h at 37°C. Plates were maintained at room temperature for 6–12 h, and a single colony then inoculated into 10 mL of liquid YPD and incubated with shaking at 37°C for 2 h. One microlitre (c. 10^2 cells) of this culture was then inoculated into 50 mL of the chlamydospore-induction medium contained in a parafilm-sealed 100-mL conical flask or Petri dish (Fig. 5.1 A, B). Increasing the size of the inoculum resulted in a reduction in the level of chlamydospore formation.

5.2.2. Media for increased *C. albicans* chlamydosporulation

Chlamydospore production is routinely induced in *C. albicans* by incubation on solid corn meal agar; however, the yield of chlamydospores under these conditions is relatively low. In order to maximize chlamydospore production by *C. albicans*, corn meal agar and a liquid formulation of this medium were supplemented with 1% Tween 80 (v/v), 0.025% (w/v) galactose and 20 mg/L (w/v) methionine. This resulted in a 20-fold increase in chlamydospore production (Fig. 5.1 C, D).

However in the liquid formulation of the medium significant production of true hyphae was also present. In order to obtain higher levels of chlamydospore production, *C. albicans* was also cultured on solid corn meal agar, 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg/l (w/v) methionine plates between two BioRad polycarbonate membranes with pores of 0.2 μ m in diameter, positioned at the top of the agar. Further addition of 0.025% (v/v) YNB w/o a.a., 0.05% (v/v) Tomato juice (Oxoid) and 10 mM farnesol was used to increase chlamydospore production in some *C. albicans* clinical isolates. With the use of these nutrient permeable sheets it was possible to stimulate the production of higher numbers of chlamydospores due to contact with a solid surface and the microaerophilic conditions. In addition, the use of these sheets prevented invasion of the agar by the hyphae and allowed easy transfer of the colonies into new culture media.

5.2.3. Evidence of metabolic activity in mature chlamydospores

It has been demonstrated from previous studies (Martin *et al.*, 2005) that after a chlamydospore is formed it has to remain attached to the mycelium for a time sufficient for the transportation of glycogen, other cellular material and for the migration of the genetic material before it can be considered a structure distinct from the suspensor cell. The nuclear division process that was found to occur inside the suspensor cells and the successive nuclear migration into the chlamydospore takes approximately 4-5 days (Martin *et al.*, 2005). Therefore after 5 days a chlamydospore can be considered mature. One marker for the metabolic activity of a cell is staining with the vital dye FUN-1. In metabolically active cells, cylindrical intravacuolar structures (CIVS) are produced after less than 1 hour exposure to the dye. These structures are orange-red when excited at 470-590 nm. Formation of CIVS requires both plasma membrane integrity and the metabolic capacity of viable cells. A cell showing a green diffuse cytoplasmic colouration has low or no metabolic activity. In order to investigate the metabolic activity of chlamydospores, liquid-grown *C. albicans* SC5314 and *C. dubliniensis* CD36 chlamydospore-rich mycelia were stained with the fluorescent probe FUN-1 every 24 h over a 30-day period. A 5 day old *C. albicans* chlamydospore rich culture stained with FUN-1 dye showed that mature chlamydospores (i.e. 5 to 10 days old chlamydospores) are metabolically active cells but, in contrast to yeast cells, their metabolism is seen to decrease over time. Staining with FUN-1 of the same culture after 14 days of growth showed that these chlamydospores had lower metabolic activity than 5 day old ones (Fig. 5.2) and by 30 days metabolic activity was undetectable (Fig. 5.3). In contrast, yeast cells do not exhibit a reduction in activity during the same time period. Identical results were obtained with *C. dubliniensis* chlamydospores and yeast cells (data not shown).

5.2.4. Induction of chlamydospore chain growth

Metabolically-active chlamydospores (5/15 day old chlamydospores) can be induced to produce other chlamydospores by refreshing the medium with new chlamydospore inducing medium and incubating at room temperature under microaerophilic conditions without shaking. Mycelia grown in these conditions were rich in chlamydospores, with chlamydospores appearing to generate daughter

Fig. 5.1

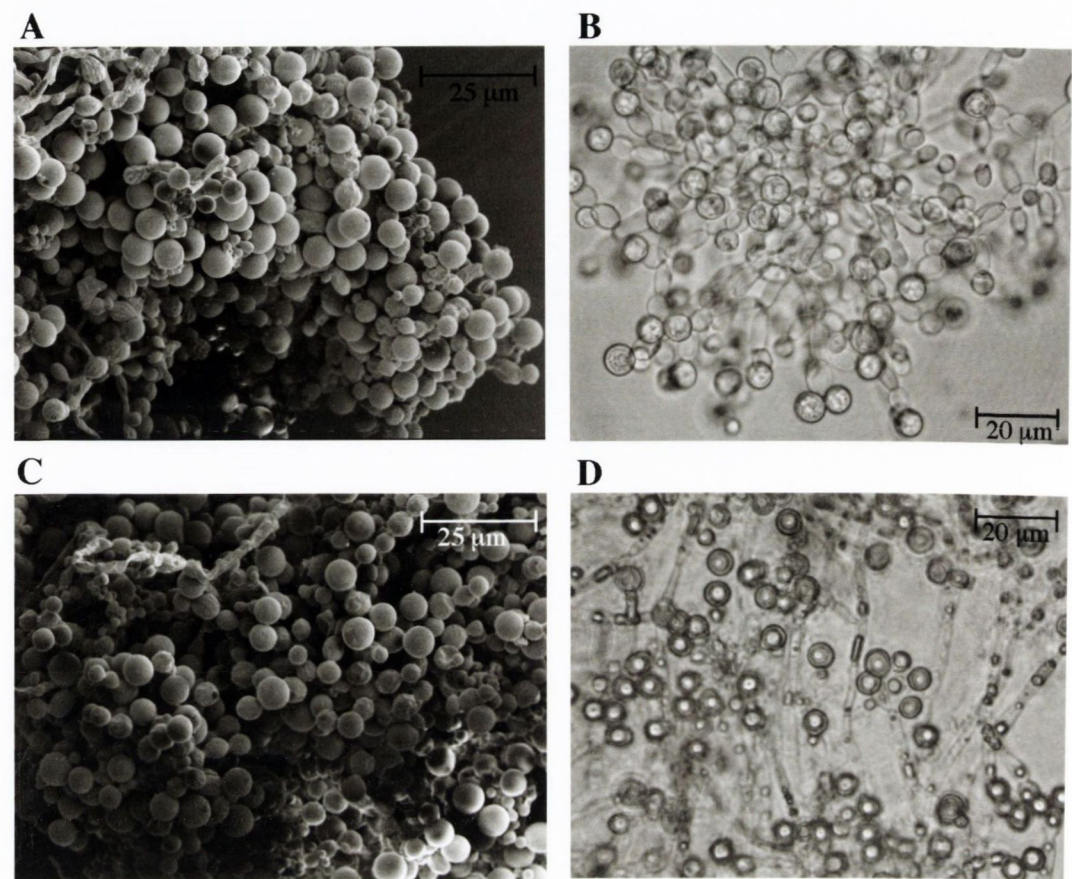


Fig. 5.1 Production of chlamydospores by *C. albicans* and *C. dubliniensis* in defined liquid media.

Scanning electron micrographs (A and C) and light micrographs (B and C) of *C. dubliniensis* CD36 (A and B) following incubation in YNB supplemented with 20mg/L methionine and 0.025%(w/v) galactose and a *C. albicans* clinical isolate (CAY5292; this study) (c and d) incubated in corn meal broth supplemented with 1%(v/v) Tween 80, 0.025% (w/v) galactose and 20 mg/L methionine for 5 days.

Fig. 5.2

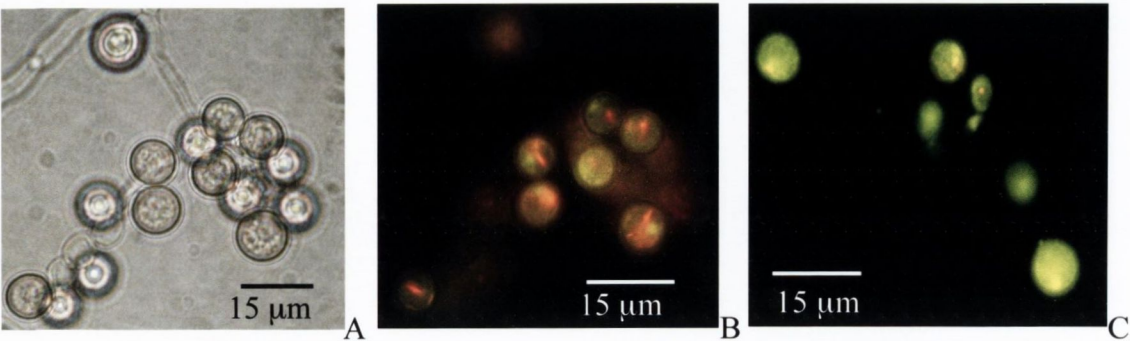


Fig. 5.2 Metabolic activity of chlamydospores detected with FUN-1.

Candida albicans SC5314 chlamydospore-rich mycelium produced following incubation in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 25 mg L⁻¹ methionine for 5 (A and B) and 16 (C) days. Chlamydospores were observed by bright-field microscopy (A) and by fluorescence microscopy following staining with FUN-1 (B and C) (x 1000 magnification).

Fig. 5.3

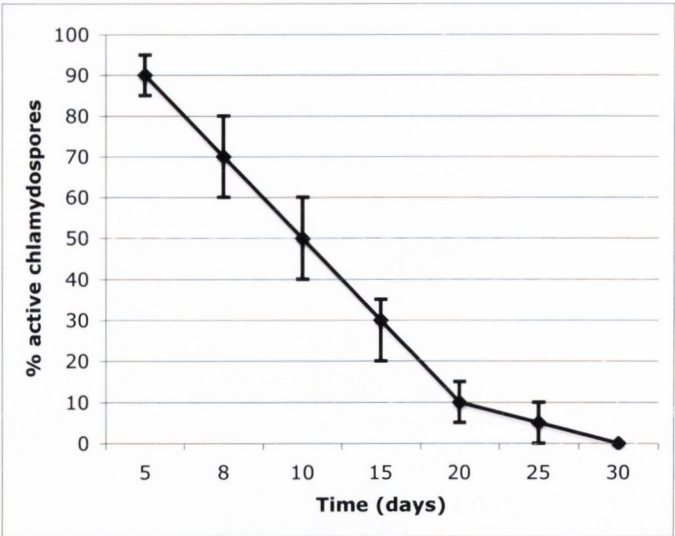


Fig. 5.3 Viability of *C. albicans* SC5314 mycelium attached chlamydospores overtime In order to investigate the metabolic activity of chlamydospores, liquid-grown *C. albicans* SC5314 chlamydospore-rich mycelia were stained with the fluorescent probe FUN-1 every 24 h over a 30-day period.

chlamydospores that grew atypically, forming chains in a process similar to budding. Chains and clumps of chlamydospores, characterised by the absence of hyphae or blastospores were frequently produced (Fig. 5.4 A, B). Transmission electron micrograph showed that the outer walls of two adjacent chlamydospores in a chain are contiguous (Fig. 5.4, C).

5.2.5. Purification of chlamydospores

When *C. albicans* and *C. dubliniensis* mycelia are grown in liquid or solid chlamydospore induction medium, there are varying proportions of yeast, hyphae, pseudohyphae and suspensor cells chlamydospores present. In order to purify the chlamydospores it is necessary to selectively remove the other cell types. This is facilitated by the thickness of the chlamydospore cell wall which in mature (5-7 day old) chlamydospores is around 0.5 to 0.9 μm , reaching a thickness of 1.3 to 1.5 μm in aging chlamydospores (14-20 day old), compared to yeast cells whose walls are approx. 0.1, 0.2 μm thick. On solid media an additional problem is the agar invasion of the hyphae and pseudohyphae to which chlamydospores are attached. In order to purify chlamydospores from solid media a method based on the use of BioRad filter sheets has been developed (with this method chlamydospore-rich mycelia can also be easily transferred to different solid media to study the chlamydospore response to the shift in environmental conditions). Chlamydospore-rich mycelia growing between the nutrient-permeable filters can not invade the agar and can be easily separated from the filters, by washing them off, and collected for further purification from the mycelial component.

Chlamydospores can be separated from other cell types by sonication, however a higher degree of purity was achieved using a zymolyase treatment prior to sonication. Due to their resistance to the zymolyase treatment chlamydospores can be isolated from the cells to which they are connected with the selective disruption of the mycelial component. Transmission electron microscopy of purified chlamydospores showed no corruption of the membrane due to the treatment (Fig. 5.5). Staining with FUN-1 indicated the presence of metabolic activity in around 40% of the purified chlamydospores. (Fig. 5.6).

5.2.6. Viability of chlamydospores: reactivation, Budding and germination

When 6–15-day-old purified or mycelium-attached chlamydospores with low metabolic activity were incubated in nutrient-rich medium (e.g. YNB with a.a. and 2% (w/v) glucose, YPD or DMEM) and incubated at 37°C, metabolic activity (detected using FUN-1 staining) was observed to increase after 6–12 h (Fig. 5.7) and the chlamydospores were observed to bud, producing nonrefractile, yeast-sized cells. Scanning electron micrographs showed the tight association between the mother chlamydospore and the daughter cell (Fig. 5.8 A, B). Transmission electron micrographs showed that the mother chlamydospore had a thick double wall while the daughter cell had a single wall typical of yeast cells (Fig. 5.8 C, D). To prove that the chlamydospore was the cell generating daughter yeast cells and not vice-versa the budding process was analyzed by time lapse photography (Fig. 5.9). The daughter yeast cells were metabolically active (detected by FUN-1 staining Fig. 5.10), contained genetic material (detected using DAPI staining see paragraph 5.2.8) and were capable of replication, producing a population of free blastospores (data not shown).

When mature chlamydospores were incubated in YPD or DMEM medium supplemented with serum (10% v/v), they were observed to produce germ tube-like structures (Fig. 5.11). Time-lapse photography was carried out to follow the development of the germ tubes from chlamydospores. It was found that when the experiment was performed in the presence of DMEM supplemented with 10% (v/v) human serum at room temperature the germ tubes led to the development of pseudohyphae (Fig. 5.12). When the same experiment was performed in the presence of 5% CO₂ (v/v), the germ tubes resulted in the formation of structures resembling true hyphae (Fig. 5.13). When *C. albicans* was incubated on solid chlamydospore induction medium for 6 days between two filter sheets and then transferred onto solid DMEM supplemented with 10% (v/v) human serum and incubated for 15–20 h at 37°C in the presence of 5% CO₂ (v/v) chlamydospores were observed to produce true hyphae (Fig. 5.14). The germination of mycelium attached chlamydospores was more rapid and more effective than the germination of the purified chlamydospores. Thirty percent of 5-day-old (decreasing to 5–10% of 15-day-old) mycelium-attached chlamydospores were found to bud or germinate, while only around 5% of purified chlamydospores of the same age were observed to bud or germinate.

Fig. 5.4

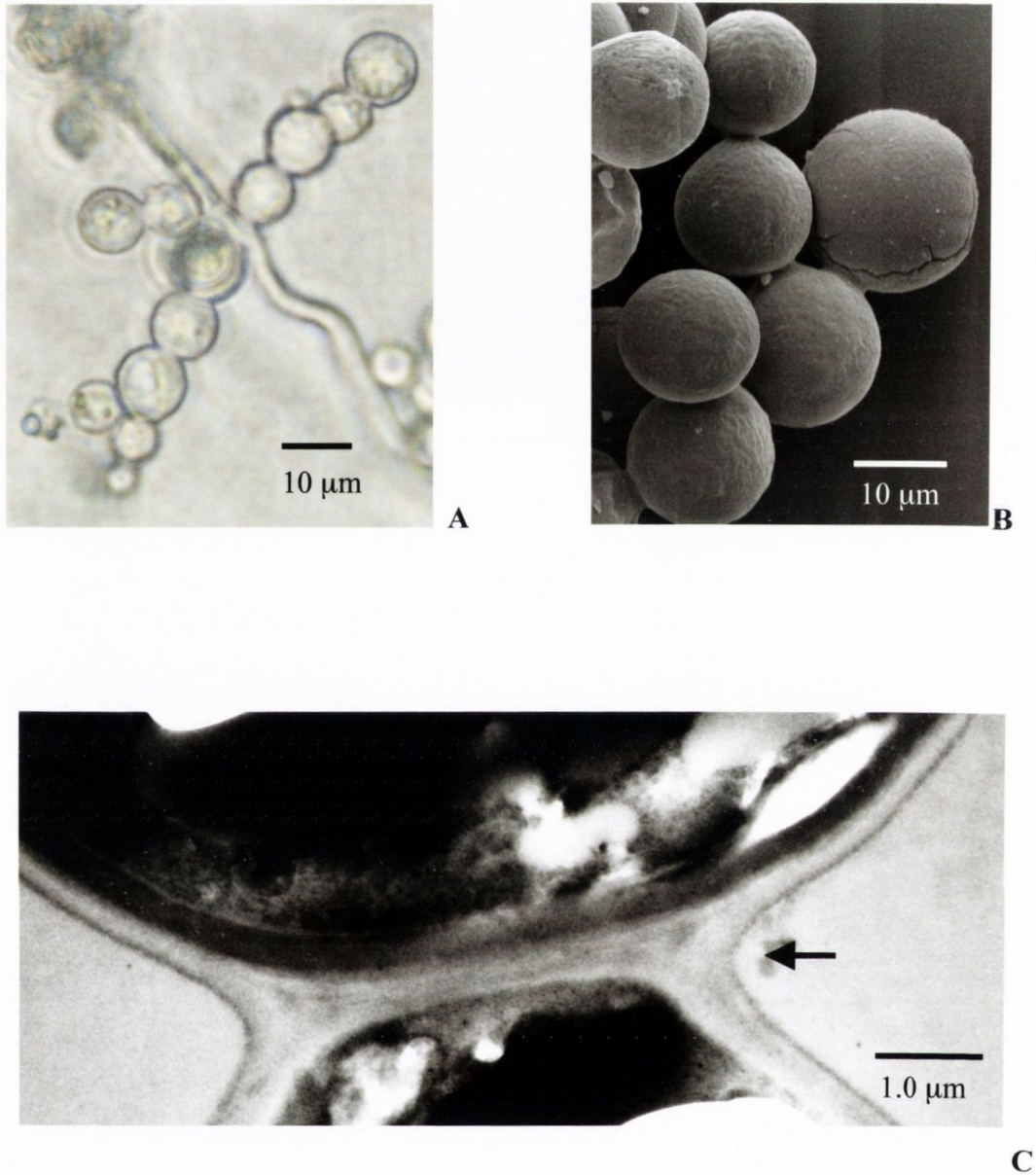


Fig. 5.4 Chlamydospore chains.

Fifteen-day-old *C. albicans* SC5314 cultures were induced to generate daughter chlamydospores by refreshment of the induction medium every 6 days. (A) Light micrograph (X 400 magnification) shows chains of chlamydospores, (B) Scanning electron micrograph shows a clump of attached chlamydospores, (C) Transmission electron microscopy showing in particular the tight association of two chlamydospores in a chain.

Fig. 5.5

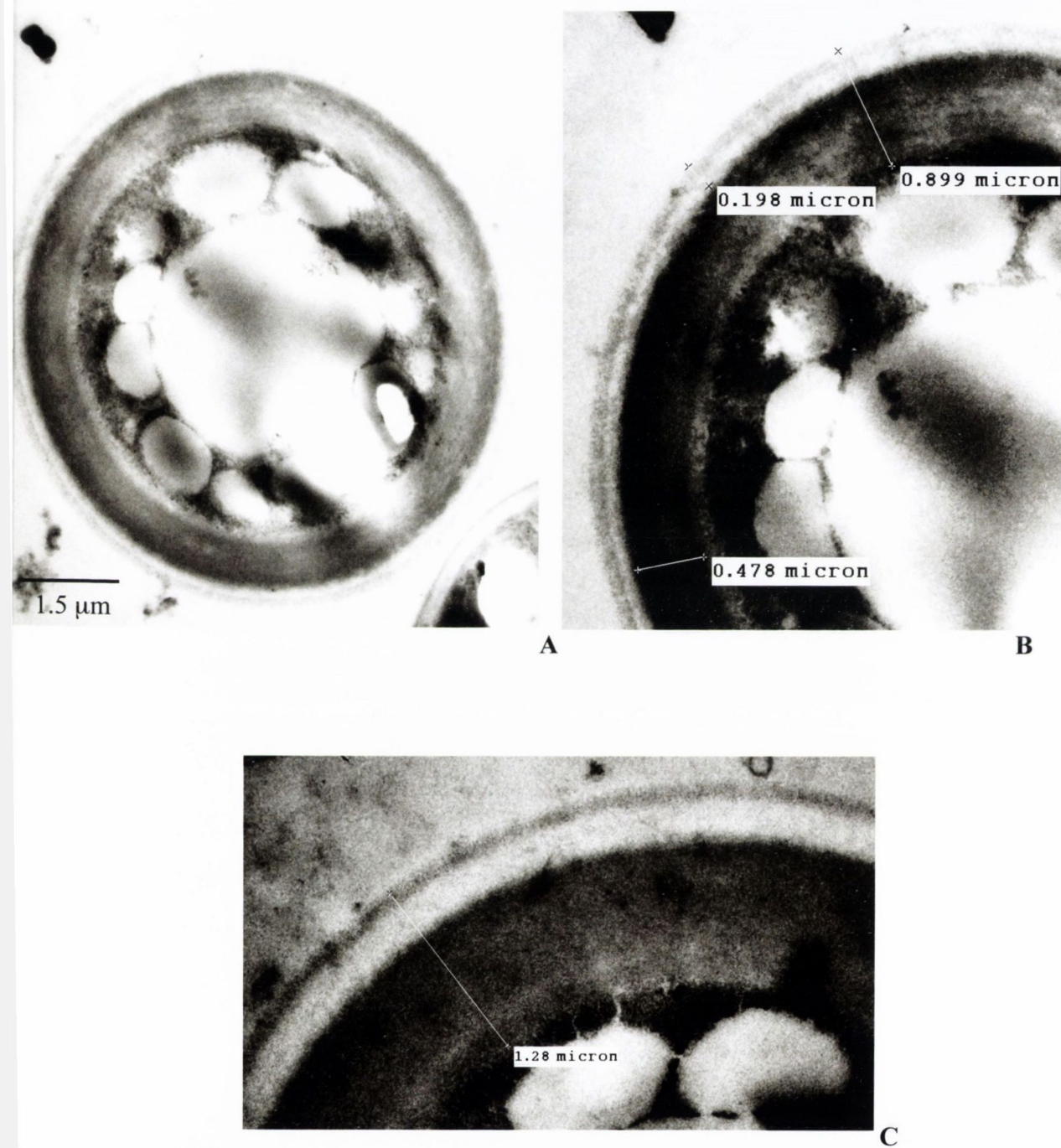


Fig. 5.5 Appearance of single purified *C. albicans* SC5314 chlamydospores analyzed by transmission microscopy.

A, Individual 7 day old chlamydospore. B, magnification of the cell in panel A showing the thickness of various layers evident in the chlamydospore cell wall. C, thickness of the wall of a 14 day old purified chlamydospore.

Fig. 5.6

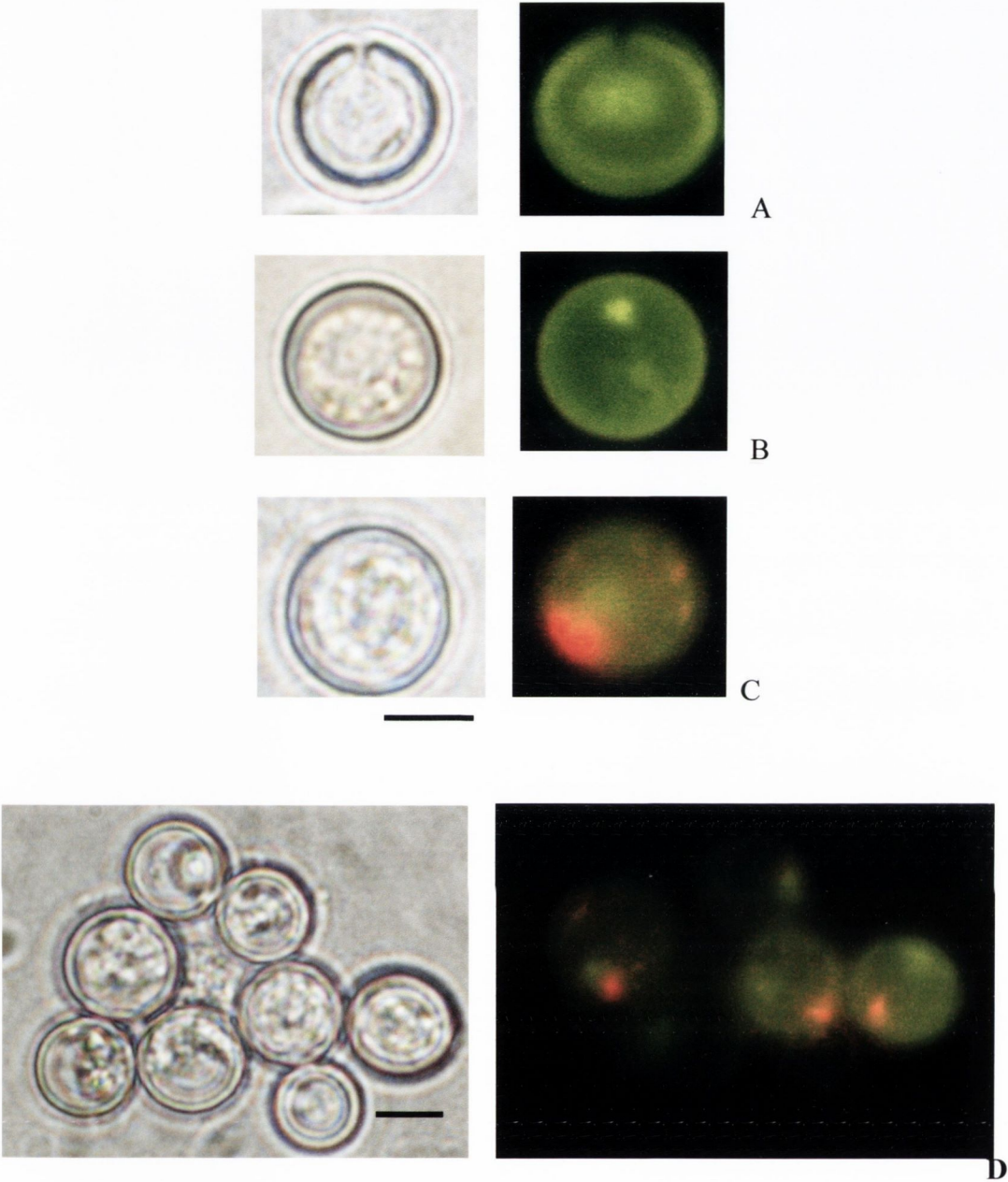


Fig. 5.6 FUN-1 staining of purified chlamydospores.

Purified 6-day-old *C. albicans* SC5314 chlamydospores were observed by bright field and fluorescence microscopy following staining with FUN-1. The majority of chlamydospores (approx 90%) appear intact following purification (B, C, D), less than 10% appeared to be damaged (A) (size bar 5 μm). Staining with FUN-1 indicates metabolic activity in more than 40% of chlamydospores (C,D), (size bar 10 μm).

Fig. 5.7

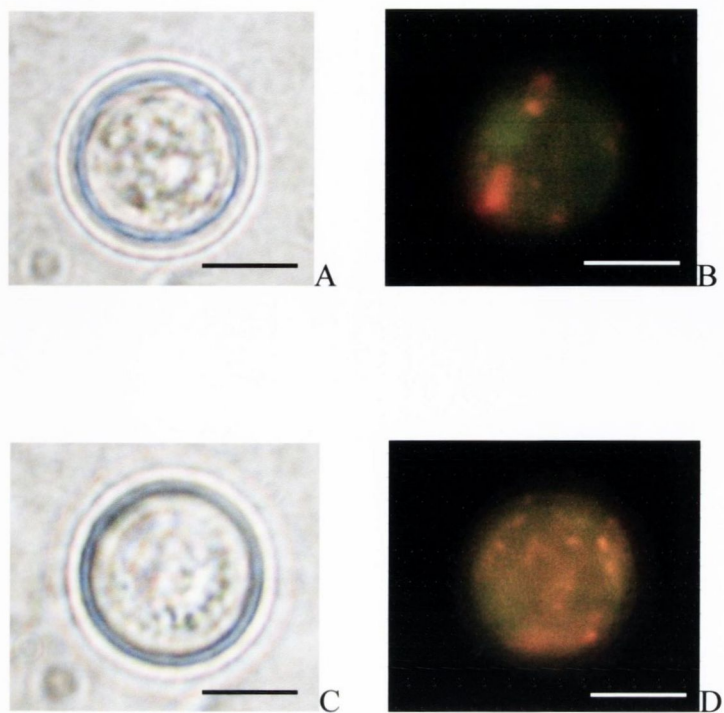


Fig. 5.7 FUN-1 staining of purified chlamydospores upon exposure to a rich environment.

Purified 6-day-old *C. albicans* SC5314 chlamydospores were observed by bright field (A, C), and fluorescence microscopy (B, D) following staining with FUN-1, upon incubation in DMEM for B, 0 h, and D, 6 h (size bar 5 μ m, X 1000 magnification).

Fig. 5.8

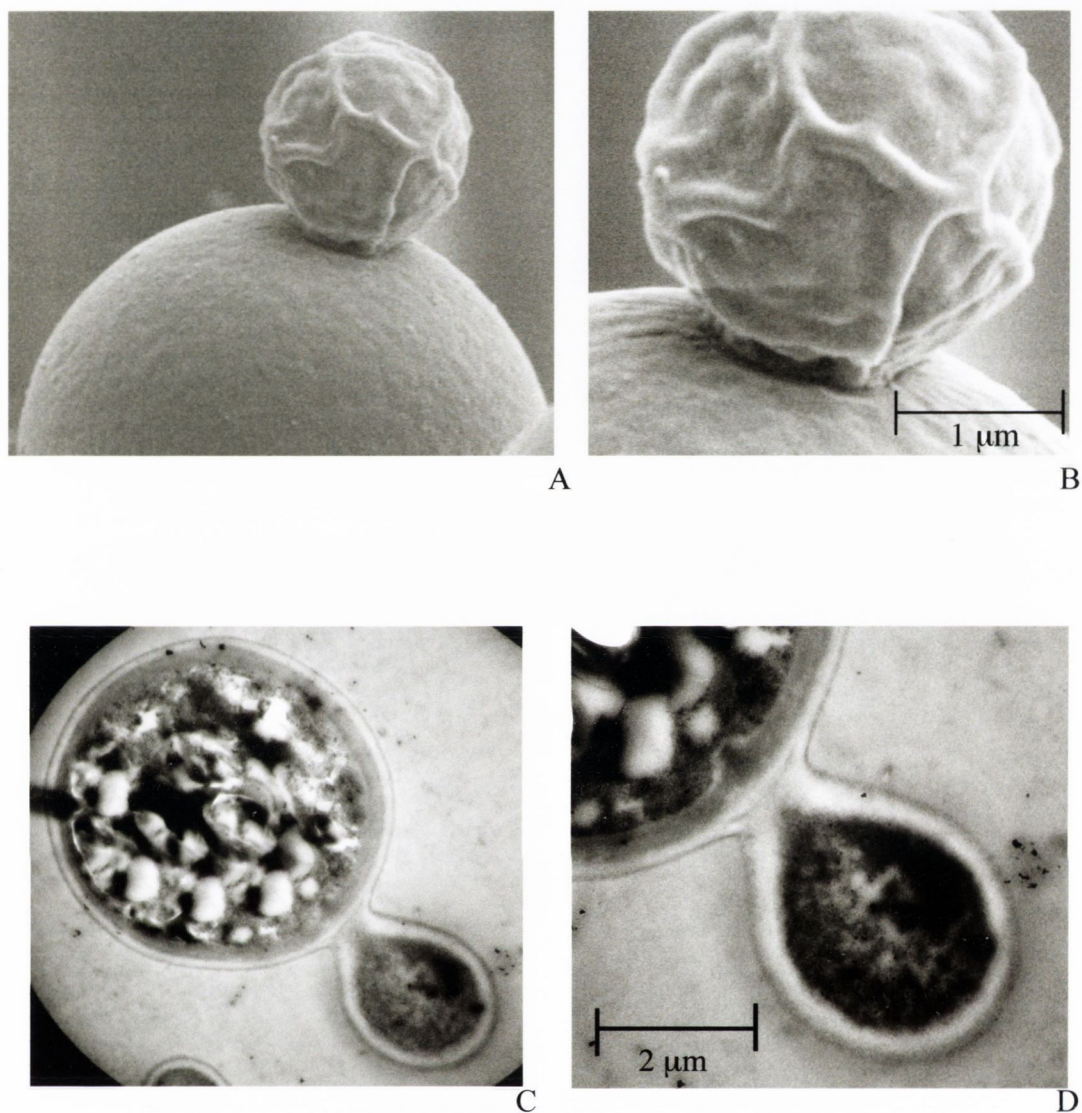


Fig. 5.8 Budding of chlamydospores.

When mycelium attached (A, B) or purified (C, D) 5-day-old *C. albicans* SC5314 chlamydospores were incubated in nutrient-rich medium supplemented with 10% (v/v) human serum, after 6–12 h the chlamydospores were observed to bud, producing yeast-like cells. (A, B) scanning electron micrograph. (C, D) transmission electron micrograph. (B and D show a higher magnification of respectively A and C).

Fig. 5.9

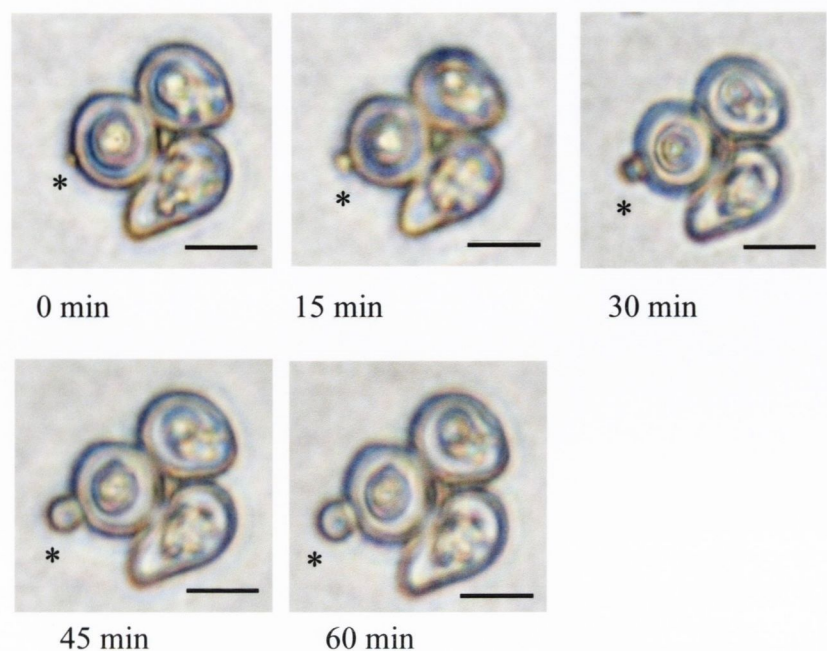


Fig. 5.9 Time-lapse photography of a budding *C. albicans* chlamydospore. Light micrographs showing the production of a bud (indicated by and asterisk) by a *C. albicans* SC5314 purified chlamydospore, triggered to germinate at room temperature following reactivation at 37°C in the presence of DMEM (size bar 5 μ m, X 400 magnification).

Fig. 5.10

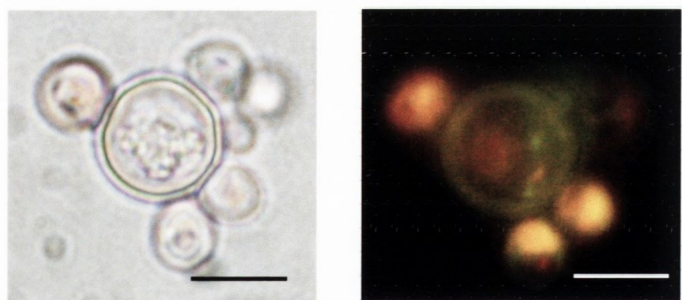


Fig. 5.10 FUN-1 staining of purified chlamydospores bearing primary buds. Purified 7-day-old budding *C. albicans* SC5314 chlamydospores were observed by bright field (A), and fluorescence microscopy following staining with FUN-1 (B) after incubation at 37°C in the presence of DMEM (size bar 5 μ m, X 1000 magnification).

Fig. 5.11

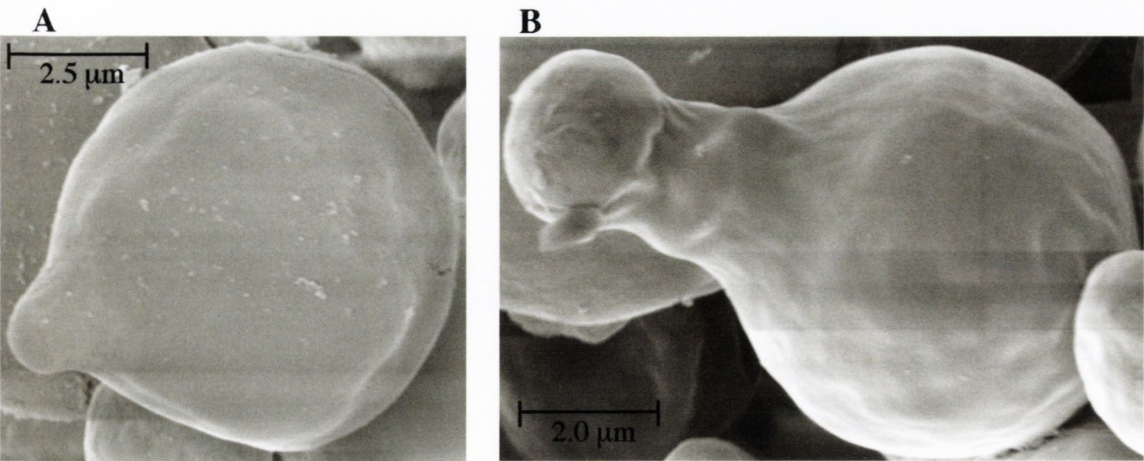


Fig. 5.11 Scanning electron micrographs of chlamydospores induced to form germ-tube-like structures. *C. albicans* SC5314 chlamydospores were incubated at 37°C in the presence of DMEM supplemented with 10% (v/v) human serum for (A) 60 min and (B) 90 min.

Fig. 5.12

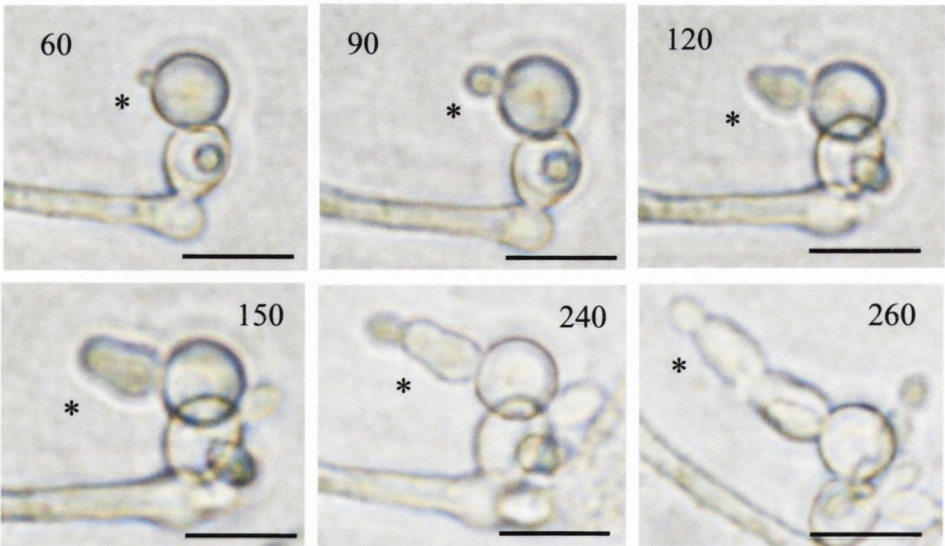


Fig. 5.12 Time-lapse photography of a germinating *C. albicans* chlamydospore. Light micrographs showing the production of a pseudohypha (indicated by an asterisk) by a *C. albicans* SC5314 mycelium-attached chlamydospore triggered to germinate at room temperature following reactivation at 37°C in the presence of DMEM supplemented with 10% (v/v) human serum. The process was observed at 60, 90, 120, 150, 240, 270 min, (size bar 10 µm, X 400 magnification).

Fig. 5.13

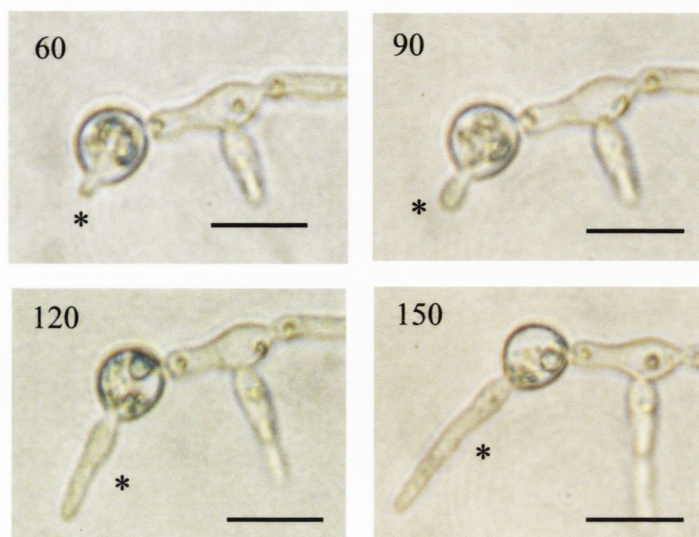


Fig. 5.13 Time-lapse photography of a germinating *C. albicans* chlamydospore. Light micrographs showing the production of true hyphae (indicated by an asterisk) by a *C. albicans* SC5314 mycelium-attached chlamydospore, triggered to germinate in the presence of DMEM supplemented with 10% (v/v) human serum at 37°C in presence of 5% (v/v) CO₂. The germination process was observed at 60, 90, 120, 150 min. (size bar 10 µm, X 400 magnification).

Fig. 5.14



Fig. 5.14 Production of true hyphae by *C. albicans* chlamydospores incubated on solid media.

C. albicans SC5314 was incubated on solid chlamydospore induction medium for 6 days between two filter sheets. To induce germination the filter containing chlamydospore rich mycelia was then removed from the chlamydospore induction medium and placed on solid DMEM medium supplemented with 10% (v/v) human serum and incubated at 37 °C in the presence of 5% CO₂ (v/v). Plates were then analyzed after 15 h incubation (400x magnification).

Chlamydospores greater than 30 days old could not be induced to resume their metabolic activity and bud or germinate, indicating that the structures are unlikely to have a resting role.

5.2.7. *Chlamydospores lose their viability through mitochondrial dysfunction and degradation*

MitoTracker Red CMXRos is a red-fluorescent dye that stains active mitochondria in live cells and its accumulation is dependent upon membrane potential. The probe does not fluoresce until it enters live cells, where it is oxidised to the corresponding fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. Cells with active mitochondria stain red while cells with inactive mitochondria have a diffuse weak blue colouration. In order to investigate the mitochondrial activity of chlamydospores, liquid-grown *C. albicans* SC5314 mature (i.e 5 days old), ageing (i.e. 10 days old) and old (i.e 20 days old) chlamydospore-rich mycelia were stained with MitoTracker Red. A 5 day old *C. albicans* chlamydospore-rich culture stained with MitoTracker Red dye showed that more than 80% of mature chlamydospores stained red, indicating that they possessed metabolically active mitochondria. Staining of the same culture after 10 days of growth showed that only around 40% of chlamydospores possessed active mitochondria. By 20 days the majority of chlamydospores possessed inactive mitochondria showing a weak blue fluorescence, only less than 5% were found to show red colouration upon staining (Fig. 5.15).

Ultrastructural studies confirmed these findings and demonstrated the loss of the mitochondrial inner membrane, lamellae and shape in ageing chlamydospores (Fig. 5. 16). In old mycelia (i.e. 20 days old) a drastic reduction or complete absence of mitochondria in the chlamydospore was found. Transmission electron microscopy showed that young chlamydospores possessed numerous mitochondria that possess all the features of the functional organelle. However ageing chlamydospores possessed fewer mitochondria and old chlamydospores did not show the presence of healthy mitochondria (Fig. 5.16). The endoplasmic reticulum and residual mitochondria, when found, resulted compressed against the membrane wall by large lipid inclusions in old chlamydospores (greater than 20 days). Lipid inclusions and

vacuoles were found to increase in number and size with the age of the chlamydospore (Fig. 5.17).

5.2.8. Nuclear staining of chlamydospores during germination

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing high specificity for genomic DNA. When DAPI binds to DNA, its fluorescence is strongly enhanced. Because of this property DAPI was used as a tool for cytochemical investigations during chlamydospore budding. DAPI staining demonstrated nuclear duplication inside the chlamydospore, possibly promoted by the addition of nutrients and serum. Although activated chlamydospores can have two or more nuclei, some activated chlamydospores were anucleate (Fig. 5.18). The presence of multinucleate chlamydospores suggests the possibility of the migration of one nucleus from the mother chlamydospore to the released cell (Fig. 5.19).

5.2.9. Effect of macrophages on chlamydospore viability

The ability of chlamydospores to survive in the presence of macrophages was investigated. Acridine orange dye was used as a tool to distinguish intact chlamydospores from corrupt ones and also to observe the effect of macrophages on chlamydospore viability. Acridine orange (AO) is a weak base and metachromatic, fluorescent cationic dye that stains live and fixed tissue with variable fluorescence. At the cellular level, AO relocation and colour change has been widely used as a test for cell viability, and for studying pH gradients across vesicular, as well as lyso- and endosomal membranes (Robbins & Marcus, 1963; Robbins *et al.*, 1964). When large amounts of the dye enter a cell with a corrupted membrane, the dye can form dimers (binding to RNA or DNA) that give a red fluorescence. After entry into an acidic granule, or a structure at low pH, AO becomes protonated and thus trapped within the granule membrane. The resulting high concentrations in the granules or organelles at low pH results in a red colouration. As a result of this AO is a good marker to investigate the loss of viability in chlamydospores and also the pH/oxidative mediated killing activity of the macrophages.

Fig. 5.15

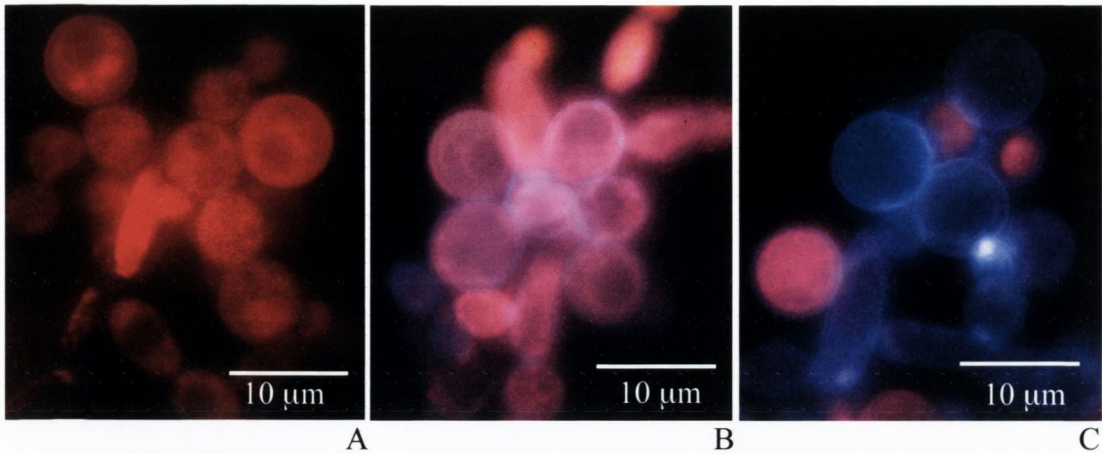


Fig. 5.15 MitoTracker Red staining of chlamydospore at different age.
Candida albicans SC5314 chlamydospore-rich mycelium produced following incubation in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg/L methionine for 5 (A), 10 (B), and 20 (C) days. Chlamydospores were observed by fluorescence microscopy following staining with MitoTracker Red CMXRos (x1000 magnification).

Fig. 5.16

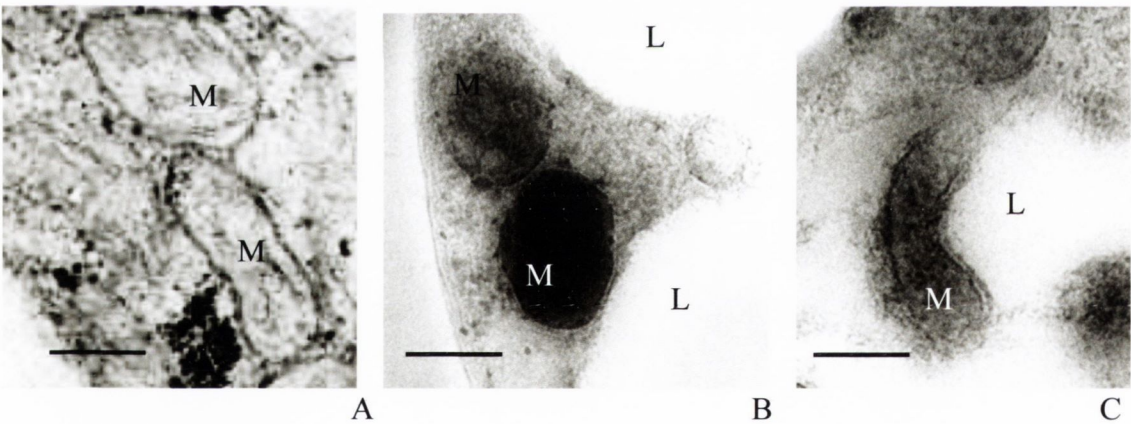


Fig. 5.16 Transmission electron micrograph of endoplasmatic reticulum of chlamydospores of different age.
Candida albicans SC5314 chlamydospore produced following incubation in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg/L methionine for 5 (A), 10 (B), and 20 (C) days. Transmission micrograph showed presence of abundant mitochondria (M) in mature 5 day old chlamydospores. Number and shape of organelles that resembled mitochondria (M) varies with the age of the chlamydospore. Lipid inclusion (L), (size bar 100 nm).

Fig. 5.17

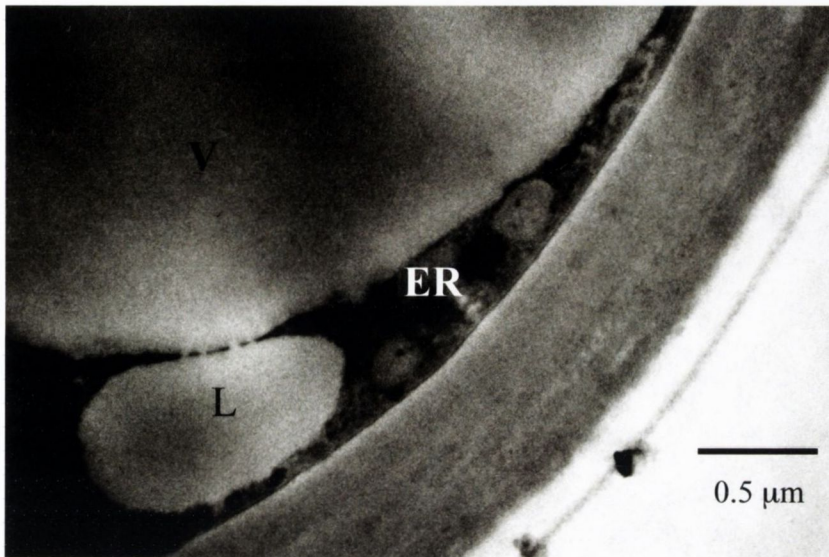


Fig. 5.17 Ultrastructural appearance of aged chlamydo spores.

C. albicans SC5314 chlamydo spore incubated in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg L⁻¹ methionine for 30 days. Large vacuole (V) and free lipids inclusions (L) occupy the major part of the intracellular volume in old chlamydo spores. Endoplasmic reticulum is restricted in the spaces between the cell wall and the vacuole.

Fig. 5.18

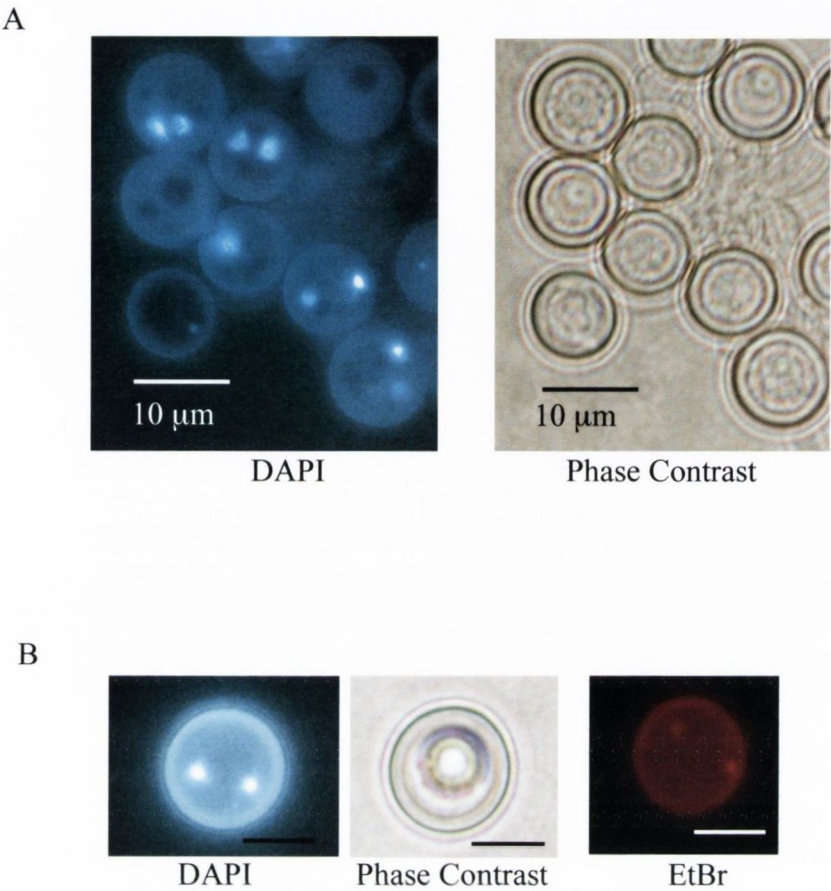
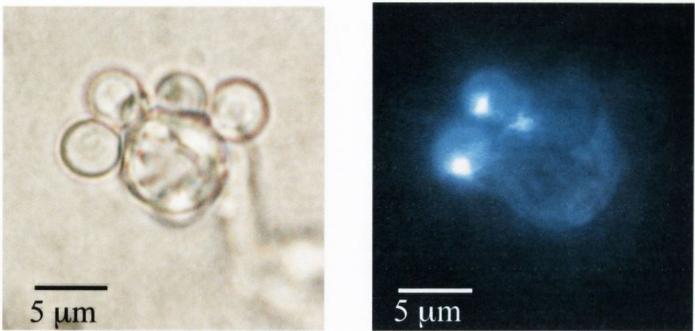


Fig. 5.18 Nuclear staining of chlamydospores using DAPI.

A, Mycelium-attached chlamydospores stained with DAPI.
B, Purified chlamydospores stained with DAPI and ethidium bromide, (size bar 5 µm, X 1000 magnification).

Fig. 5.19

A



B

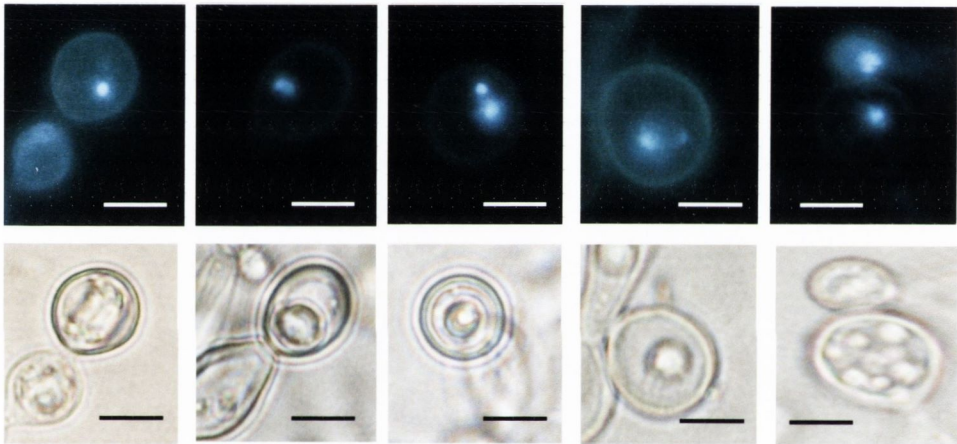


Figure 5.19 DAPI stained nuclei of budding chlamydospores.
A, Presence of genetic material in the cells released by a *C. albicans* mycelium-attached chlamydospore triggered to bud at 37°C in nutrient rich medium.
B, Overview of the possible process of nuclear duplication inside the chlamydospore and successive budding (size bar 5 µm, X 1000 magnification).

5.2.9.1. Dormant *C. albicans* and *C. dubliniensis* chlamydospores in co-culture with macrophages

A 70% pure suspension of 1×10^4 mycelium-free chlamydospores was inoculated onto a 90% confluent layer of the murine macrophage cell line RAW264.7. A high rate of chlamydospore internalization by macrophages was observed within 2–4 h. After 6–7 h almost all the chlamydospores including those forming clumps were phagocytosed. Some macrophages were found to have engulfed up to five to 10 chlamydospores. Staining of the chlamydospores and macrophages with acridine orange showed that at the moment of interaction with the macrophages, the dormant chlamydospores were viable and intact. Following phagocytosis, the dye showed metachromism (red/orange colour), indicative of loss of chlamydospore viability. Following an overnight co-culture with macrophages, phagocytosed chlamydospores lost their round shape and appeared to be dead (Fig. 5.20).

5.2.9.2. Pre-activated *C. albicans* and *C. dubliniensis* chlamydospores germinate when co-cultured with macrophages

Following an activation step of 15–20 hours in nutrients and serum and incubation at 37°C in the presence of CO₂, some (2–5%) of the re-activated chlamydospores were able to germinate in the presence of macrophages after 4–5 hours and escape phagocytosis by producing hyphae. Staining with acridine orange showed that the chlamydospore cell walls were intact and that the hyphae emerging from them were also viable (Fig. 5.21).

5.2.10. Chlamydospores are intolerant of environmental stress

To investigate whether some of the attributes typical of fungal resting spores (e.g. long-term survival under nutrient-poor conditions and dry stress resistance) were present in chlamydospores, these structures were subjected to a variety of stresses and their viability (measured by the ability to bud or germinate) was analyzed and quantified. Five-day-old mycelium-attached chlamydospores and yeast cells recovered from cultures of the same age were transferred to distilled water and their viability, upon exposure to glucose and serum, was measured for a period of 15 days. The percentage of cells producing buds or germ tubes was counted as a measure of viability (Fig. 5.22).

To assess dry stress tolerance of mycelium-attached chlamydospores and yeast cells micro-drops (100 μ l) of the two cultures were spotted on Petri dishes and allowed to dry over time at room temperature. At defined time points (0, 5, 10, 20, 30, 40, 50 hours) rich medium (i.e YEPD) and serum were added into the Petri dishes and viability was quantified by counting the percentage of cells able to produce buds or germ tubes. Chlamydospores had similar dry stress tolerance compared to yeast cells (Fig. 5.23).

5.2.11. Growth of strains of different mating type in CIM containing phloxine

Based on the fact that chlamydospores are intolerant to environmental stresses and do not appear to possess a resting role, the possibility of a link between chlamydospore formation and mating was investigated. If there is any link between these structures and the mating process, then their production might be linked to the mating loci or phenotypic switching and could also be pheromone controlled.

Phloxine has been widely used to identify white opaque switching in *C. albicans*. To assess if *C. albicans* and *C. dubliniensis* cells were undergoing the white/opaque switching before producing chlamydospores and to test if chlamydospore production was affected when a strain of one mating type was plated close to a strain of the opposite mating type, 1% phloxine was added to the solid medium used for chlamydospores induction in *C. dubliniensis*. Four *C. dubliniensis* α/α homozygous strains (Egf200, P7718, SAIO5, P2) 4 *a/a* homozygous strains (Ansa28, J11, P7276, 9097) and 4 *a/\alpha* heterozygous strains (CD 36, Egf207, P6785, D38) were plated adjacent to each other in all possible combinations. It was found that all mating type of *C. dubliniensis* produced chlamydospores. It was also found that chlamydospore production was repressed in the area between the two strains. Chlamydospores were instead present in the outer part of each streaked strains where the two strains were not facing each other. Interestingly when Egf 200 (α/α) was plated together with the strain Egf 207 (*a/\alpha*) unusual hyphal arrangements were observed in the space between the two strains (Fig. 5.24).

Fig. 5.20

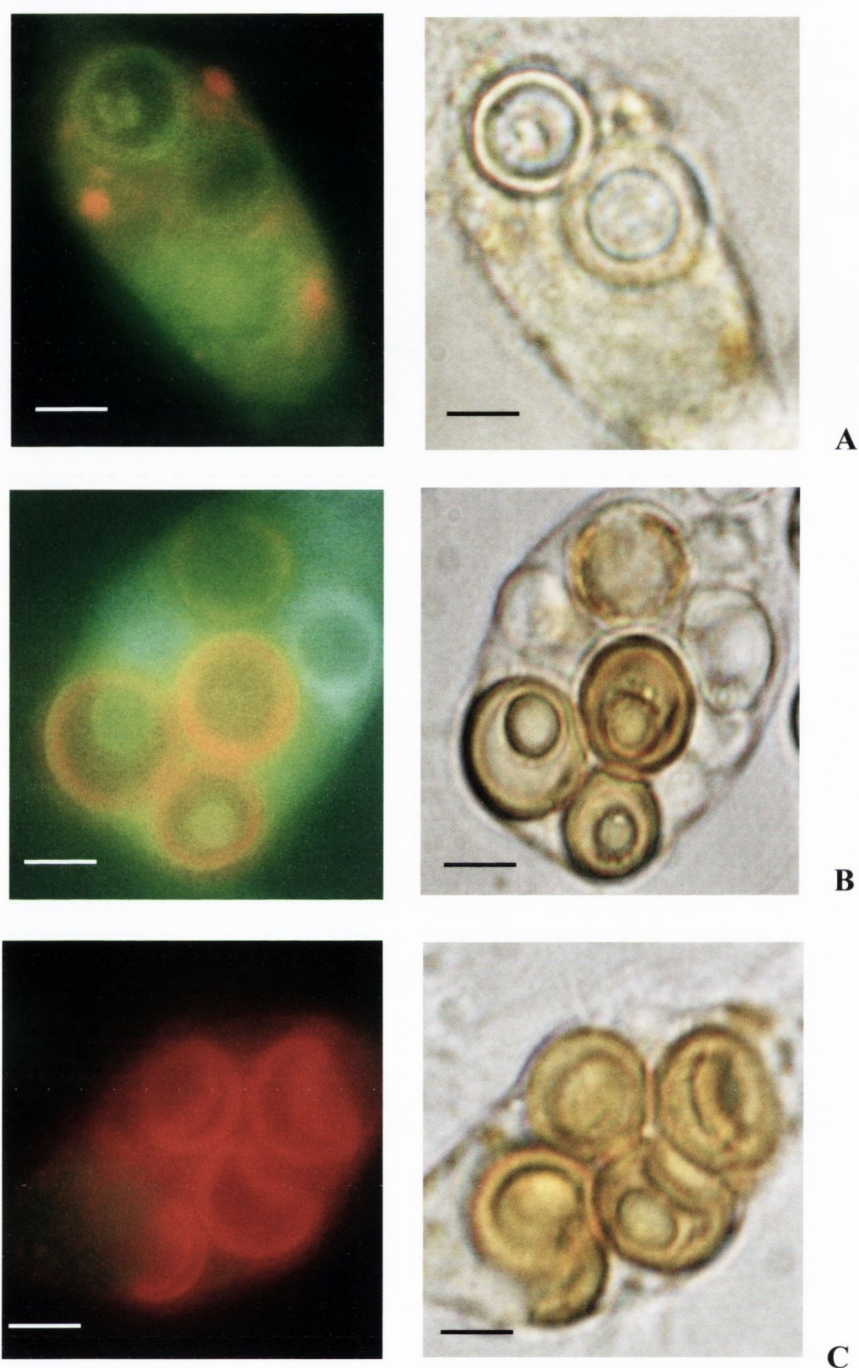


Fig. 5.20 Viability of dormant *C. albicans* SC5314 chlamydospores following phagocytosis by murine macrophage cells.

The murine cell line RAW264.7 was co-incubated with *C. albicans* chlamydospores. Following 2-4 hours (A) the chlamydospores were phagocytosed. Staining with Acridine Orange indicates that chlamydospores are alive inside the macrophage. By 6 hours (B) the chlamydospores exhibited an orange-red coloration localized near the cell wall suggestive of cell wall damage.

Between 14 and 20 hours (C) the chlamydospores were severely damaged, a diffuse red coloration was indicative of loss of viability (size bar 5 μm , 1000x magnification).

Fig. 5.21

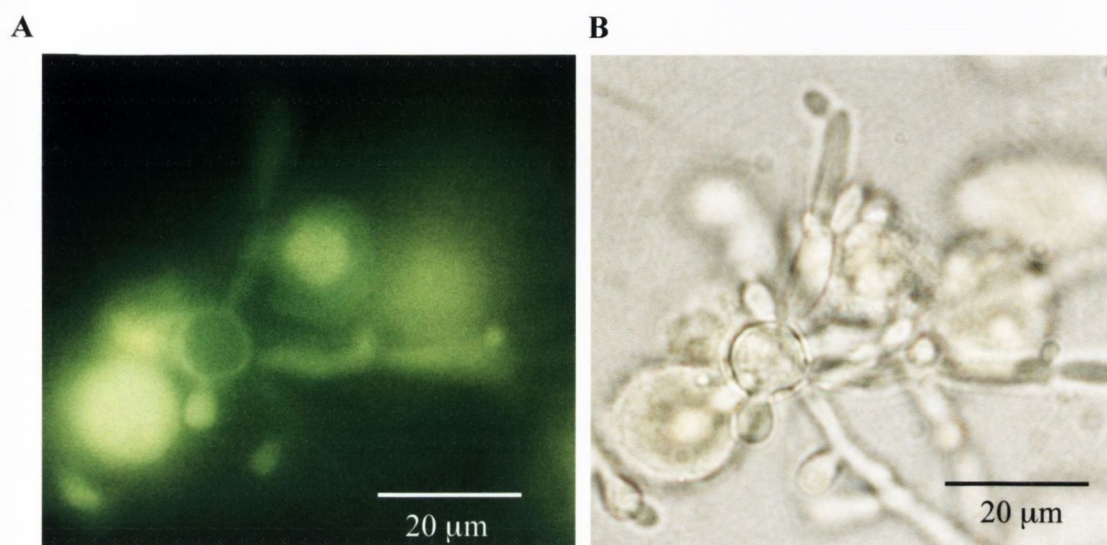


Fig. 5.21 Viability of pre-activated *C. albicans* SC5314 chlamydospores in the presence of murine macrophages.

Chlamydospores were first activated for 15-20 hours in nutrient rich medium plus serum before co-culture with RAW264.7 macrophages. Following 3-4 hours exposure to the macrophages chlamydospore began to germinate following phagocytosis (**A**, acridine orange; **B**, bright field) (1000x magnification).

Fig. 5.22

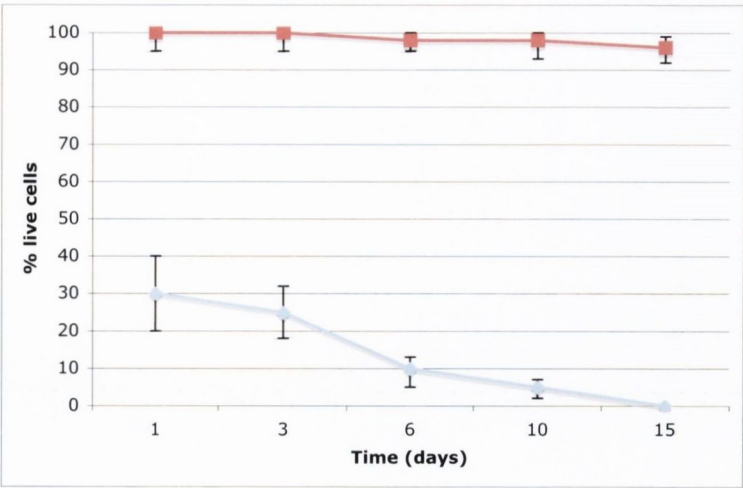


Fig. 5.22 Comparative survival of yeast cells and chlamydospores in water. Five-day-old mycelium-attached chlamydospores (◆) and yeast cells (■) from cultures of the same age were incubated in water and viability (ability to produce daughter cells) was assessed over time.

Fig. 5.23

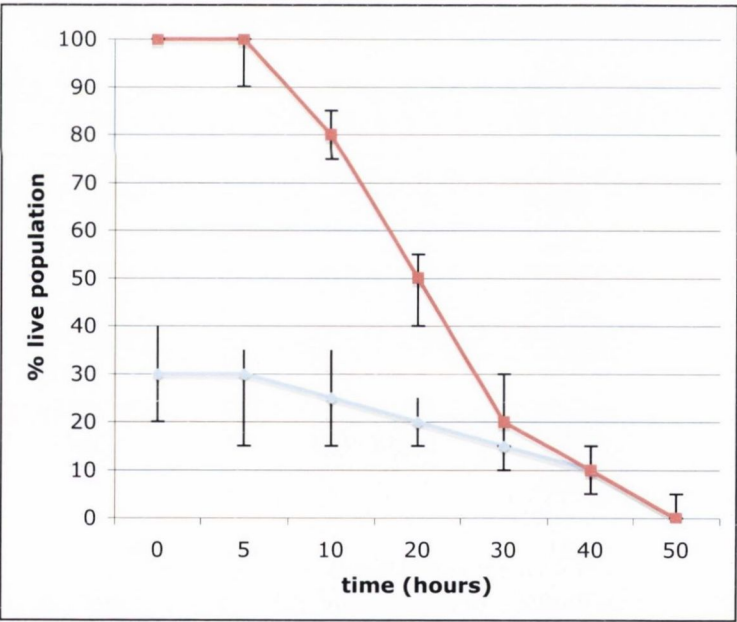


Fig. 5.23 Comparative survival of yeasts and chlamydospores in dry environment. Five-day-old mycelium-attached chlamydospores (◆) and yeast cells (■) from culture of the same age were exposed to dry stress and viability (ability to produce daughter cells) was assessed over time.

Fig. 5.24

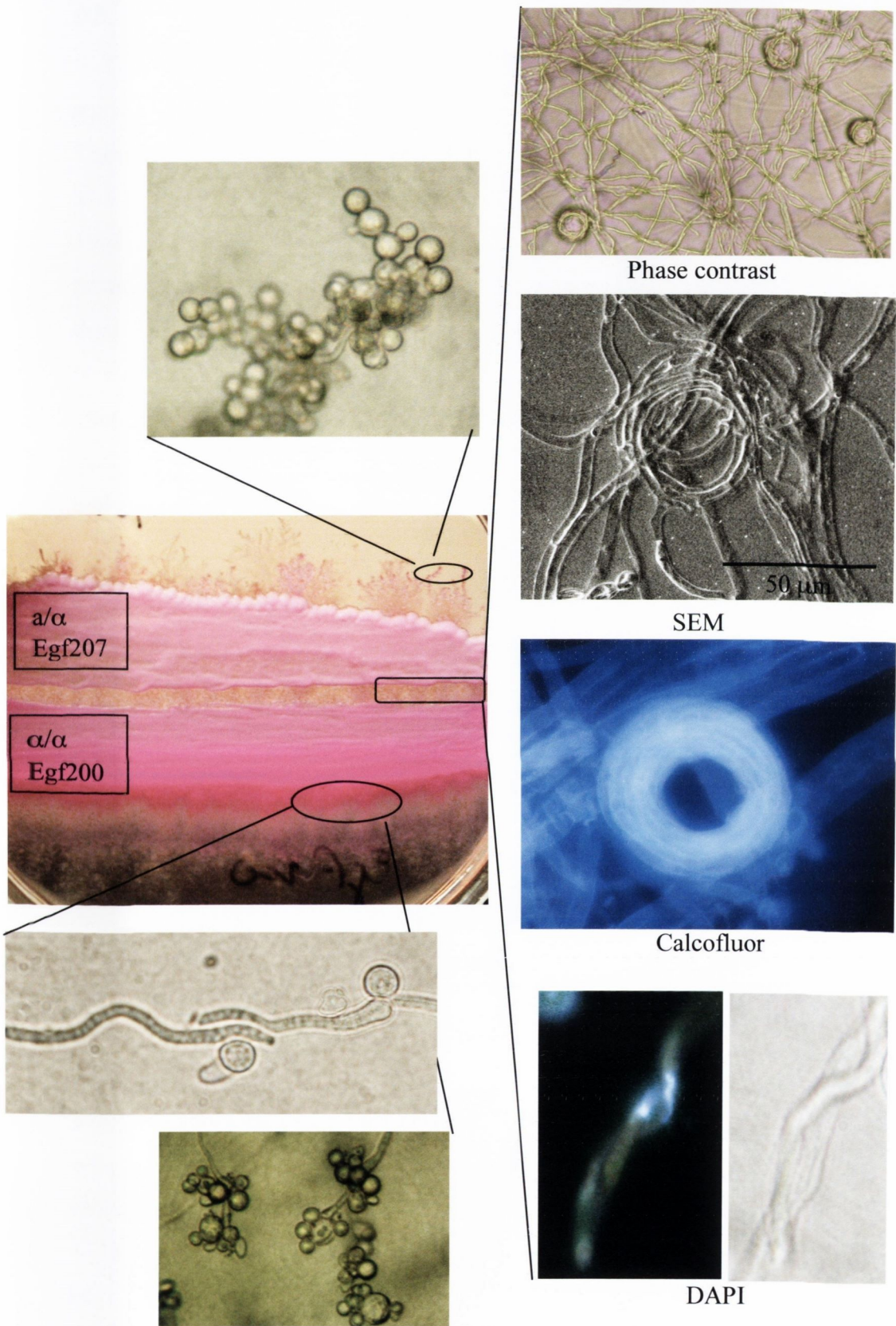


Fig. 5.24 Appearance of the strains Egf200 (α/α) and Egf 207 (a/α) plated together in CIM added with phloxine

5.3. Discussion

Candida albicans and *C. dubliniensis*, whose natural habitats are humans (and some animals) are the only *Candida* species known to produce chlamydospores. These thick-walled cells are only produced under very specific conditions *in vitro* and have rarely been observed *in vivo* (Chabasse *et al.*, 1988; Wilborn & Montes, 1980; Schonborn & Schmidt, 1971; Heineman *et al.*, 1961). Due to the difficulty in producing these cells in sufficient numbers, they have been poorly investigated and their role in the life cycle of *C. albicans* and the pathogenesis of candidal infections is not known. The main problems encountered when studying the function of chlamydospores include the low numbers of chlamydospores produced using the traditional culturing methods, the agar invasion of chlamydospore-producing mycelia and the tight association of chlamydospores with their parental suspensor cells.

We have developed a chemically defined solid and liquid medium, containing galactose and methionine at low concentration, which induces high levels of chlamydospore induction by the majority of *C. dubliniensis* strains tested. Under these conditions, *C. albicans* failed to produce chlamydospores. However, the addition of 0.025% (w/v) galactose and 20 mg/L methionine and YNB in traces to a liquid formulation containing corn meal and 1% (v/v) Tween 80 resulted in the production of large volumes of chlamydospores by this species. The observation that the addition of glucose and galactose at high concentrations inhibited the production of chlamydospores in liquid and in solid media in both species suggests that low nutrient availability may play a role in the induction of chlamydospores. In chapter 3 we have demonstrated that glucose or galactose and methionine at low concentrations have an effect on the species-specific regulation of the transcriptional factor *NRG1* in *C. dubliniensis*, in the CIM there was a down-regulation of *NRG1* expression in *C. dubliniensis* that was absent in *C. albicans* (see 3.2.4). However, other factors such as low temperature and oxygen limitation are also likely to play a role in the process of chlamydospore formation.

In order to investigate the viability and metabolic activity of chlamydospores, the molecular probe FUN-1, which detects metabolic activity in cells, was used to demonstrate that mature chlamydospores (5-7 days-old) possess high metabolic activity, and that this metabolic activity decreases overtime under nutrient-poor

conditions. After 30 days incubation metabolic activity was almost undetectable. This process resembles the dormancy in fungal spores with a resting function, which is characterised by a progressive loss of metabolic activity that does not affect viability. This period can be interrupted and metabolic activity induced to resume following exposure of the spore to specific external stimuli. In chlamydospores however, unlike resting spores, this stage of low metabolic activity can only be interrupted in chlamydospores that are not older than 15-20 days. Relatively young chlamydospores can be reactivated, resuming metabolic activity, following exposure to nutrient-rich environmental condition and the presence of serum finally resulting in the ability to bud or germinate. Following the incubation of mature chlamydospores (i.e. 6-15 days old) in rich media containing 2% (w/v) glucose and 1% (v/v) human serum, approximately 30% of mycelium-attached chlamydospores and 5% of purified chlamydospores started to bud within 24 h.

When dormant chlamydospores were incubated in rich medium containing 2% (w/v) glucose and 10% human serum at 37°C, the chlamydospores produced either pseudohyphae or true hyphae (depending on the presence or absence of 5% CO₂ (v/v)) in a manner similar to blastospores. Indeed mature pre-activated *C. albicans* chlamydospores have the ability to germinate and escape from macrophages. In the conditions tested it was found to be easier to interrupt the dormancy of mycelium-attached chlamydospores (6-15 days old) than that of purified chlamydospores of the same age. In order to understand the reason for this loss of viability ultra-structural and biochemical studies were performed on chlamydospores of different ages. Mitochondria are the organelles involved in the aerobic respiration of the cells and can be considered the power generators of the cell, converting oxygen and nutrients into adenosine triphosphate (ATP). Because in addition to their role in cellular energy metabolism, mitochondria are now recognised as central players in cell death, we further concentrated on the study of these organelles in chlamydospores. When a mitochondrion is not functional, ATP becomes depleted and cells die by necrosis. Mitochondria also play a central role in apoptosis through the release of pro-apoptotic proteins contained in the intermembrane space (Cheng *et al.*, 2008). These include cytochrome c that activates the caspase cascade whose activation is responsible for the changes to cell structure and function that occur in apoptosis. During mitochondrial energy transduction, reactive oxygen species (ROS) are generated as by-products. These molecules are able to damage all cellular

compounds leading to cellular dysfunctions. Within certain limits cells are able to cope with ROS-related problems, however if damage of essential components is too severe, cells may induce specific pathways to compensate for the corresponding impairments. This allows mitochondria to determine not only whether a cell should die, but also the nature of that death. Analysis of global gene expression in chlamydospores compared to yeast cells (described in chapter 6) we found a specific regulation in chlamydospores of genes related to mitochondrial metabolic activities and of genes involved in response to oxidative stress. This suggests the possibility that these organelles are involved in regulating the life span of chlamydospores (see chapter 6). Analysis of chlamydospore mitochondrial function using MitoTracker Red dye revealed that a 5 day old *C. albicans* chlamydospore-rich culture showed that more than 80% of mature chlamydospores possessed metabolically active mitochondria but by day 20 the majority of chlamydospores possessed inactive mitochondria. This study was verified by transmission electron microscopy that showed that mitochondria lose their shape (i.e. membrane and lamellae) during chlamydospore ageing. This suggests that chlamydospores may lose their viability through mitochondrial dysfunction and degradation.

The incubation of chlamydospores in nutrient poor media for long periods results in a significant reduction in the number of those capable of resuming their metabolic activity. In particular mycelium-free chlamydospores appear to completely lose their viability if incubated in water for more than two weeks after their purification. This suggests that these chlamydospores are less tolerant of long term storage in water than blastospores. It was also observed that mycelium-attached and purified chlamydospores have almost the same ability to resist dry stress as yeast cells, losing viability after exposure to dry environment for more than two days. These data suggest that candidal chlamydospores are unlikely to act as spores for long-term (i.e. months or years) survival in nutrient poor or dry conditions.

The observation that chlamydospores can exhibit metabolic activity, coupled with their ability to produce, blastospores, hyphae and chlamydospores, suggests that these cells are alternative growing forms of *C. albicans* and *C. dubliniensis*. All these findings suggest that candidal chlamydospores are not “dead end” or storage structures. We have demonstrated instead, that chlamydospores are an alternative and specialised growth form of the two species resulting from major changes in gene expression and morphology in the blastospore and mycelial cell forms. The different

dynamic of nuclear duplication inside a budding chlamydospore, with the migration of one nucleus from the mother chlamydospore to the released cell, also suggests that this particular growing form is very different from the yeast and hyphae phases and may have very specific role in the *C. albicans* and *C. dubliniensis* life cycles.

Chapter 6

Analysis of global gene expression in chlamydospores

6.1. Introduction

6.1.1. Microarray technology as a method to evaluate gene expression in chlamydo spores

Very little is currently known concerning the molecular pathways expressed during chlamydo spore formation and maturation. We hypothesised that comparison of global gene expression in chlamydo spores and yeast cells could provide insights into the possible role of chlamydo spores in the *Candida* life cycle and may also explain the differences in life span, cellular organization, and stress resistance in those cells. The technique chosen for this analysis was two colour microarray analysis. A DNA microarray consists of an arrayed series of spots of DNA oligonucleotides or PCR fragments of specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize with a cDNA sample under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorescence- or chemiluminescence-labels. The probes are attached to a solid surface, normally glass, by a covalent bond. Two-colour microarrays are hybridised with single-stranded cDNA, prepared from two samples to be compared (e.g. yeast cell versus chlamydo spores). The RNA is copied by reverse transcription and labeled with two different fluorescent dyes. Florescent dyes commonly used for cDNA labelling include Cyanine3 (Cy3), which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and cyanine5 (Cy5) with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum). When only very small amounts (ng) of RNA are available, labeled cRNA target can be prepared in sufficient quantities for expression array hybridization by including a linear amplification step after first-strand cDNA synthesis. The two Cy-labelled cDNA (or c-RNA) samples are mixed and hybridised to a single microarray slide that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes. Normalization is used to attenuate the effect of variation in intensity levels of the dye within and between replicated spots of the same gene or within and between array slides. Much of the variation in intensity levels can arise

from technical rather than biological causes. Lowess normalization is normally used in experiments with two fluorescent dyes. This kind of normalization merges two-colour data, applying a smoothing adjustment that removes variation. Identifying genes that are differentially expressed under two or more treatment conditions is a primary goal of the array study. The two main issues in assessing differential expression are determining a method for assessing the extent of differential expression (e.g., fold change, *t*-test, ANOVA) and adjusting the method for the effects of multiple comparisons, since typically there are thousands of genes being studied. The multiplicity problem arises from the fact that thousands of hypotheses are tested simultaneously; this increases the chances of false positives arising. For expression studies it has become common practice to focus on control of the false discovery rate (FDR). The false discovery rate is the expected proportion of incorrect rejections among the rejected hypotheses. One commonly used statistic test is the Benjamini-Hochberg multiple correction test to determine which gene sets are selected with a particular false discovery rate. After differentially regulated genes have been found genes can be sorted by their function or the process in which they are involved.

Functional annotation of genes is a way of assigning roles and functions to genes and is useful in identifying genes involved in related processes in large datasets. By making annotations to a common, shared set of vocabularies, the Gene Ontology (GO) resource provides a powerful way to analyse information in a way that is independent of species. At present over 87,000 species have some GO annotation. There are three GO vocabularies, each providing a specific type of information about a gene or protein: (i) its molecular function, (ii) the broader biological processes it is involved in and (iii) the cellular compartment it acts in.

Array results can be influenced by each step of the complex assay, from array manufacturing to sample preparation (extraction, labeling, hybridisation) and image analysis. Other methods of verification of the expression of some genes are normally used alongside the array. Among the many methods currently available for quantifying mRNA transcript abundance, reverse transcription-polymerase chain reaction (RT-PCR) has proved to be the most sensitive and is currently the most accurate and reproducible approach to gene quantification. Starting with a very small amount of material (usually total RNA), it is possible to copy the RNA by reverse transcription (RT) to produce cDNA. The cDNA is more stable than RNA and can

then be amplified by PCR and quantified to determine the relative abundance of expressed genes within and between sample groups. The procedure follows the general principle of PCR. The amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. RT-PCR evaluates product accumulation during the log-linear phase of the reaction. Two of the most common uses of this technology are: (i) to compare relative or absolute levels of expression of genes between control and treatment groups and for confirmation of gene expression (mRNA) changes identified in a primary screen (e.g., hybridization array analysis). Two common methods of quantification are used; the first uses fluorescent dyes that intercalate with double-stranded DNA (e.g. SYBR Green I), and the second uses modified DNA oligonucleotide probes that fluoresce when hybridised with a complementary DNA (e.g. Taq Man probe). Recently, several protocols for real-time relative RT-PCR using the reporter dye SYBR Green have been developed. In these methods, sample and control mRNA abundance is quantified relative to an internal reference RNA whose abundance is known not to change under the different experimental conditions (house keeping gene). Relative concentrations of DNA present during the exponential phase of the reaction are determined by plotting fluorescence against cycle number (cycle number log-based fluorescence).

These two techniques together could provide some clues concerning the possible role of chlamydospores in the *Candida* life cycle. Chlamydospores are not produced by the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, so there is limited understanding of the pathways that govern their development. It is known that perturbation of the classical signal transduction pathways, including the MAP Kinase pathway and the cAMP pathway inhibit the production of chlamydospores. Sonneborn *et al.* (1999) found that the *efg1/efg1* mutant was completely deficient for chlamydospore formation. Eisman *et al.* (2006) showed that the Hog1 mitogen-activated protein (MAP) kinase plays a role in chlamydospore formation as the homozygous mutant is unable to form chlamydospores. Staib & Morschhauser (2005) identified *NRG1* as the main transcriptional regulator involved in chlamydospore production on Staib medium. Nobile *et al.* (2003) based on the phenotypes of homozygous insertion mutants identified 6 genes (i.e. *ISW2*, *MDS3*, *RIM13*, *RIM101*, *SCH9* and *SUV3*) that are required for efficient chlamydospore formation. *SUV3*, *SCH9*, and *ISW2*, are involved in mitochondrial function, glycogen accumulation, and chromatin remodelling, respectively. On the basis of these

findings we expected to find sets of genes regulated by Nrg1, Efg1 and Hog1 during chlamydospore development and we expected some of these genes to govern the processes of dormancy in chlamydospores and other characteristic feature of these intriguing structures.

6.2. Results

6.2.1. Microarray Experiment design: growth conditions and strains used for the comparison

C. dubliniensis CD36 was used in this study due to its ability to produce very large numbers of chlamydospores in defined liquid environmental conditions. In order to compare yeast cell and chlamydospore gene expression patterns, cell culture conditions were chosen to ensure maximum levels of chlamydospore or yeast cell formation with as few differences in media composition as possible. We could achieve this by simply altering the content of galactose in CIM. This could allow a consistent comparison of gene expression between chlamydospore inducing-cultures and yeast inducing-cultures. The quantity of chlamydospores produced in CIM by *C. dubliniensis* was found to be strongly dependent upon the concentration of galactose in the medium and also dependent on the size of the inoculum and hypoxic conditions. When 1×10^7 exponentially growing yeast cells were inoculated in 50 ml of a formulation of CIM containing 0.5% (w/v) galactose for 4 days at room temperature shaking at 80 rpm, the cultures were predominantly comprised of blastospores (Fig. 6.1, A). When the inoculum was reduced to 1×10^2 cells and cells were incubated in a formulation of the medium containing 0.025% (w/v) galactose, high levels of chlamydospores were formed (Fig. 6.1, B). Blastospores were removed from the latter culture by pelleting only chlamydospores with time limiting centrifugation.

6.2.2. The microarray slides and platform

The slides used in the microarray experiment were designed by Dr. Gary Moran based on the *C. dubliniensis* genome sequence generated at the Sanger Centre in Cambridge. The *C. dubliniensis* oligonucleotide microarray slide comprised a set of

5,999 predicted ORFs from the CD36 genome used to design a *C. dubliniensis* expression microarray. Two unique 60mer oligonucleotides were designed specific for each ORF using the Agilent eArray probe design tool. Whole genome microarrays were printed on glass slides by Agilent technologies. Each 60mer was printed in quadruplicate.

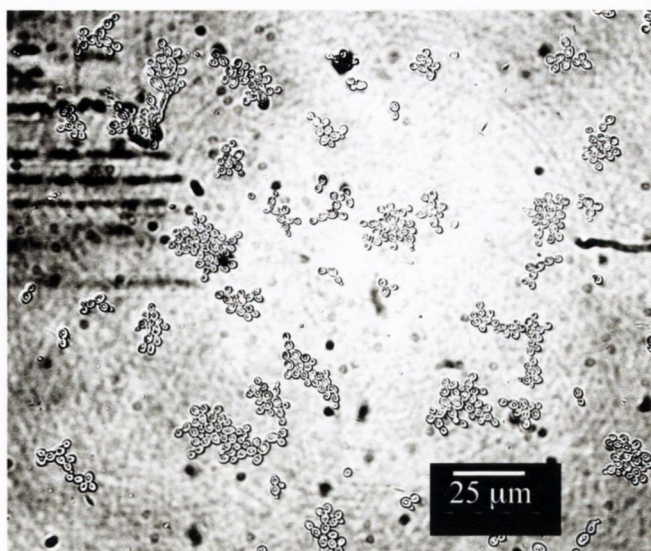
6.2.3. The microarray comparison and results

The mRNA was extracted from chlamyospore rich cultures and from yeast cell rich cultures on 3 separate occasions and processed for the array, using the Agilent two-colour Microarray-based Gene expression analysis platform. The mRNA was reverse-transcribed to form cDNA. In a second reaction the resulting cDNA was transcribed and amplified and simultaneously labelled with Cy3 or Cy5 (cRNA). cRNA generated from chlamyospore rich-cultures labelled with one cyanine dye was co-hybridised with cRNA generated from yeast culture that was labelled with the other cyanine dye on a 4x44k *C. dubliniensis* genome array slide. A dye swap experiment in which the labelling dye was changed in both the cRNA generated from chlamyospores and yeast cells was also performed to evaluate the possibility of differential incorporation of the two dyes. Slides were scanned with a Gene Pix Personal 4100A scanner (Axon) and data were extracted with Gene Pix Pro 6.1. Spots were flagged absent and excluded from analysis if the fluorescent signal was less than background plus 2 standard deviations. Raw data were imported into GeneSpring GX10 (Agilent technologies) and normalised using Lowess normalization. A ratio of the fluorescent signal in chlamyospores relative to yeast cell cultures was determined for each spot on the array. Genes exhibiting a 2-fold difference in expression were selected for further analysis. A *t* test was performed between the two data sets using the variance derived from replicate spots. The genes with a *p* value <0.025 that passed the Benjamini-Hochberg multiple correction test to remove false differential gene expression are listed in Appendix I.

In total 550 genes were found to be differentially regulated (greater than 2-fold up or 2-fold down-regulation) in chlamyospores compared to yeasts. The genes differently regulated (> 2-fold up and > 2-fold down) between yeast and chlamyospores were analyzed with the program "Go Slim Mapper" tool to evaluate the percentage of the various cellular processes in which these genes may be

Fig. 6.1

A



B

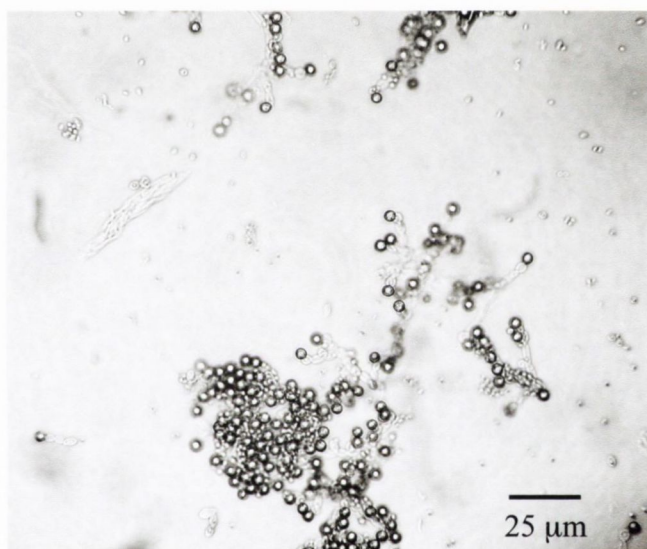


Fig. 6.1 Growth morphology of *C. dublinensis* CD36 when incubated CIM supplemented with different concentrations of galactose.

(A) 1×10^7 exponentially growing yeast cells were inoculated in CIM containing 0.5% (w/v) galactose for 4 days at room temperature with moderate shaking.

(B) 1×10^2 exponentially growing cells were incubated in CIM containing 0.025% (w/v) galactose for 4 days at room temperature without shaking.

involved in (Fig. 6.2). These greater than 2-fold upregulated genes were analysed with the program Go Term Finder to find significant gene ontology terms shared between the two differentially regulated categories of genes and discover what process they may be involved in (Table 6.1). Genes implicated in iron and metal ion transport, including *RBT5*, *ZRT2*, *ALR1*, *SIT1* and *FRE9* and genes involved in assimilation of sulphate including *CBF1*, *MET3* and *MET6* were found to be greater than 2-fold upregulated in chlamydospores. Genes involved in carbohydrate catabolic processes, including *CDC19*, *PGK1*, *GND1*, *IFE2* and *YMX6*, including genes involved in the pentose phosphate pathway (*ZWF1*) and glycolysis (*GPM2*, *ENO1*) were upregulated. In addition genes involved in protein catabolism (*PRE2*, *PRE6* and *PRE10*) were also found to be upregulated. In addition to genes involved in metal transport and catabolism a large group of genes responsive to stress and chemical-pheromone stimulus were found to be upregulated. These included *CDR4*, *FAV2*, *ERG24*, *FMO1*, *FMP45*, *MAD2*. Genes involved in chromatin remodelling (*HHO1*, *HHT2*, *HHF22*, *PDS5*) in DNA repair-recombination (*DMC1*, *DLH1*, *SMC1*, *MSH2*) and other DNA metabolic processes (*RFA1*, *RNH35*, *RNR1*, *RNR22*) were found to be up-regulated in chlamydospores (or down-regulated in yeast cells). Categories of genes downregulated in chlamydospores included genes involved in translation (*IFM1*, *MEF2*, *MRPL3*, *MRPL8*) oxidative phosphorylation (*QCR2*, *QCR7*, *QCR8*, *CYT1*, *RIP*), the Krebs (TCA) cycle (*ACO1*, *ACO2*, *SDH2*) and the glyoxylate cycle (*MDH1*, *MDH2*, *MDH3*). In addition to genes involved in metabolism, genes encoding amino acids transporters (*CAN2*, *HIP1*, *GAP1*, *AGP2*, *GNP3*) were found to be downregulated in chlamydospores. Another set of genes regulating mitochondrial biogenesis and the respiratory electron transport chain (*NDH51*, *CYC1*, *COX4*, *COX5*, *CYT1*) and mitochondrial ATP synthesis (*ATP17*) were found to be downregulated in chlamydospores. Further analysis was carried out to identify the most highly differentially regulated processes in chlamydospores and yeast cells. To examine these processes, we selected genes regulated up and down 4-fold in each conditions. Again we found significant upregulation of metal ion and catabolism related genes but also genes involved in biofilm formation and some genes that encode highly antigenic proteins (Table 6.2).

6.2.3.1. Identification of genes controlled by the transcriptional regulator *NRG1* that are up-regulated in chlamydospores

The transcriptional regulator *NRG1* has been found to be involved in controlling the process of chlamydospore formation as a repressor, and its downregulation promotes the formation of these structures (Staib & Morschhauser, 2005a, 2007). However no previous experiments have investigated the regulation of the genes that are under the control of this transcriptional factor in mature chlamydospores. In order to verify the downregulation of *NRG1* in chlamydospores, the expression of the transcriptional regulator was studied using a *pNRG1-GFP* strain that allowed a comparison of the relative fluorescence of yeast cells and chlamydospores, indicative of the *NRG1* expression, upon visualization with fluorescence microscopy. Yeast cells were found to be highly fluorescent indicating high expression of *NRG1* in this form of growth, whereas chlamydospores were completely non-fluorescent suggesting that *NRG1* was downregulated in the spores compared to yeast (Fig. 6.3). Previously Moran *et al.* (unpublished) carried out a comparison of the transcriptomes of the *C. dubliniensis* parental strain with that of a strain with a homozygous deletion in the *NRG1* under in vitro growth conditions (YPD broth, 30°C) in order to determine the set of genes regulated by *CdNRG1*.

In the present study a comparison between the genes that are up-regulated in the *C. dubliniensis* strain with a homozygous deletion of the *NRG1* and the genes that are upregulated in the chlamydospores of the *C. dubliniensis* CD36 was carried out with the aim of identifying the proportion of *NRG1*-repressed genes that are upregulated in chlamydospores. In addition, in order to identify any possible correlation between the genetic programs of chlamydospore formation and filamentation and how *NRG1* regulates both of these genetic programs, the genes found by Moran *et al.* to be upregulated when *C. dubliniensis* was grown in 10% calf serum were also included in this comparison. We found that 24 genes were commonly upregulated genes in the $\Delta nrg1$ background and in chlamydospores (13% of the > 2-fold upregulated genes in the $\Delta nrg1$ background and the 11% of the genes > 2-fold up-regulated in chlamydospores). The 24 genes identified included genes involved in glucose metabolism and glucose transport (*HGT5*, *GRE3*, *ZWF1*, *ARA1*, *PFK1*) genes involved in oxidative stress response (*GRP2*) and *RAM2* the homolog

Table 6.1 Grouping of genes differentially regulated between chlamydo spores and yeast cells.

Significantly upregulated (2-fold) genes (A) and downregulated (B) in the *C. dubliniensis* chlamydo spores compared to yeast were analysed using the CGD Gene Ontology Term Finder (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

A

GO_term	Cluster frequency	Background frequency	Corrected P-value	Gene(s) annotated to the term
carbohydrate catabolic process	12 out of 320 genes, 3.8%	54 out of 6435 background genes, 0.8%	0.00581	GPM2, XKS1, PDC11, CDC19, ENO1, PFK1, GRE3, ZWF1, GCA1, IFE2, YMX6, orf19.6816
chromatin assembly or disassembly	10 out of 320 genes, 3.1%	46 out of 6435 background genes, 0.7%	0.03963	HTA2, orf19.1052, HHF1, HHT21, HHT2, HHF22, TOP2, CBF1, NHP6A, HTA1
catabolic process	43 out of 320 genes, 13.4%	486 out of 6435 background genes, 7.6%	0.07478	orf19.1058, GPM2, GAD1, XKS1, RAD23, PXP2, LIP4, PRE2, orf19.2401, ADH4, orf19.2733, orf19.2755, PDC11, MSH2, HTA3, DUT1, VTC4, orf19.3447, CDC19, ENO1, PFK1, GRE3, ZWF1, LIP10, LIP6, GCA1, IFE2, LAP3, PRE6, orf19.5614, YMX6, orf19.5752, orf19.5862, FDH1, RNH35, PRE10, orf19.6816, orf19.7196, orf19.732, orf19.7593, FDH3, DUR1,2, orf19.850
transition metal ion transport	10 out of 320 genes, 3.1%	50 out of 6435 background genes, 0.8%	0.08362	CFL1, orf19.1534, ZRT2, SIT1, FET31, CCC2, orf19.4690, FTH1, FRP1, YCF1

B

GO_term	Cluster frequency	Background frequency	Corrected P-value	Gene(s) annotated to the term
mitochondrion organization	27 out of 329 genes, 8.2%	161 out of 6435 background genes, 2.5%	1.50E-05	orf19.1483, orf19.1650, orf19.185, orf19.2984, orf19.3946, orf19.4096, orf19.4190, orf19.4294, MSW1, orf19.4932, orf19.51, orf19.5143, IFM1, orf19.5394.1, orf19.5444, QCR7, TUF1, orf19.6062, MRPL8, orf19.6374, ACO1, orf19.6524, orf19.6565, orf19.6698, orf19.680, YHM1, PAM16
electron transport chain	12 out of 329 genes, 3.6%	33 out of 6435 background genes, 0.5%	1.63E-05	COX4, CYC1, QCR2, SDH12, CYT1, SDH4, orf19.4096, QCR8, COX5, QCR7, RIP1, SDH2
fatty acid metabolic process	13 out of 329 genes, 4.0%	62 out of 6435 background genes, 1.0%	0.0051	CTF1, orf19.2599, FAT1, orf19.4121, AGP2, FAD3, MDH1-3, orf19.5420, PEX5, ANT1, orf19.6443, ECI1, orf19.819
succinate metabolic process	4 out of 329 genes, 1.2%	6 out of 6435 background genes, 0.1%	0.04278	SDH12, orf19.345, SDH4, SDH2

Table 6.2 Grouping of genes differentially regulated between chlamydospores and yeast cells.

Greater than 4 fold upregulated genes (A) and downregulated (B) in the *C. dubliniensis* chlamydospores compared to yeast were analysed using the CGD Gene Ontology Term Finder (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

A

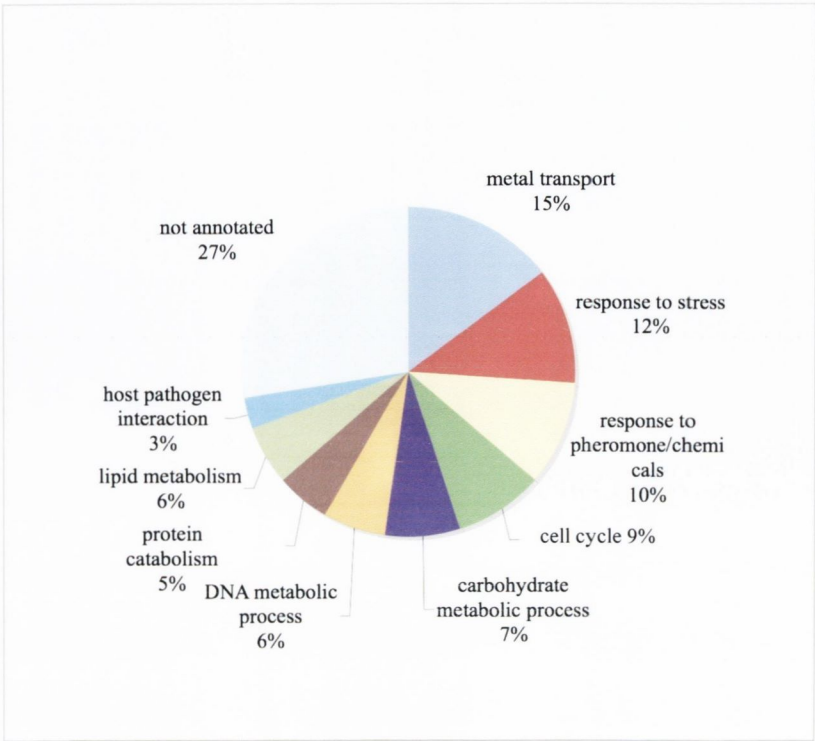
GO_term	Cluster frequency	Background frequency	Corrected P-value	Gene(s) annotated to the term
metal ion transport	12 out of 194 genes, 6.2%	68 out of 6435 background genes, 1.1%	0.00026	orf19.1534, ZRT2, ALR1, SIT1, FRE9, CTR1, FET31, orf19.4690, FTH1, FRP1, HAK1, FTR1
glucose catabolic process	8 out of 194 genes, 4.1%	28 out of 6435 background genes, 0.4%	0.00042	GPM2, CDC19, PGK1, ENO1, ZWF1, GND1, IFE2, YMX6
biofilm formation	9 out of 194 genes, 4.6%	51 out of 6435 background genes, 0.8%	0.00676	IFD6, SUN41, CSR1, CSH1, GCA1, RBT5, PGA10, PBR1, CSA1
modulation of host defense response	6 out of 194 genes, 3.1%	22 out of 6435 background genes, 0.3%	0.01346	MET6, PRA1, CDC19, PGK1, ENO1, HSP70

B

GO_term	Cluster frequency	Background frequency	Corrected P-value	Gene(s) annotated to the term
amino acid transport	6 out of 121 genes, 5.0%	40 out of 6435 background genes, 0.6%	0.01824	CAN2, HIP1, GAP1, AGP2, GNP3, orf19.7566

Fig. 6.2

A



B

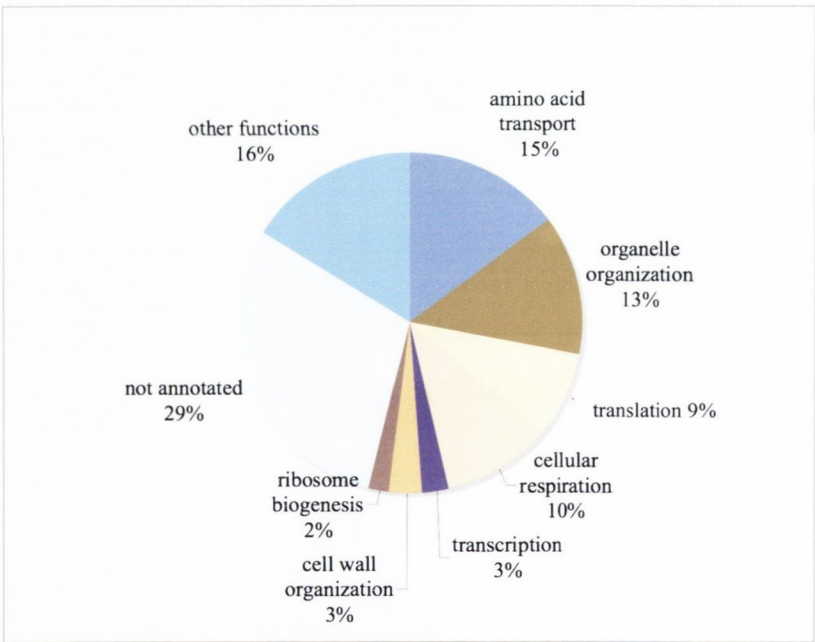


Fig. 6.2 Pie chart indicating the various cellular processes involving the genes differentially regulated between chlamydospores and yeast cells.

The 550 differentially regulated genes > 2-fold upregulated (A) and > 2-fold downregulated (B) in chlamydospores compared to yeast cells were analysed with the program Go Slim Mapper (<http://www.candidagenome.org/cgi-bin/GO/goTermMapper>).

Fig. 6.3

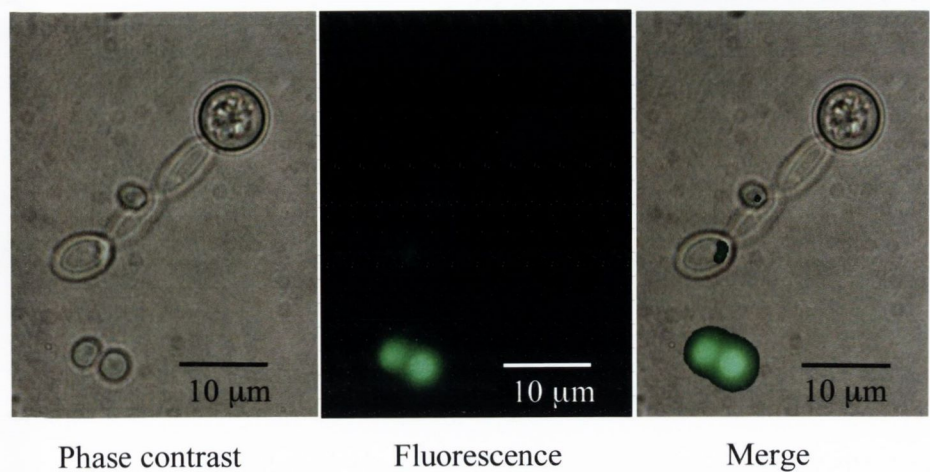


Fig. 6.3 Appearance of the strain *C. dubliniensis* WU284 *pNRG1-GFP* when incubated IN CIM.

The strain WU284 *pNRG1-GFP* was incubated in CIM for 4 days to induce chlamydospores production. The relative *NRG1* expression was studied in the yeasts, filaments and chlamydospores by examining fluorescence with a Nikon Eclipse E 600 fluorescence microscope equipped with a GFP emission filter.

The picture is shown with the permission of Dr Gary Moran who performed the experiment.

of an essential *S. cerevisiae* gene that encodes the two polypeptide components of the farnesyltransferase that prenylates a-factor and Ras proteins. These data indicate that in CIM these genes may be induced due to removal of *NRG1* repression (in fact this transcriptional regulator was found to be downregulated in a species-specific manner in CIM). A different set of genes was found to be commonly regulated by serum exposure and in the $\Delta nrg1$ background (estimated to be 12% of genes > 2-fold upregulated upon exposure to serum and 14% of the greater than two fold upregulated genes in the $\Delta nrg1$ background). These included genes well known to be under the control of *NRG1*, such as the hypha specific genes *HWPI* and *RBT1* and genes involved in oxidative stress response including *SOD5*. Eight genes were co-regulated in chlamydospores and upon exposure of *C. dubliniensis* to serum; these included mainly cell wall proteins involved in adhesion and in biofilm formation, (*INT1*, *SCW11*, *PHM7*, *SUN41*). Only one gene, *orf19.8361*, encoding a hypothetical protein, possibly homologue of the *S. cerevisiae* Ald6, required for conversion of acetaldehyde to acetate that locates to the mitochondrial outer surface upon oxidative stress, was found to be commonly upregulated in all the 3 different conditions. A Venn diagram illustrating the number of genes co-regulated in the 3 different conditions was created (Fig. 6.4).

6.2.4. Verification of the upregulation in chlamydospores of selected genes using the quantitative real time approach

In this study the housekeeping gene *ACT1* (Cd36_12690) was used as internal control. The analysis was carried out using the $2^{-\Delta\Delta Ct}$ model (Schmittgen & Livak, 2008). The genes examined were *HHD2* (Cd36_32340), *RBT5* (Cd36_40190), *MSH6* (Cd36_12230) and *CBF1* (Cd36_46060). The amplification efficiency of the targeted genes and the reference gene was assessed by comparing the changes in C_T observed over several template dilutions. According to the Livak and Schmittgen method several dilutions (1, 1/2, 1/4, 1/8,) of the *C. dubliniensis* genomic DNA (starting from a concentration of 0.5 μ g) were made. The slope for the different amplification was calculated using linear regression. Upon this analysis it was found that all the genes had approximately equal ($\leq 10\%$) amplification efficiencies (i.e. *ACT1* 1.373, *HHD2* 1.435, *RBT5* 1.66, *CBF1* 1.25, *MSH6* 1.39).

Expression analysis of the genes relative to *ACT1* in chlamydospores and yeast cells was investigated using RNA from the above mentioned growth conditions. According to the array results upregulation of the investigated genes was found in chlamydospores using RT PCR (Fig. 6.5).

6.3. Discussion

The chlamydospore is a specialised structure produced by *C. dubliniensis* and *C. albicans* in a combined response to specific stimuli. In the CIM medium the specific stimuli included methionine and carbohydrate limitation. Upon analysis of the expression of the transcriptional regulator *NRG1* using the *pNRG1-GFP* strain (see paragraph 3.2.4) it was found that galactose had a concentration dependent effect on the expression of the transcription factor. In the chlamydospore induction conditions (0.025% (w/v) galactose) used in the array experiment the expression of *NRG* was reduced compared to its expression in yeast cells grown in the same formulation of the medium but containing galactose in non limiting concentration (i.e. 2% (w/v) galactose). Therefore, *NRG1* is downregulated in response to galactose and methionine limitation under the conditions in which chlamydospores form readily in *C. dubliniensis*. Downregulation of *NRG1* may be considered a necessary event for chlamydospore formation to occur in the low methionine, low glucose medium. Due to this it was expected that some of the genes upregulated in chlamydospores would be *NRG1* repressed. Indeed, we found that 13% of the 2-fold upregulated genes in the *nrg1Δ* background and 11% of the genes 2-fold upregulated in chlamydospores coincided. These genes included genes involved in glucose metabolism and glucose transport, including *HGT5*, *GRE3*, and *ZWF1*. *HGT5* is a glucose transporter, whose transcription is glucose concentration dependent, possessing 12-transmembrane domains and a predicted signalling sequence possibly involved in morphogenesis. *GRE3*, the homolog of the *S. cerevisiae* *ScGRE3* encodes an aldose reductase which converts galactose to galactitol, and has a double role in galactose metabolism and in stress response. *ZWF1* which encodes a Glucose-6-phosphate dehydrogenase that catalyses the first step of the pentose phosphate pathway and is also involved in adapting to oxidative stress. In *S. cerevisiae* the *ZWF1* deletion strain displays decreased sporulation and increased sensitivity to oxidative stress. The pentose phosphate pathway plays an important role in generating NADPH, an important

Fig. 6.4

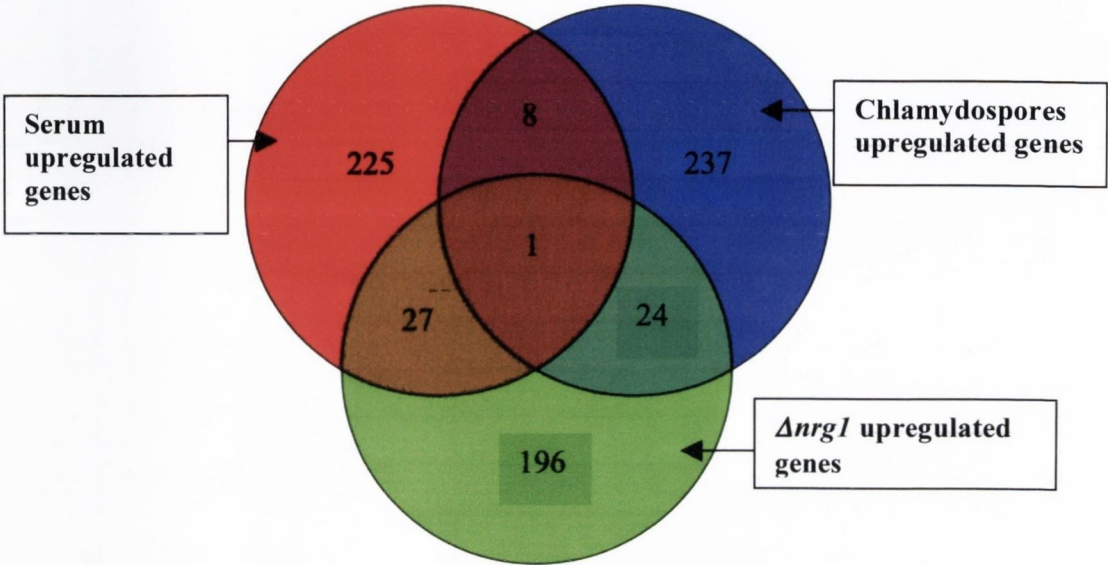


Fig. 6.4 Venn diagram illustrating the number of genes upregulated in the *nrg1* Δ background, the chlamydospores and upon exposure to serum and the proportion of the genes upregulated in common in these conditions.

Fig. 6.5

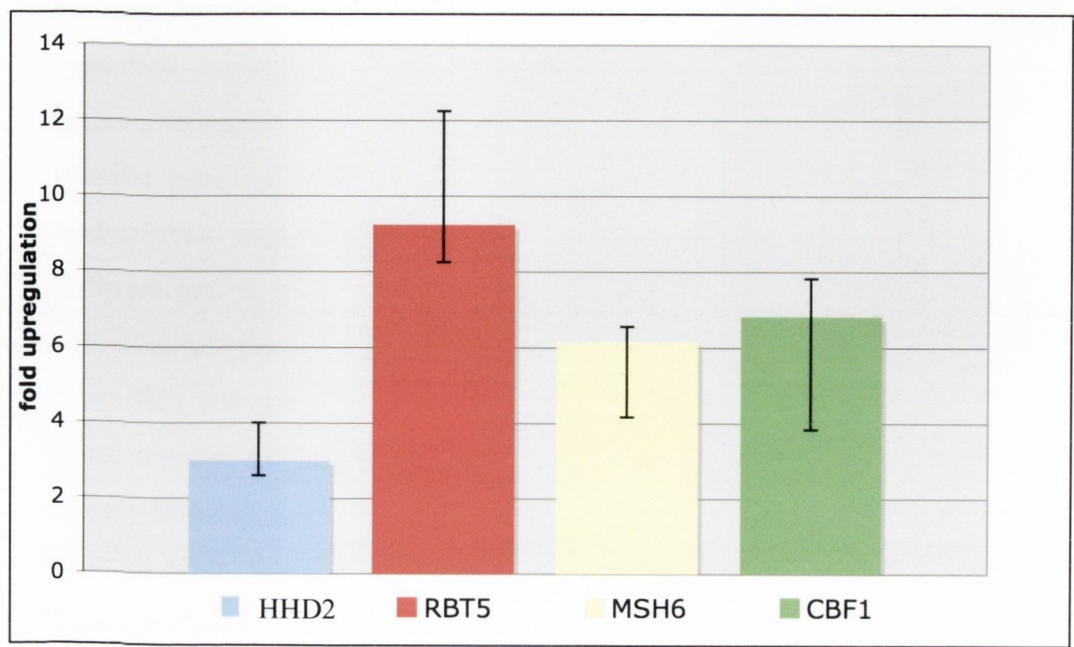


Fig. 6.5 Comparative quantitative real-time analysis of expression of selected genes between chlamydospores and yeast cells.

Relative expression of HHD2, RBT5, MSH6 and CBF1 in chlamydospores compared to yeast cells. The y axis indicate the fold upregulation of the transcript in chlamydospores compared to yeast cells.

coenzyme for glutathione and thioreductase dependent enzymes. Interestingly comparison of the genes upregulated in serum with those upregulated in the *nrg1Δ* background identified a different set of common up regulated genes from those identified during spore formation. These genes included hypha-specific genes and genes involved in the oxidative stress response. These are well characterised hypha-specific gene known to be under the regulation of *NRG1*. This suggests that this transcription factor can assume two different roles, regulating two completely different sets of genes, one in the genetic program at the basis of the yeast-to-hypha transition and the other in the genetic program activated during chlamydosporulation. From this comparison it is possible to conclude that *NRG1* downregulation is a necessary but not sufficient condition for chlamydospores production in CIM. In chlamydospores a set of genes involved in stress response, chromatin remodelling and cell cycle, were identified which did not appear to be Nrg1 regulated. However these genes may be under the control of the transcription factors Cap1, Efg1 and Hog1. These transcriptional regulators have previously been shown to control stress responses in blastospores and their subsequent morphogenesis (Cheetham *et al.*, 2007; Eisman *et al.*, 2006; Alonso-Monge *et al.*, 2003; Bahn & Sundstrom, 2001; Leng *et al.*, 2001). It has previously been shown that these transcription factors, in addition to *NRG1*, also play a role in the early phases of chlamydospore formation (Nobile *et al.*, 2003). Perhaps an interaction between the Nrg1, Cap1, Efg1 and Hog1 is at the basis of the transcript profile of chlamydospores, in particular of genes regulating cellular processes like cell cycle control, chromosome rearrangements and DNA repair, all of which are involved in cellular proliferation. In particular, genes involved in cell cycle check-point regulation (*MEC1*), chromatin rearrangement and histone regulation, acetylation/ deacetylation (*HHO1*, *HHT2*, *HTA2*, *HHT2*, *HHF22*, *PDS5*), were upregulated in chlamydospores. These genes and the genes involved in DNA repair-recombination (*RAD51*, *DMC1*, *DLH1*, *SMC1*, *MSH2*, *MSH6*) were also upregulated in spores. These genes may govern the process of nuclear duplication inside the chlamydospores which is associated with their budding and has been found to be different from the classical nuclear duplication during blastospores proliferation. In yeast nuclear duplication occurs at the neck between the mother and the daughter cell, however in chlamydospores, we found that nuclear duplication was happening in the mother chlamydospore with the subsequent migration of one

nucleus in the daughter cell (Fig. 5.18, 5.19). Some of the genes involved in DNA replication in chlamydospores have homologue in *S. cerevisiae* that are implicated in meiosis. For example *ScPDS5* is required for sister chromatid cohesion, *ScDMC1* and *ScDMC2* are required for meiotic recombination and *ScMEK1* is a meiosis-specific kinase that promotes recombination between homologous chromosomes. In *C. dubliniensis* and in *C. albicans* these genes may have evolved different roles, possibly related to genome replication or recombination functions that may confer evolutionary advantages to *Candida*. The upregulation of chromatin condensation genes may indicate that gene silencing may play a role in the shut down of genes associated with protein translation and whole translation process in chlamydospores. We found that many genes that regulate metabolism in chlamydospores were downregulated. Genes that encode amino acid permeases (*GAP1*, *AGP2*) were repressed, as were genes involved in translation. In particular enzymes involved in oxidative phosphorylation (*QCR2*, *QCR7*, *QCR8*, *CYT1*, *RIP1*, *NDH5*), fatty acid oxidation (*ECI1*, *FAA4*, *FAT1*, *PXP2*), the enzymes involved in the TCA cycle (*ACO1*, *ACO2*, *SDH2*) and in the glyoxylate cycle (*MDH1*, *MDH13*, *ACO3*) appeared down regulated. This finding was consistent with the process of reduction of metabolic activity related to age that is seen in chlamydospores (see Figs. 5.2, 5.3) and suggest that a precise genetic program is controlling the decrease in metabolic activity. We also found however some evidence for residual metabolic activities: the genes involved in the pentose phosphate pathway are upregulated in chlamydospores. In particular *ZWF1* that encodes a putative glucose-6-phosphate dehydrogenase and *GND1* that encodes a putative 6-phosphogluconate dehydrogenase are upregulated. Their upregulation could be related to the production of NADH and glyceraldehyde-3-phosphate from stored sugar or (indirectly) from lipid sources, in which chlamydospores are rich (Fig. 5.17). Genes implicated in proteins catabolism (*PRE2*, *PRE10*) were upregulated. It appears that metabolism in the mature chlamydospore is shifted towards endogenous catabolism rather than anabolism using lipids stored during maturation and degrading proteins. Alternatively the up-regulation of genes in chlamydospores that are implicated in sugar metabolism may be just a direct consequence of the difference in galactose content between the media for chlamydospore induction and yeast growth. Interestingly some authors studying chlamydospore structure found that these structures were rich in RNA (Jansons & Nickerson, 1970b). Our studies show the

progressive process of decrease in metabolic activity including repression of translation, and of transcription it is possible also to suppose a regulation of protein synthesis at the RNA level. In chlamydospores RNA could be stored in the cytoplasm and only translated in protein when stimuli for germination are present.

We found that the respiratory functions in chlamydospores appeared to be downregulated. Analysis of the differentially regulated genes with the Gene Ontology Term Finder further revealed that genes implicated in the respiratory electron transport chain (*NDH51*), mitochondrial ATP synthesis (*ATP17*) and mitochondrial organization processes were found downregulated in chlamydospores. The cytochrome c isoform 1 (*CYCI*), encoding an electron carrier of the mitochondrial intermembrane space that transfers electrons from ubiquinone-cytochrome c oxidoreductase to cytochrome c oxidase during cellular respiration (Janbon *et al.*, 1997) was found to be differentially regulated between chlamydospores and yeast cells suggesting that mitochondrial respiratory functions and biogenesis were down-regulated in these structures. This observation is reinforced by the fact that chlamydospores have been found to lose viability, upon ageing, through mitochondrial dysfunctions (see paragraph 5.2.7 and Figs. 5.15, 5.16).

Although methionine was at the same limiting concentration (i.e. 20 mg/L) in the media for both chlamydospores and yeast cells, some genes involved in methionine biosynthesis were found differentially regulated between chlamydospores and yeast cells. Met6 and Cbf1 appeared to be up-regulated in chlamydospores (or down-regulated in yeast cells). The role of the methionine transcriptional activator Cbf1 in *C. albicans* virulence and to its resistance to oxidative stress was analysed and described in chapter 4. The methionine synthase, Met6, catalyzes the transfer of a methyl group from tetrahydrofolate to homocysteine producing methionine. Burt *et al.* first identified this enzyme and found that its expression was related to various stresses and to the addition of estrogens to the culture medium (Burt *et al.*, 1999). Sulliman *et al.* knocked out both copies of the gene and found that Met6 was essential for *C. albicans* (Suliman *et al.*, 2007). Znaidi *et al.* found that Met6 was under the control of the transcriptional regulator Upc2 which is a key regulator of ergosterol metabolism and plays a role in *Candida* azole resistance and its upregulated in hypoxic conditions (Znaidi *et al.*, 2008). It is possible that the hypoxic conditions (absence of shaking and parafilm-covered flasks) in which the

chlamydospores were produced modulated the expression of the transcriptional activator Upc2. Murillo *et al.* found that Cbf1 regulates the first stages of biofilm formation (Murillo *et al.*, 2005). It is possible that upregulation of this transcriptional activator in chlamydospores may also control the expression of genes related to biofilm that we found upregulated in chlamydospores.

A number of genes implicated in stress response and also inducible by the presence of azoles or other drugs (e.g. *ECM33*, *GRP2*, *RAM1*) were also found differentially regulated in chlamydospores compared to yeast cells. This is in agreement with the work of Cole *et al.* that found induction of chlamydospores in mice upon administration of immunocompromising drugs (cyclophosphamide plus cortisone acetate) to animals with persistent GI infection (Cole *et al.*, 1991). Upon histological examination of the gastric mucosa the authors found invasive hyphal elements and yeast as well as multiple chlamydospore-like cells. They also described the effect of the cyclophosphamide in stimulating chlamydospore formation *in vitro*. Interestingly, a set of genes that encode for iron permeases and haemoglobin utilization enzymes were found to be up-regulated. However the iron was not at a limiting concentration in the chlamydospore induction medium. *C. albicans*, and likely *C. dubliniensis* among many pathogenic fungi and bacteria, are able to utilize haemin and haemoglobin as iron sources. Weissman *et al.* found that *RBT5* is strongly induced by iron starvation, and that deletion of *RBT5* is sufficient to significantly reduce the ability of *C. albicans* to utilize haemin and haemoglobin as iron sources (Weissman & Kornitzer, 2004). *RBT5* is under the control of the transcriptional activator Rim101 (Bensen *et al.*, 2004), Nobile *et al.* demonstrated the essentiality of this transcriptional activator in the process of chlamydospore formation (Nobile *et al.*, 2003). Setiadi *et al.* showed a considerable increase of *RBT5* when *C. albicans* was grown in hypoxic conditions also in non-limiting iron environment; the author also identified a linkage of oxygen sensing and Efg1p-regulatory network (Setiadi *et al.*, 2006). Some of the culture conditions used by these authors are similar to the chlamydospore culturing conditions. The upregulation of *RBT5* may be a consequence of a yet unidentified link between low carbohydrate concentrations, hypoxic condition and the transcription factor Upc2 with the pathway of iron assimilation. The ability to acquire iron from host tissues is a major virulence factor of pathogenic microorganisms, and this finding could link

chlamydospore formation to the ability of *Candida* to cause disease in certain host niches.

Pheromone stimulation may also be implicated in chlamydospore formation. Genes that respond to pheromones and genes implicated in mating are up-regulated (*MAT α* , *SAP30*, *AKR1*, *CPPI*, *OPY2*). The *MAT α* locus encodes for the α pheromone that is implicated in triggering the pheromone response in cells of the opposite mating type. In addition *SAP30* encodes an endopeptidase involved in degradation of alpha pheromone; that is functional equivalent of *S. cerevisiae* Bar1p a barrier activity protein that degrades α pheromone. Its expression is specific to a-cells and its transcription is induced in response to alpha pheromone. *C. albicans* mutants lacking Sap30 also exhibit a striking mating defect in a cells, but not in alpha cells, due to overstimulation of the response to alpha pheromone (Schaefer *et al.*, 2007). It appears that an autocrine response to pheromone is triggered by the incubation in the CIM in the a/ α CD36 cells with which the experiment was performed. Recently Alby *et al.* investigated the ability of *Candida* to undergo homothallic mating in absence of another strain by the activation of an autocrine mating signalling pathway (Alby *et al.*, 2009). This hypothesis could relate these structures for the first time to mating in *C. albicans* and *C. dubliniensis*. Experiments designed to test the genetic response of different mating types (and relative knock outs) of *C. albicans* and *C. dubliniensis* to synthetic α pheromone in chlamydospore induction conditions and if its administration in the medium influences chlamydospore formation could elucidate if chlamydospore formation is controlled by pheromone stimulation. Testing the ploidy of chlamydospores and daughter cells could bring insight of the relation of these structures and mating.

At the moment these results suggest that a chlamydospore is a specific growing form possibly related to a survival stage in the host in adverse conditions.

Chapter 7

General discussion

7. General discussion

7.1. The effect of methionine and glucose on *C. albicans* and *C. dubliniensis* morphogenesis and chlamydospore formation

Amino acids, in combination with glucose and other carbohydrates, are important morphogens in *C. albicans* but to date there is a paucity of studies describing their effects on their ability to trigger the yeast-to-hypha transition in this pathogen, and in *C. dubliniensis*.

The effect of altering the concentration of glucose/galactose and methionine in growth media where these are the sole source of carbohydrate and amino acids provided evidence for the role of the sulphur amino acids, in particular methionine, in combination with low carbohydrate concentrations, on the morphogenesis of *C. albicans* and *C. dubliniensis* (i.e. yeast-to-hypha transition) with some significant differences evident between the two species. While *C. albicans* was found to produce hyphae under a broad range of carbohydrate concentrations (e.g. 1% to 0.025% (w/v)), most *C. dubliniensis* strains produced hyphae only in low carbohydrate concentrations (i.e. 0.025% (w/v)). For the first time we have shown that glucose-induced hyphal expression can also be influenced by amino acid concentrations; in particular cysteine and methionine at high concentrations had an inhibitory effect on hypha production in *C. albicans* that was absent in *C. dubliniensis*. The absence of these amino acids from the growth media induced both species to grow in the yeast form. This raises the possibility of an interaction between the sensing of glucose and the sensing of these amino acids. Together these findings point to a complex process by which the sulphur amino acids, in combination with carbohydrates, trigger morphogenesis in the two species and that factors downstream of the uptake of methionine and glucose may be involved in the different phenotypes exhibited by the two species. Separate sensor proteins for carbohydrate and methionine and effectors could converge on components of the regulator pathway required for hypha formation.

During an infection *C. albicans* and *C. dubliniensis* become exposed to a range of concentrations of carbohydrates and amino acids, and as successful pathogens, the two species would have to change their morphology in response to nutritional requirements. It appears that *C. albicans* has evolved its mechanism of sensing-

signalling to respond to a wider range of concentrations of methionine and sugars. In contrast the reduced capacity of *C. dubliniensis* to respond to these changes in the growth medium may reflect a lack of sensitivity to environmental conditions and may contribute to the reduced virulence of *C. dubliniensis* or to its restricted capability to colonise different body sites (e.g. the gastrointestinal tract) compared to *C. albicans*. We have also described the identification of a defined condition (i.e. YNB containing 0.025% (w/v) glucose or galactose and 20 mg/L methionine pH 3.7) which causes *C. albicans* to grow as a yeast while *C. dubliniensis* grows as hyphae, pseudohyphae and chlamydospores. This medium, generated highly reproducible results in a large number of strains of both species and therefore, can be used as a means to differentiate between *C. albicans* and *C. dubliniensis*. We have called this medium chlamydospore induction medium (CIM). The main advantages of this defined medium, compared to other methods of discrimination between the two species based on their colony morphology (e.g. Tobacco agar or Staib agar), are that the medium is inexpensive and simple to prepare.

The different phenotypes of the two species may be attributed to a fine species-specific regulation of factors downstream of glucose and methionine uptake, that in turn would regulate well described transcriptional regulators involved in the yeast-to-hyphal transition and in chlamydospore production. One of the best described transcriptional regulators involved in both processes is the repressor *NRG1*. This transcriptional regulator was also found to be involved in the differential morphogenesis of *C. albicans* and *C. dubliniensis* in Staib agar. We further analysed its regulation in CIM and we found its species-specific downregulation in *C. dubliniensis*. This fact could also explain the increased virulence of some *C. dubliniensis* strains in the RHE model, and their increased ability to escape the macrophage killing mechanism upon incubation in CIM. These findings are also in agreement with those of Moran *et al.* (2007) who found that deletion of *NRG1* enhanced filamentation and survival of *C. dubliniensis* in co-culture with murine macrophages. These data reveal, at least in *C. dubliniensis*, the existence of a previously undiscovered link between the amino acid methionine and the transcriptional activator *NRG1*. To analyse what genes of the methionine biosynthetic pathway could be responsible for the differences in morphology between the two species in the media (in which glucose/galactose and methionine

were the sole source of carbohydrate and amino acids) we carried out an analysis of the regulators of methionine biosynthesis.

Almost all of the methionine biosynthetic genes in *C. albicans* and *C. dubliniensis* are $\geq 90\%$ identical. Interestingly, the two most divergent genes are *CBF1* and *MET4* (70% and 80% identical at the amino acid level, respectively), which are the regulatory proteins that control the biosynthesis of methionine. In addition, these proteins in *S. cerevisiae* also control aspects of general metabolism (through the activity of S-adenosyl methionine (SAM)) and oxidative stress (through the synthesis of glutathione). In order to investigate the role of these regulators we decided to study the phenotype of the *C. albicans* $\Delta cbf1$ mutant, to integrate the *CaCBF1* gene in the *C. dubliniensis* genome and to inactivate the *CaMET4* gene.

Heterologous expression of the *C. albicans* *CBF1* gene in *C. dubliniensis* did not have any detectable effect on the phenotype of this species. This suggests two possibilities, firstly that the *C. dubliniensis* gene is fully-functional and the expression of the *C. albicans* gene in this species does not complement any defect. The second possibility is that the degree of divergence in the oligomerisation domain between the two homologues is so high that *C. albicans* Cbf1 can not carry out all its original functions in *C. dubliniensis*.

Cbf1, the methionine main transcriptional activator was found to play an important role in *C. albicans* virulence affecting its ability to resist oxidative stress and also partially affecting its ability to produce hyphae and to cause damage in the RHE infection model. The *C. albicans* $\Delta cbf1$ mutant was found to be unable to produce hyphae when grown on BSA agar. The inability of $\Delta cbf1$ to form hyphae in this specific medium may be indicative of a possible relationship between the transcriptional activator and the mechanism involved in the sensing of proteins in the environment. A biofilm assay on *C. albicans* wild type and $\Delta cbf1$ strain was performed because whole transcriptome analysis in the early stages of biofilm formation identified an up-regulation of almost all the genes in the methionine biosynthesis pathway by 3-5 fold (Yeater *et al.*, 2007). The lower ability of the $\Delta cbf1$ to form biofilm, compared to the wild type, indicates that (also in medium containing methionine in non limiting concentration, in which the assay was performed) in addition to activating genes related to methionine biosynthesis Cbf1 regulates proteins involved in the early stages of biofilm formation. In the RHE model the

Δcbf1 mutant also exhibited attenuated virulence in comparison to the wild type. It was found that the *Δcbf1* mutant was still able to undergo the yeast-to-hypha transition following interaction with the epithelial cells but strangely the hyphae formed by the mutant were short and not able to invade the epithelium, instead forming a non-invasive layer on top of the cells. Together these data suggested that the methionine biosynthesis pathway plays an important role in *C. albicans* and in *C. dubliniensis* virulence as has been observed for other pathogenic fungi (Pascon *et al.*, 2004; Yang *et al.*, 2002; Maresca & Kobayashi, 1989; Boguslawski & Stetler, 1979).

Thomas *et al.* showed that in *S. cerevisiae* induction of the sulphate assimilation pathway and methionine biogenesis is not achieved solely by Cbf1p. This induction also requires another trans-acting factor, encoded by the *MET4* gene, that forms heterodimers with the Cbf1 protein (Thomas *et al.*, 1992). In *S. cerevisiae* the Met4 transcription factor (and its co-factor Cbf1) not only controls the expression of sulphur metabolism but also regulates expression of oxidative stress response proteins (e.s. glutathione and the methyl donor protein, S-adenosylmethionine) (Wheeler *et al.*, 2003). In order to assess the function of the Met4 transcriptional activator in *C. albicans* we have identified an ORF (19.5312) in the *C. albicans* genome that encodes for a putative protein annotated as Met4. We deleted both alleles of this gene in *C. albicans* by targeted mutagenesis and investigated the effect of this inactivation on the phenotype of the resulting mutant. To our surprise the *C. albicans Δmet4* mutant was not auxotrophic for methionine. Further analysis of the mutant using the Biolog phenotype MicroArray revealed that there was no difference in the ability of the mutant and wild type strains to grow on a wide range of nitrogen, carbon and phosphorus sources. To investigate the possible role of Met4 in glutathione biosynthesis the *Δmet4* mutant was tested, in comparison with the wild type, in a range of stress tolerance tests. In addition, the ability of the mutant to survive in co-culture with macrophages was tested. No differences between the wild type and the mutant were found in any of these analyses.

As was previously mentioned, these data suggest two possibilities; the first is that the role of Met4 in *C. albicans* is very different from its role in *S. cerevisiae* and that transcriptional rewiring has occurred in these pathways as appears to have happened in the galactose pathway (Martchenko *et al.*, 2007; Rokas & Hittinger, 2007). The other possibility is that the *C. albicans* ORF 19.5312 that has been annotated as Met4

is not in fact the homolog of the *S. cerevisiae* *MET4* gene despite the 40% identity shared by the two proteins.

Glucose and methionine also have the peculiarity of triggering chlamydospore formation in *C. dubliniensis* and to increase chlamydospore production in *C. albicans* when added to commonly used media for chlamydosporulation in this species. In contrast to hyphae which are also triggered in the two species by these two compounds and are considered virulence attributes, the role of chlamydospores in *C. albicans* and *C. dubliniensis* virulence and life cycle is not known. The novel media described (i.e. CIM for *C. dubliniensis* chlamydosporulation and the liquid medium corn meal broth 1% (w/v) tween 80 with of 0.025% (w/v) galactose and 20 mg/L methionine for *C. albicans* chlamydosporulation) were an invaluable tool for the study of chlamydospore characteristics. Using the molecular probe FUN-1 it was demonstrated that chlamydospores are metabolically active cells. Mature chlamydospores (5-7 days-old) were found to possess high metabolic activity, and this metabolic activity decreases over time under nutrient-poor conditions. After 30 days of incubation metabolic activity was almost undetectable. This period can be interrupted and metabolic activity induced to resume following exposure of the chlamydospores to specific external stimuli. Unlike in resting spores, this stage of low metabolic activity can only be interrupted in chlamydospores that are not older than 15-20 days. Relatively young chlamydospores can be reactivated, resuming metabolic activity, following exposure to a nutrient-rich environment and serum and produce daughter yeast cells or germ tubes. Interestingly, it was noticed that the number of the mature chlamydospores that underwent reactivation was far higher than the number of chlamydospores that germinated. This can be indicative of the fact that rich medium and serum are sufficient for a proper reactivation, but for more efficient germination additional and specific environmental stimuli, for example contact with the host tissue, should be tested.

When germination was achieved in rich medium with serum at 37°C in presence of CO₂, hyphae were found emanating from the chlamydospores. Since hyphae are associated with pathogenesis in the two species, this observation, for the first time suggests that chlamydospores of *C. albicans* and *C. dubliniensis* may play a role in virulence. Interestingly, activated chlamydospores of the two species were able to produce filaments when exposed to murine macrophages.

The incubation of chlamydospores in starvation conditions for long periods resulted in a strong reduction in the number of chlamydospores capable of resuming metabolic activity. Chlamydospores appear to lose viability if incubated in water for more than a week. This reduction in viability was not seen in a suspension of yeast cells when incubated in the same conditions.

We also discovered that chlamydospores have almost the same ability to resist dry stress as yeast cells, losing their viability after exposure for more than two days to a dry environment. This is not consistent with the hypothesis that *C. albicans* and *C. dubliniensis* could produce chlamydospores outside the human body in response to specific adverse conditions, (temperature switching and nutrient limitation) and act as a resting spore. If chlamydospores are true vegetative spores, it would be necessary for the spore to undergo long periods of dormancy and be able to resist to dry stress. Chlamydospores, at least in *in vitro* experiments, did not show these abilities and so must perform another function in the life cycle of *C. albicans* and *C. dubliniensis*.

In order to understand the reason for loss of viability ultra-structural and biochemical studies were performed on chlamydospores of different stages of maturation. When the mitochondrial function of chlamydospore was investigated using MitoTracker Red dye it was found that a 5 day old *C. albicans* chlamydospore-rich culture stained with this dye showed that more than 80% of mature chlamydospores possessed metabolically active mitochondria but by day 20 the majority of chlamydospores possessed inactive mitochondria. These results were reinforced by transmission electron microscopy that showed that mitochondria lose the shape of their membrane and lamellae and consequently their metabolic activity during chlamydospore ageing. This demonstrated that chlamydospores probably lose their viability through mitochondrial dysfunction and degradation.

Gene expression analysis in chlamydospores, compared to yeast cells can provide some clues concerning their possible role in the *Candida* life cycle. The technique that we used for this analysis was the two colour microarray technique. *C. dubliniensis* CD36 was used in this study due to its ability to produce very large numbers of chlamydospores in CIM. Because *C. dubliniensis* chlamydosporulation was dependent upon the concentration of galactose and the starting inoculum in CIM two very similar culturing conditions were used one promoting the growth of *C. dubliniensis* in the yeast form, the other enhancing chlamydospore formation (i.e.

0.025% (w/v) galactose and a starting inoculum of 1×10^2 cells to promote chlamydospore formation and 0.5% (w/v) galactose and a starting inoculum of 1×10^7 to promote growth in the yeast form). We found that around 11% of the genes two fold up-regulated in chlamydospores were genes regulated by *NRG1*. These genes included mainly genes involved in glucose metabolism, glucose transport and genes involved in oxidative stress response. *NRG1* controls instead a different set of genes during the yeast-to-hypha transition (i.e. upon serum exposure). This suggests that *NRG1* is implicated in two completely distinct genetic programs, in which it regulates completely different sets of genes, depending on the environmental conditions, one that triggers the yeast-to-hypha transition and the other that induce chlamydosporulation. Other upregulated genes involved in stress response, were found to be under the control of the transcription factors Cap1, Efg1 and Hog1. This indicates the importance of these transcriptional activators not only in the yeast-to-hypha transition process but also in the formation of chlamydospore and also explains why the $\Delta efg1$ and the $\Delta hog1$ mutants are defective in chlamydospore formation whereas the $\Delta nrg1$ mutant hyperproduce chlamydospores. We also found that genes that regulate general metabolism in chlamydospores were down-regulated, (e.g. genes that encoding amino acid permeases, oxidative phosphorylation, fatty acid oxidation, TCA cycle and glyoxylate cycle enzymes). Genes related with mitochondrial respiratory functions and biogenesis were also downregulated in these structures. We also found however some evidence for residual metabolic activities: the genes involved in the pentose phosphate pathway, in the utilisation of lipid sources and genes involved in the reutilisation of amino acids from proteins were upregulated in chlamydospores. It appears that metabolism in the mature chlamydospore is shifted towards endogenous catabolism rather than anabolism using lipids stored during maturation and degrading proteins. Testing the chemical composition of the lipid inclusions found with transmission microscopy in the chlamydospores cytoplasm could lead us to understand what type of metabolite these structures accumulate and possibly use (when mature) to survive. This would give value to the hypothesis by which chlamydospores could allow survival within specific niches in the host consuming stored nutrient sources and only germinate when specific stimuli are present (or inhibitory stimuli are absent). This hypothesis is also in agreement with the work of Cole *et al.* (1991) who found production of

chlamydospores in mice upon administration of immunocompromising drugs to animals with persistent GI infection and that the same drug induced chlamydospores production in *in vitro* experiments. The upregulation of sets of genes involved in the response to a variety of stresses (oxidative, genotoxic and chemical) found in chlamydospores could help these structures to survive harsh environments *in vivo* despite the results of our *in vitro* tests. Other studies in which chlamydospores and yeast cells will be challenged with a wide range of different stresses may reveal some distinctive characteristic and survival potential of chlamydospores that are absent in yeast cells. Scanning and transmission electron microscopy studies have shown the chlamydospore cell wall to be different in dimension and organisation compared to that of yeast cells. Investigation into its composition may also bring insight to the possible advantages that these structures may confer to *C. albicans* and *C. dubliniensis* during the interaction with the host.

One of the most intriguing findings related to gene expression in chlamydospores is the revelation that pheromone stimulation appears to be involved in chlamydospore formation as genes that respond to pheromones and genes involved in mating are upregulated. Amongst the genes found to be upregulated in chlamydospores that govern cellular processes like cell cycle control, chromatin regulation, chromosome rearrangements and DNA repair-recombination, are found some homologues of the genes that in *S. cerevisiae* are implicated in meiosis. In *C. albicans*, which is not known to undergo meiosis, these genes may have evolved different roles, possibly related to genome recombination functions and may also govern the unique nuclear duplication process found in chlamydospores. This may confer evolutionary advantages to *C. albicans* and *C. dubliniensis*.

Testing the ploidy and the chromosomal arrangement of chlamydospores and their daughter cells may reveal DNA recombination events that may confer advantages in the two species during host colonisation. What the role of these cells is in the normal life cycle of *C. albicans* and *C. dubliniensis* is still not clear. Indeed, the paucity of reports of chlamydospores in clinical samples suggests that they are unlikely to play an important role in pathogenesis. However, the fact that these two species have maintained the ability to produce these complex structures, which very likely require complex signalling and regulatory pathways, since their divergence approximately 20 million years ago, suggests that they may indeed have important functions.

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Fold change	Common Name	GeneName	orf
280.3		Cd36_41000	orf19.4690
129.5		Cd36_15800	orf19.2038
117.5		Cd36_81460	orf19.1691
50.7	INO1	Cd36_35120	orf19.7585
43.5	HAP3	Cd36_41400	orf19.4647
30.7	ZRT2	Cd36_17380	orf19.1585
29.5	SUL2	Cd36_42530	orf19.2738
28.4		Cd36_12860	
27.7	SOD3	Cd36_70320	orf19.7111.1
27.2		Cd36_24424	
24.9	CSR1	Cd36_44490	orf19.3794
22.9		Cd36_04400	orf19.781
21.6	FTH1	Cd36_08870	orf19.4802
20.8		Cd36_41700	orf19.4612
20.8	SIT1	Cd36_22250	orf19.2179
20.0		Cd36_43110	orf19.1438
19.8	PGA23	Cd36_27260	orf19.3740
19.1		Cd36_18660	orf19.822
19.1	HSP31	Cd36_01930	orf19.3664
18.6		Cd36_09760	orf19.4907
18.0	MNN1	Cd36_52400	orf19.4279
16.6	SEO1	Cd36_32190	orf19.700
16.2	RBT5	Cd36_40190	orf19.5636
15.8	FRP1	Cd36_40170	orf19.5634
15.7		Cd36_28410	orf19.2397
14.5		Cd36_16960	orf19.1534
14.1		Cd36_52340	orf19.4270
13.7	DUT1	Cd36_01230	orf19.3322
13.3		Cd36_10650	orf19.668
13.3		Cd36_86160	
12.5	IFD6	Cd36_03780	orf19.1048
12.1		Cd36_01980	
11.7	RNR1	Cd36_17930	orf19.5779
11.5		Cd36_63190	orf19.5565
11.3	AHP1	Cd36_42290	orf19.2762
11.2	LAP3	Cd36_29830	orf19.539
11.1		Cd36_63680	orf19.5620
11.1		Cd36_04190	orf19.6837
10.4		Cd36_20100	orf19.6877
10.3		Cd36_21800	orf19.1874
10.0		Cd36_17890	orf19.5784
9.3		Cd36_22640	orf19.3627
9.2		Cd36_29580	orf19.513
9.2		Cd36_32760	orf19.732
9.1		Cd36_03760	orf19.4476
9.1	SAP8	Cd36_82480	orf19.242
8.8	CSA1	Cd36_70300	orf19.7114
8.3		Cd36_33200	
8.2		Cd36_43680	orf19.5290
8.1	PGA7	Cd36_40180	orf19.5635
8.0		Cd36_63900	orf19.5602
8.0	NHP6A	Cd36_41610	orf19.4623.3

7.8	MCD4	Cd36_11430	orf19.5244
7.8	IFE2	Cd36_30670	orf19.5288
7.8	PST1	Cd36_21170	orf19.2241
7.7	OSM1	Cd36_20140	orf19.6882
7.7	GRE2	Cd36_21020	orf19.3150
7.7		Cd36_86300	
7.6	ESC4	Cd36_16260	orf19.1445
7.5		Cd36_52975	
7.5	FET31	Cd36_60390	orf19.4213
7.3		Cd36_16140	orf19.1998
7.1		Cd36_18390	orf19.850
7.1		Cd36_20810	
7.0	SAP30	Cd36_15430	orf19.2082
6.9	ADH4	Cd36_82790	orf19.271
6.8	AXL2	Cd36_43700	orf19.5292
6.8		Cd36_22770	
6.7		Cd36_42450	orf19.2745
6.7		Cd36_46710	
6.6		Cd36_23460	orf19.4095
6.6	SOD4	Cd36_15610	orf19.2062
6.6	RHD3	Cd36_43810	orf19.5305
6.6		Cd36_01920	
6.5	YMX6	Cd36_63450	orf19.5713
6.4	PGA6	Cd36_08570	orf19.4765
6.4	GPM2	Cd36_04070	orf19.1067
6.3	CSH1	Cd36_03770	orf19.4477
6.1	PRA1	Cd36_46450	orf19.3111
6.1		Cd36_82570	orf19.251
6.1	PGA60	Cd36_64000	orf19.5588
6.1		Cd36_45360	
6.1		Cd36_23910	orf19.1368
6.0	SCW11	Cd36_53810	orf19.3893
6.0	ARG3	Cd36_63830	orf19.5610
6.0	GRP2	Cd36_52640	orf19.4309
6.0	LIP10	Cd36_09030	orf19.4822
6.0		Cd36_22800	
5.8		Cd36_17580	orf19.5826
5.8		Cd36_23550	
5.8	GCA1	Cd36_09690	orf19.4899
5.7		Cd36_09930	
5.7		Cd36_87800	
5.6		Cd36_43870	orf19.1430
5.6		Cd36_24210	orf19.1765
5.6	DSE1	Cd36_22620	orf19.3629
5.6	HHT21	Cd36_04010	orf19.1061
5.5		Cd36_46170	orf19.3139
5.5		Cd36_72840	orf19.2123
5.4	HSP70	Cd36_12490	orf19.4980
5.3		Cd36_51470	orf19.584
5.3	ZWF1	Cd36_08460	orf19.4754
5.3	IFF9	Cd36_29150	orf19.465
5.2	CSA2	Cd36_46390	orf19.3117
5.2	SUC1	Cd36_34590	orf19.7319

5.2		Cd36_15810	orf19.2037
5.1		Cd36_60040	
5.1		Cd36_23940	orf19.1365
5.1		Cd36_81870	
5.1		Cd36_01530	orf19.3356
5.0		Cd36_87550	orf19.6720
4.9	PGA10	Cd36_40510	orf19.5674
4.9		Cd36_41150	orf19.4673
4.9	ECM331	Cd36_52240	orf19.4255
4.8		Cd36_08780	orf19.4791
4.8	ENO1	Cd36_08010	orf19.395
4.8		Cd36_22450	
4.8		Cd36_44800	
4.8		Cd36_04820	
4.8	HHO1	Cd36_72820	orf19.5137.1
4.8	HHF22	Cd36_32340	orf19.1854
4.7		Cd36_40900	orf19.4707
4.7	MSH6	Cd36_12230	orf19.4945
4.7		Cd36_81940	
4.7		Cd36_40910	orf19.4706
4.7	LIP4	Cd36_72950	orf19.2133
4.6	FAV2	Cd36_53480	orf19.1120
4.6	CDC19	Cd36_19920	orf19.3575
4.6		Cd36_05660	orf19.2446
4.6		Cd36_70700	orf19.1208
4.5	DLH1	Cd36_11740	orf19.3760
4.5		Cd36_86820	orf19.6816
4.5		Cd36_31080	orf19.6641
4.5	RNR22	Cd36_21850	orf19.1868
4.5		Cd36_22790	
4.4		Cd36_42600	orf19.2726
4.4	FGR6-3	Cd36_07280	orf19.4712
4.4		Cd36_05810	orf19.2429
4.4	TOP2	Cd36_46080	orf19.2873
4.3	RBE1	Cd36_13090	orf19.7218
4.3	FGR6-4	Cd36_24200	orf19.3490
4.3		Cd36_45380	orf19.1267
4.3		Cd36_05370	orf19.2481
4.3	RAD51	Cd36_27150	orf19.3752
4.3	IFD3	Cd36_01150	orf19.3311
4.2	MET6	Cd36_26580	orf19.2551
4.2	FGR41	Cd36_09790	orf19.4910
4.2		Cd36_82120	orf19.1632
4.2		Cd36_04810	
4.1		Cd36_83580	orf19.355
4.1	HHF1	Cd36_04000	orf19.1059
4.1	FGR6-10	Cd36_45130	orf19.1234
4.1		Cd36_87420	orf19.6737
4.1		Cd36_44770	orf19.8361
4.0	SUN41	Cd36_60800	orf19.3642
4.0	HHT2	Cd36_32350	orf19.1853
4.0	PGA52	Cd36_15090	orf19.1911
3.9		Cd36_61470	orf19.3448

3.9	FGR24	Cd36_72500	orf19.7275
3.9		Cd36_87190	orf19.6770
3.9		Cd36_02070	orf19.3679
3.9		Cd36_44500	orf19.3793
3.9		Cd36_03940	orf19.1052
3.9	PSA2	Cd36_12220	orf19.4943
3.9		Cd36_31460	orf19.3852
3.9		Cd36_81930	
3.8		Cd36_81030	orf19.2515
3.8		Cd36_40350	orf19.5655
3.8		Cd36_17120	orf19.1571
3.8	INT1	Cd36_52250	orf19.4257
3.8	PRE10	Cd36_71300	orf19.6582
3.8		Cd36_61480	orf19.3447
3.8		Cd36_31690	orf19.3871
3.8	HTA1	Cd36_83890	orf19.6924
3.8		Cd36_62950	orf19.5536
3.7		Cd36_08980	orf19.4816
3.7		Cd36_53270	
3.7		Cd36_61580	orf19.3437
3.7		Cd36_34795	orf19.6605
3.7		Cd36_29610	orf19.516
3.6		Cd36_80040	
3.6	JEN2	Cd36_43830	orf19.5307
3.6		Cd36_82690	orf19.260
3.6		Cd36_28350	orf19.2401
3.6	GAD1	Cd36_10950	orf19.1153
3.6		Cd36_16860	orf19.1523
3.5	HTA2	Cd36_03930	orf19.1051
3.5		Cd36_70790	orf19.7032
3.5		Cd36_85300	orf19.6968
3.5		Cd36_10760	
3.5		Cd36_15690	orf19.2049
3.5		Cd36_15880	orf19.2030
3.5		Cd36_42860	orf19.2693
3.5		Cd36_30530	orf19.649
3.4	SPR28	Cd36_52310	orf19.4266
3.4		Cd36_33530	orf19.3714
3.4		Cd36_32090	orf19.916
3.4	ECM331	Cd36_02990	orf19.4255
3.4	SMC1	Cd36_28880	orf19.4367
3.4		Cd36_25410	
3.4		Cd36_85200	
3.4		Cd36_01960	orf19.3667
3.4		Cd36_43670	orf19.1208
3.4		Cd36_44960	orf19.1290
3.4		Cd36_42230	orf19.2769
3.4	RFA1	Cd36_15350	orf19.2093
3.3	DUR1,2	Cd36_04410	orf19.780
3.3	CBF1	Cd36_46060	orf19.2876
3.3		Cd36_18400	orf19.849
3.3		Cd36_41580	orf19.4626
3.3	CSP37	Cd36_26450	orf19.2531

3.3		Cd36_46550	orf19.3100
3.3	CCC2	Cd36_52780	orf19.4328
3.2		Cd36_24430	
3.2		Cd36_64050	
3.2	MSI3	Cd36_05770	orf19.2435
3.2		Cd36_22820	
3.2	MOH1	Cd36_43220	orf19.3369
3.2		Cd36_12630	orf19.5003
3.2		Cd36_81040	orf19.2516
3.2		Cd36_09010	orf19.4820
3.2		Cd36_86540	orf19.7445
3.2		Cd36_81180	orf19.1729
3.2	AKR1	Cd36_12270	orf19.4950
3.2		Cd36_73440	orf19.7204
3.2	PGA12	Cd36_35200	orf19.7597
3.2	CFL1	Cd36_45410	orf19.1263
3.1	MRF1	Cd36_11010	orf19.1149
3.1		Cd36_55090	
3.1	PDC11	Cd36_46050	orf19.2877
3.1		Cd36_52940	orf19.4347
3.1		Cd36_60050	
3.1		Cd36_87240	orf19.6758
3.1		Cd36_64290	orf19.5752
3.0		Cd36_73100	
3.0	CYP1	Cd36_72170	orf19.6472
3.0		Cd36_04670	orf19.750
3.0	VTC4	Cd36_43130	orf19.3363
3.0		Cd36_04330	orf19.6852
3.0		Cd36_15010	
2.9		Cd36_32110	
2.9	MP65	Cd36_24090	orf19.1779
2.9		Cd36_24040	orf19.1355
2.9	RAM2	Cd36_08990	orf19.4817
2.9	PGA38	Cd36_42320	orf19.2758
2.9		Cd36_52980	
2.9		Cd36_45200	orf19.1240
2.9		Cd36_84150	orf19.5862
2.9		Cd36_64490	orf19.5775
2.9	SSR1	Cd36_70800	orf19.7030
2.9	FGR6-3	Cd36_72390	orf19.4712
2.9	ERG24	Cd36_23520	orf19.1598
2.8		Cd36_44440	orf19.3799
2.8		Cd36_72690	orf19.5158
2.8		Cd36_51230	orf19.1961
2.8	ECM42	Cd36_71970	orf19.6500
2.8	CTF8	Cd36_50720	orf19.576
2.8		Cd36_23840	orf19.1374
2.8		Cd36_30250	orf19.6307
2.8	MPH1	Cd36_45720	orf19.2919
2.8		Cd36_11750	orf19.3759
2.8		Cd36_23340	orf19.4080
2.8		Cd36_09050	orf19.4824
2.8	LIP6	Cd36_09040	orf19.4823

2.7		Cd36_19300	orf19.194
2.7	FGR22	Cd36_17390	orf19.1586
2.7		Cd36_34580	orf19.7317
2.7	GCS1	Cd36_07400	orf19.5059
2.7	CRH11	Cd36_42770	orf19.2706
2.7	SRB1	Cd36_87780	orf19.6190
2.7		Cd36_87260	orf19.6756
2.7	MSH2	Cd36_46590	orf19.3093
2.7		Cd36_09230	orf19.4845
2.7		Cd36_80730	orf19.3071
2.7		Cd36_20380	orf19.5216
2.6		Cd36_73550	orf19.7196
2.6		Cd36_17410	orf19.1588
2.6		Cd36_26890	orf19.2600
2.6	IFG3	Cd36_50460	orf19.944
2.6		Cd36_50940	orf19.4131
2.6	FDH3	Cd36_35225	orf19.7600
2.6	MAD2	Cd36_03840	orf19.1040
2.6		Cd36_63110	orf19.5572
2.6		Cd36_17350	
2.5		Cd36_17230	orf19.1557
2.5		Cd36_65060	
2.5		Cd36_12180	orf19.4939
2.5		Cd36_30260	orf19.6306
2.5	PI-PLC	Cd36_17810	orf19.5797
2.5		Cd36_42640	orf19.2721
2.5	IDP1	Cd36_20310	orf19.5211
2.5		Cd36_64760	orf19.1095
2.4		Cd36_64780	orf19.1096
2.4		Cd36_41030	orf19.4686
2.4	SAH1	Cd36_53940	orf19.3911
2.4	CDR4	Cd36_07580	orf19.5079
2.4	YCF1	Cd36_72120	orf19.6478
2.4	RNH35	Cd36_71460	orf19.6562
2.4		Cd36_35170	orf19.7593
2.4	HGT5	Cd36_85250	orf19.6005
2.4		Cd36_22530	orf19.1434
2.4		Cd36_63760	orf19.5614
2.4	SNQ2	Cd36_64350	orf19.5759
2.4		Cd36_23570	orf19.1605
2.4		Cd36_82050	orf19.1639
2.4	PFK1	Cd36_54420	orf19.3967
2.4		Cd36_08690	orf19.4779
2.4	PRE6	Cd36_29910	orf19.544.1
2.4		Cd36_86840	orf19.6813
2.4		Cd36_60490	orf19.4203
2.3	IFF8	Cd36_50710	orf19.570
2.3		Cd36_33720	orf19.6366
2.3	WSC4	Cd36_13330	orf19.7251
2.3	SMC2	Cd36_22690	orf19.3623
2.3		Cd36_82070	orf19.1637
2.3	RAD23	Cd36_16680	orf19.1494
2.3	UTR2	Cd36_81610	orf19.1671

2.3	PRE2	Cd36_21120	orf19.2233
2.3	DYN1	Cd36_85190	orf19.5999
2.3		Cd36_46700	
2.3		Cd36_42350	orf19.2755
2.3		Cd36_06850	orf19.4436
2.3		Cd36_00020	
2.3	MDM34	Cd36_05840	orf19.1826
2.3	PGA19	Cd36_15850	orf19.2033
2.3	LEM3	Cd36_19620	orf19.3542
2.3		Cd36_52710	
2.3		Cd36_74020	orf19.8197
2.3		Cd36_22590	orf19.3633
2.3		Cd36_22710	orf19.3621
2.2		Cd36_40420	orf19.5663
2.2		Cd36_61710	orf19.3425
2.2	FLC2	Cd36_32680	orf19.1813
2.2	PHM7	Cd36_22320	orf19.2170
2.2	PXP2	Cd36_81790	orf19.1655
2.2		Cd36_64690	orf19.1092
2.2		Cd36_31770	orf19.3879
2.2	SLA2	Cd36_73470	orf19.7201
2.2		Cd36_07830	orf19.5110
2.2	RBT4	Cd36_06570	orf19.6202
2.2		Cd36_09670	orf19.4897
2.2		Cd36_26300	orf19.3225
2.2		Cd36_33100	orf19.6117
2.2	FMP45	Cd36_72040	orf19.6489
2.2		Cd36_85850	orf19.7372
2.2		Cd36_72300	orf19.6453
2.2		Cd36_10750	
2.2		Cd36_81840	
2.2		Cd36_80020	
2.2	GRE3	Cd36_52690	orf19.4317
2.1		Cd36_32750	orf19.733
2.1		Cd36_87460	orf19.6731.1
2.1		Cd36_42480	orf19.2733
2.1		Cd36_50930	orf19.4132
2.1		Cd36_33490	orf19.3712
2.1	PDR16	Cd36_03560	orf19.1027
2.1	LEU2	Cd36_70610	orf19.7080
2.1		Cd36_00720	
2.1	ERG9	Cd36_22740	orf19.3616
2.1		Cd36_03990	orf19.1058
2.1	DUN1	Cd36_54700	orf19.4002
2.1	HTA3	Cd36_83230	orf19.327
2.1		Cd36_17260	orf19.1551
2.0		Cd36_05360	orf19.2483
2.0		Cd36_09210	orf19.4843
2.0		Cd36_23950	
2.0		Cd36_83930	orf19.2812
2.0		Cd36_16570	orf19.1482
2.0		Cd36_18860	
2.0		Cd36_41570	orf19.4627

2.0		Cd36_08150	orf19.4722
2.0		Cd36_05170	orf19.419
2.0	FDH1	Cd36_30420	orf19.638
2.0		Cd36_04800	
2.0	ARA1	Cd36_22310	orf19.2172
2.0		Cd36_12300	
0.50		gb_Cd36_64900	orf19.1080
0.50	SMP3	gb_Cd36_17840	orf19.5792
0.50	MAS1	gb_Cd36_03100	orf19.3026
0.50		gb_Cd36_84530	orf19.5915
0.50		gb_Cd36_20110	orf19.6879
0.50		gb_Cd36_22660	
0.50		gb_Cd36_54740	orf19.3625
0.50	LYS21	gb_Cd36_04470	orf19.4006
0.50	RPL13	gb_Cd36_02820	orf19.772
0.50	RPS15	gb_Cd36_84630	orf19.2994
0.50		gb_Cd36_84720	orf19.5927
0.50		gb_Cd36_29750	orf19.5938
0.50		gb_Cd36_82020	orf19.529
0.50	RPL8B	gb_Cd36_85230	orf19.1643
0.50		gb_Cd36_85870	orf19.6002
0.50		gb_Cd36_10850	orf19.7375
0.47	TIF35	gb_Cd36_13230	orf19.1163
0.47		gb_Cd36_35140	orf19.7236
0.47		gb_Cd36_71120	orf19.7588
0.47		gb_Cd36_13120	orf19.6908
0.47		gb_Cd36_00450	orf19.7222
0.47	YST1	gb_Cd36_85350	orf19.6056
0.47		gb_Cd36_08930	orf19.6975
0.47		gb_Cd36_30620	orf19.4811
0.47	MRPL3	gb_Cd36_07440	orf19.1007
0.47		gb_Cd36_26050	orf19.5064
0.47	PTC7	gb_Cd36_40400	orf19.3250
0.47		gb_Cd36_86710	orf19.5661
0.47	ARO3	gb_Cd36_16830	orf19.6829
0.47		gb_Cd36_35294	orf19.1517
0.47	ATP17	gb_Cd36_25290	orf19.7617
0.47		gb_Cd36_32730	orf19.7509.1
0.47		gb_Cd36_08000	orf19.11
0.47	MRPS9	gb_Cd36_11560	orf19.394
0.47		gb_Cd36_40890	orf19.5230
0.47		gb_Cd36_86640	orf19.4711
0.47		gb_Cd36_29820	orf19.7456
0.47		gb_Cd36_02910	orf19.538
0.47	RPL3	gb_Cd36_23540	
0.47		gb_Cd36_21860	orf19.1601
0.47		gb_Cd36_04420	orf19.1867
0.47	FGR10	gb_Cd36_44520	orf19.779
0.47		gb_Cd36_40380	orf19.3791
0.47		gb_Cd36_28430	orf19.5660
0.47		gb_Cd36_22230	orf19.2395
0.47		gb_Cd36_07670	orf19.2181
0.47		gb_Cd36_06440	orf19.5090
0.47	DRG1	gb_Cd36_07620	

0.47		gb_Cd36_45630	orf19.5083
0.47	TRP4	gb_Cd36_46560	orf19.4420
0.47		gb_Cd36_86190	orf19.3099
0.47	MDH1-3	gb_Cd36_24490	orf19.7403
0.44	DFR1	gb_Cd36_72780	orf19.5323
0.44		gb_Cd36_23380	orf19.5142
0.44		gb_Cd36_83760	orf19.4086
0.44		gb_Cd36_71800	orf19.6940
0.44	YFH1	gb_Cd36_44010	orf19.6522
0.44		gb_Cd36_07590	orf19.1413
0.44		gb_Cd36_01870	
0.44		gb_Cd36_30690	orf19.4524
0.44		gb_Cd36_83050	orf19.5287
0.44		gb_Cd36_12780	orf19.304
0.44		gb_Cd36_03080	orf19.5016
0.44		gb_Cd36_71780	orf19.3022
0.44		gb_Cd36_11820	orf19.6524
0.44	SDH4	gb_Cd36_54880	orf19.6359
0.44		gb_Cd36_53450	orf19.4022
0.44		gb_Cd36_80570	orf19.1124
0.44		gb_Cd36_44400	orf19.5394.1
0.44		gb_Cd36_71130	orf19.3804
0.44		gb_Cd36_10530	orf19.6909
0.44		gb_Cd36_15410	orf19.680
0.44		gb_Cd36_50280	orf19.2087
0.44		gb_Cd36_52360	orf19.970
0.44		gb_Cd36_61560	orf19.4273
0.44		gb_Cd36_72770	orf19.3439
0.44		gb_Cd36_11350	orf19.5143
0.44		gb_Cd36_40110	orf19.5253
0.44		gb_Cd36_28920	orf19.371
0.44		gb_Cd36_50860	orf19.4370
0.44		gb_Cd36_72660	orf19.4144
0.44		gb_Cd36_65460	orf19.5161
0.44		gb_Cd36_19190	orf19.2149
0.44	QCR8	gb_Cd36_18680	orf19.4490.2
0.44		gb_Cd36_34280	orf19.819
0.41	ECI1	gb_Cd36_81080	orf19.6445
0.41	RPL24A	gb_Cd36_44530	orf19.3156
0.41	ELC1	gb_Cd36_04290	orf19.3789
0.41	PXA1	gb_Cd36_25450	orf19.6849
0.41		gb_Cd36_52460	orf19.7500
0.41		gb_Cd36_87340	orf19.4286
0.41		gb_Cd36_42000	orf19.6747
0.41		gb_Cd36_51330	orf19.4580
0.41	PGA55	gb_Cd36_23160	orf19.1972
0.41		gb_Cd36_54240	orf19.207
0.41		gb_Cd36_35566	orf19.3946
0.41	SCP1	gb_Cd36_20760	orf19.7675
0.41	FUS1	gb_Cd36_10920	orf19.17
0.41		gb_Cd36_54000	orf19.1156
0.41		gb_Cd36_82170	orf19.3920
0.41		gb_Cd36_09720	orf19.1625
0.41		gb_Cd36_05410	orf19.4903
0.41		gb_Cd36_83470	orf19.2478

0.41	ARO7	gb_Cd36_10790	orf19.345
0.41		gb_Cd36_01060	orf19.1170
0.41		gb_Cd36_07320	orf19.3301
0.41	ZCF25	gb_Cd36_42110	orf19.5050
0.41	PHA2	gb_Cd36_82420	orf19.4568
0.41		gb_Cd36_01030	orf19.234
0.41		gb_Cd36_65180	orf19.3297
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RESEARCH ARTICLE

Purification and germination of *Candida albicans* and *Candida dubliniensis* chlamydo-spores cultured in liquid media

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Received 26 March 2009; revised 7 April 2009; accepted 11 May 2009.
Final version published online 16 June 2009.

DOI:10.1111/j.1567-1364.2009.00533.x

Editor: Frank Odds

Keywords

chlamydo-spore; *Candida albicans*; *Candida dubliniensis*.

Abstract

Candida albicans and *Candida dubliniensis* are the only *Candida* sp. that have been observed to produce chlamydo-spores. The function of these large, thick-walled cells is currently unknown. In this report, we describe the production and purification of chlamydo-spores from these species in defined liquid media. Staining with the fluorescent dye FUN-1 indicated that chlamydo-spores are metabolically active cells, but that metabolic activity is undetectable in chlamydo-spores that are > 30 days old. However, 5–15-day-old chlamydo-spores could be induced to produce daughter chlamydo-spores, blastospores, pseudohyphae and true hyphae depending on the incubation conditions used. Chlamydo-spores that were preinduced to germinate were also observed to escape from murine macrophages following phagocytosis, suggesting that these structures may be viable *in vivo*. Mycelium-attached and purified chlamydo-spores rapidly lost their viability in water and when subjected to dry stress, suggesting that they are unlikely to act as long-term storage structures. Instead, our data suggest that chlamydo-spores represent an alternative specialized form of growth by *C. albicans* and *C. dubliniensis*.

Introduction

Candida albicans and *Candida dubliniensis* are unique among members of the genus *Candida* in their ability to produce chlamydo-spores. The production of these large (c. 6–10 µm) refractile cells was formerly a commonly used diagnostic feature of *C. albicans*. Induction of chlamydo-spore production can be achieved *in vitro* by culturing yeast cells in a nutrient-poor medium supplemented with a detergent (e.g. Tween 80) and incubating them at room temperature preferentially under oxygen limitation and in the dark (Dujardin *et al.*, 1980). Solid media containing complex carbohydrates, such as corn meal and rice extract agar, are the best-known chlamydo-spore-inducing media for both *C. albicans* (Casal & Linares, 1981) and *C. dubliniensis*, with the latter species reported to produce significantly greater numbers of chlamydo-spores (Sullivan *et al.*, 1995). It has since been reported that *C. dubliniensis* produces chlamydo-spores when cultured on Pal's and Staib agar, unlike *C. albicans*, which grows exclusively as yeasts on both of these media (Staib & Morschhauser, 1999; Al Mosaid *et al.*, 2001, 2003). This phenotypic difference has been shown to be due to the differential expression of the

transcriptional repressor Nrg1p (Staib & Morschhauser, 2005a). The role of chlamydo-spores, if any, in the normal life cycle or pathogenicity of *C. albicans* and *C. dubliniensis* is yet to be established, and they have only been observed in tissue samples on very rare occasions (Heineman *et al.*, 1961; Schonborn & Schmidt, 1971; Wilborn & Montes, 1980; Chabasse *et al.*, 1988; Cole *et al.*, 1991). It has been shown, however, that chlamydo-spore production is controlled by the morphogenetic pathways that control hypha formation [e.g. the Efg1 (Sonneborn *et al.*, 1999) and Hog1 (Eisman *et al.*, 2006) pathways] and that farnesol increases the levels of chlamydo-spore induction. However, chlamydo-spore formation is clearly distinct from other forms of candidal morphogenesis (Martin *et al.*, 2005).

Chlamydo-spores develop at the tip of suspensor cells in a budding fashion and a septum is formed between the suspensor cell and the chlamydo-spore (Martin *et al.*, 2005). Because this budding process is technically blastic conidogenesis, chlamydo-spores are sometimes referred to as 'chlamydoconidia'. Mature chlamydo-spores are rich in RNA and possess a single nucleus (Vidotto *et al.*, 1996). It has been demonstrated that nuclear division during chlamydo-spore

formation from the suspensor cell occurs within the suspensor cell, followed by the migration of one nucleus into the immature chlamydospore. The process of nuclear migration and maturation takes around 3–5 days (Martin *et al.*, 2005). Nuclear division across the suspensor cell–chlamydospore junction has never been detected, whereas this is typical at the necks of budding yeast cells. Electron micrographs of chlamydospores have shown that chlamydospore cell walls are double layered, consisting of a thin, electron-transparent outer layer surrounding a thick electron-dense inner layer (Shannon, 1981). The thickness of the inner layer increases with age and is around 400 nm in mature chlamydospores. The outer layer of the chlamydospore is contiguous with the wall of the suspensor cells. The composition of the chlamydospore cell wall is currently unknown. However, it has recently been shown that *C. albicans* Δ dit2 mutants defective in the formation of dityrosine, an essential component of the ascospore wall in *Saccharomyces cerevisiae*, are unable to form chlamydospores, suggesting that this compound is an important component of *C. albicans* chlamydospores (Melo *et al.*, 2008).

Previously, attempts have been made to investigate the characteristics of *C. albicans* chlamydospores produced on traditional solid culture media. However, the results of these studies were inconsistent. Jansons & Nickerson (1970) described the viability of young (30–40 h) *C. albicans* chlamydospores based on the observation of budding. However, other authors (e.g. Martin *et al.*, 2005) do not consider a chlamydospore a structure distinct from a suspensor cell at this stage. In contrast, Bakerspigel & Burke (1974) were unable to induce germination of chlamydospores, leading them to propose that chlamydospores have a role as storage cells.

In order to fully investigate the biology of chlamydospores, it is first of all necessary to be able to purify them in sufficient numbers from their associated suspensor cells, hyphae and pseudohyphae. In the past, using chlamydospores cultured on solid media, attempts were made to separate chlamydospores from mycelial cells by sulphuric acid treatment (Vidotto *et al.*, 1988), sonication and enzymatic treatment with β -glucuronidase (Fabry *et al.*, 2003). These methods allowed high levels of purity to be achieved; however, the density of chlamydospores in the starting cultures was low (Simonetti & Strippoli, 1971; Gunasekaran & Hughes, 1978; Vidotto *et al.*, 1988). The ability to harvest high yields of chlamydospores from liquid culture would greatly facilitate the purification and analysis of these structures. To date, only one study has observed this, when Staib and colleagues recently described a rapid method to produce chlamydospores in liquid formulations of Staib and Pal's media (Staib & Morschhauser, 2005b), both of which are complex media prepared from plant seed extracts.

The purpose of the present study was to develop defined culture conditions to generate high yields of chlamydospores to facilitate their analysis and purification.

Materials and methods

Candida strains and culture

Candida albicans SC5314 and *C. dubliniensis* CD36 and representative clinical isolates were routinely incubated on YPD [1% (w/v) yeast extract, 2% (w/v) Bacto™ neopeptone, 2% (w/v) glucose; pH 5.5] agar or broth at 37 °C.

Culture of *C. albicans* and *C. dubliniensis* chlamydospores on solid and liquid media

A defined solid medium comprising 6.7 g L⁻¹ yeast nitrogen base (without amino acids; with ammonium sulphate) (YNB w/o aa), 0.025% (w/v) galactose, 25 mg L⁻¹ methionine and 2% (w/v) Bacto agar (Difco Labs) (pH 3.7) was found to induce high levels of chlamydospore production by *C. dubliniensis* when incubated in the dark at room temperature for 3–4 days. A liquid chlamydospore inducing medium comprising 6.7 g L⁻¹ YNB w/o aa, 0.5% (w/v) galactose, 25 mg L⁻¹ methionine and 5% (v/v) newborn calf serum was used to induce high levels of chlamydospore production in *C. dubliniensis*. Inocula were made from *C. dubliniensis* blastospores incubated on YPD agar for 24–48 h at 37 °C. Plates were maintained at room temperature for 6–12 h, and a single colony then inoculated into 10 mL of liquid YPD and incubated with shaking at 37 °C for 2 h. One microlitre (*c.* 10² cells) of this culture was then inoculated into 50 mL of the chlamydospore-induction medium contained in a parafilm-sealed 100-mL conical flask or Petri dish. Cultures were then incubated statically at room temperature in the dark. *Candida albicans* chlamydospores were induced by incubation in liquid and solid media containing corn meal broth, 1% (v/v) Tween 80, 0.025% (w/v) galactose and 20 mg L⁻¹ methionine. The starting inocula were prepared as for *C. dubliniensis*. Unless otherwise specified, media and chemicals were obtained from Sigma. Chlamydospores were identified by their size, refractile cell wall and staining with lactophenol cotton blue.

Chlamydospore reactivation and germination conditions

Liquid YPD or Dulbecco's modified Eagle's medium (DMEM) supplemented with 5–10% (v/v) human serum were used for the reactivation of dormant chlamydospores in both species. Mycelium-attached or purified chlamydospores of both species were centrifuged at 20 000 g for 2 min. The resulting pellet was then resuspended in YPD or DMEM and incubated at 37 °C for 3–12 h to allow resumption of

metabolic activity. After reactivation, incubation in YPD with or without serum or DMEM with serum was carried out at room temperature or at 37 °C with or without 5% (v/v) CO₂ to induce germination.

Purification of chlamydospores

Chlamydospores and mycelia grown in liquid chlamydospore induction medium were centrifuged at 20 000 g for 2 min. The resulting pellet was then resuspended in 1 M sorbitol in citrate phosphate buffer (pH 5.6). Purification of chlamydospores from the mycelial component was obtained by repetitive sonication of the suspension for 10 min in a Vitasonic II sonicating bath at 25 kHz. In order to achieve a higher level of purification of chlamydospores from hyphal and yeast cells, sonicated samples were incubated with 700 U mL⁻¹ zymolyase 20T (Seikagaku Corp., Tokyo, Japan) for 2 h at room temperature. Samples were then sonicated again as described above and the chlamydospores were pelleted by centrifugation (3000 g for 5 min). Purified chlamydospores were resuspended in phosphate-buffered saline (PBS), and the purity was assessed by microscopy.

Detection of metabolic activity

Cells were stained with the fluorescent probe 2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinoliniumiodide (FUN-1; Molecular Probes, OR) to determine the level of metabolic activity in isolated chlamydospores and in germinated cells. Chlamydospores at different stages of exposure to nutrient-rich media and serum (0, 3, 6, 12, 24 h time points) were stained with 0.5 µM FUN-1 in 10 mM Na-HEPES solution (pH 7.2) supplemented with 2% (w/v) glucose (GH solution) in the dark at room temperature for 30 min. Fluorescence was measured using a fluorescence microscope Nikon Eclipse 600 equipped with a filter with excitation at 470–590 nm.

4',6-diamidino-2-phenylindole (DAPI) and calcofluor staining

To stain the cell wall and the nucleus of chlamydospores, 100-µL samples of mycelia-attached or purified chlamydospores were either washed twice in PBS or fixed in 5% paraformaldehyde and then resuspended in 100 µL calcofluor white solution (1 mg mL⁻¹) and 10 µL of DAPI solution (10 mg mL⁻¹). Five-microlitre volumes of the samples were then mounted on glass slides and cells were analysed by fluorescence microscopy with a DAPI filter.

Phagocytosis assay

Murine RAW264.7 macrophages were seeded at a concentration of 1.5×10^6 cells in the wells of a six-well plate containing sterile coverslips, on the top of the coverslip.

Macrophages were cultured overnight at 37 °C in the presence of 5% (v/v) CO₂. The monolayer was then washed with warm DMEM (Sigma), and 1 mL of the medium was added to each well. Purified dormant or activated chlamydospores of *C. albicans* and *C. dubliniensis* (1×10^3 , 1×10^4) were then added to the monolayer. Dormant chlamydospores were incubated in PBS or water following purification whereas reactivated chlamydospores were incubated in YPD supplemented with 5% serum. Chlamydospores were cocultured with macrophages for 3, 5, 10, 15 and 20 h. At each time point, the medium was removed and acridine orange (Sigma) at 0.1% (v/v) in DPBS was added to the wells. After 10-min staining, the dye was removed and the coverslips were washed twice with PBS. Coverslips were examined by fluorescence microscopy with a DAPI filter.

Electron microscopy

Chlamydospore-rich mycelia or chlamydospore suspensions were fixed overnight in PBS containing 2.5% (v/v) glutaraldehyde and 2% (w/v) formaldehyde. The cells were washed briefly in PBS, dehydrated in graded concentrations of ethanol and critical point-dried in 100% CO₂. Specimens were mounted on aluminium stubs, coated with gold and viewed using a Zeiss Supra 35 variable pressure field emission scanning electron microscope.

Results

Culture of *C. albicans* and *C. dubliniensis* chlamydospores in a defined liquid medium

We observed that when incubated on a defined medium comprised of 6.7 g L⁻¹ YNB w/o aa agar supplemented with 0.025% (w/v) glucose or galactose and 20 mg L⁻¹ methionine (pH 3.7), at room temperature for 3–5 days, a wide range of *C. dubliniensis* strains tested grew as rough colonies, producing copious hyphae, pseudohyphae and chlamydospores, while all *C. albicans* strains incubated under these conditions grew as smooth colonies without hyphae. The levels of the chlamydospore-forming mycelia produced were higher when galactose was used as a carbon source. When cultured in a liquid formulation of this medium, *C. dubliniensis* strains grew exclusively as pseudohyphae producing large numbers of chlamydospores when incubated at room temperature in parafilm-sealed 100-mL conical flasks without shaking for 4–5 days (Fig. 1). A medium comprised of 6.7 g L⁻¹ YNB w/o aa, 0.5% (w/v) galactose, 25 mg L⁻¹ methionine and 5% (v/v) serum was found to induce maximal levels of chlamydospore production in *C. dubliniensis*. In contrast, as with the solid medium, all *C. albicans* strains tested grew solely in the yeast form when incubated under these conditions. Replacement of methionine with a similar concentration of cysteine also resulted in the production of

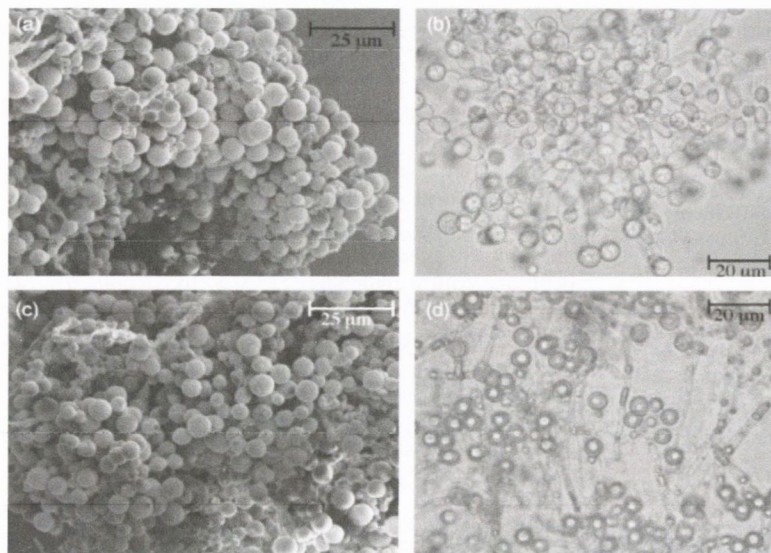


Fig. 1. Production of chlamydozoospores by *Candida albicans* and *Candida dubliniensis* in defined liquid media. Scanning electron micrographs (a and c) and light micrographs (b and d) of *C. dubliniensis* CD36 (a and b) following incubation in YNB supplemented with 25 mg L⁻¹ methionine and 0.025% (w/v) galactose and a *C. albicans* clinical isolate (CAY5292; this study) (c and d) incubated in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 25 mg L⁻¹ methionine for 5 days.

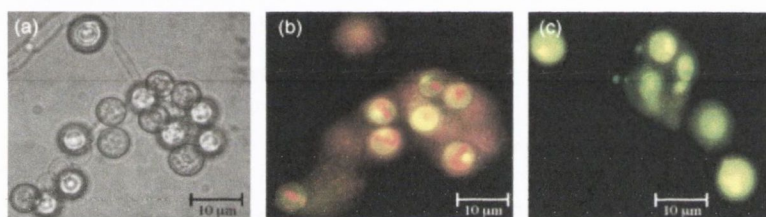


Fig. 2. Metabolic activity of chlamydozoospores detected with FUN-1. *Candida albicans* SC5314 chlamydozoospore-rich mycelium produced following incubation in corn meal broth supplemented with 1% (v/v) Tween 80, 0.025% (w/v) galactose and 25 mg L⁻¹ methionine for 5 (a and b) and 16 (c) days. Chlamydozoospores were observed by bright-field microscopy (a) and by fluorescence microscopy following staining with FUN-1 (b and c) ($\times 1000$ magnification).

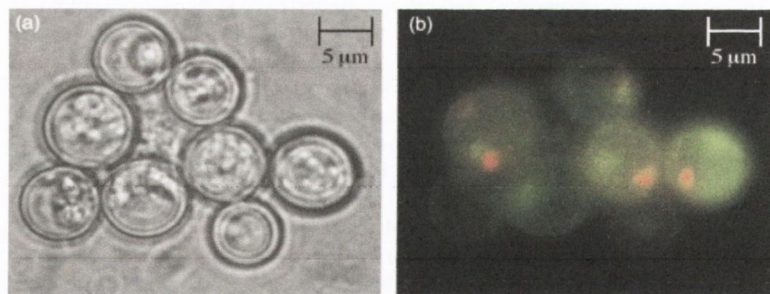


Fig. 3. FUN-1 staining of purified chlamydozoospores. Purified 6-day-old *Candida albicans* SC5314 chlamydozoospores were observed by bright field (a), and fluorescence microscopy following staining with FUN-1 (b) ($\times 1000$ magnification).

high levels of chlamydozoospores by *C. dubliniensis*; however, replacement with proline or lysine resulted in a failure to produce any chlamydozoospores (data not shown).

Chlamydozoospore production is routinely induced in *C. albicans* by incubation on corn meal agar; however, the

yield of chlamydozoospores under these conditions is relatively low. In order to maximize chlamydozoospore production by *C. albicans*, corn meal agar and a liquid formulation of this medium were supplemented with 1% Tween 80 (v/v), 0.025% (w/v) galactose and 20 mg L⁻¹ methionine. This

Fig. 4. Production of daughter chlamydospores. Fifteen-day-old *Candida albicans* SC5314 cultures were induced to generate daughter chlamydospores by refreshment of the induction medium every 6 days. A light micrograph ($\times 400$) shows chains of chlamydospores (a) and a scanning electron micrograph shows a clump of attached chlamydospores (b).

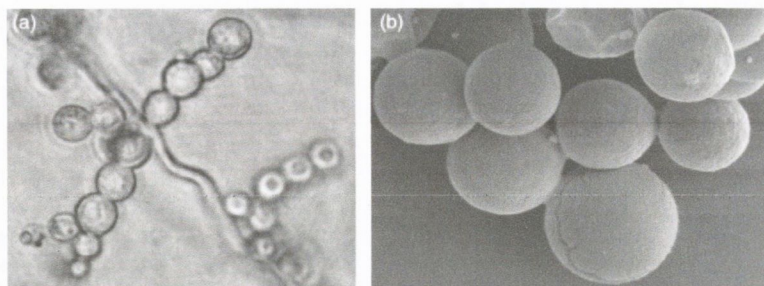
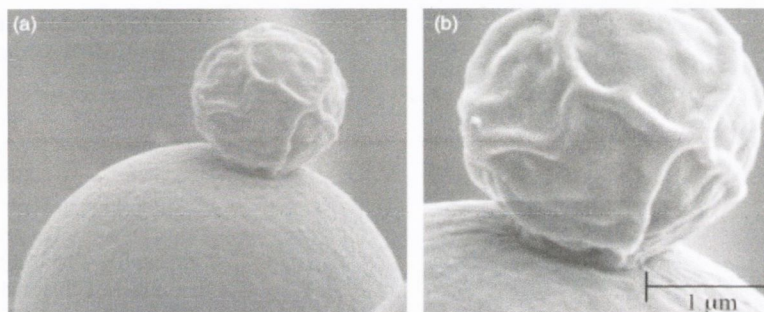


Fig. 5. Budding of mycelium-attached chlamydospores. When 5-day-old *Candida albicans* SC5314 chlamydospores were incubated in nutrient-rich medium supplemented with 10% (v/v) human serum, after 6–12 h the chlamydospores were observed to bud, producing yeast-like cells (b shows a higher magnification of a).



resulted in an *c.* 20-fold increase in chlamydospore production (Fig. 1). In both *C. albicans* and *C. dubliniensis*, increasing the concentration of glucose or galactose resulted in a reduction in chlamydospore formation (data not shown).

Evidence for metabolic activity in mature chlamydospores

Once formed, the chlamydospore has to remain attached to the mycelium for a time sufficient for the migration of genetic material before it is considered as a distinct structure from the suspensor cell. The nuclear division process that occurs inside the suspensor cells and subsequent nuclear migration in the chlamydospore takes *c.* 3–5 days (Martin *et al.*, 2005). In order to investigate the metabolic activity of chlamydospores, liquid-grown *C. albicans* SC5314 and *C. dubliniensis* CD36 chlamydospore-rich mycelia were stained with the fluorescent probe FUN-1 every 24 h over a 30-day period. Metabolic activity was detected by the production of red fluorescence in young *C. albicans* (e.g. 5 days old) chlamydospores (Fig. 2). However, this metabolic activity decreased over time. Staining with FUN-1 of the same chlamydospore sample after 14 days of incubation showed a significantly lower level of metabolic activity (Fig. 2), and by 30 days, metabolic activity was undetectable. In contrast, yeast cells incubated under the same conditions did

not exhibit a reduction in metabolic activity during the same time period (data not shown).

Purification of chlamydospores

Chlamydospores could be separated from other cells by sonication; however, a higher degree of purity was achieved using a zymolyase treatment before the brief sonication. Microscopic analysis confirmed that the majority of chlamydospores ($> 95\%$) appeared intact following purification. Staining with FUN-1 indicated the presence of metabolic activity in *c.* 40% of the purified chlamydospores (Fig. 3).

Chlamydospores can generate daughter chlamydospores

Metabolically active mycelium-attached *C. albicans* and *C. dubliniensis* chlamydospores (3–15-day-old chlamydospores) were induced to produce daughter chlamydospores by refreshing the medium with the addition of new chlamydospore-inducing medium and incubating at room temperature under microaerophilic conditions without shaking. Mycelia grown under these conditions were rich in chlamydospores, with chlamydospores appearing to generate daughter chlamydospore cells. Chains and clumps of chlamydospores, characterized by the absence of hyphae or blastospores, were frequently produced (Fig. 4).

Chlamydospores can bud, forming daughter yeast cells

When 6–15-day-old purified or mycelium-attached chlamydospores (i.e. with low metabolic activity) were incubated in

nutrient-rich medium (e.g. YNB with aa and 2% (w/v) glucose, YDP or DMEM) supplemented with 10% (v/v) human serum and incubated at 37 °C, metabolic activity (detected using FUN-1 staining) was observed to increase after 6–12 h and the chlamydospores were observed to bud, producing nonrefractile, yeast-sized cells (Fig. 5). The released yeast cells were metabolically active (detected by FUN-1 staining), contained genetic material (detected using DAPI staining) and were capable of replication, producing a population of free blastospores (data not shown). Chlamydospores > 30 days old could not be induced to resume metabolic activity.

Chlamydospores can germinate and produce pseudohyphae and hyphae

When mature chlamydospores were incubated in YPD medium supplemented with serum (10% v/v), they were observed to produce germ tubes, which led to the development of pseudohyphae within c. 15 h (Figs 6 and 7). When the same experiment was performed in the presence of 5% CO₂ (v/v), the germ tubes resulted in the formation of true hyphae (data not shown). The germination of mycelium-attached chlamydospores was more rapid and more effective than the germination of the purified chlamydospores. Thirty percent of 5-day-old (decreasing to c. 10% of 15-day-old) mycelium-attached chlamydospores were found to bud or germinate, while only c. 5% of purified chlamydospores of the same age were observed to bud or germinate.

Chlamydospores are intolerant to environmental stress

To investigate whether some of the attributes typical of fungal resting spores (e.g. long-term survival under nutrient-poor conditions, dry stress resistance and heat shock resistance) were present in chlamydospores, these structures were subjected to a variety of stresses and their viability (measured by the ability to bud or germinate) was analyzed and quantified. Five-day-old mycelium-attached chlamydospores and yeast cells recovered from cultures of the same age were transferred to distilled water and their viability, upon exposure to glucose and serum, was measured for a period of 15 days. The percentage of cells producing buds or germ tubes was counted as a measure of viability. While

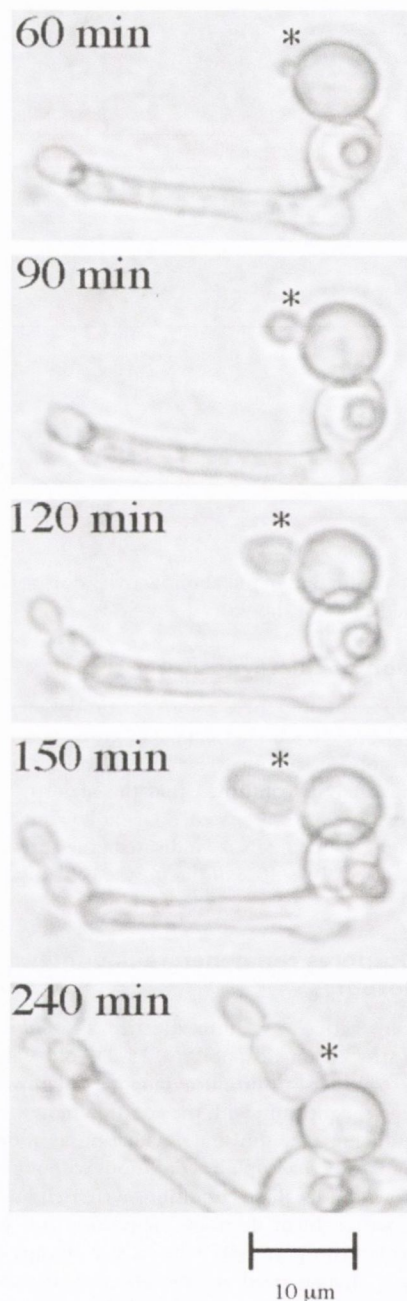


Fig. 6. Time-lapse photography of a germinating *Candida albicans* chlamydospore. Light micrographs showing the production of a pseudohypha (indicated by an asterisk) by a *C. albicans* SC5314 mycelium-attached chlamydospore, triggered to germinate at room temperature following reactivation at 37 °C in the presence of DMEM supplemented with 10% (v/v) human serum was observed at 60, 90, 120, 150 and 240 min under $\times 400$ magnification.

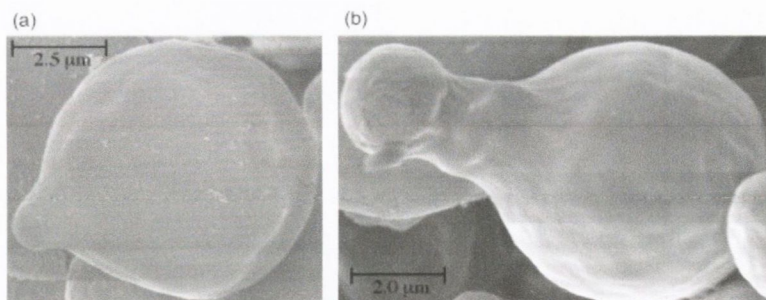


Fig. 7. Scanning electron micrographs of chlamydo spores induced to form germ-tube-like structures. Purified *Candida albicans* SC5314 chlamydo spores were incubated at 37 °C in the presence of DMEM supplemented with 10% (v/v) human serum for (a) 60 min and (b) 90 min.

yeast cells maintained their viability over the 15-day time period, purified and nonpurified chlamydo spores rapidly lost their viability when incubated in water.

To assess dry stress tolerance of mycelium-attached chlamydo spores and yeast cells, drops (100 µL) of the two cultures were spotted onto Petri dishes and allowed to dry over time at room temperature. At defined time points (0, 5, 10, 20, 30, 40 and 50 h), nutrient-rich medium (i.e. YPD) and serum were added into the Petri dishes and viability was quantified by counting the percentage of cells able to produce buds or germ tubes. Chlamydo spores and yeast cells showed no detectable viability by the 50-h time point.

Interaction between chlamydo spores and murine macrophages

The ability of purified 5–15-day-old chlamydo spores to survive in coculture with murine macrophages RAW264.7 was investigated. Acridine orange dye was used as a tool to distinguish intact from damaged chlamydo spores and to observe the effect of macrophages on chlamydo spore viability. A 70% pure suspension of 1×10^4 mycelium-free chlamydo spores was inoculated onto a 90% confluent layer of the murine macrophage cell line RAW264.7. A high rate of chlamydo spore internalization by macrophages was observed within 2–4 h. After 6–7 h almost all the chlamydo spores including those forming clumps were phagocytosed. Some macrophages were found to have engulfed up to five to 10 chlamydo spores. Staining of the chlamydo spores and macrophages with acridine orange showed that at the moment of interaction with the macrophages, the dormant chlamydo spores were viable and intact. Following phagocytosis, the dye showed metachromism (red/orange colour), indicative of loss of chlamydo spore viability. Following an overnight coculture with macrophages, phagocytosed chlamydo spores lost their round shape and appeared to be dead (Fig. 8).

When purified chlamydo spores were preincubated for 15–20 h under the conditions described above that induced

hypha formation, before coculture with RAW264.7 cells, the chlamydo spores were phagocytosed, but after 3–5 h, a small proportion (c. 5%) was observed to produce hyphae and escape from the macrophages. Staining with acridine orange showed that the chlamydo spore cell walls were intact and that the hyphae emerging from them were also viable (Fig. 8).

Discussion

Candida albicans and *C. dubliniensis*, whose natural hosts are humans (and some animals), are the only *Candida* species that produce chlamydo spores. These thick-walled cells are only produced under very specific conditions *in vitro* and have rarely been observed *in vivo*. Because of the difficulty in producing these cells in sufficient numbers, they have been poorly investigated, and their role, if any, in the life cycle of *C. albicans* and the pathogenesis of candidal infections is not known. The main problems encountered while studying the function of chlamydo spores include the low numbers of chlamydo spores produced by traditional culturing methods, the agar invasion of chlamydo spore-producing mycelia and the tight association of chlamydo spores with their parental suspensor cells.

In the present study, we have developed chemically defined solid and liquid media, containing galactose and methionine, which induce high levels of chlamydo spore formation by *C. dubliniensis* strains. Under these conditions, *C. albicans* failed to produce chlamydo spores. However, the addition of 0.025% (w/v) galactose and 25 mg L^{-1} methionine to a liquid formulation containing corn meal and 1% (v/v) Tween 80 resulted in the production of high levels of chlamydo spores by this species. The observation that the addition of glucose and galactose at high concentrations inhibited the production of chlamydo spores in liquid and in solid media in the two species suggests that low nutrient availability may play a role in the induction of chlamydo spores. However, other factors such as pH, low temperature and oxygen limitation are also likely to play a role. Similarly, methionine and cysteine, unlike other amino acids, appear

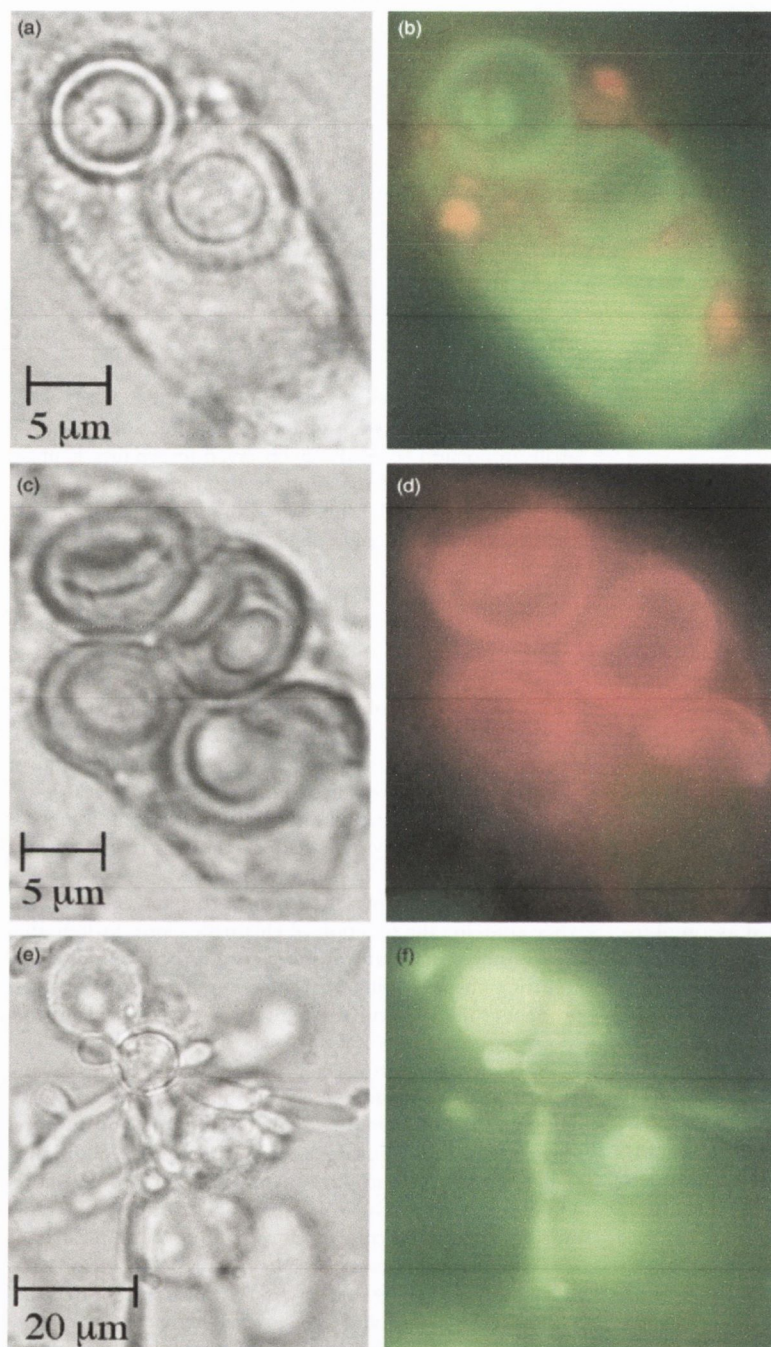


Fig. 8. Viability of purified chlamydospores following phagocytosis by murine macrophage cells. The murine cell line RAW264.7 was coincubated with purified *Candida albicans* SC5314 chlamydospores. After 2–4 h (a, b) chlamydospores were phagocytosed. Staining with acridine orange (b) indicates the presence of viable chlamydospores inside the macrophage. Following coincubation for > 7 h (c, d) the chlamydospores were severely damaged and the red fluorescence (d) indicates a loss of viability. When chlamydospores were preincubated in YPD medium supplemented with 10% (v/v) serum (e, f), following 3–4 h coculture, chlamydospores were seen to germinate following phagocytosis ($\times 1000$ magnification).

to be important inducers of the morphogenetic pathways required for chlamydo spore formation.

In order to investigate the viability and metabolic activity of chlamydo spores, experiments were performed on chlamydo spores attached to mycelia and on chlamydo spores that had been enzymatically separated and purified from hyphal and yeast cells. The molecular probe FUN-1 detects metabolically active cells and was used to show that mature chlamydo spores (≥ 5 days old) possess high metabolic activity, but that this metabolic activity decreases over time under nutrient-poor conditions. After 30 days of incubation metabolic activity was undetectable, and these chlamydo spores could not be revived by the addition of fresh media, suggesting that these cells were nonviable. A dormancy period of up to 1 year has been reported in resting spores. This period can be interrupted and metabolic activity induced to resume following exposure of the spore to specific external stimuli. In chlamydo spores, unlike resting spores, this stage of low metabolic activity can only be interrupted in chlamydo spores that are not older than 15–20 days. Relatively young chlamydo spores can be reactivated, resuming metabolic activity, following exposure to a nutrient-rich environment and serum. Under the conditions tested it was found to be easier to interrupt the dormancy of mycelium-attached chlamydo spores than that of purified chlamydo spores of the same age, possibly as a result of damage to the chlamydo spores caused by the purification method. The incubation of chlamydo spores in nutrient-poor media for longer periods resulted in a significant reduction in the number of cells that have the potential to resume their metabolic activity. In particular, chlamydo spores appear to completely lose their viability if incubated in water for > 2 weeks. This suggests that these chlamydo spores are less tolerant to long-term storage in water than blastospores. It was also observed that mycelium-attached and purified chlamydo spores have almost the same low ability to resist dry stress as yeast cells (Kashbur *et al.*, 1980), losing viability after exposure to a dry environment for > 2 days. These data suggest that candidal chlamydo spores are unlikely to act as spores for long-term (i.e. months or years) survival under nutrient-poor or dry conditions.

Following the incubation of mature chlamydo spores (i.e. 5–15 days old) in rich media containing 2% (w/v) glucose and 1% (v/v) human serum, c. 30% of mycelium-attached chlamydo spores and 5% of purified chlamydo spores started to bud within 24 h. When mature chlamydo spores were incubated in a rich medium containing 2% (w/v) glucose and 10% human serum at 37 °C, the chlamydo spores produced either pseudohyphae or true hyphae [depending on the presence or absence of 5% (v/v) CO₂] in a manner similar to blastospores. Purified mature chlamydo spores (5–15 days old) are phagocytosed and killed following an

overnight coculture with macrophages. However, some preactivated *C. albicans* chlamydo spores have the ability to germinate and escape from macrophages. These data suggest that these cells are alternative growing forms of *C. albicans* and *C. dubliniensis* and that they may replicate *in vivo*.

Our data suggest that candidal chlamydo spores may not be, as has been previously suggested, 'dead end' or long-term survival structures. We have demonstrated instead that chlamydo spores are an alternative and specialized growth form of the two species (resulting from major changes in gene expression and morphology in the blastospore and mycelial cell forms). The role of these cells in the normal life cycle of *C. albicans* and *C. dubliniensis* is still not clear. The paucity of reports of chlamydo spores in clinical samples suggests that they may be unlikely to play an important role in pathogenesis. However, the fact that both these species have retained the ability to produce these complex structures (which very likely require complex signalling and regulatory pathways) since their divergence suggests that they may indeed have an important function, which remains to be identified.

Acknowledgement

This work was supported by the EU grant MRTN-CT-2004-512481 (CanTrain) and by the Dublin Dental School and Hospital.

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Letter

Comparative genomics of the fungal pathogens *Candida dubliniensis* and *Candida albicans*

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Candida dubliniensis is the closest known relative of *Candida albicans*, the most pathogenic yeast species in humans. However, despite both species sharing many phenotypic characteristics, including the ability to form true hyphae, *C. dubliniensis* is a significantly less virulent and less versatile pathogen. Therefore, to identify *C. albicans*-specific genes that may be responsible for an increased capacity to cause disease, we have sequenced the *C. dubliniensis* genome and compared it with the known *C. albicans* genome sequence. Although the two genome sequences are highly similar and synteny is conserved throughout, 168 species-specific genes are identified, including some encoding known hyphal-specific virulence factors, such as the aspartyl proteinases Sap4 and Sap5 and the proposed invasin Als3. Among the 115 pseudogenes confirmed in *C. dubliniensis* are orthologs of several filamentous growth regulator (FGR) genes that also have suspected roles in pathogenesis. However, the principal differences in genomic repertoire concern expansion of the *TLO* gene family of putative transcription factors and the *IFA* family of putative transmembrane proteins in *C. albicans*, which represent novel candidate virulence-associated factors. The results suggest that the recent evolutionary histories of *C. albicans* and *C. dubliniensis* are quite different. While gene families instrumental in pathogenesis have been elaborated in *C. albicans*, *C. dubliniensis* has lost genomic capacity and key pathogenic functions. This could explain why *C. albicans* is a more potent pathogen in humans than *C. dubliniensis*.

[Supplemental material is available online at <http://www.genome.org>. The sequence data from this study have been submitted to EMBL (<http://www.ebi.ac.uk/emb/>) under accession nos. FM992688–FM992695.]

Candida species are pathogenic yeasts and are the most prevalent cause of opportunistic fungal infections in humans. The diseases caused by these yeasts include superficial infections of the oral cavity and vagina (commonly known as thrush) and deep-seated systemic infections that can affect a wide range of organs and are associated with high levels of morbidity and mortality. *Candida albicans* is the most frequent cause of superficial and systemic candidosis and is widely recognized as the most pathogenic yeast species. *Candida dubliniensis* is the most closely related species to *C. albicans* (Sullivan et al. 1995, 2004) and was routinely misidentified as *C. albicans* prior to its recognition, since it can produce germ tubes and chlamydospores, traits previously only associated with *C. albicans*. However, while *C. albicans* causes ~50%–60% of cases of systemic candidosis, *C. dubliniensis* is not

usually associated with haematogenous infection and accounts for only ~2%–3% of *Candida* species recovered from blood samples (Kibbler et al. 2003; Odds et al. 2007).

Epidemiological and infection model data suggest that *C. dubliniensis* is less prevalent than *C. albicans* because it is substantially less pathogenic. In two separate studies using the murine systemic infection model, mice infected with *C. dubliniensis* have longer survival times than *C. albicans* (Gilfillan et al. 1998; Vilela et al. 2002). Similarly, in an infant mouse oral intragastric model of infection, *C. dubliniensis* was far less capable than *C. albicans* of colonizing the intestinal tract and disseminating to other organs (Stokes et al. 2007). The reasons for the apparently lower virulence of *C. dubliniensis*, in comparison with *C. albicans*, are not yet known. However, it is known that the species differ in their tolerance of various environmental stresses (Pinjon et al. 1998; Alves et al. 2002; Vilela et al. 2002; Enjalbert et al. 2009). Furthermore, although *C. dubliniensis* has the ability to produce hyphae, widely accepted as being an important virulence factor

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Article published online before print. Article and publication date at <http://www.genome.org/cgi/doi/10.1101/gr.097501.109>.

for *C. albicans*, it does so less efficiently than *C. albicans* under a wide range of hypha-induction conditions (Stokes et al. 2007) and exhibited low levels of filamentation in infection model experiments (Vilela et al. 2002; Moran et al. 2007; Stokes et al. 2007).

Recently, Butler et al. (2009) compared the genomes of seven *Candida* species with other parasitic and free-living yeasts, but not including *C. dubliniensis*. These data encompassed the global diversity of *Candida* spp. and identified genomic changes that have accompanied the evolution of parasitism, such as expansions of secreted and cell-wall proteins, including the secreted aspartyl proteinase (SAP) and agglutinin-like sequence (ALS) families. However, in addressing such ancient events associated with the origins of *Candida* and candidosis, the study could not identify those specific features of *C. albicans* that explain its particular virulence and medical relevance. Moran et al. (2004) analyzed the *C. dubliniensis* and *C. albicans* genomes using comparative genome hybridization (CDH). They identified numerous species-specific genes, indicating that a comparative analysis could elucidate the genetic factors responsible for reduced virulence in *C. dubliniensis*. Therefore, we have sequenced the genome of the type *C. dubliniensis* strain and compared it with the *C. albicans* genome sequence (Jones et al. 2004) to further characterize their differences and identify possible mechanisms of virulence in *C. albicans*. In doing so, we have exposed a much more recent episode in *Candida* evolution than was possible in the pan-*Candida* genome comparison, revealing disparities in copy number of gene families that, while present in both species, have evolved subtly different repertoires and may be involved in the increased pathogenicity of *C. albicans*.

Results

Genome order and content in *C. dubliniensis* and *C. albicans* are highly similar

The *C. dubliniensis* genome (see Supplemental Table S1) was produced by whole genome shotgun sequencing to 11-fold average coverage, assembled de novo, and manually finished. Compared to *C. albicans*, the 14.6-megabase (Mb) diploid genome has a complex karyotype comprising 10 haploid and three diploid chromosomes (Magee et al. 2008). Various chromosomal translocations have occurred since the separation of *C. dubliniensis* and *C. albicans*, meaning that homologous chromosomes, or chromosomal blocks, may occupy two distinct genomic positions, as shown in Figure 1. For instance, the entire length of chromosome 5 in *C. albicans* corresponds to part of chromosome VIII in *C. dubliniensis*, along with a part of chromosome R. In addition, sequences corresponding to chromosome 5 in *C. albicans* are also found in chromosomes IV and I in *C. dubliniensis*. Furthermore, the sequences of chromosomal homologs are so similar that it was not possible to separate them

into a true diploid representation of the sequence. Instead, for each chromosome a single haploid sequence was produced up to the point where two alternative junctions in the assembly were possible. Without duplicating the sequence, or arbitrarily splitting reads between the two assembly choices, a single junction was reconstructed by choosing the one that most closely resembled *C. albicans*. By ordering *C. dubliniensis* contigs onto the *C. albicans* genome sequence, we produced eight haploid chromosomal sequences that correspond to the eight-chromosome *C. albicans* karyotype. The resultant eight pseudochromosomes were submitted to EMBL (<http://www.ebi.ac.uk/embl/>) under accession numbers FM992688–FM992695, inclusive. Subsequent PCR walking demonstrated that these pseudochromosomes were real. The remaining chromosomes from the complex karyotype that are not represented were shown to exist through sequencing of selected BACs, as shown in Figure 1. Hence, the *C. dubliniensis* genome sequence consists of eight pseudochromosomes that reconstruct eight genuine chromosomes: Five haploid (II, VI, VIII, IX, and XII) and three diploid (III, VII, and XIII). While the remaining five haploid chromosomes are not shown, no genome sequence was discarded since each pseudochromosome is a haploid consensus of both chromosomal homologs. So for example, chromosome “S” in the *C. dubliniensis* genome sequence reconstructs chromosome VIII of the complex karyotype, but also incorporates sequence from chromosomes IV and I that are not represented structurally, essentially because they differ from chromosomal arrangements in *C. albicans*.

Since the *C. dubliniensis* genome sequence is a consensus of both chromosomal homologs, we can assess the scale and distribution of nucleotide heterogeneity. Polymorphism is unevenly

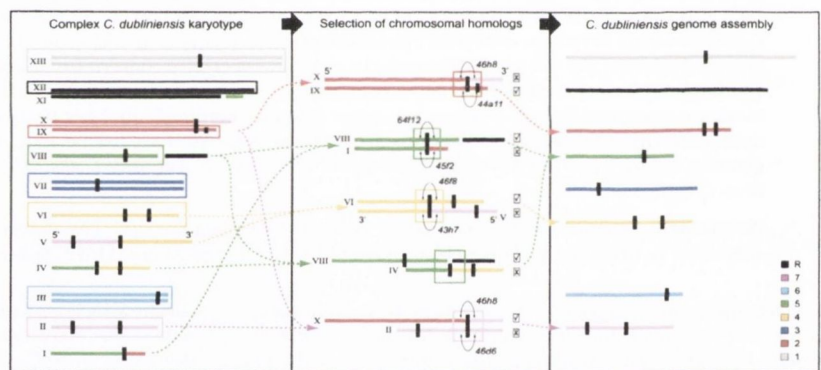


Figure 1. The *C. dubliniensis* complex in vivo karyotype and genome sequence. The 14.6-Mb *C. dubliniensis* genome (left panel, redrawn with permission from Magee et al. 2008) has a complex karyotype of 10 haploid and three diploid chromosomes (Roman numerals), showing multiple chromosomal rearrangements relative to *C. albicans*. Vertical black marks denote MRS regions. In order to avoid redundancy in the assembly and to maximize comparability with *C. albicans*, the sequence was assembled into eight “pseudochromosomes” (right panel), which are numbered according to the karyotype of *C. albicans* strain SC5314 (Arabic numerals) and color-coded after *C. albicans* in order to illustrate the extent of chromosomal rearrangement in vivo. Note that each pseudochromosome in the right panel is a haploid consensus of the distinct chromosomal homologs apparent in the left panel. Components of the in vivo karyotype selected for the genome sequence are boxed. *C. dubliniensis* chromosomal homologs often adopted two alternative configurations, relative to the *C. albicans* karyotype. The middle panel illustrates the selection process by which one haploid chromosome was chosen over its homolog, to reproduce the *C. albicans* karyotype. Tick boxes indicate which chromosomal configuration was selected in the final genome representation (as submitted to the EMBL database). BAC clones were sequenced that bridged the two distinct chromosomal configurations to confirm that both existed (BAC clone identifiers are noted in italics where appropriate). Note that chromosome V is inverted.

distributed across all chromosomes of the *C. albicans* genome sequence and occurs at a frequency of one SNP per 330–390 bases (Butler et al. 2009). The distribution of sequence polymorphisms across the *C. dubliniensis* genome is presented in Figure 2. While the distribution pattern is not the same as in *C. albicans*, polymorphism is similarly distributed unevenly and does not correlate with major repeat sequence (MRS), centromeric, or telomeric-proximal regions in any distinctive manner. There are large chromosomal tracts, as in *C. albicans*, where polymorphism is entirely absent. In general, the *C. dubliniensis* genome is much less polymorphic than most *Candida* genome published to date, with SNP frequency ranging from every 635 bases (chromosome 6) to every 12,555 bases (chromosome 1); the latter rate is comparable with *C. parapsilosis*, which is virtually homozygous (one SNP per 15,553 base pair [bp]; Butler et al. 2009). Polymorphisms are available in Supplemental Table S2 of the Supplemental material.

Approximately 6% of the 5758 identified genes are spliced and putative functions were manually assigned to over 4000. Aside from karyotypic differences, the composition and gene order of the *C. dubliniensis* genome sequence is very similar to *C. albicans*. The

conservation of genome structure extends to complex repeat regions, such as the subtelomeres and the MRSs (Joly et al. 2002). The level of finishing achieved here allows us to fully elaborate the structure of the chromosome end in *C. dubliniensis* for two instances: the left end of chromosome 6 and the right end of chromosome R (see Supplemental Fig. S1). The subtelomeres of *C. dubliniensis* possess an end-proximal mosaic of repetitive regions, including the terminal 22-mer repeat, which differ by only 1 base from that observed in *C. albicans* (Sadhu et al. 1991). The subtelomeres also contain various transposable elements (see below) and a subclass of RecQ-like helicase pseudogenes, which are most similar to the *Saccharomyces cerevisiae* Y' helicase (as well as homologous subtelomeric RecQ helicases in *C. albicans*), rather than to internally located helicases. However, all but one RecQ helicase in *C. dubliniensis* were gene relics; the intact copy, on the right arm of chromosome 6, differs in its predicted transcriptional orientation, pointing away from the chromosome terminus, while all others point toward it.

The MRS is a feature unique to the genomes of *C. albicans* and *C. dubliniensis*, and may contribute to karyotypic variation in these species by acting as a “hot-spot” for chromosomal translocation (Lephart et al. 2005). The situation in *C. dubliniensis* is described in Supplemental Table S3 and noted in Figure 1. Each chromosome carried at least one MRS element, except for chromosome R, and the conserved HOK and RB2 units flanking the central RPS domains were also present. This is consistent with the situation previously described in *C. albicans* (Jones et al. 2004), except that *C. albicans* has no MRS on chromosome 3 and sequencing revealed an additional, smaller MRS on chromosome 2 in *C. dubliniensis*. The single MRS on chromosome 3 was very small, containing only a single RPS domain, and was flanked by a transposable element on either side; however, we found no evidence to associate the movement of MRS with transposable element insertion sites.

The absence of any substantial variation in these dynamic genomic features was matched by the generally high identity between protein-coding genes; of the 5569 orthologous gene pairs, 44.4% (2470) are >90% identical at the nucleotide level and 96.3% (5363) are >80% identical. Gene order is also largely collinear, with 98.1% (5647) of all *C. dubliniensis* putative genes positionally conserved in both species. This background of similarity means that any breaks in chromosomal collinearity are obvious and manual inspection of such putative species-specific loci identified 111 genes in *C. dubliniensis* and 191 in *C. albicans* that have either no heterospecific sequence match, or no reciprocal top BLASTX hit. This compares well with the 247 species-specific genes identified by CGH, which is sensitive only where a gene is absent from one species or where sequence divergence exceeds

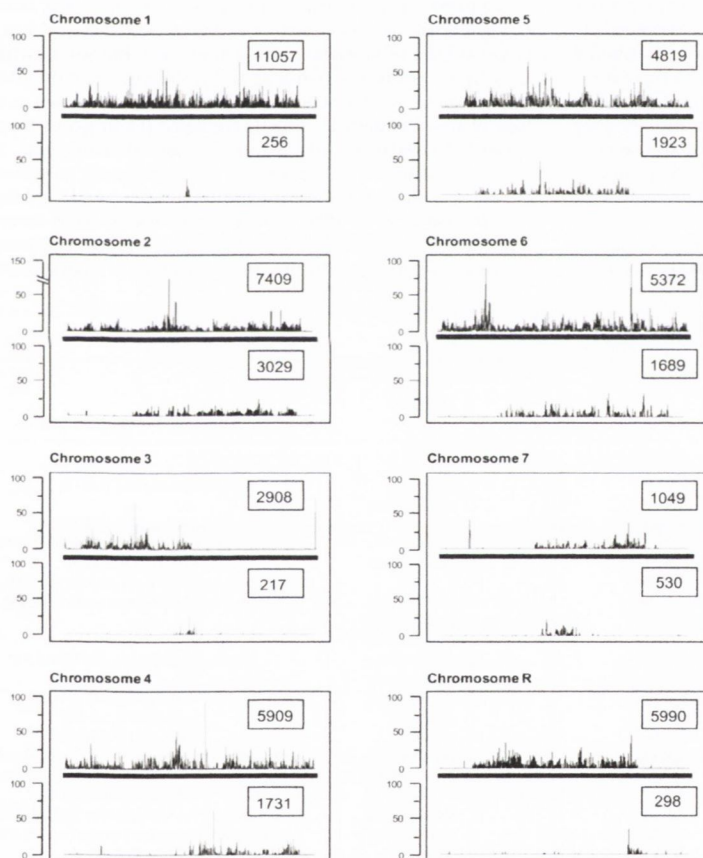


Figure 2. Comparison of sequence polymorphism (SNP) distribution between homologous chromosomes in *C. albicans* and *C. dubliniensis*. Each panel describes the distribution of SNPs along the haploid consensus for each chromosome in *C. albicans* (top) and *C. dubliniensis* (bottom). The absolute numbers of polymorphisms per chromosome are boxed in each panel.

60% (Moran et al. 2004). However, after removing transposable element genes, the number of “verified” species-specific genes (i.e., those with recognized homology) fell to 29 and 168, respectively (see Supplemental Table S4). Among these putative genes are factors with established roles in candidosis, which might contribute to increased virulence in *C. albicans*.

Exceptions to genomic collinearity concern genes with known associations to pathogenesis.

While the *C. dubliniensis* and *C. albicans* genomes largely correspond, there are various inversion, insertion-deletion, and transposition events that disrupt the otherwise collinear gene order. Importantly, some of these differences have affected genes known to have roles in *Candida* pathogenesis. Table 1 lists 21 major inversion events of between 8.5 and 185 kilobases (kb), (average ~ 50 kb) that were observed across the genome. One of these events co-occurs with genes of the *SAP* family, which mediates hydrolytic responses to host tissues in various developmental stages and physical conditions, and is among the most important virulence factors in *C. albicans* (Naglik et al. 2003). Four *SAP* loci are found in close proximity on chromosome 6 in *C. albicans* and Figure 3 shows that, of these, *C. dubliniensis* lacks two loci, *SAP4* and *SAP5*. One of the two existing *SAP* genes in *C. dubliniensis* is orthologous to *SAP1* in *C. albicans*, while the second (labeled *SAP456* in Fig. 3A) has partial affinity with *SAP4-6*, so there is no strict ortholog in *C. albicans*. We suggest a hypothesis, depicted in Figure 3B, in which two segmental inversions have modified an ancestral tandem gene pair (retained in *C. dubliniensis*) to create the observed differences. This hypothesis predicts that *SAP4-6*, but not *SAP1*, in *C. albicans* should be monophyletic, which was confirmed by phylogenetic trees (data not shown).

Breaks in chromosomal collinearity were more commonly caused by insertion-deletion events, and most of these were due to

transposable elements. The *C. dubliniensis* genome contains numerous families of LTR and non-LTR retrotransposons and two types of DNA transposon; these are uniformly distributed, and have close affinity to those in *C. albicans* (see Supplemental Table S5). Although almost all elements are inactive, no elements were identified at the same locus in the two species, suggesting a complete loss of individual insertions from the last common ancestor, and extensive transposition subsequently. Beyond transposon activity, the remaining breaks in collinearity were due to insertions or deletions of individual genes, most of which are captured in the global gene family analysis below. For instance, *C. dubliniensis* lacks a member of the *IFF* gene family, the hyphal-associated *HYR1* (orf19.4975). The *IFF* gene family encodes highly decorated, repetitive cell-wall proteins, which are induced during hyphal differentiation in *C. albicans* (Bailey et al. 1996). One member, IFF11 is secreted and contributes to virulence (Bates et al. 2007). Phylogenetic trees (data not shown) indicate that *HYR1* is a relatively old lineage and not a recent duplication of an existing locus in *C. dubliniensis*, but rarely observed in *C. albicans*. Furthermore, genes continue to be lost from the *C. dubliniensis* genome, relative to its inferred ancestor; 115 pseudogenes were observed (see Supplemental Table S6), of which 78 have intact positional orthologs in *C. albicans*. These pseudogenes affect various gene functions but several were identified as orthologs of the filamentous growth regulator (FGR) genes that have suggested roles in *C. albicans* morphogenesis (Uhl et al. 2003). Table 2 describes 16 FGR genes that are functionally modified in *C. dubliniensis*, among them are eight pseudogenes and six deletions.

In contrast, *C. albicans* displayed various novel insertions, relative to *C. dubliniensis* and *C. tropicalis*; again, most of these were subsequently observed by the global gene family analysis described

Table 1. Chromosomal inversions in the *C. dubliniensis* genome

Chromosome	Inverted region		Size (bp)	No. of genes	Derived species ^a	Duplicated inversion points
	Start	End				
2	458,616	519,886	61,270	28	<i>C. albicans</i>	—
	540,030	597,468	57,438	30	<i>C. albicans</i>	Aminotransferase (orf19.5842)
	2,215,803	2,276,205	60,402	31	<i>C. dubliniensis</i>	—
3	1,446,253	1,514,616	68,363	27	—	ALS protein (orf19.7414)
4	541,852	565,613	23,761	12	—	Transcriptional regulator (orf19.2743)
	742,045	797,620	55,575	15	<i>C. dubliniensis</i>	HP (orf19.3375)
	882,592	931,640	49,048	20	—	Conserved hypothetical protein (orf19.1430)
5	1,292,316	1,330,825	38,509	15	<i>C. dubliniensis</i>	Zinc-finger protein (orf19.1255)
	193,915	377,298	183,383	77	<i>C. dubliniensis</i>	—
	378,010	424,313	46,303	16	<i>C. dubliniensis</i>	—
	865,203	876,049	10,846	6	<i>C. dubliniensis</i>	Methyltransferase (orf19.6676)
6	19,938	36,194	16,256	13	<i>C. dubliniensis</i>	—
	205,015	213,686	8671	6	<i>C. dubliniensis</i>	Nucleotidase (orf19.105)
	571,384	647,815	76,431	32	<i>C. albicans</i>	<i>SAP</i> (orf19.5542)
	638,962	762,406	123,444	49	<i>C. albicans</i>	<i>SAP</i> (orf19.5585)
7	954,721	993,584	38,863	14	<i>C. dubliniensis</i>	(possibly involved with ALS, orf19.4555)
	18,887	52,607	33,720	19	<i>C. dubliniensis</i>	—
	842,213	868,563	26,350	12	—	IFA (orf19.6690)
R	418,551	430,515	11,964	7	<i>C. dubliniensis</i>	Oligopeptide transporter (orf19.2583)
	745,642	780,274	34,632	16	<i>C. dubliniensis</i>	Histidine-triad superfamily (orf19.2376)
	1,071,448	1,113,813	42,365	16	<i>C. albicans</i>	PIR (orf19.654)

^aThe derived species is that which has experienced evolutionary change relative to the ancestral state; this was inferred through comparison of both species with the character state in an outgroup species (*C. tropicalis*), e.g., corresponding gene order in *C. tropicalis* around the regions inverted on chromosome 5 is consistent with *C. albicans*, indicating that the inversion occurred in *C. dubliniensis*.

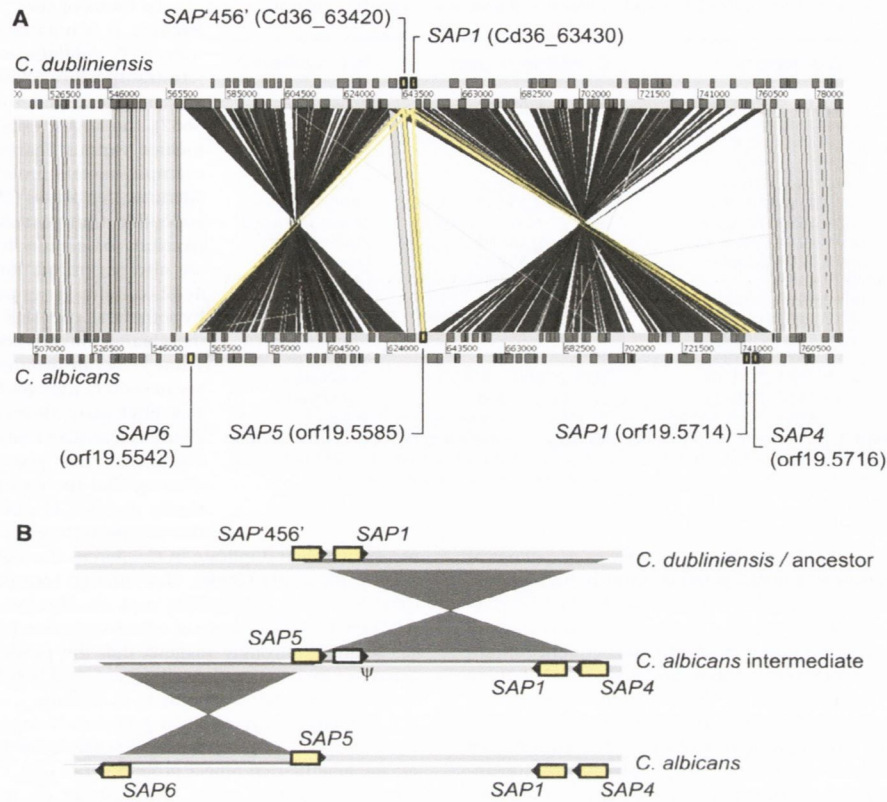


Figure 3. Segmental inversion and the evolution of secreted aspartyl proteinases (SAP). (A) Comparison of SAP genes on chromosome 6 (0.5–0.8 Mb) in *C. albicans* and *C. dubliniensis* generated using the Artemis Comparison Tool (ACT); DNA strands are represented by horizontal gray bars (scale in base pairs); genes are indicated by darker boxes. SAP genes are shaded yellow and linked to systematic IDs. Significant TBLASTX hits between genes are shown by vertical bars (gray, sense; black, anti-sense; yellow, SAPs). (B) A cartoon of the hypothesis that two segmental inversions created two additional SAP loci in *C. albicans*. The first inversion duplicated the original tandem gene pair ("*C. dubliniensis*/ancestor") creating paralogs of SAP1 and SAP'456' in the opposite orientation ("*C. albicans* intermediate"); this is consistent with SAP1 and SAP4 in *C. albicans* and demands that the original SAP1 copy was subsequently lost (ψ). The second inversion duplicated the remaining SAP'456' copy (i.e., SAP5) to create a single gene in the opposite orientation (i.e., SAP6).

below. One particular example of note concerns the invasin-like ALS3 (orf19.1816; Hoyer et al. 1998; Phan et al. 2007; Almeida et al. 2008). ALS encodes a large family of repetitive cell-surface proteins that function primarily in host cell adhesion (Fu et al. 2002; Zhao et al. 2003; Sheppard et al. 2004; Zhao et al. 2007); they are found throughout *Candida* (Hoyer 2001) and are instrumental in the initiation and maintenance of infection (Hoyer et al. 2008). ALS3 is found on chromosome R in *C. albicans*, but is entirely absent from the corresponding position in *C. dubliniensis*. A maximum likelihood phylogeny of all ALS genes in *C. albicans*, *C. dubliniensis*, and *C. tropicalis* was estimated from an alignment of the conserved 5' domain and is shown in Figure 4B. It indicates that ALS3 clusters with ALS1 and 5, although it is most similar to ALS1, consistent with the origin of ALS3 in *C. albicans* through a unique transposition event from chromosome 6 to R.

Subtle differences in *C. dubliniensis* and *C. albicans* ALS gene family repertoire

Transposition of ALS3 is not the only change to have affected this vital gene family. Superficially, *C. dubliniensis* appears to have

a very similar ALS repertoire to *C. albicans*, in which eight ALS genes are distributed across three chromosomes (Jones et al. 2004; Sheppard et al. 2004). As shown in Figure 4, A and C, six *C. albicans* loci are shared with *C. dubliniensis*: ALS1, ALS2, ALS4, ALS6, ALS7, and ALS9. ALS3, as we have seen, has no corresponding locus, and neither do ALS5 or Cd36_64800. So, based on genomic position only, both species have evolved novel ALS. Turning to the apparently orthologous ALS genes, Figure 4B shows that: (1) *C. albicans* and *C. dubliniensis* sequences for ALS4, ALS6, ALS7, and ALS9 are siblings, supporting their orthology; (2) conversely, ALS1 and ALS2 cluster together rather than with their putative orthologs in *C. dubliniensis*; (3) Cd36_64800 is almost identical to the positional ortholog of ALS2 (Cd36_65010); and (4) ALS1 and ALS5 cluster together, suggesting a recent tandem duplication in *C. albicans*. Hence, phylogenetic analysis of *C. albicans* and *C. dubliniensis* ALS repertoires reveals a mixture of some positional orthologs displaying sequence orthology as expected (ALS4, ALS6, ALS7, and ALS9), and others showing none; either because they are species-specific acquisitions (ALS3, ALS5, Cd36_64800), or because structure has been altered post hoc (ALS1 and ALS2, Cd36_65010).

Table 2. Differences between *C. dubliniensis* and *C. albicans* filamentous growth regulator (FGR) genes

Chromosome	<i>C. albicans</i> gene	<i>C. dubliniensis</i> gene	In <i>C. dubliniensis</i>
1	orf19.4549 (FGR38)	Cd36_01600	Pseudogene
	orf19.4712 (FGR6-3)	Cd36_07280	Pseudogene
	orf19.4786 (FGR43)	Cd36_08745	Pseudogene
	orf19.7275 (FGR24)	NA	Missing/deleted
2	orf19.156 (FGR51)	Cd36_19290	Pseudogene
	orf19.218 (BUD20)	Cd36_23060	Other ^a
4	orf19.1596 (FGR28)	NA	Missing/deleted
	orf19.1234 (FGR6-10)	Cd36_45130	Pseudogene
5	orf19.3209 (FGR42)	Cd36_53735	Pseudogene
	orf19.4055	NA	Missing/deleted
6	orf19.6339 (NRG2)	NA	Missing/deleted
	orf19.3413 (FGR37)	Cd36_61825	Pseudogene
R	orf19.559 (FGR14)	NA	Missing/deleted
	orf19.562 (FGR13)	NA	Missing/deleted
	orf19.3884 (FGR50)	Cd36_31800 + 31850	Duplicated
	orf19.7557 (FGR46)	Cd36_34968	Pseudogene

^aCd36_23060 and orf19.218 appear to be positional orthologs, but there is almost no sequence similarity and so no evidence of homology between the putative proteins. It may be that orf19.218 is missing from the *C. dubliniensis* genome, or that the two genes have diverged beyond recognition. NA, Not available.

There is evidence that recombination between *ALS* genes at different loci is responsible for *ALS* genes at corresponding genomic positions lacking the expected sequence similarity. The phylogenetic network shown in Figure 4D presents a consensus of all competing relationship patterns within the sequence alignment for *ALS* genes on chromosome 6, and suggests that there are conflicting phylogenetic signals. For example, while *ALS1* is most closely related to *ALS2*, there are some characters (highlighted in red) that still support orthology with its positional ortholog in *C. dubliniensis* (Cd36_64210). A Pairwise Homoplasy Index confirmed that the alignment contained significant phylogenetic incompatibility ($P < 0.0001$; Bruen et al. 2006). This apparent mosaicism among *ALS* 5' regions was corroborated by the distribution of C terminus types, shown in Figure 4B; some closely related genes have dissimilar C termini (e.g., *ALS9* and Cd36_64220), while some unrelated sequences have identical C termini (e.g., *ALSS* and 6).

Principal differences in gene family repertoire concern hypothetical proteins with unspecified functions

For a more comprehensive comparison of genomic content, we used OrthoMCL (Li et al. 2003) to cluster all homologous sequences by reciprocal BLAST searches. This reproduced the differences described above (*ALS*, cluster 5; *IFE*, cluster 5204; *SAP*, cluster 1125), but also identified the principal disparities, in terms of copy number, between the *C. dubliniensis* and *C. albicans* gene families. In Table 3, gene clusters for which there is a difference are ordered by disparity, with those in excess in *C. dubliniensis* at the top. Two general points can be made about these results. First, the principal interspecific differences do not concern the familiar cell-wall gene families with established roles at the host–pathogen interface; indeed, a survey of predicted GPI-anchored proteins produced only four differences (see Supplemental Table S7). Second, through comparison with *C. tropicalis* it was possible to determine the polarity of evolutionary change and show that excesses in *C. dubliniensis* were due to specific gains and not losses in *C. albicans*, while excesses in *C. albicans* were due to both *C. dubliniensis* losses and *C. albicans* gains.

In terms of specific gene family differences, it is notable that while expansions in *C. dubliniensis* typically concern retrotransposons (clusters 1504 and 9) and associated genes (clusters 3369 and 3687), the largest excesses in *C. albicans* include various characterized genes, for example, metalloproteases (cluster 433), allantoate permeases (cluster 48), and oligopeptide transporters (cluster 14). Expansions of certain hypothetical genes are also suggested, for instance, cluster 4642 contains genes predicted to encode hypothetical proteins with four transmembrane domains and a signal peptide. These genes are arranged in a tandem array in both *C. albicans* and *C. dubliniensis*. The phylogeny shown in Supplemental Figure S2 describes four orthologous clades consistent with genomic position, indicating that the four paralogs are structurally distinct. The only departure from this scheme is the expansion of the fourth

and last tandem duplicate in *C. albicans* through tandem duplication (to form orf19.3906), and through transposition within the array (to form orf19.3903) and to chromosome 4 (to form orf19.4961). Maintenance of orthology between tandem duplicates in the arrays, strongly suggests that the paralogs perform subtly different functions on the cell surface, and points to functional innovation of the last copy only in *C. albicans*.

The largest disparities in gene family copy number favoring *C. albicans* concern two uncharacterized gene families in particular: the leucine-rich repeat protein genes (*IFA*, cluster 11) and the telomere-associated genes (*TLO*, cluster 8), which are now described in detail.

The *IFA* gene family has undergone widespread gene loss in *C. dubliniensis*

The *IFA* genes encode a large family of putative transmembrane proteins with weak affinity to “LPT” leucine repeat-rich proteins in viruses. Table 4 describes the 22 loci defined in *C. albicans* and *C. dubliniensis*. Locus 16 (*IFA19/25*) is conserved in both species on chromosome 7, as well as *C. tropicalis*. All other loci were either shared by *C. albicans* and *C. dubliniensis* (15), specific to *C. albicans* (6), or specific to *C. dubliniensis* (2). However, a substantial component of the *C. dubliniensis* repertoire was predicted to be fragmentary or nonfunctional (14/21 loci, compared with 6/31 in *C. albicans*). A maximum likelihood phylogeny estimated from the 648 amino acid multiple alignment, shown in Figure 5 indicates that (1) derived gene duplication in *C. albicans* has created several new loci; (2) gene relics at various stages of mutational decay demonstrate that gene loss is on-going in *C. dubliniensis*; and (3) while many positional orthologs cluster together as expected (e.g., locus 13, indicated by a blue square), other cases are poorly related suggesting that gene conversion may affect loci in close proximity (e.g., locus 17, purple stars). This is supported by the phylogenetic distribution of C-terminal types, which are described as “acidic” or “basic” (see Fig. 5B). As with the *ALS* C termini previously, the distribution is incompatible with the phylogenetic relationships; for instance, at locus 2, Cd36_05810 and orf19.2430 (red triangles) are positionally and structurally orthologous, but do not share the

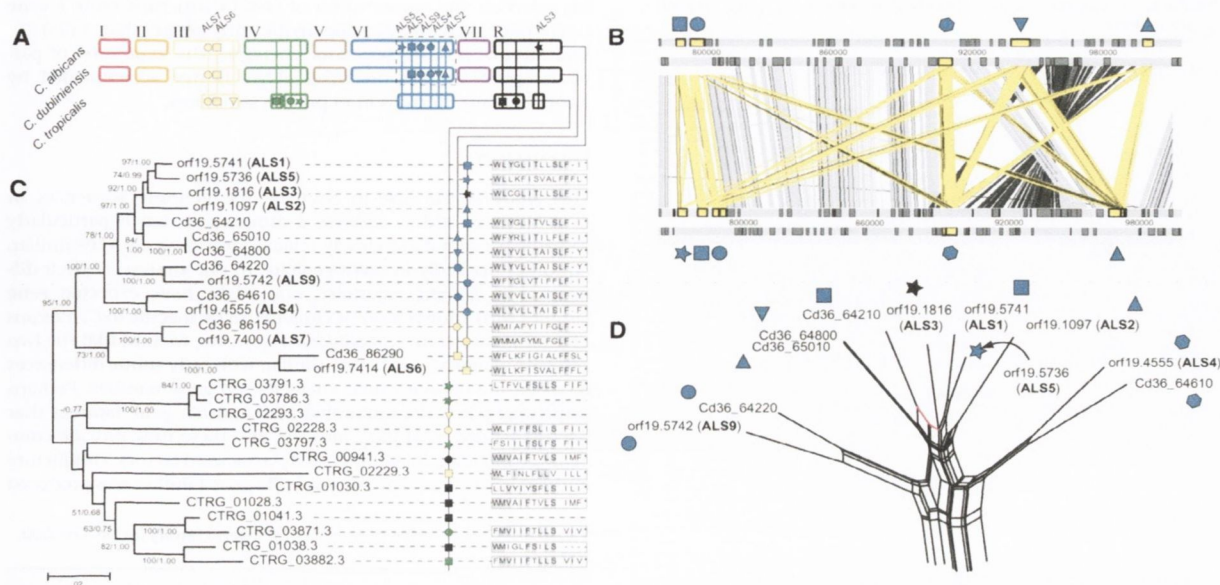


Figure 4. Comparative genomics and phylogeny of agglutinin-like sequences (ALS) in *Candida* spp. (A) A cartoon showing genomic distribution of ALS loci in *C. albicans*, *C. dubliniensis*, and *C. tropicalis*. The positions of loci are indicated by vertical lines and the presence of a gene(s) is represented by a unique symbol (which is also applied to positional orthologs in other species). A dashed line indicates the region of chromosome 6 expanded in panel C. Note that the chromosomes are drawn based on the *C. albicans* karyotype, with *C. dubliniensis* and *C. tropicalis* pseudochromosomes drawn for comparison, reflecting their conserved gene order, but not precise karyotypes. (B) Maximum likelihood phylogeny of ALS N-terminal nucleotide sequences in *C. albicans* (for which gene names ALS1-9 are given), *C. dubliniensis* (prefixed Cd36_) and *C. tropicalis* (prefixed CTRG_). Branch lengths are proportional to evolutionary change and measured in substitutions/site. This topology was concordant with an alternative Bayesian consensus tree; each node is attended by nonparametric bootstrap values and posterior probabilities from likelihood and Bayesian analyses respectively. Terminal nodes are labeled with gene IDs and locus symbols (as used in A), and then to the 14 amino acid sequence of the C terminus (where available). (C) ACT representation of ALS loci on chromosome 6 in *C. dubliniensis* (top) and *C. albicans* (bottom). ALS genes are shaded yellow and marked as in A. Significant TBLASTX hits between genes are represented by vertical bars (gray, sense; black, anti-sense; yellow, ALS). (D) Phylogenetic network showing a consensus of all possible relationships among chromosome 6 genes simultaneously. Splits supporting monophyly of Cd36_64210 and ALS1 are shown in red.

same C terminus type. In summary, the *IFA* gene family shows a marked dichotomy between evolutionary expansion in *C. albicans* and depletion in *C. dubliniensis*.

The *TLO* gene family has been uniquely elaborated in *C. albicans*

15 *TLO* genes were identified at the subtelomeres of the original *C. albicans* genome sequence (van het Hoog et al. 2007). As the OrthoMCL analysis suggests, *C. dubliniensis* lacks these genes and only two *TLO* homologs were identified: *CdTLO1* (Cd36_72860; internally on chromosome 7) and *CdTLO2* (Cd36_35580; subtelomerically on chromosome R). For the purposes of providing an outgroup, a single homolog was found in *C. tropicalis* (CTRG05798.3). Figure 6A shows that the only locus occupied by all three species is at the right arm of chromosome R. This is termed the “ancestral” locus, since a single gene at this position is the probable ancestral state. The phylogeny in Figure 6B shows that (1) *C. albicans* and *C. dubliniensis* copies are reciprocally monophyletic, supporting independent *TLO* expansions; (2) *C. albicans* copies are genetically homogeneous (average nucleotide identity = 96.5%), except for the length of repeat sequences and the apparent alternative splicing of a subset of sequences, whereas the two distinct *C. dubliniensis* paralogs are relatively well diverged (nucleotide identity = 74.9%); (3) in contradiction of the species tree, *C. dubliniensis* sequences are closer in average amino acid identity over 193 aligned positions to *C. tropicalis* (73.6%) than to *C. albi-*

cans (67.2%); and (4) despite its shared position with the ancestral locus in *C. dubliniensis* and *C. tropicalis*, orf19.7680 does not branch basally, but instead is barely distinguishable from its paralogs. These observations indicate that beginning with a single gene at the ancestral locus, additional loci have evolved independently in *C. dubliniensis*, through a transposition event to create Cd36_72860 on chromosome 7, and in *C. albicans*, through the expansion to almost all telomeres.

As the principal disparity in gene content between *C. dubliniensis* and *C. albicans*, we sought to investigate *TLO* function by creating null mutants in *C. dubliniensis*. Null mutants for *CdTLO1* showed a major reduction in hyphal formation in response to serum, as shown in Figure 7. Complementation of the *CdTLO1Δ* with either of two *C. albicans* *TLO* genes (i.e., *TLO11* and *CTA24* [also known as *TLO12*]) restored the defect in hyphal formation, indicating that these *TLO* genes have a common role in morphogenesis in both species. Further experiments are on-going to determine if all *TLO* gene copies have the same effects when ablated and restored, and to define the precise function of the *TLO* families in *C. albicans* and *C. dubliniensis* and their role in pathogenesis.

Absence of adaptive evolution among orthologous gene sequences

Orthologous gene sequences in *C. albicans* and *C. dubliniensis* are generally well conserved, as described previously. While the rates

Table 3. Disparities in gene family size between *C. albicans* and *C. dubliniensis*

Cluster	Copy number: <i>C. dubliniensis</i>	<i>C. albicans</i>	Disparity ^a	Description
1504	15	2	-13	Non-LTR retrotransposon, reverse transcriptase
9	16	6	-10	Non-LTR retrotransposon, polyprotein
1	7	2	-5	Subtelomeric helicase ^b
3369	4	0	-4	Retrotransposon-associated subtelomeric
3687	3	0	-3	Retrotransposon-associated subtelomeric
3972	3	0	-3	Hypothetical protein
24	10	8	-2	Major repetitive sequence <i>NR6</i> protein
3294	3	1	-2	DNA-binding protein
3791	6	4	-2	Non-LTR retrotransposon protein
5220	2	0	-2	Retrotransposon-associated subtelomeric
5437	2	0	-2	Retrotransposon-associated subtelomeric
5698	2	0	-2	Hypothetical protein
5758	2	0	-2	Retrotransposon-associated subtelomeric
123	3	2	-1	Vacuolar amino acid transporter
243	2	1	-1	Dinucleoside triphosphate hydrolase
244	2	1	-1	RNA polymerase II mediator complex subunit
245	7	6	-1	Peptidyl-prolyl cis-trans isomerase precursor
246	2	1	-1	U3 small nucleolar ribonucleoprotein protein
305	2	1	-1	Spindle pole body component
381	4	3	-1	Ammonium transporter
476	2	1	-1	Biosynthesis of nicotinic acid (BNA) protein
1093	2	1	-1	Dipeptidyl aminopeptidase
2649	2	1	-1	Glutamate decarboxylase
3599	2	1	-1	Hypothetical protein
3600	2	1	-1	Hypothetical protein
3646	23	22	-1	Hexose transporter
3869	5	4	-1	Diacylglycerol pyrophosphate phosphatase
5162	2	1	-1	Retrotransposon-associated subtelomeric
5668	2	1	-1	Retrotransposon-associated hypothetical protein
5	7	6	-1	At 5 family protein (see Figure 4)
115	9	10	1	Succinate-semialdehyde dehydrogenase
157	8	9	1	Formate dehydrogenase
479	2	3	1	Oxidoreductase
533	1	2	1	Hypothetical protein
918	1	2	1	Protease
2693	1	2	1	Trans-aconitate methyltransferase
3111	0	1	1	Hypothetical protein
3114	0	1	1	Hypothetical protein
3119	0	1	1	c-regulated endoplasmic reticulum protein
3279	11	12	1	LOC family protein
3472	4	5	1	Putative GPI-anchored protein
3480	1	2	1	Zinc cluster transcription factor
3683	1	2	1	Hypothetical protein
3721	6	7	1	Esterase/lipase
3848	7	8	1	YFP family protein
4049	3	4	1	Aminotransferase
4050	0	1	1	Dethiobiotin synthetase
4521	5	6	1	Cysteine-rich hypothetical protein
5175	6	7	1	Mannosidase
5204	11	12	1	<i>HYR1</i> family protein (<i>HYR1</i>)
4	4	6	2	ABC transporter
14	7	9	2	Oligopeptide transporter
73	0	2	2	Allantoin permease
126	1	3	2	Vacuolar membrane protein
1125	8	10	2	Secreted aspartyl protease (see Figure 3)
4037	0	2	2	Hypothetical protein
4040	0	2	2	Non-LTR retrotransposon, reverse transcriptase
4041	0	2	2	GPI-anchored cell surface glycoprotein
4043	1	3	2	Hypothetical protein
4044	0	2	2	Non-LTR retrotransposon
433	1	4	3	Metalloprotease
48	13	16	3	Allanate permease
4885	4	7	3	Mutator family transposase
4842	4	7	3	Hypothetical protein (see Figure S2)
58	2	7	5	Cir family transposase
11	21	31	10	<i>IFA</i> family protein (see Figure 5)
8	2	14	12	<i>TLO</i> family protein (see Figure 6)

Gene clusters were generated from all annotated gene models in *C. dubliniensis* and *C. albicans*, including pseudogenes. Clusters were then manually curated to ensure that all family members were included (see Methods).

^aDisparity expresses the copy number in *C. dubliniensis* subtracted from the number in *C. albicans*. Cell shading reflects the polarity of evolutionary change, inferred through phylogenetic and comparative analyses (see Methods): *C. albicans* gain (blue), *C. albicans* loss (red), *C. dubliniensis* gain (pink), *C. dubliniensis* loss (purple), combination of *C. albicans* gain, and *C. dubliniensis* loss (light blue). Unshaded cells indicate that the direction of change could not be determined.

^bY'-related helicases are found in subtelomeric regions in *C. dubliniensis*; evidence suggests that these are also present in *C. albicans* and that the apparent disparity in helicase copy number is due to the omission of subtelomeric regions from the *C. albicans* assembly.

of both synonymous and nonsynonymous (i.e., amino acid replacing) substitutions were low, the ratio of these two rates could still reveal the action of adaptive evolution. However, the d_N/d_S rates shown in Supplemental Table S8 demonstrate that there is little evidence for positive selection among 5569 orthologous gene pairs. For most genes (5177) $d_N/d_S < 0.3$, indicating strong purify-

ing selection and conservation of protein structure. Only 1 gene (orf19.6601) had a d_N/d_S value significantly greater than 1 (3.077), indicative of positive selection. Therefore, the expansion of particular gene families described above is not accompanied by a concomitant innovation in protein structures.

Discussion

This study is the first to compare the genome sequences of *C. dubliniensis* and *C. albicans*, a comparison that is particularly valuable because these closely related organisms are very similar, but differ markedly in pathogenicity. In our analysis of their differences in genetic repertoire, one might have expected gene families with known roles in virulence to be specific to *C. albicans* or nonfunctional in *C. dubliniensis*. However, we find that the two gene complements are highly similar, with only subtle differences in repertoire between such characterized gene families. Perhaps surprisingly, it is the unfamiliar *TLO* and *IFA* gene families that have expanded most in *C. albicans*, and these now provide compelling candidates for pathogenicity-associated factors. This picture of the relative complement of specific gene families was produced

Table 4. Comparative genomics of *IFA* gene family loci in *Candida* spp.

Locus	Alias	Chromosome	Species identifier		
			<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. tropicalis</i>
1	IFA10	1	orf19.4549	Cd36_01600 ^a	x
2	IFA9	1	orf19.2430	Cd36_05810	x
		1	orf19.2663		
3	IFA15	1	orf19.996	x	x
4	IFA22	1	orf19.1002	x	x
		1	orf19.1005		
5	IFA5	1	orf19.6353	x	x
		2	orf19.4507 ^a		
6	IFA4	2	orf19.4510	Cd36_19060 ^a	x
		2	orf19.4511		
7	IFA1	2	orf19.156	Cd36_19290 ^a	x
8		3	x	Cd36_80890 ^a	x
9	IFA7	4	orf19.1326	x	x
10	IFA11	4	orf19.1596	x	x
		5	orf19.931	Cd36_50580 ^a	x
11		5	orf19.3919	Cd36_53990 ^b	x
12		6	x	Cd36_63200	x
13		6	orf19.5619	Cd36_63710 ^a	x
14	IFA12	6	orf19.5177	x	x
15	IFA6	7	orf19.5138	Cd36_72790 ^b	
		7	orf19.5139		
16	IFA25	7	orf19.5140	Cd36_72800	CTO05211.3
		7	orf19.5141	Cd36_72810 ^b	
17		7	orf19.1330	Cd36_73110	
		7	orf19.6704 ^a	Cd36_73120 ^b	x
18		7	orf19.6690	Cd36_73230 ^a	
		7	orf19.6690	Cd36_73260	x
19		7	orf19.5508 ^a	Cd36_73360	x
		7	orf19.6703		
20	R	R	orf19.6641 ^a	Cd36_31080 ^a	x
21	R	R	orf19.3877	Cd36_31750 ^a	
		R	orf19.3879 ^a	Cd36_31770 ^a	x
22	R	R	orf19.7550	Cd36_34930	x
Loci			23	18	1
Genes			31	21	1

An "x" indicates corresponding positions without genes or pseudogenes.

^aAn annotated pseudogene.

^bA gene relic (i.e., noncoding region with significant similarity to an IFA gene, yet extensively corrupted to such as extent that a gene model could not be composed).

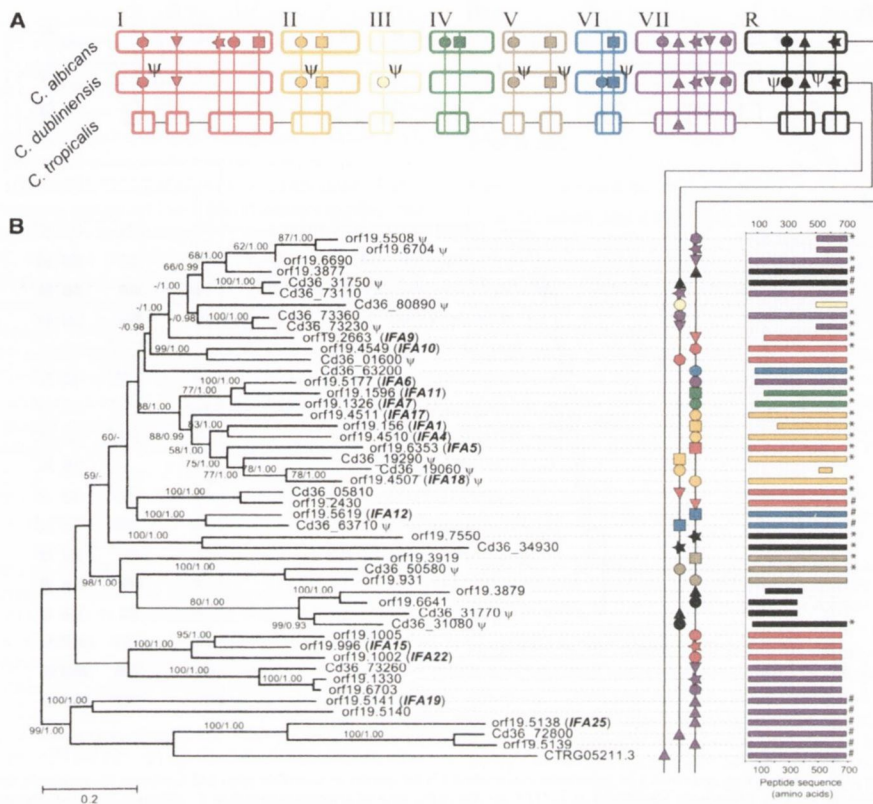


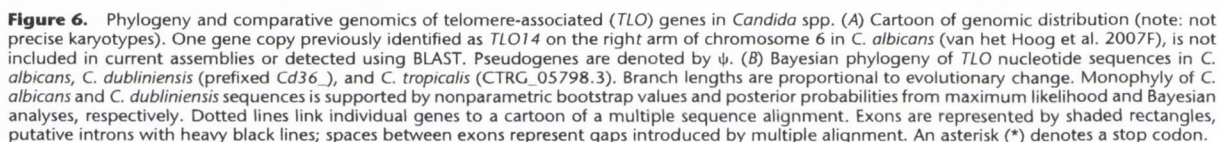
Figure 5. Phylogeny and comparative genomics of the IFA gene family in *Candida* spp. (A) Cartoon of genomic distribution (note: not precise karyotypes). Pseudogenes are denoted by Ψ . (B) Maximum likelihood phylogeny of IFA protein sequences in *C. albicans* (prefixed orf19.), *C. dubliniensis* (prefixed Cd36.), and *C. tropicalis* (CTRG05211.3). Branch lengths are proportional to evolutionary change and measured in substitutions/site. This topology was concordant with an alternative Bayesian consensus tree. Dotted lines link gene IDs to a cartoon of a multiple sequence alignment. C termini are labeled according to amino acid composition: (#) long, acidic-type; (*) a shorter, nonacidic-type C-terminal. No label indicates that neither type is present or the C terminus is absent.

by an earlier genomic analysis using CGH (Moran et al. 2004), indicating that this technique can provide an accurate impression of genomic differences.

C. albicans infection is associated with the developmental transition from the yeast to hyphal growth stage, and genes regulating, or specifically expressed during hyphal differentiation, are therefore implicated in pathogenesis. Both *C. dubliniensis* and *C. albicans* possess various hydrolytic enzymes, such as secretory lipases and phospholipase B, which are expressed at the onset of hyphal differentiation and when infection is established (Naglik et al. 2003). Both genomes also include diverse SAP gene families, although additional SAP genes have evolved in *C. albicans*, which we propose is a result of sequential segmental inversions. Duplication of genes at the breakpoints of chromosomal inversions has been observed previously, for instance, in *Drosophila melanogaster* (Matzkin et al. 2005) and *Anopheles gambiae* (Sharakhov et al. 2006), and in yeast, recently duplicated genes co-occur with chromosomal breakpoints more frequently than expected by chance (Gordon et al. 2009). This is thought to occur during the repair of chromosomal breaks, either single- or double-stranded, during which nonreciprocal recombination can occur with the template used in DNA repair, which is proposed to contain the

duplicated gene (Sharakhov et al. 2006; Ranz et al. 2007; Meisel 2009). In this case, the genes concerned, *SAP5* and *SAP6*, encode proteins known to have hypha-specific expression and may have increased the ability of *C. albicans* to cause systemic infection. In almost all respects *C. dubliniensis* and *C. albicans* correspond in their repertoires of GPI-anchored proteins, which mediate interactions at the host-pathogen interface. Thus, their common ancestor was probably a competent opportunist, with the molecular tools to cause disease, among them at least six *ALS* genes that are instrumental in host cell adhesion and are unambiguously associated with pathogenesis (Hoyer 2001; Hoyer et al. 2008).

Given the prominent role of *ALS* in disease (Fu et al. 2002; Zhao et al. 2007), their known functional differentiation (Sheppard et al. 2004; Zhao et al. 2004; Cheng et al. 2005; Zhao et al. 2005) and genetic variability, perhaps even hypervariability (Zhang et al. 2003; Zhao et al. 2003; Oh et al. 2005; Zhao et al. 2007), it is important to clearly and correctly interpret genomic variation in *ALS* repertoire. While *ALS* loci are positioned in a roughly similar manner in *C. dubliniensis* and *C. albicans*, the phylogeny in Figure 4B often showed that there is no simple orthology between *ALS* genes on chromosome 6. *ALS2* and 4 from *C. albicans* did not cluster with their positional orthologs in



Since it shared an ancestor with *C. albicans*, *C. dubliniensis* has experienced reductive evolution that has probably diminished the genetic repertoire it inherited. With the exception of conspicuous transposon activity, there has been little expansion of existing gene families, or derivation of novel genes. Instead, *C. dubliniensis*

Strain	% Hypha formation
SC5314	~82
Wu284	~45
CdTLO Δ	~1
CdTLO Δ + TLO11	~62
CdTLO Δ + CTA24	~68

Figure 7. Formation of hyphae by wild-type *C. albicans* (SC5314), wild-type *C. dubliniensis* (Wü284), *Cdt101Δ* (Cd36_35580 deleted), *Cdt101Δ* expressing *CaTLO11* (orf19.5700), and *Cdt101Δ* expressing *caCTA24* (orf19.4054). The percentage of yeast cells producing germ tubes was calculated following incubation at 37°C for 1 h.

clearly originated in *Candida*, and could be a specific adaptation to parasitism (Butler et al. 2009). *C. dubliniensis* and *C. albicans* are known to differ in their IFA gene repertoire (Moran et al. 2004); the exhaustive analysis presented here shows that 14 out of 21 loci are predicted to be nonfunctional. While some genes are corrupted by only one or a few frame-shifts, others are genuine relics with heavily corrupted coding sequences that betray a recent history of mutational decay. However, this process of unmistakable degradation in *C. dubliniensis* is only half the story: it cannot explain the greatest differences between the species.

For its part, *C. albicans* has lost relatively little and has expanded its genomic repertoire of selected gene families. In Table 3, only one case of excess copy number in *C. dubliniensis* is due to a *C. albicans* loss (glutamate decarboxylase; cluster 2649). Relatively minor evolutionary acquisitions, such as *SAP4* and 5 through inversion, or *ALS3* through duplicative transposition, may yet turn out to be of great importance. But it is the *TLO* gene family that stands out as the largest copy number disparity that has evolved specifically in *C. albicans*, rather than through deletion in *C. dubliniensis*. This disparity was suggested in previously reported CGH experiments (Moran et al. 2007), but was not observed in pan-*Candida* genomic comparisons (Butler et al. 2009), despite only one *TLO* gene being present in *C. tropicalis*. *TLO* are thought to encode transcription factors (Kaiser et al. 1999; van het Hoog et al. 2007), since they contain a putative Med2 domain (Hallberg et al. 2004; Pfam: PF11214), which is associated with the RNA polymerase II mediator complex in yeast (Björklund and Gustafsson 2005). In preliminary assays, we have shown that loss of *TLO1* in *C. dubliniensis* significantly reduces hyphal formation in response to serum, and that this can be restored by complementation with certain *C. albicans* *TLO* genes. Therefore, *TLO* genes may have a regulatory function during morphogenesis in both species, perhaps affecting their relative abilities to cause disease.

As Figure 6 suggests, *C. albicans* has derived novel *TLO* gene copies through duplication at its subtelomeres, perhaps through telomeric exchange. Since their sequences are virtually identical, this suggests that subtelomeric *TLO* genes diverge as a unit under concerted evolution, and evolved for dosage rather than functional diversity. Contrary to expectation, the *C. albicans* *TLO* gene sequence is less related to the *C. dubliniensis* homolog than the *C. tropicalis* gene. Substitution patterns within the *TLO* multiple alignments (see Supplemental Fig. S3) show that this unexpected similarity is due to amino acid motifs shared by *C. dubliniensis* and *C. tropicalis* (shown in red), which have been modified in *C. albicans*. Hence, the "closeness" of *C. dubliniensis* and *C. tropicalis* *TLO* sequences, which contradicts the overall species relationships, probably owes more to the derived state of *C. albicans*: in short, the *TLO* family has been uniquely modified in number and structure in *C. albicans*, while *C. dubliniensis* retains a *TLO* repertoire not unlike the ancestral state.

The differences between the genomic repertoires of *C. albicans* and *C. dubliniensis* demonstrate that they have taken divergent evolutionary trajectories since speciation. While *C. albicans* has augmented its parasitic capacity with expansions of the *SAP*, *ALS*, and *IFA* gene families (among many others), *C. dubliniensis* has experienced widespread deletions, many of which have affected its capacity to cause disease, at least in humans. Since there is no comparable innovation in the *C. dubliniensis* genome, this suggests a process of reductive evolution and the deletion of redundant loci after *C. dubliniensis*. We exploited the diminished pathogenicity of *C. dubliniensis* to identify the candidate genes in *C. albicans* that could account for the phenotypic disparity between these other-

wise very similar organisms. We have shown that the principal disparities favoring *C. albicans* concern two relatively unknown gene families, the *TLO* and *IFA* proteins, providing substantial new leads in the molecular characterization of candidal virulence.

Methods

Genome sequencing and assembly

C. dubliniensis has a complex diploid karyotype consisting of 13 haploid chromosomes, some of which correspond in their entirety to a *C. albicans* chromosome, and some of which are chimaeras of *C. albicans* chromosomal blocks (Magee et al. 2008). This karyotype could not be recovered during genome sequencing, or reproduced in the final assembly, because chromosomal homologs are virtually indistinguishable. Therefore, the *C. dubliniensis* genome sequence is a haploid consensus of both chromosomal homologs, which was assembled de novo without reference to the *C. albicans* genome sequence. Genome assembly produced 1110 contigs with an N50 value of 86 kb, meaning that at least half of all bases belonged to contigs of this size or greater. Given that the actual karyotype could not be resolved, and the purpose of assembly was for comparison with *C. albicans*, at this point the order and orientation of *C. dubliniensis* contigs was established by mapping them onto the *C. albicans* genome sequence, to produce pseudochromosomes. This inevitably meant that those haploid components of the *C. dubliniensis* karyotype that departed from the eight chromosome karyotype of *C. albicans* were not represented in our *C. dubliniensis* assembly. However, no genome data were discarded since these "missing" chromosomes contributed their nucleotide sequences to the haploid consensus. PCR walking was used to validate the contig scaffolds produced in reference to *C. albicans*. Specific BACs were sequenced to demonstrate that those haploid components contained in the in vivo karyotype, but omitted from our genome assembly, did exist.

Sequence polymorphism

To identify heterozygous sites in the *C. dubliniensis* assembly, we realigned all of the shotgun reads using SSAHA2 (Ning et al. 2001) onto the final genome sequence. We then identified all reads that uniquely align and are paired in the correct orientation within the expected insert size. Sites were identified where a base differed from the reference base and where the cumulative *phred* score for the alternative base was greater than 42, as described previously (Jeffares et al. 2007). This was also done for *C. albicans* reads for comparison.

Gene prediction and annotation

Open reading frames (ORFs) over 300 bp were marked up in Artemis (Rutherford et al. 2000). ORFs were manually checked using BLASTX to select the most plausible coding sequence where multiple overlapping ORFs existed, and to identify likely start and stop codons. All splice donor and acceptor sequences and lariat (TACTAAC) sites were manually marked up, enabling a refinement of intron-exon structures of appropriate gene models. Additional coding sequences were annotated, and existing gene models were refined based on a base-wise comparison with *C. albicans* using the Artemis Comparison Tool (ACT; Carver et al. 2005). Functions were ascribed by searching UNIPROT and Pfam databases for homologous proteins using FASTA and BLASTP analyses. All protein alignments were manually reviewed. Pseudogenes were only confirmed after exhaustive manual base checking to prove that both alleles were predicted to be nonfunctional. Where only one allele was nonfunctional, the haploid consensus, (which contains

sequences from both chromosomal homologs), was resolved into two haplotypes and the full-length allele was retained in the consensus.

Annotation of the major repeat sequence (MRS)

A gene-poor region of chromosome 2 was found to contain a cluster of five SfiI restriction sites, known to be located in the *C. albicans* MRS (Chibana et al. 1994; Jones et al. 2004), and to contain a coding sequence which, in *C. albicans*, was described as being located in the MRS RB2 region. Dot-plot analysis (Dotter) of this region showed that it contained four tandem repeat units of 2106 bp each, plus one partial repeat of 1535 bp. Each of these repeat units contained a single SfiI site. This represents the RPS "core" of the MRS region. A single RPS unit from this region was compared to each *C. dubliniensis* chromosome in turn using BLASTN, in order to locate the other MRS regions. The conserved flanking HOK and RB2 regions, previously noted in *C. albicans* MRS (Chindamporn et al. 1998), were located by aligning 10 kb of sequence upstream and downstream of the core RPS unit from each MRS using ClustalW (Larkin et al. 2007).

Comparative analysis of genome content

The OrthoMCL algorithm (Li et al. 2003; Chen et al. 2006) was used to obtain a global view of the differences between *C. dubliniensis* and *C. albicans* genomic complement. OrthoMCL groups homologous sequences into clusters that range in size from single species-specific genes to large gene families conserved across species. We used OrthoMCL to obtain a preliminary list of gene clusters that was then manually curated to identify gene families with interspecific disparities in copy number. After inspecting all such clusters using BLASTX to ensure that gene families were not subdivided into distinct clusters, and that all family members were included, we then inspected the genomic positions of each cluster member in ACT to confirm presence or absence in *C. dubliniensis* and *C. albicans*. The preliminary list of disparities produced by OrthoMCL did not include many species-specific singleton genes that were evident from chromosomal comparisons. Therefore, we manually inspected each chromosome for disruptions to the colinear gene order. *C. dubliniensis* coding sequences (including transposable element genes, but not pseudogenes) at these breakpoints were then compared with the *C. albicans* genome using BLASTX to obtain the top sequence match. Genes without matches in *C. albicans*, or where the top hit in *C. albicans* was not reciprocal, were confirmed as *C. dubliniensis* specific. The process was repeated for putative *C. albicans*-specific genes. Where possible, the polarity of evolutionary change (i.e., the species in which it occurred) was assessed through comparison with the character state in an outgroup, in this case, the draft *C. tropicalis* genome sequence (Butler et al. 2009).

Comparative analysis of predicted GPI-anchored proteins

Genes that encode putative GPI proteins were selected by identifying coding sequences that were predicted to have an N-terminal signal peptide and a GPI-anchor attachment site, but were negative for transmembrane spanning regions. C-terminal GPI signal peptides were predicted using Big-PI (Eisenhaber et al. 2004) and a complementary pattern search method that searched for the GPI-specific sequence (gasndc)(gasvietkdif)(gasv)-x(4,19)-(filmvagspctcywn) (Prosite format) using the fuzzpro (de Groot et al. 2003) program from EMBOSS. Absence of internal transmembrane domains was analyzed using TMHMM (Krogh et al. 2001), and presence of an N-terminal signal peptide predicted using SignalP (Emanuelsson et al. 2007). Proteins fulfilling the criteria described above were

analyzed further by BLAST to identify *C. albicans* orthologs. The *C. dubliniensis* genome was surveyed for orthologs to all known GPI-anchored proteins and protein families in *C. albicans*.

Phylogenetic analysis

Comparative genomics and phylogenetic estimation were used to characterize the evolutionary changes affecting several gene families (*TLO*, *IFA*, *ALS*, *IFF*, *SAP*, and cluster 4629). Sequences were retrieved through BLASTX analysis and subsequent inspection of each locus in ACT. Where possible, outgroup sequences for *C. albicans* and *C. dubliniensis* were provided by homologs from *C. tropicalis* (Butler et al. 2009). All gene sequences were translated and aligned using ClustalW (Larkin et al. 2007) and then edited manually. It was necessary to remove highly divergent repeat regions from the *TLO* (789 bp) and *ALS* (1326 bp) alignments. The *SAP* (1857 bp), *IFA* (1941 bp), *IFF* (1311 bp), and cluster 4642 (577 bp) alignments comprised the entire coding sequences after minor adjustments to ensure equal length. Phylogenies for the *TLO*, *ALS*, and cluster 4642 nucleotide alignments were estimated using maximum likelihood (ML) and Bayesian inference (BI). The ML phylogeny was estimated with PHYML v2.4.4 (Guindon and Gascuel 2003; Guindon et al. 2005), using a general-time reversible (GTR) model (Yang 1994) with six rate categories. Empirical base frequencies and a gamma distribution parameter (α) were optimized from the data. The BI phylogeny was estimated with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Four parallel MCMC chains were run for 1,000,000 generations, with a sample frequency of 100 generations; a burn-in of 1000 generations was found to be sufficient to achieve stationary model parameters using Tracer v1.4.1 (<http://tree.bio.ed.ac.uk/software/tracer/>). Due to greater sequence divergence and available characters, phylogenies for the *SAP*, *IFA*, and *IFF* families were estimated from protein sequences. ML and BI phylogenies employed a WAG model (Whelan and Goldman 2001) with an additional rate heterogeneity parameter. Otherwise, operating conditions were as described previously. In all cases, topological robustness was assessed through 100 nonparametric bootstrap replicates (Felsenstein 1985).

Experimental disruption of *TLO* genes

Growth and transformation of *C. dubliniensis* strain Wü284 was performed by electroporation as described previously (Moran et al. 2007). Disruption of the *CdTLO1* gene was achieved using the SAT1-flipper cassette system (Reuß et al. 2004). A deletion construct was created by amplifying the flanking regions of *CdTLO1* with the primer pairs CTA21KF/CTA1X and CTA1S/CTA21SIR (see Supplemental Table S9), followed by ligation of the products into plasmid pSFS2A to yield plasmid pTY101. This construct was then used to transform *C. dubliniensis* Wü284, and deletion of the *CdTLO1* gene was confirmed by Southern analysis. Reintroduction of wild-type *TLO* genes was achieved by amplification of the entire ORF plus their upstream and downstream regulatory sequences using primer pairs *CdTLO1FP/CdTLO1RP* (for *CdTLO1*), *CdTLO2FP/CdTLO2RP* (for *CdTLO2*), *CaTLO11FP/CaTLO11RP* (for *CaTLO11*), and *CaTLO12FP/CaTLO12RP* (for *CaTLO12*) (see Supplemental Table S9) and ligation of these into the *C. dubliniensis* integrating vector pCDRI to yield plasmids pCdTLO1, pCdTLO2, pCaTLO11, and pCaTLO12, respectively. These plasmids were transformed into the *CdTLO1Δ* strain as described above.

Calculation of d_N/d_S ratios

Five thousand five hundred sixty-nine (5569) pairs of orthologous gene sequences were extracted from the OrthoMCL analysis

described above. Each of these pairs was aligned using ClustalX. The ratio of the nonsynonymous substitutions per site to synonymous substitutions per site (d_N/d_S) was estimated for each orthologous pair, averaged over the entire alignment, using K_a/K_s Calculator v1.2 (Zhang et al. 2006). This program implements several candidate models of codon substitution in a maximum likelihood framework; we used the NG method to estimate d_N/d_S values.

Acknowledgments

We thank Lee Murphy and members of the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute (WTSI). This work was supported by the Wellcome Trust (WT085775/Z/08/Z) through their support of the Pathogen Genomics Group at the WTSI, and grants from Science Foundation Ireland (O4/IN3/B463), the European Union (MRTN-CT-2004-512481), and the Irish Health Research Board (RP/2004/235). A.P.J. is supported by a Postdoctoral Fellowship from the Wellcome Trust Sanger Institute. C.A.M. was supported by a Medical Research Council New Investigator Award. We thank two anonymous referees for their helpful comments.

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Received June 26, 2009; accepted in revised form August 30, 2009.

