Fungal azole resistance and the role of cytochrome P450.

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Fungal Azole Resistance and the Role of Cytochrome P450

BY

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This thesis is dedicated to my Parents:

José and Ida Rodrigues de Melo;

Henry and Robert O'nions

Summary

The incidence of fungal infections and particularly infections caused by non-*albicans Candida* species has increased considerably in the past two decades. The azole antifungal drugs are central to the management of fungal infections and like the use of other antibiotics drug resistance has emerged. Factors associated with antifungal resistance were investigated including azole susceptibility, drug accumulation and membrane sterol composition. Intracellular drug accumulation in *Candida* strains and species was examined and a clear correlation to microbial sensitivity was not observed. Also the membrane sterol profiles of some resistant strains were also altered with an increase in intermediates sterols, but no clear evidence of new mutants of sterol biosynthetic enzymes was uncovered. Some clinical strains were found to be resistant to specific azoles where other strains were resistant to a broad spectrum of azoles. The fungal cell wall and its biosynthesis are essential for cell viability and as such are potential targets for novel antifungal agents. *CYP56* in *S. cerevisiae* encodes a cytochrome P450 enzyme that catalyses the biosynthesis of dityrosine during spore wall biogenesis. The role of the *C. albicans CYP56* gene in growth and drug susceptibility was investigated. The full-length *CYP56* DNA sequence was determined and the gene disrupted using the SAT1-flipper technique. The *cyp56*Δ mutant exhibited increased susceptibility to caspofungin and nikkomycin compared to the wild-type parental strain, whereas susceptibility to azoles and other metabolic inhibitors tested was unaffected. Phenotypically, the wild-type and mutant strains were morphologically similar on rich media, however in minimal media the *cyp56*Δ mutant exhibits hyphal growth in contrast to the yeast only form of the parental wild-type strain. Also the mutant failed to form chlamydospores. Over-expression of CYP56 protein in *E. coli* produced a membrane-associated cytochrome P450 enzyme that catalysed the conversion of *N*-formyl tyrosine to *N*-formyl dityrosine when reconstituted with a fungal cytochrome P450 reductase in liposomes. The present study aimed to further the understanding of the biological, genetic and physiological aspects azole effect on clinical strains, on fungal CYPs (including CYP56) and for sterol biology associated with fungi, particularly in *Candida* species.

DECLARATION

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Publications

1. MELO, Nadja Rodrigues de, VILELA, Maria M. S, CULHARI, Victoria V. P., TAGUCHI, Hideaki, MIYAJI, Makoto, Philip GROENEVELD, Andrew WARRILOW, Diane E. KELLY, Steven L. KELLY. Antifungal cross-resistance in an oral *Candida* isolate from a Brazilian HIV 1-infected child. FEMS Yeast Research submitted 2006.
2. MELO, Nadja Rodrigues de, CULHARI, Victoria V. P., TAGUCHI, Hideaki, SANO, A, MIYAJI, Makoto, KELLY, Steven L., VILELA, Maria M. S. *Candida dubliniensis* in a Brazilian family with an HIV-infected child. *Brazilian Journal of Microbiology*, 2006, 37:223-227.
3. MELO, Nadja Rodrigues de, VILELA, Maria M. S, JORGE, Jacks, J FUKUSHIMA, K., NISHIMURA, K., Kamei, K., MIYAJI, Makoto, KELLY, Steven L., TAGUCHI, Hideaki. Effect of HIV protease inhibitor and antifungal agents combination on the growth rate of single hyphae of *C. albicans* by a bio-cell tracer system. *Brazilian Journal of Microbiology*, 2006, 37:1-7.

Abstracts

1. MELO, Nadja Rodrigues de, MORAN, Gary, COLEMAN, David, SULLIVAN, Derek, SMITH, Steve, WARRILOW, Andrew G. S., KELLY, Diane E., LAMB, D. C., KELLY, S. L. Role of DIT2 in *C. albicans* growth and drug susceptibility. **Oral presentation**: in British Society for Medical Mycology, annual meeting March 2007.
2. MELO, Nadja Rodrigues de, WARRILOW, Andrew G. S., KELLY, Diane E., LAMB, D. C., KELLY, S. L. Expression studies and azole binding to *C. albicans* and *A. fumigatus* CYP51. Poster in: 8TH International Symposium on Cytochrome P450 Biodiversity and Biotechnology, July 2006, Swansea, Wales.
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KELLY, Steven L. KELLY. Antifungal cross-resistance in oral *Candida*. Poster in: British Society for Medical Mycology, annual meeting April 2005, Canterbury, UK.

6. MELO, Nadja Rodrigues de, VILELA, Maria M. S, CULHARI, Victoria V. P., TAGUCHI, Hideaki, SANO, A, MIYAJI, Makoto. Follow up – Oral Candida flora from Brazilian family with HIV-infected children in the HAART Era. **Oral presentation** in: 1st ABEP/Oxford Centre for Brazilian Studies Conference. Oxford, pag. 73, March 19th 2004. http://www.bioresearchers.com/2/a_oral_microbiology.htm
7. MELO, Nadja Rodrigues de, VILELA, Maria M. S, CULHARI, Victoria V. P., TAGUCHI, Hideaki, MIYAJI, Makoto. Philip GROENEVELD, Andrew WARRILOW, Diane E. KELLY, Steven L. KELLY. Azole cross-resistance in oral HIV-infected child. Poster in: 1st ABEP/Oxford Centre for Brazilian Studies Conference. Oxford, pag. 72, March 19th 2004. http://www.bioresearchers.com/2/3/nadja_r_melo.htm

Contents

Chapter 2

Materials and Methods

List of Tables and Figures

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Abbreviations

The abbreviations used in this thesis are listed below:

A	absorbance
aa	amino acid
AIDS	acquired immunodeficiency syndrome
ALA	δ-aminolaevulinic acid
ATCC	american type culture collection
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
BSTFA	bis (trimethylsilyl) trifluoroacetamide
°C	degree centigrade
CO	carbon monoxide
cm	centimetre
Da	Dalton
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
5FC	5-flucytosine
g	grams
HCL	hydrochloric acid
H_3PO_4	phosphoric acid
HPLC	High performance liquid chromatography
IPTG	isopropylthio-β-D-galactoside
IS	inoculum size
kbp	kilo base pairs
kDa	kilo Dalton
kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
K_2HPO_4	dipotassium hydrogen orthophosphate
K_2SO_4	potassium sulphate
KOH	potassium hydroxide
L	litre
M	molecular mass
μ	micron
μg	micrograms
μl	microlitres
μM	micromolar
$MgCL_2$	magnesium chloride
mg	milligrams
ml	millilitres
mM	millimolar
$MnCl_2$	mangenese chloride

Mops	morpholinepropanesulphonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
N	nitrogen
NaN_3	sodium azide
Ni^{2+}-NTA	nickel-nitrilotriacetic acid
nm	nanometres
nmol	nanomoles
OD	optical density
PCR	polymerase chain reaction
rpm	revolutions per minute
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	NNN'N'-tetramethylethylenediamine
TLC	Thin-Layer Chromatography
Tris	(hydroxymethyl) aminomethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

Chapter 1

Introduction

1.1 Introduction

In recent years, fungal infections have increased markedly and one of the main agents is the opportunistic pathogen *Candida albicans* (Colombo et al., 2006, Capoor et al., 2005). The first class of antifungal agents used in the medical field were polyenes reported in the 1950s (Holt, 1980). For about 30 years, amphotericin B was the one drug available to control severe systemic fungal infections. Although the first reports of the antifungal properties of N-substituted imidazoles were published in the late 1960s, only in the 1980s was the imidazole ketoconazole and then the triazole fluconazole approved for use in the clinic that allowed further advances in fungal chemotherapy (Beck-Sague and Jarvis, 1993, Holt, 1980). However, the widespread use of the azoles resulted in resistance emerging. Consequently new antifungal drugs are now required (Kelly et al., 1993, Sanglard and Odds, 2002, Howard et al., 2006). Triazoles and their newer derivatives derive their antifungal activity through the inhibition of cytochrome P450-dependent 14α-sterol demethylation, an essential step in fungal sterol biosynthesis. Furthermore, combined therapies including azoles and non-azoles antifungal drugs have been examined as an alternative treatment to overcome resistance (Steinbach *et al.*, 2003). The development of new antifungal drugs with novel modes of action is required to combat the mortality rate associated with fungal infection and overcome resistance. The present work examines the prevalence of resistance mechanisms in *Candida* spp. and investigates the biology of a novel *C. albicans* cytochrome P450 as a new potential antifungal drug target.

1.1.1. Nomenclature of Cytochromes P450

Cytochrome P450 (CYP) is a generic term for a haem-containing superfamily of related, but distinct, oxidative enzymes important in biology. CYP was first described in 1958 (Klingenberg, 1958). The name refers to "pigment at 450 nm", so named for the characteristic Soret peak formed by absorbance of light at wavelengths near 450 nm when the haem iron is reduced (e.g. with sodium dithionite) and complexed to carbon monoxide. CYP nomenclature, based on divergent evolution of the P450 superfamily, was proposed and developed by Nebert and coworkers (Nelson et al., 1996). Specifically, CYPs are classified into the same family (e.g. CYP1, 2, 3 etc) if they have >40% amino acid identity and the same subfamily if they share >55% amino acid identity (e.g. CYP1A, 2A, 3A etc). The rules can be more flexible if CYPs have the same function. (Gotoh, 1993, Roberts et al., 2002). Genes encoding for the CYP enzymes are designated with the abbreviation CYP, followed by an Arabic numeral indicating the gene family, a capital letter indicating the subfamily, and another numerals for the individual gene. The convention is to italicise when referring to the gene, but not the protein. For example, *CYP2E1* is the gene that encodes for the enzyme CYP2E1 – one of the enzymes involved in ethanol metabolism.

1.1.2. CYP function and properties

CYPs represent a great of phylogenetically diverse enzymes performing an array of endogenous and exogenous metabolism (Kelly et al., 2003, Nelson, 2003). CYPs play key roles in steroid hormone and sterol biosynthesis, the activation and

detoxification of many xenobiotics, the metabolism of polyunsaturated fatty acids (such as arachidonic acid and prostaglandins), activation of vitamins A and D_3 to biologically active molecules, the synthesis of a vast array of secondary metabolites in plants, insects and microorganisms as well as the metabolism of contaminating environmental chemicals and carcinogens (Szczebara et al., 2003, Smith et al., 1993). CYP mixed-function monooxygenase systems are probably the most important element of metabolism of xenobiotics in animals. To metabolize such diverse substrates, CYP shows considerable molecular multiplicity. CYP sequence homologues have been determined in all lineages of life, including mammals, birds, fish, insects, worms, sea squirts, sea urchins, plants, fungi, slime moulds, bacteria and archaea (Gonzalez and Nebert, 1990, Nelson, 2003). However, many bacteria lack CYPs. It is believed that the multiple forms of CYPs may have been derived from a common ancestor by gene duplication and mutation during evolution (Kelly et al., 2001).

1.1.3. CYP Localization

Most eukaryotic CYPs are membrane-associated proteins, either in the inner membrane of mitochondria or in the endoplasmic reticulum (ER) of cells. Endoplasmic reticulum located CYPs metabolise thousands of endogenous and exogenous compounds (Kelly et al., 2003) whilst mitochondrial CYPs are mostly involved in steroid biosynthesis. In humans the liver has the highest concentration of, CYPs and their substrates include drugs and toxic compounds as well as metabolic biproducts such as bilirubin (a breakdown product of hemoglobin). In contrast, bacterial cytochromes P450 are usually soluble and are involved in the production of

energy as well as participating in secondary metabolite pathways. Mitochondrial and bacterial CYP systems combine with NADPH-ferredoxin-dependent reductase and ferredoxin to form an electron transport chain. In contrast endoplasmic reticulum CYPs use NADPH dependent cyochrome P450 reductase (CPR) to transport electrons to allow completion of the catalytic cycle (Fig. 1.1). Reducing equivalents in the form of electrons are required for the molecular splitting of atmospheric dioxygen, with one atom being inserted into the substrate molecule and the secod atom being reduced to a water molecule. Due to their membrane-binding properties, eukaryotic CYPs have been difficult to purify and crystallize, but this has been achieved recently (Williams et al., 2000).

1.1.4. CYP structural architecture

The resolved crystal structures of many CYPs have now been reported beginning with P450cam (CYP101) from *Psuedomonas putida* (Poulos and Howard, 1987, Xiao et al., 2004). The CYP molecule is a protein shaped like a triangular prism (Fig. 1.2). The number of CYP structures solved has increased considerably (Poulos et al., 1987, Podust et al., 2001, Lepesheva et al., 2006). The structure of CYPs is conserved however the position of various structural elements differs considerably contributing to their substrate specificity and stereo- and region-selectivity. Although the sequence identity between any two CYPs with resolved crystal structure may be only < 20%, the overall topology of the proteins has general similarity, with some differences in the orientations of various helices and protein domains. The most dramatic variations observed between CYP structures are found in regions responsible for substrate access, binding and catalysis.

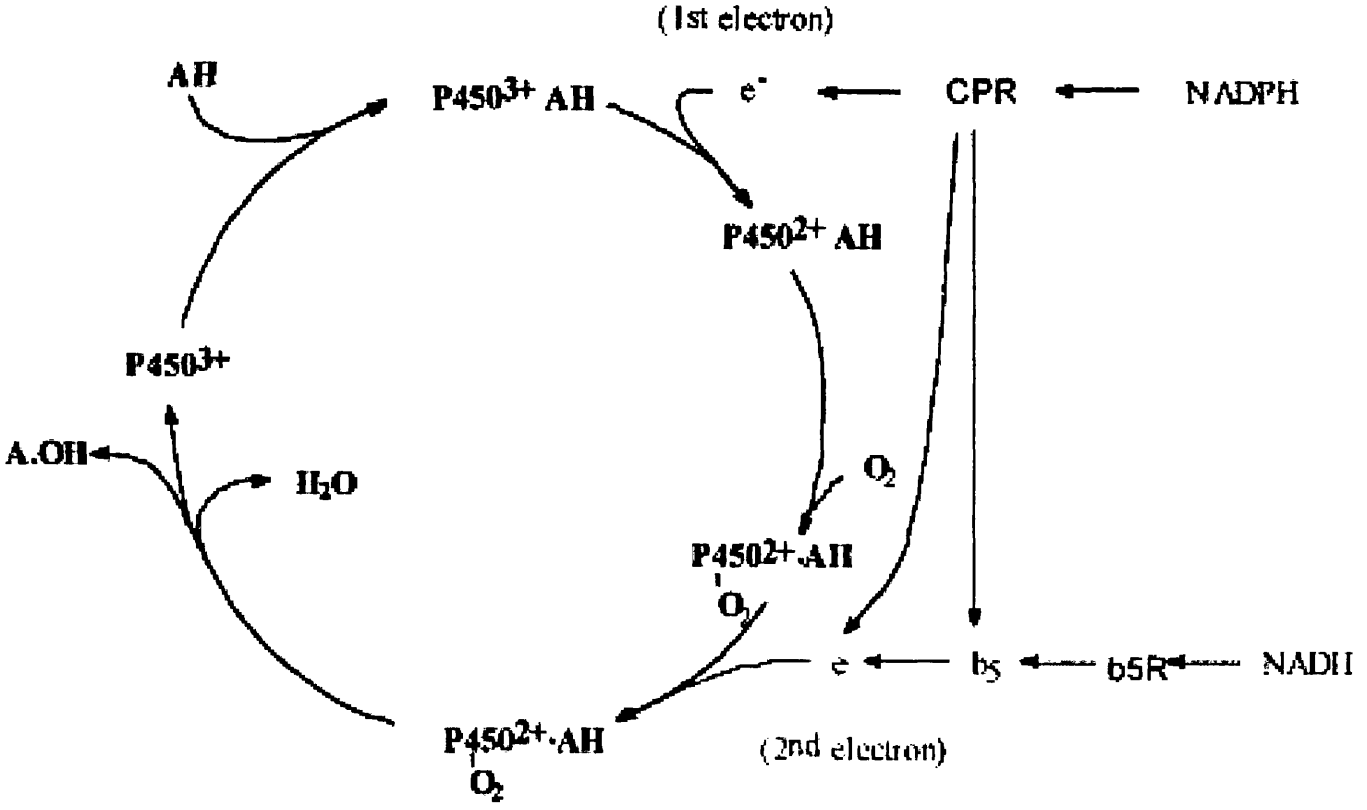


Fig. 1.1. The catalytic cycle of cytochrome P450. NADPH cytochrome P450 reductase (CPR) supplies two electrons to P450 for catalysis via NADPH. Cytochrome *b*5/NADH cytochrome *b*5 reductase complex has been implicated as a component in this electron transfer cycle for some cytochromes P450, being involved in the transfer of the second electron to P450, but not the first. The catalytic cycle is initiated by binding of the substrate (AH) to the oxidised cytochrome (P450^{3+}).

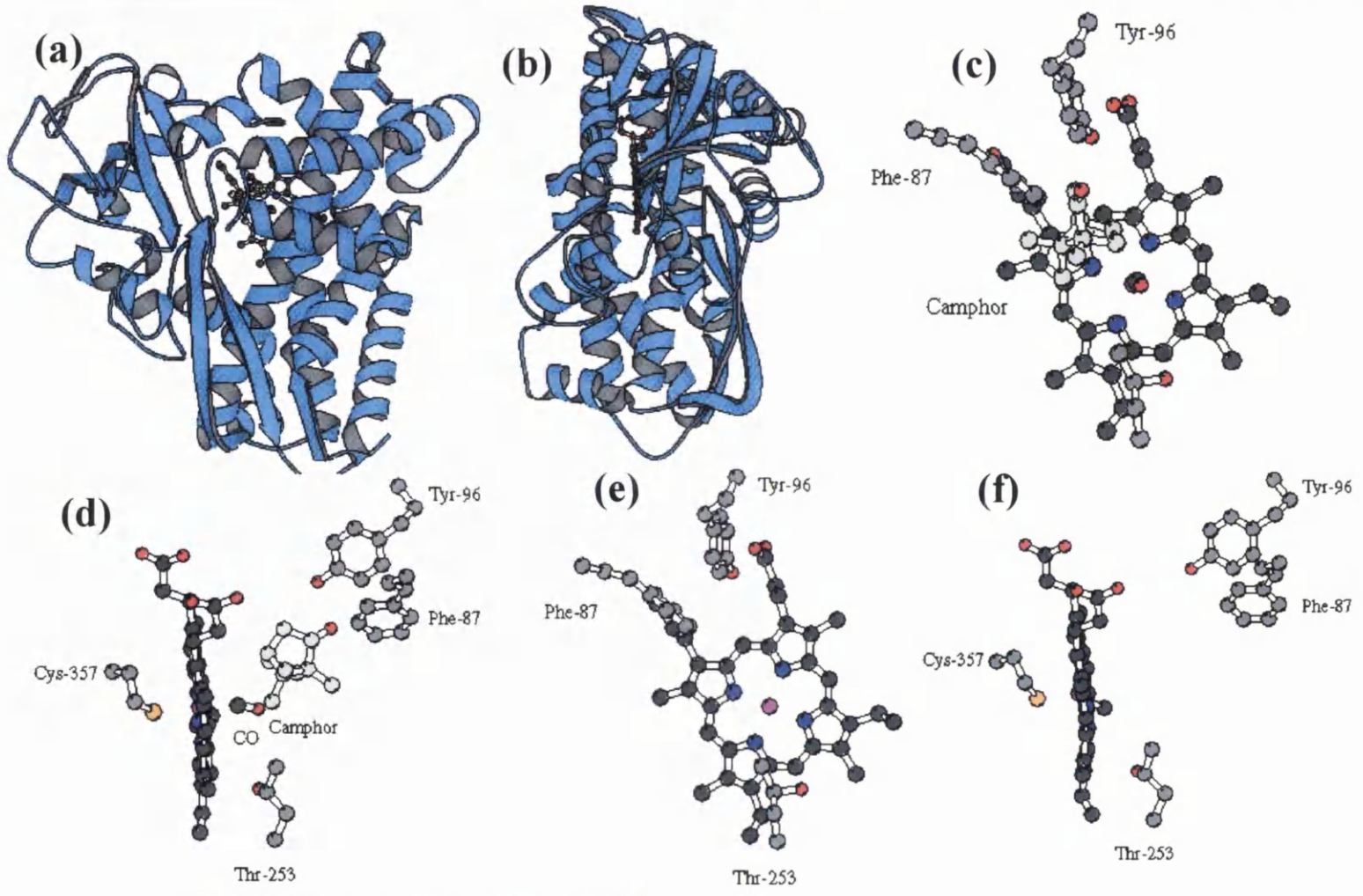


Fig. 1.2. Structure of cytochrome P450.
(a) Ribbon representation of the *Pseudomonas putida* P450cam complex with CO and camphor structure.
(b) As a, rotated 90° around *y* axis.
(c) Active site structure: haem, bound CO and camphor, Phe-87, Tyr-96 and Thr-253.
(d) As c, plus Cys-357 (haem iron proximal ligand); rotated 90° around *y* axis.
(e) Active site structure of substrate-free P450cam.
(f) As e, plus Cys-357; rotated 90° around *y* axis.
http://metallo.scripps.edu/PROMISE/P450.html.

1.1.5. Molecular biology of CYPs

CYPs are mono-oxygenases and consisting of approximately 500 amino acids and containing a haem group at the active site. Many can metabolise multiple substrates as well as catalysing multiple reaction types (eg. Hydroxylation, epoxidation, demethylation, desaturation, dehalogenation) which accounts for their central importance in metabolising the potentially endless variety of endogenous and exogenous molecules. Generally, CYPs with endogenous biosynthetic functions seemingly have restricted substrate ranges and dedicated roles (eg CYPs involved in steroid biosynthesis).

Table 1.1. A list of organisms with *CYP*s in the database (October 2006) (http://drnelson.utmem.edu/CytochromeP450.html). The gene sequences for CYP enzymes deposited include approximately 6422 genes between the 708 families.

	CYPs genes	**Families**
Animals	2279	99
Plants	2311	94
Fungi	1001	282
Bacteria	621	177
Protists	210	51
Archaebacteria	8	5
Total	6422	708

1.1.6. Fungal CYP diversity

Fungal CYP was first identified in *Saccharomyces cerevisiae* (Lindenmayer and Smith, 1964) and this yeast system was extensively characterized by Yoshida and colleagues (eg. Yoshida and Aoyama, 1984). The P450 identified in this characterization was sterol 14α-demethylase and subsequently named CYP51 when the gene was cloned in the Loper laboratory (Kalb et al., 1987). CYP51 is the target for azole antifungal agents. In late 1970s, azole antifungal agents were shown to inhibit sterol 14α-demethylase in *Ustilago maydis* and *C. albicans* (Henry and Sisler, 1978, Van den Bossche et al., 1978). Various azole compounds have been developed as potent antifungal agents (Sanati et al., 1997, Walsh et al., 2000, Wolff et al., 2006). Knowledge of fungal CYP51 has become indispensable in our understanding of the mechanism of action of azole antifungal compounds and for the development of more effective and safe azole derivatives. Since initial isolation of the CYP51 gene in yeast, CYP51 has been found to be widespread in eukaryotes and in some bacteria and hence is the only CYP family found in all the Kingdoms of Life (Fig. 1.3) (Stromstedt et al., 1996, Bak et al., 1997).

Although *S. cerevisiae* contained only three CYPs, it was anticipated that filamentous fungi would contain many more CYPs considering their abitility to produce diverse secondary metabolites and their capability in biotransformation. However, it was still very surprising when it was found that *Aspergillus* spp. contained over 100 *CYP*s, more than the 57 found in humans (Nelson, 2003, Mellado et al., 2002). Besides CYP51, fungal genome sequences also contain another CYP, CYP61, required to form the C-22(23) double bond in the sterol side chain in ergosterol biosynthesis (Kelly et al., 1995, Hata et al., 1981).

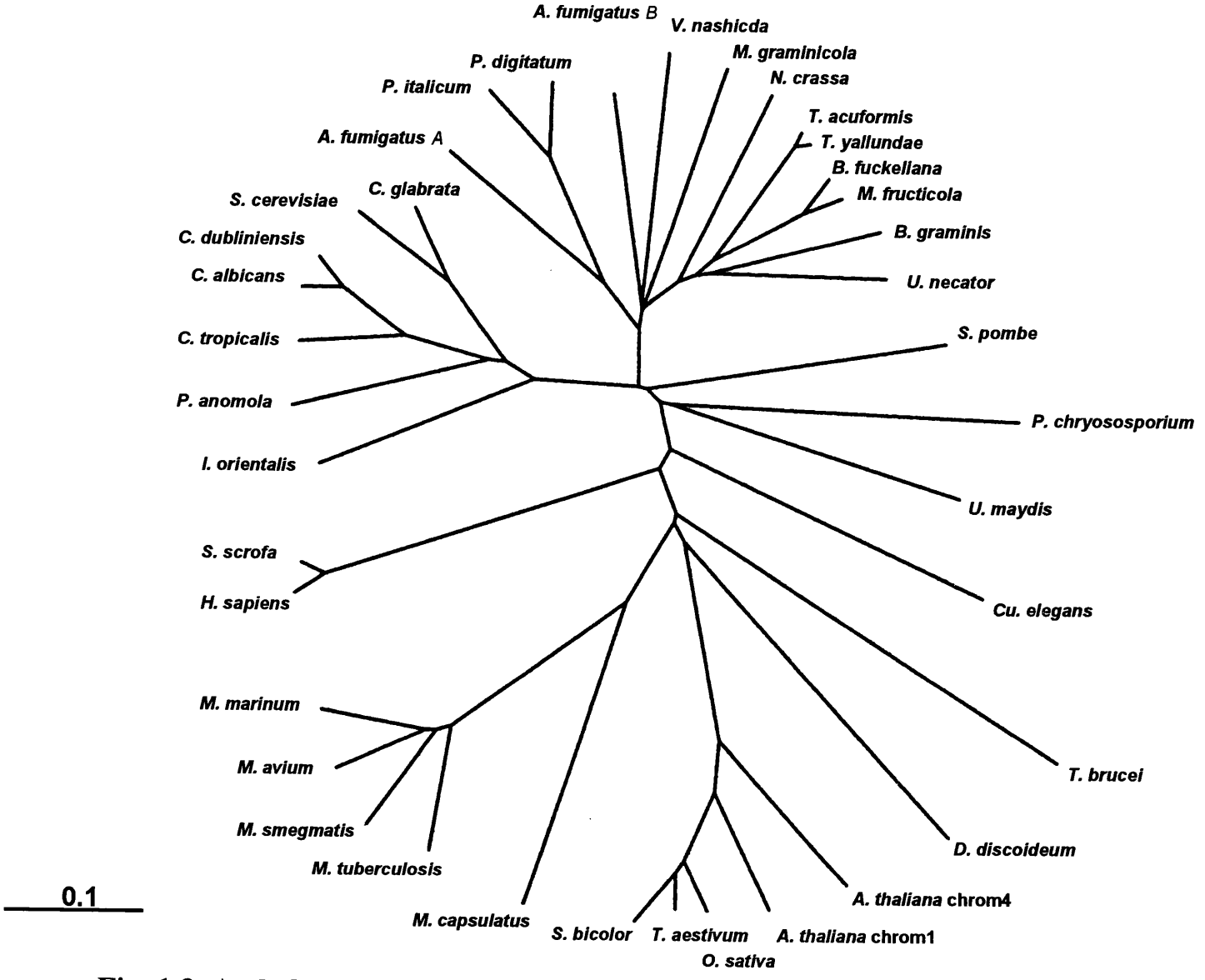


Fig. 1.3. A phylogenetic tree of CYP51s was produced using clustal X 1.8 and Tree View1.6.1.

Other characterised fungal CYPs include CYP52 (responsible for the terminal hydroxylation in the metabolism of alkanes and fatty acids, Sanglard and Loper, 1989), CYP53 (responsible for benzoate hydroxylation), CYP54 (unknown function, van Gorcom et al., 1990), and CYP56 (a CYP catalysing dityrosine biosynthesis in the spore wall of *S. cerevisiae*, Briza et al., 1994, Smail et al., 1995). It was also suprising that a second *CYP51* gene was discovery in the genome of *A. fumigatus* (Gollapudy et al., 2004, Mellado et al., 2001).

Infections caused by *A. fumigatus* have increased substantially in recent years, particularly with high mortality and morbidity in the immunocompromised host (Latge, 1999, Howard et al., 2006). *A. fumigatus* is intrinsically resistant to fluconazole which is used to treat *C. albicans* infections. However, the newer triazoles such as itraconazole, voriconazole and posaconazole are potent inhibitors *in vitro* and *in vivo* of this species (Mellado et al., 2004). Unfortunately, a number of *A. fumigatus* itraconazole resistant isolates have now been reported (Alcazar-Fuoli et al., 2006, Denning et al., 1997). This may be due to the multiplicity of CYP51 in *A. fumigatus* due to their being two distinct and related *CYP51* genes (Mellado et al., 2001). Ergosterol depletion, coupled with the accumulation of methylated sterol precursors, has been indicated to affect both membrane integrity and the function of some membrane-bound proteins, including chitin synthase (Vanden Bossche et al., 1987). This leads to an inhibition of fungal cell growth. In *A. fumigatus* azole resistance has been associated with reduced intracellular accumulation due to either increased expression of drug transporters as well as mutation of the cellular target CYP51, which decreases the affinity of the enzyme for azoles compounds (Alcazar-Fuoli et al., 2006, Mellado et al., 2004).

1.1.6.1. The role of CYP56 in fungal biology

The cell wall is the outermost structure of yeast cells and it confers substantial mechanical strength with a dynamic plasticity, thus guaranteeing cell survival in a fluctuating environment. The yeast cell wall also constitutes a permeability barrier that has dramatic influence on the tolerance of yeast cells to damaging environmental agents. The combination of both characteristics requires a very complex cell wall whose biosynthesis and composition is only relatively recently beginning to be understood (Ram et al., 1998).

The *C. albicans* cell wall is composed mainly of mannan (linked with protein to form mannoprotein), α-glucan, β-glucan, and chitin, which are complex polymers of mannose, glucose, and *N*-acetylglucosamine, respectively. The content and distribution of these components vary with cell age, culture conditions, and morphology (Brown et al., 2000, Bogengruber et al., 1998, Ruiz-Herrera et al., 2006). The linkages between the glucan, chitin, and mannoprotein components are poorly understood but presumably play a critical role in maintaining the integrity of the cell wall and hence the viability of the organism.

Yeast cells have a thick cell wall that maintains cell shape, protects against osmotic stress and requires modification for both oval and dimorphic cell growth. The polysaccharide scaffold that strengthens the cell wall consists of a flexible network of branched 1,3-β-glucan, to which 1,6-β glucan and chitin are attached by their reducing ends, with some chitin attached directly to 1,6-β-glucan (Ruiz-Herrera et al., 2006). The fate of proteins targeted for extracellular localization is varied, as are the changes

associated with modification of the cell wall in the dimorphic yeast *C. albicans* during morphogenesis (Nombela et al., 2006). The evidence accumulated during the past decade, using the two current yeast models *S. cerevisiae* and *C. albicans,* clearly shows that many proteins that lack an N-terminal signal peptide also reach the cell surface. The initial discovery of these proteins outside the plasma membrane was totally unexpected. Recent evidence suggests that these peptides may be involved in several processes, including cell-wall dynamics and interactions with host components. Due to the data not being consistent with the classical export route, which is based on canonical N-terminal signal peptides, it can only be interpreted in terms of other mechanisms.

As a response to nitrogen starvation, in the presence of a poor carbon source, *MAT***a**/*MAT-alpha* diploid cells of the baker's yeast *S. cerevisiae* exit the cell cycle, undergo meiosis, and form haploid spores (Coluccio and Neiman, 2004). These spores are a quiescent, stress-resistant cell type that can survive until nutrients become available. The spore wall is a stratified extracellular matrix that is more complex than the normal vegetative cell wall (Klis et al., 2006, Smits et al., 2001). The vegetative wall consists primarily of an inner layer (closest to the plasma membrane) of β-glucans interspersed with a small amount of chitin and an outer layer of heavily mannosylated proteins (mannans) (Smits et al., 1999, Klis et al., 2006). By contrast, the spore wall consists of four distinct layers. The first two strata consist of an innermost layer composed primarily of mannan and a second layer of β-1-3-linked glucans, which are similar in composition to the vegetative wall but are reversed in position with respect to the spore plasma membrane (Kreger-Van Rij, 1978). The outer portion of the spore wall is comprised of two polymers that are unique to the

spore and confer much of the spore's resistance to environmental damage (Pammer et al., 1992, Briza et al., 1994). Immediately outside of the β-glucan is a layer composed primarily of chitosan, a glucosamine polymer synthesized by the deacetylation of chitin (Mishra et al., 1997, Pammer et al., 1992). Outside of the chitosan is a layer that consists largely of cross-linked dityrosine molecules (Briza et al., 1988, Briza et al., 1990a, Briza et al., 1994). In addition to being more complex than the vegetative cell wall, the spore wall is also unique in that it is constructed without a preexisting matrix to act as a template.

Studies of the *Candida* cell surface (Shepherd, 1987) have been of particular interest for a number of reasons including its importance in antigenicity (Calderone and Braun, 1991, Smail and Jones, 1984), in adherence to epithelial and endothelial cells (Calderone and Braun, 1991), and in interactions with the host immune system (Smail and Jones, 1984, Smail et al., 1988). Furthermore, the mechanisms of cell wall biosynthesis are critical for our understanding of cell morphogenesis and may provide novel targets for the development of new antifungal agents (Hector, 1993). The linkages between the glucan, chitin, and mannoprotein are poorly understood and dityrosine is well known as a crosslinking agent that is involved in the resistance of several fungal species to adverse environmental conditions. Smail et al. 1995 (Smail et al., 1995) described observations which indicated that under certain conditions the cell wall of *C. albicans* contains dityrosine, but this has hitherto not been described experimentally.

S. cerevisiae mutants deficient in spore wall dityrosine were isolated by Pammer *et al.* (Pammer et al., 1992). Most of the mutant spores lacked only the outermost,

dityrosine-rich layer of the spore wall. However, mutant *dit101* was found to be lacking the chitosan layer of the spore wall. Chemical measurements showed that this mutant does not synthesize chitosan during sporulation. The decrease in the amount of chitin in vegetative cells and the absence of chitosan in spores suggested that mutant *dit101* could be defective in a chitin synthase. Indeed, a genomic yeast clone harboring the gene, CSD2, sharing significant sequence similarity with yeast chitin synthases I and II (Bulawa, 1992), complemented their mutant and was shown to correspond to the chromosomal locus of *dit101*. *S. cerevisiae* contains only 2-3% of chitin in its cell walls. However, the synthesis of this polymer seems to be essential for cell survival (Selvaggini et al., 2004).

The proposed pathway of dityrosine biosynthesis in sporulating *S. cerevisiae* cells indicated that two tyrosine molecules are covalently cross-linked to form dityrosine, and the enzyme responsible for this reaction is a member of the cytochrome P450 superfamily (named Dit2p also CYP56, Briza et al., 1994). CPR was essential for dityrosine formation because it provides reducing equivalents to CYP56 which are essential for catalysis. The first description of the chemical synthesis of dityrosine formation was in 1959 by Gross and Sizer (Gross and Sizer, 1959), who achieved this feat by the oxidation of tyrosine with hydrogen peroxide and peroxidase. Dityrosine is an intensely fluorescent compound and thought to arise via the cross-linkage of two tyrosyl radicals (Briza et al., 1986). Cross-links can be formed between existing tyrosine residues in structural and non-structural proteins, and also between soluble tyrosine residues (Amado et al., 1984). Dityrosines can also be obtained under a variety of laboratory-based conditions. Dimerization of the monomer can occur via oxidative coupling with reagents such as VOF_3. Alternatively, dimerization can occur

via reductive coupling using potassium hexacyanoferrate (III). These methods can dimerize simple tyrosines, however they fail to dimerize full-length peptides.

1.1.7. Mechanism of reaction of CYPs

During typical eukaryotic monooxygenation of substrates, CYP requires NADPH, a reductase partner (usually CPR) and molecular oxygen. The CYP catalytic cycle is illustrated in Fig. 1.1 The catalytic reaction is initiated by the binding of the substrate (AH) with the oxidized form (Fe^{3+}) CYP. Subsequently, the P450(Fe^{3+})-AH bound complex is reduced by the first electron and and the haem can then bind 0_2 to form the P450(Fe^{2+})-AH-0_2 ternary complex. Carbon monoxide interferes with binding of 0_2 to P450-AH and inhibits the catalytic cycle at this first step. The second electron is then transferred into the ternary complex. The oxygen is activated (P450(Fe^{2+})-AH-0_2^-), and one oxygen atom attacks the substrate to form an oxygenated product AOH whilst the other is reduced to H_20 with P450(Fe^{3+}) being restored. The two electrons consumed during the catalytic reaction are usually supplied from NADPH via CPR although the cytochrome b5/NADH cytochrome b5 reductase system can replace CPR in *S. cerevisiae* (Venkateswarlu et al., 1998). In the case of CYP51 the catalytic reaction was demonstrated previously (Shyadehi et al., 1996) and is illustrated in the Fig. 1.4.

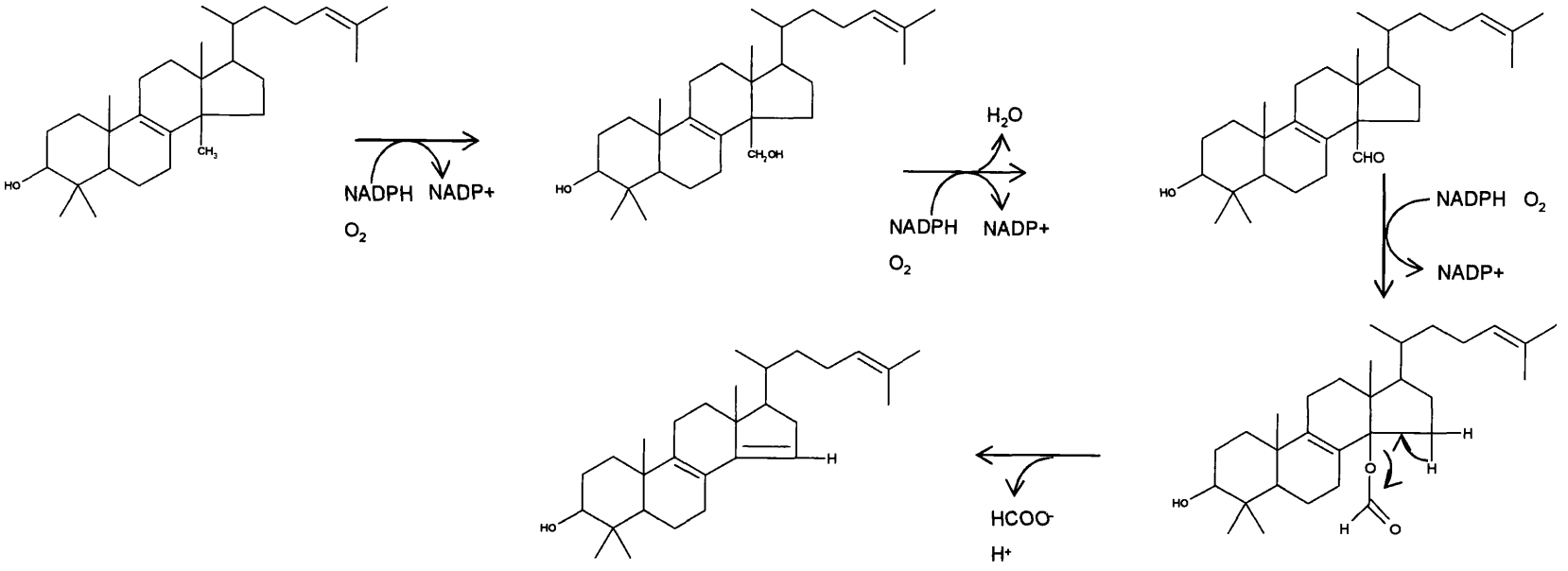


Fig. 1.4. The sequence of reactions catalysed by CYP51.

1.1.8. Spectral change of CYPs on substrate binding

Binding of a substrate to oxidized CYP generally causes spectral change as measured spectrophotometrically between the wavelengths 500nm – 400nm. A substrate spectral change can be classified into two categories, Type I and II, which are based on the absorbance minimum and maximum of the resulting spectra. The substrate spectral change is originated by the displacement of a sixth ligand water molecule of the oxidized CYP and the binding of the substrate to the active site. The type I spectral change represents the low to high spin state transition with a spectral minimum absorption at approximately 420 nm and spectral maximum absorption at approximately 390 nm (Sibbesen et al., 1995). In contrast, a type II spectrum exhibits a spectral maximum absorption at approximately 430 nm and a spectral minimum absorption at approximately 410 nm corresponding to a ligand-bound low spin complex and results from an atom containing a lone electron pair in the binding molecule interacting directly with the Fe atom in the haem. Substrate spectral changes provide information on the mode of interaction of a substrate/ inhibitor with CYP. Usually, potential CYP substrate molecules induce Type I spectra whilst CYP inhibitors induce Type II spectra. An example of an azole binding spectrum to P450 is illustrated in the Fig. 1.5.

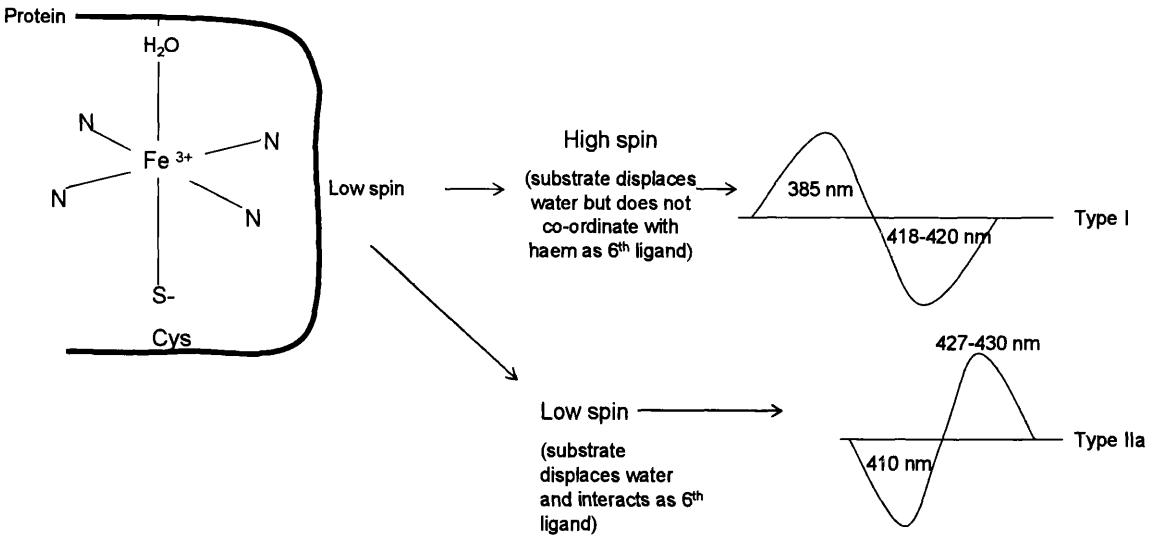


Fig. 1.5. Difference spectra originated from substrate binding to cytochromes P450. Type I binding spectra resulted when substrate does not act as 6th ligand, but displaces co-ordinating water molecule, resulting in change from low to high spin state. Type II binding spectra obtained when substrate binds as 6th ligand and Iron is in a low spin state. Type IIa is seen when the spin state was originally low, type IIb is seen when the spin state was originally high.

1.2. The biology of *Candida* spp.

1.2.1. General aspects

In 1839, Lagenbeck was the first to demonstrate the presence of a yeast-like fungus growing as a parasite on oesophageal mucosa in a patient who had died from typhoid fever. He also discovered that the fungus was able to cause thrush,. The genus *Candida*, species *albicans*, was first described by Christine Marie Berkhout. She described the fungus in her doctoral thesis at the University of Utrecht in 1923. Over the subsequent years the classification of the genus and species has evolved. Obsolete names for this genus include *Mycotorula* and *Torulopsis*. The species has also been known in the past as *Monilia albicans* and *Oidium albicans*. The current classification is *nomen conservandum*, which means the name is authorized for use by the International Botanical Congress (IBC). The genus *Candida* includes approximately 150 different species. Many different *Candida* spp have been implicated in causing human infections. *C. albicans* is the most significant of these species (Odds, 1984, Ruchel, 1989). Other *Candida* species responsible for human disease include *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida lusitaniae*.

In the 1970s and 1980s, *C. albicans* isolates were shown to exist in a diploid state and there was no evidence suggesting a sexual reproductive cycle (Olaiya and Sogin, 1979, Whelan et al., 1980, Riggsby et al., 1982). This was surprising given the close relationship of *C. albicans* to other sexually reproducing yeast species such as *S. cerevisiae* and *Kluyveromyces lactis*. Furthermore, by the mid-1990s several *S.*

cerevisiae genes known to function in mating and meiosis were found to have close orthologues in *C. albicans* (Bennett and Johnson, 2005). Some of the *C. albicans* orthologues can even substitute for their disrupted *S. cerevisiae* counterparts when expressed in *S. cerevisiae* (Magee and Magee, 2000, Weber et al., 2002). In recent years, this list has grown much longer owing to the sequencing of the *C. albicans* genome. However, since the discovery of *C. albicans* mating in 2000 (Magee and Magee, 2000, Hull et al., 2000), experiments carried out in several laboratories have revealed that *C. albicans* contains an elaborate mating program that has many similarities but also many major differences when compared with those of other fungi.

1.2.2. *Candida* taxonomic classification

The origin of the fungi is very ancient. They first appeared in the fossil records, coincidentally with the appearance of the land plants, in the Devonian period around 400 million years ago. The early microscopists found that fungi were simple to study and they first observed division of cells by observing yeasts grow under the microscope. Early biological science was focused with categorizing microrganisms and researchers constructed taxonomic systems based solely on structure. However, for the fungi this created a polyphyletic group that contained microorganisms which had very different ancestors (Schauer and Hanschke, 1999). Fungi are referred to as the True Fungi, or Eumycota, and are currently divided into four major phyla on the basis of their morphology and sexual reproduction. New information based on the presence and type of mitochondria, and DNA sequencing of ribosomal RNA, place the members of the fungi into a complex phylogenetic tree. For example, in taxonomic terms *C. albicans* is: Kingdom: Fungi, Phylum: Ascomycota, Subphylum:

Ascomycotina, Class: Ascomycetes, Order: Saccharomycetales, Family: Saccharomycetaceae and Genus: Candida. Synonymy: *Saccharomyces albicans* (C.P. Robin) Reess, (1877), *Monilia albicans* (C.P. Robin) Zopf, (1890), *Parasaccharomyces albicans* (C.P. Robin) Mello & L.G. Fern. (1918), *Procandida albicans* (C.P. Robin) E.K. Novák & Zsolt, Acta Botanica Hungarica 7: 133 (1961), today *Candida albicans* (C.P. Robin) Berkhout 1923 (http://www.indexfungorum.org/).

1.2.3. *Candida* spp. and clinical infections

The genus *Candida* includes around 154 species (Odds, 1984, Sullivan et al., 1995). While *Candida albicans* is the most abundant and significant infectious species, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitaniae* are also isolated and deemed causative agents of *Candida* infections (Nguyen et al., 1996b). HIV-infected patients often suffer severe forms of oropharyngeal candidosis, mainly caused by *C. albicans*, although over the last decade the reported incidence of infections caused by other *Candida* species has increased significantly (Nguyen MH, 1996). *C. dubliniensis* is a recently identified yeast and closely related to *Candida albicans* phylogenetically, (Schorling et al., 2000, Sullivan et al., 1995, Sano et al., 2000) and has been mostly isolated in HIV-infected individuals with oral candidosis (Sullivan et al., 1995, Sano et al., 2000). *C. dubliniensis* has also been recovered from the oral cavities of asymptomatic and symptomatic immunocompetent individuals, although to a much lesser extent. A major obstacle in the treatment of *Candida* infections is the spread of antifungal drug resistance mainly in patients chronically subjected to antimycotic therapy. Patients

receiving fluconazole prophylaxis are particularly at risk of developing infections. Furthermore, the diversity of *Candida* spp. that are encountered in infections is expanding and the emergence of other species that were rarely in play as pathogens in the past is now observed (Nguyen et al., 1996a, Melo et al., 2004, Agarwal et al., 2004).

1.2.4. *Candida* Macroscopic and Microscopic Features

The colonies of *Candida* spp. are cream /yellowish coloured and grow rapidly in 2 days. The texture of the colony may be pasty, smooth, glistening or dry, wrinkled and dull, depending on the species. The microscopic features of *Candida* spp. also show species-related variations. All species produce blastoconidia singly or in small clusters (Sudbery et al., 2004). Most species produce pseudohyphae which may be long, branched or curved. True hyphae and chlamydospores are produced by strains of some *Candida* spp., including *C. albicans*. Most *Candida* species are dimorphic, showing biochemical and structural as well as morphological differences between the different forms.

Environmental regulation of morphology is complex and incompletely understood (Calderone and Fonzi, 2001). The yeast form is a spherical or ovoid budding unicellular form and can be found living commensally on the body surface or in the gastrointestinal tract and/or vagina. *Candida* spp. can switch to (True) hyphae (mycelia filaments), which can be found invading tissue during a disease process and in the laboratory under certain conditions of growth, e.g., 37°C, neutral pH, presence of serum (approximate conditions during tissue invasion). Considering the association

of temperature with dimorphism, *C. albicans* behaves in an opposite manner to the systemic dimorphic fungal pathogens (that are hyphal at room temperature and yeast-like at 37°C). In addition *C. albicans* can also grow as pseudohyphae, with chains of elongated yeasts, generally budding in a polar fashion with occasional branches. Germ tubes (extensions) arise from yeast cells (Gow, 1997), and they are different from a bud arising from a mother cell, and can extend to form hyphae or pseudohyphae. In any particular clinical specimen, a variety of forms may be observed. Yeast cells may be associated with normal commensal existence but also with some disease processes. The other forms (hyphae, pseudohyphae, and germ tubes) may be grouped together and are associated with deep tissue invasion (Gow et al., 2002, Calderone and Fonzi, 2001).

1.2.5. General aspects of Candidosis

Infections caused by *Candida* spp. are in general referred to as candidosis. *Candida* exist as a member of the normal flora of skin, mouth, vagina, and stool. As well as being pathogens, other *Candida* species are found in the environment, particularly on leaves, flowers, water, and soil.

Systemic fungal infections (fungaemias) have emerged as important causes of morbidity and mortality in immunocompromised patients (*e.g.*, AIDS, cancer chemotherapy, organ or bone marrow transplantation) (Campisi et al., 2002, Kovacicova and Krcmery, 2001, Krcmery et al., 1999, Krcmery and Barnes, 2002, Matsumoto et al., 2002). In addition, hospital-related fungal infections in patients not

previously considered at risk (*e.g.* patients in intensive care units) has become a major cause of health concern (Collin et al., 1999).

The clinical spectrum of candidosis is extremely diverse (Weinberger et al., 2005). Almost any organ or system in the body can be affected. Candidosis may be superficial and local or deep-seated and disseminated. Disseminated infections arise from the haematogenous spread from the primarily infected locus (Calderone and Fonzi, 2001, Cassone et al., 1981). The ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from the yeast to hyphal phase are the major determinants of its pathogenicity. Several host factors predispose a patient to candidosis such as physiological (pregnancy, age), trauma (maceration, burn, infection), haematological (neutropenia, AIDS, immunodeficiency), endocrinological (diabetes mellitus, hypoparathyroidism), iatrogenic (chemotherapeutics, corticoteroids, oral contraceptive, catheters) (Sobel and Vazquez, 2003, Matsumoto et al., 2002) and others (intravenous drug addiction, malnutrition) (Attia and Zaoutis, 1999). Candidosis is mostly an endogenous infection, arising from overgrowth of the fungus inhabiting in the normal flora. However, it may occasionally be acquired from exogenous sources (such as catheters or prosthetic devices) or by person-to-person transmission (such as oral candidosis in neonates of mothers with vaginal candidosis or endophthalmitis following corneal transplantation from an infected donor) (Frezzini et al., 2005).

1.2.6. The completed sequence of the *C. albicans* genome

The *C. albicans* genome was sequenced using the strain SC5314 and results deposited in the databases in 2004 (Nantel, 2006, Jones et al., 2004). One of the most

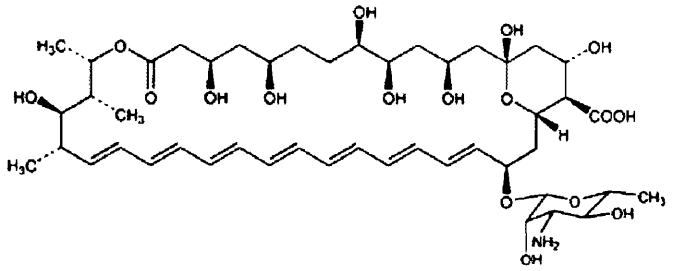
interesting features of the *C. albicans* genome was the occurrence of structural chromosomal rearrangements as means of generating genetic diversity, chromosome length polymorphisms (contraction/expansion of repeats), reciprocal translocations, chromosome deletions and trisomy of individual chromosomes (Jones et al., 2004). These karyotypic alterations lead to changes in phenotype, which is a survival adaptation strategy for this fungus. *C. albicans* genome sequence confirmed eight chromosomes (historically named 1-7 and R) and these constitute a haploid genome size of 14,851 kilobases (kb), containing 6,419 open reading frames (ORFs) longer than 100 triplets, of which some 20% have no known counterpart in other available genome sequences. *C. albicans* is a diploid organism with no known haploid phase, and for a long time it was considered to be asexual. Early assemblies of the *C. albicans* genome sequence revealed a mating-type (MAT-like) locus that led to the engineering of mating competent strains (Hull and Johnson, 1999). The assembly of a complete *C. albicans* diploid genome sequence for SC5314 has allowed a reliable estimate of the frequency of heterozygosities which occurred 4.21 polymorphisms per kb, or 1 polymorphism per 237 bases (Jones et al., 2004). These heterozygosities are distributed across the *C. albicans* genome, with the highest prevalence being found on chromosomes 5 and 6. Genome sequencing has altered our understanding of this organism and allowed numerous experimental approaches to be undertaken including some described in this thesis.

1.4. *Candida* spp. antifungal resistance

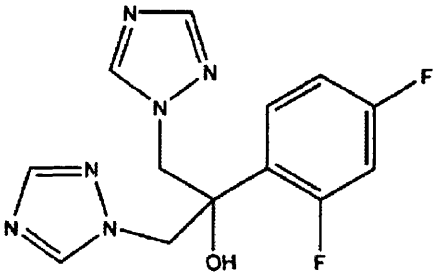
The term "microbial resistance" describes the relative insensitivity of a microbial organism to an antimicrobial drug as tested *in vitro* and when compared with other

isolates of the same species. In contrast, clinical failure describes the failure of an appropriate therapy for a certain indication to result in a clinical response. The reason for clinical failure may be, for example, antifungal resistance, an impaired immune function, poor bioavailability of the drug given, or an accelerated metabolism of the drug (Rex et al., 1997, Rex et al., 2001, Oakley et al., 1998, Verweij et al., 1998, Dannaoui et al., 2000).

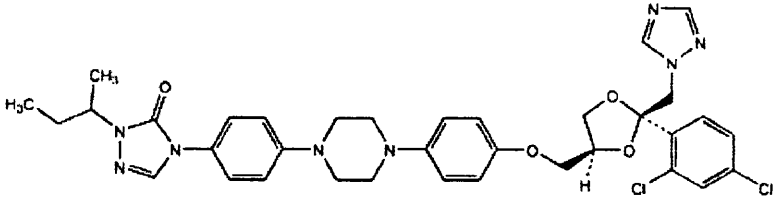
Different types of antifungal resistance have also been defined. Primary (intrinsic) resistance occurs in organisms never previously exposed to the drug of interest. In contrast, secondary (acquired) resistance arises only after exposure of the organism to the drug.,In addition, the term "clinical resistance" can be used to describe failure of therapy or relapse of infection. *In vitro* and *in vivo* correlations for microbial susceptibility in relation to therapeutic response abound in mycology, which emphasise the predictive value of standardized testing (Rex et al., 2001, Pfaller et al., 2005). The National Committee for Clinical Laboratory Standards has published a standardized method (M27-A) for antifungal susceptibility testing of yeast (National Commitee for Clinical Laboratory Standard, 2002) and has proposed a standardized method for testing filamentous fungi (M38-P). The antifungal therapeutic choice is based on criteria such as the immune status of the patient, site of infection, characteristics of the infection (the fungal species and its susceptibility to different antifungal drugs), and the pharmacokinetic characteristics of the drug (e.g., absorption, elimination, and toxicity) (Calderone and Fonzi, 2001, Gonzalez et al., 2005, Maertens, 2004). Only a limited number of antimycotic drugs are available for the treatment of systemic fungal infections (Fig. 1.6).



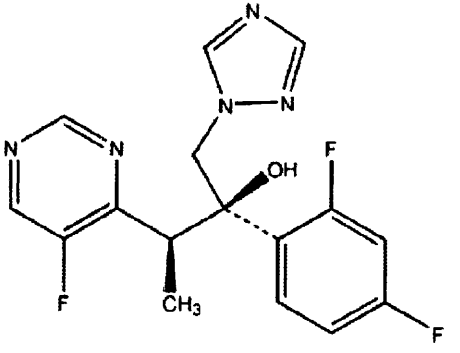
amphotericin B



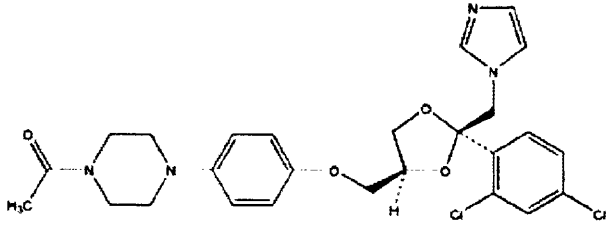
fluconazole



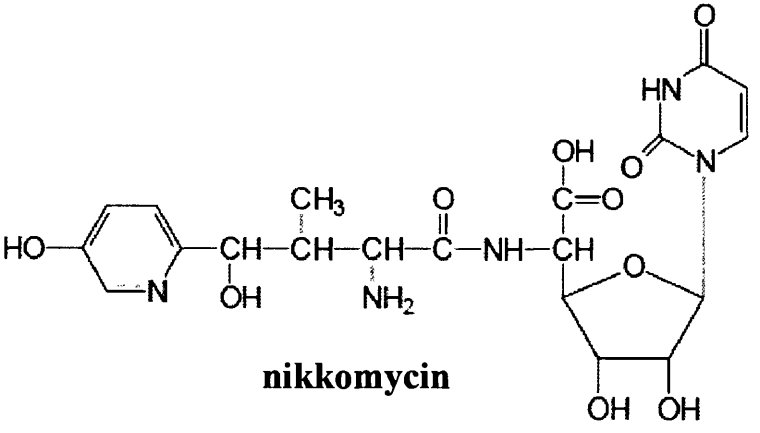
itraconazole



voriconazole



ketoconazole



nikkomycin

Fig. 1.6. Chemical structures of various antifungal agents.

The mode of action of the most important antifungal drugs can be divided into five classes (Vanden Bossche et al., 1987, Ryder, 1991). These are as follows: (1) polyene macrolides that lead to an alteration of membrane functions (e.g. amphotericin B); (2) sterol biosynthesis inhibitors; (a) inhibitors of CYP51 (e.g. azole derivatives - fluconazole, itraconazole, and voriconazole),) and (b) inhibitors of squalene epoxidase (e.g. allylamines - naftifine, terbinafine); (3) DNA and RNA synthesis inhibitors (e.g. flucytosine); (4) 1,3-β-glucan synthase inhibitors (e.g. echinocandins) and (5) chitin synthase inhibitors (e.g. nikkomycin). Azole antifungals, such as fluconazole and itraconazole are most commonly used to treat *Candida* spp. infections. Due to the repeated use of these agents, especially in HIV-positive patients with recurrent oropharyngeal candidosis, treatment failures linked to the emergence of azole-resistant *C. albicans* strains are often observed. Additionally, the emergence of candidosis caused by non-*albicans* species and aspergillosis, which exhibits low antifungal susceptibility, has also been related to the spread of antifungal resistance (Howard et al., 2006, Weinberger et al., 2005).

1.4.2. Antifungal agents

1.4.3. Amphotericin B and other polyenes

Amphotericin B is a polyene antifungal agent, first isolated by Gold *et al.* from the bacterium *Streptomyces nodosus* in 1955 (Donovick et al., 1955). Amphotericin B is active against a wide variety of fungi, including yeasts and moulds such as *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*, *Zygomycetes*, dimorphic fungi, and some dematiaceous fungi. The interaction between amphotericin B and human cell

membranes containing cholesterol results in toxic side effects of the drug. The most important side effect is an impairment of renal function due to decreased filtration in the glomeruli (Holt, 1980, Ernst et al., 2002). This results in limited application of amphotericin B as an antifungal and it is commonly used as a drug of last resort.

The NCCLS method is not yet sufficient for the discrimination of amphotericin B-resistant isolates from the susceptible ones, primarily due to the narrow range of MICs that it generates for all test strains (Johnson et al., 2000). Amphotericin B binds to sterols, preferentially to the the fungal cell membrane sterol (ergosterol), resulting in the formation of pores in the membrane (Prasad, 1997). Consequently, osmotic integrity of the fungal membrane is disrupted resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites and ultimately leading to cellular death. In higher concentrations, polyenes also inhibit chitin synthase, a cell wall synthetic enzyme localized in the membrane. The classic amphotericin B deoxycholate formulation has been available since 1960. While efforts to develop a novel relevant methodology for amphotericin B susceptibility testing are in progress, amphotericin B has proven to be fungicidal against *Candida* spp. strains.

Reports of amphotericin B resistance are limited. Primary resistance to amphotericin B exists in some isolates of *Candida lusitaniae* (Yoon et al., 1999), *C. lipolytica*, and *C. guillermondii* (Redding et al., 2000, Krcmery and Barnes, 2002). Secondary resistance has occasionally been described in *Candida* spp. as well as in *C. neoformans* (Kelly et al., 1994). Whether repeated treatment with amphotericin B, or other factors such as treatment with immunosuppressive chemotherapy, is responsible for polyene resistance remains to be clarified. Reasons for recurrence of infection may

be poor penetration of the drug, inconsistent compliance with other drug regimens, or deterioration of the immune system of the host. It has been hypothesized that concurrent or previous therapy with azoles might lead to amphotericin B resistance (Johnson et al., 2000).

1.4.3.1. Mechanism of resistance of polyenes

The cause of resistance to polyenes is usually a significant alteration of the sterol composition in the fungal plasma membrane (e.g., a reduction in the ergosterol content due to a lack of the Δ(8,7) isomerase) (Hitchcock et al., 1987). This circumstance leads to a lower affinity of amphotericin B for the predominant sterols other than ergosterol in the membrane. (Joseph-Horne et al., 1996, Ghannoum and Rice, 1999). Polyene resistance through altered sterol composition in the membrane can result from mutations in the enzymes involved in fungal ergosterol biosynthesis. Kelly et al. observed cross-resistance to amphotericin B in two fluconazole-resistant *C. albicans* strains isolated from patients with AIDS(Kelly et al., 1996). These strains accumulate 14α-methylfecosterol instead of the toxic 14α-methylergosta-8,24-dien-3β,6α-diol seen in wild-type and hence can grow. This specific change results from mutations in sterol C5-desaturase so that in the absence of azole ergosterol does not accumulate (ergosta-7 and ergosta-7,22-dienol does) and thus the strains became resistant to amphotericin B. The same group reported two amphotericin B-resistant mutants of *Ustilago maydis* with cross-resistance to nystatin. An alteration of the ergosterol biosynthesis was precluded as the cause of polyene resistance in these isolates (Kelly et al., 1997b, Sanglard and Odds, 2002). Treatment with amphotericin B is often guided by toxicity rather than by therapeutic efficacy, whereas lipsosomal

formulations have reduced toxicity, thus allowing higher doses to be administered (Gokhale et al., 1993, Walsh et al., 2004).

Besides altered ergosterol content other mechanisms of resistance to amphotericin B appear to operate. For example, in a highly resistant strain of *A. terreus* it was shown that the major sterol in its plasma membrane was ergosterol (Dannaoui et al., 2000). Another cause for amphotericin B resistance may be an altered content of β-1,3 glucans in the fungal cell wall. These components, which increase the stability of the cell wall, influence the access of large molecules such as amphotericin B to the plasma membrane sterol. Seo et al. showed that alteration of glucans also led to amphotericin B resistance in *A. flavus* (Seo et al., 1999).

1.4.4. Azoles antifungal compounds

In the 1970s, a new class of antimycotic drugs was developed, the azoles. The first azole that was available for systemic use was clotrimazole. However, its use was limited largely because of inconsistent concentrations in the blood. Miconazole was the first effective azole for systemic infections, but its use was also limited because it can only be given intravenously and it offered few advantages over amphotericin, aside from lessened toxicity (Prasad and Kapoor, 2005). Recently, ketoconazole is the only member of the imidazole class of azole antfungal that is currently used for the treatment of systemic fungal infections. Ketoconazole was the first azole to be available as an oral formulation and showed consistent blood levels (Utz, 1980). Additionally, ketoconazole showed reported activity against *Coccidioides*, *Histoplasma*, *Blastomyces*, and dermatophytes. However, in addition to

ketoconazole's rare hepatotoxicity, resistance was described, especially in patients with mucocutaneous, oropharyngeal and oesophageal candidosis (Powderly et al., 1999, Korashy et al., 2007). It now used clinically as an alternative drug for specific indications. Itraconazole, a triazole antifungasl, is available for oral and intravenous administration. Itraconazole shows broad *in vitro* activity against *Candida* spp., but also against *Aspergillus* spp. and dimorphic fungi.

Fluconazole, another triazole that can be administered orally and intravenously, is used for the treatment of oropharyngeal and oesophageal candidosis in patients with AIDS as well as in neutropenic patients (Ullmann and Cornely, 2006). Fluconazole has only limited activity against moulds and other filamentous fungi. Use of fluconazole in prophylaxis has resulted in a shift of host flora to resistant species. Voriconazole, structurally related to fluconazole, shows an activity spectrum similar to that of itraconazole. It is active against *C. krusei, C. guillermondii,* and *C. lusitaniae* (Zaragoza and Peman, 2006, Maschmeyer and Ruhnke, 2004, Tortorano et al., 2003). Walsh *et al.* published a randomized, international, multicenter trial comparing voriconazole with liposomal amphotericin for empirical antifungal treatment (Walsh et al., 2002). They concluded that voriconazole is a more suitable alternative to amphotericin preparations in antifungal treatment, particularly in patients with neutropenia and persistent fever. Voriconazole was approved in the United States for limited use in May 2002.

Posaconazole and ravuconazole, two additional triazoles, are currently under investigation. Posaconazole has *in vitro* and *in vivo* activity against aspergillosis (Walsh et al., 2007), cryptococcosis, and coccidioidosis as well as candidosis

(Gonzalez et al., 2005, Nguyen et al., 1995). Ravuconazole shows *in vitro* activity against dimorphic fungi, such as *Coccidioides, Histoplasma,* and *Blastomyces,* as well as against *Aspergillus, Fusarium*, and fluconazole-sensitive and -resistant *Candida* species (e.g., *C. krusei*), and also against important dermatophytes (Gonzalez et al., 2005).

Azoles act as ergosterol synthesis inhibitors by binding to and inhibiting the activity of CYP51, an enzyme essential in the ergosterol biosynthesis pathway. Ergosterol is the most prevalent sterol in the fungal plasma membrane (Loffler et al., 2000). The active site of CYP51 contains a Fe-containing haem domain and azoles bind via a specific nitrogen atom in the azole ring nucleus to the Fe atom (Lamb et al., 1999). Azoles may also target lipids of the fungal plasma membranes and may interact in some fungi with another ergosterol biosynthetic enzyme, 3-ketosteroid reductase, an enzyme essential in C4- demethylation (Vanden Bossche et al., 1993).

1.4.4.1. Mechanisms of resistance of azoles

1.4.4.2. Alteration of the target enzyme

Alterations in the primary sequence of CYP51, defined by point mutations in the *CYP51* gene, have been described to be associated with azole resistance. Amongst many point mutations only two have been biochemically characterised. White described the presence of the amino acid substitution of lysine for arginine at position 467 (R467K) in *C. albicans* CYP51 (Lamb et al., 2000, White, 1997). This substitution is located near the haem cysteine ligand and thus probably causes structural alterations so that the azole binds with less affinity. Another substitution at

amino acid 464 (G464S) in azole-resistant *C. albicans* isolates has also been described (Sanglard et al., 1998b). Kelly et al. (Kelly et al., 1999) confirmed that this mutation results in changes in the haem-binding domain, causes fluconazole resistance through reduced affinity probably via perturbation of the haem environment. Fluconazole enters the active binding site through an access channel and mutations therein may affect the binding of inhibitors.

1.4.4.3. Alteration in *ERG3*

Altered sterol Δ(5,6) desaturase (ERG3) is another explanation for azole resistance. In azole-sensitive strains treated with azoles, the sterol 14-methyl-3,6-diol accumulates leading to a fungistatic effect, whereas in *erg3* mutants (due to mutations in the gene rendering the enzyme inactive), the substrate of sterol Δ(5,6) desaturase accumulates, which can support growth of the fungal cell. In *S. cerevisiae*, 120 fluconazole-resistant isolates were found to have altered ERG3 activity. It has been shown that *ERG3* mutations alone can cause azole resistance in *S. cerevisiae* (Watson et al., 1989, Arthington et al., 1991, Kelly et al., 1995). In *C. albicans*, Kelly et al. demonstrated that resistance was caused by defective sterol Δ(5,6) desaturase in two clinical isolates from patients with AIDS, leading to an accumulation of 14-methylfecosterol under azole treatment and ergosta-7,22-dienol without treatment (Kelly et al., 1997a). A consequence of this mechanism of resistance is that an absence of ergosterol causes cross-resistance to amphotericin as discussed previously.

amino acid 464 (G464S) in azole-resistant *C. albicans* isolates has also been described (Sanglard et al., 1998b). Kelly et al. (Kelly et al., 1999) confirmed that this mutation results in changes in the haem-binding domain, causes fluconazole resistance through throughreduced affinity probably via perturbation of the haem environment. Fluconazole enters the active binding site through an access channel and mutations therein may affect the binding of inhibitors.

1.4.4.3. Alteration in the *ERG3* genes

Altered sterol Δ(5,6) desaturas (ERG3)e is another explanation for azole resistance. In azole-sensitive strains treated with azoles, the sterol 14-methyl-3,6-diol accumulates leading to a fungistatic effect, whereas in ERG3 mutants (due to mutations in the gene *ERG3* rendering the enzyme inactive), the substrate of sterol Δ(5,6) desaturase, 14-methylfecosterol, accumulates, which can support growth of the fungal cell. In *S. cerevisiae*, 120 fluconazole-resistant isolates were found to have altered ERG3 activity. It has been shown that *ERG3* mutations alone can cause azole resistance in *S. cerevisiae* (Watson et al., 1989, Arthington et al., 1991, Kelly et al., 1995). In *C. albicans*, Kelly et al. demonstrated that resistance was caused by defective sterol Δ(5,6) desaturase in two clinical isolates from patients with AIDS, leading to an accumulation of 14-methylfecosterol under azole treatment and ergosta-7,22-dienol without treatment (Kelly et al., 1997a). A consequence of this mechanism of resistance is that an absence of ergosterol causes cross-resistance to amphotericin as discussed previously.

1.4.4.4. Alteration of drug efflux

It has been demonstrated on many occasions that *C. albicans* can respond to antifungal challenge by developing specific resistance mechanisms. Several molecular studies have identified drug transporters being responsible for resistance in clinical isolates (Sanglard et al., 1995, Sanglard et al., 1998a, Magill et al., 2006, Ferreira et al., 2005, Prasad and Kapoor, 2005).

Recent genome sequencing data and numerous biochemical and molecular genetic investigations have revealed the occurrence of families of primary and secondary transporter proteins. Two such families, ATP-binding cassette (ABC) superfamily (White et al., 2002) and the major facilitator superfamily (MFS), have been found to occur ubiquitously in all fungi. While the ABC family are primary active transporters, capable of transporting both small molecules and macromolecules in response to ATP hydrolysis (Prasad and Kapoor, 2005), the MFS transporters are secondary carriers capable only of transporting small solutes in response to chemiosmotic gradients. Although well over 100 families of transporters have now been identified and classified, the ABC superfamily and MFS account for nearly 50% of the solute transporters encoded within the genomes of microorganisms (Gbelska et al., 2006).

In *C. albicans* one of the well-documented mechanisms of resistance to azole antifungal agents is the upregulation of multidrug transporter genes (Prasad et al., 1995, Prasad and Kapoor, 2005, Niimi et al., 2006, White et al., 2002). The upregulation of multidrug transporter genes leads to the enhanced efflux of azoles and therefore results in decreased intracellular concentrations of the drug and hence

reduced inhibition of their target encoded by the *CYP51*. Cdr1p and Cdr2p from the family of ABC transporters and CaMDR1p (*C. albicans* multidrug resistance 1) from the family of MFS transporters are the principal mediators of resistance to azoles due to transport phenomena. Expression of each of the genes encoding these proteins can be upregulated in distinct clinical azole-resistant settings. The transcription of *CaMDR1*, the gene encoding CaMdr1p, is almost undetectable in azole susceptible isolates but is much higher in some azole resistant isolates. In contrast, the transcription of *CDR1*, the gene encoding Cdr1p, is detectable in azole-susceptible isolates but is increased to higher levels in some azole-resistant isolates.

1.4.5. Glucan Synthesis Inhibitors (Echinocandins)

In vitro data examining the effect of echinocandins against *Candida* spp. are promising and have been developed as clinical therapeutics. Agents of this class are fungicidal against both fluconazole-resistant and -susceptible isolates (Joseph et al., 2007). Isolates of *C. parapsilosis* consistently have elevated MICs for echinocandins to-date, but the relevance of this is unclear (Pfaller et al., 2006). Caspofungin formerly identified in the literature as L-743,872 and MK-0991, is a polypeptide antifungal. It is a glucan synthesis inhibitor of the echinocandin structural class. Caspofungin blocks the synthesis of a major fungal cell wall component, 1-3-β-D-glucan. The inhibition of glucan synthesis occurs via inhibition of 1,3-β-glucan synthase (Kurtz et al., 1996). The lack of glucan synthesis enzymes in mammalian cells without cell walls makes this an attractive target for selective antifungal activity (Maschmeyer and Ruhnke, 2004, Cassone et al., 1981).

1.4.6. Chitin synthase inhibitor

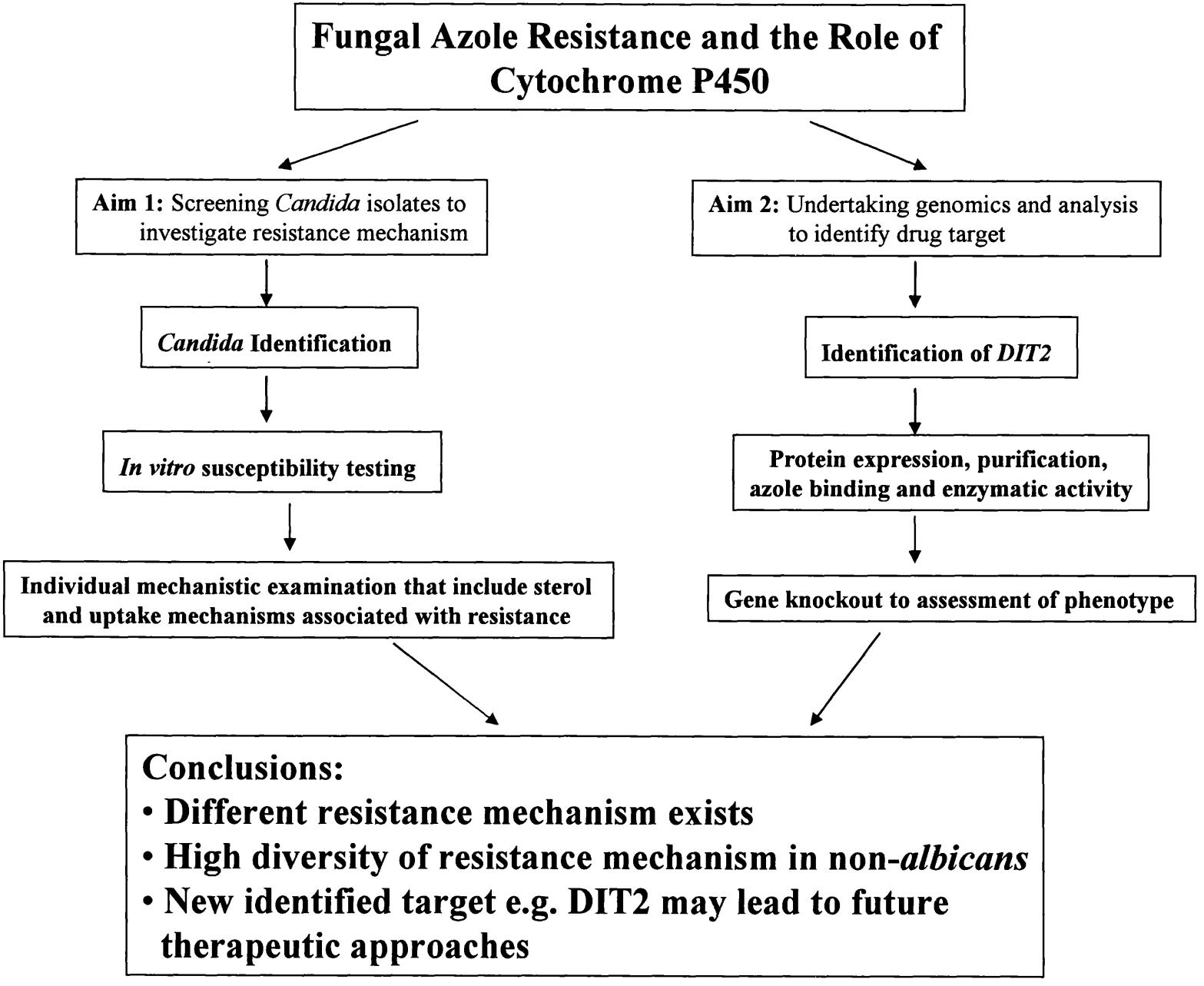
The increase in antifungal resistance and the incidence of deep-seated fungal infections in the clinic has led to the development of new agents directed at novel targets. Chitin is a major and essential component of the fungal cell wall and is absent in the human cell (Odds et al., 1974, Ram et al., 2004, Mellado et al., 2003). Thus, it is a very attractive target for designing antifungal agents although efforts over decades have not yielded effective drugs targeting the enzyme chitin synthase. Polyoxins and nikkomicins are known competitive inhibitors of chitin synthase. There is evidence that fungi may adapt to the inhibition of the synthesis of one wall component by compensatory production of another component. This again leads to the theoretical expectation that a drug that acts on two targets could produce an enhanced and desirable antifungal effect.

1.6. Concluding remarks

Candida resistance and its clinical impact have drastically changed in the last few years presenting a challenge to understand *Candida* virulence, prevention and to control resistance. Of special importance in the control of fungal infections, particularly in the clinic, is a more thorough understanding of CYPs especially as we now see huge multiplicity of these proteins in filamentous fungi. The work presented herein aims to further the understanding of the biological, genetic and physiological aspects of azole treatment on clinical strains, on fungal CYPs (including CYP51) and for sterol biology associated with fungi, particularly in *Candida* species (Flow chart). Numerous *Candida* strains are examined for their drug susceptibility in relation to

factors that could be important such as sterol composition and drug accumulation.

The role of a novel CYP56 in *C. albicans* is probed biochemically and genetically.


Fungal Azole Resistance and the Role of Cytochrome P450
Aim 1: Screening Candida isolates to investigate resistance mechanism
Aim 2: Undertaking genomics and analysis to identify drug target
Candida Identification
Identification of DIT2
In vitro susceptibility testing
Protein expression, purification, azole binding and enzymatic activity
Individual mechanistic examination that include sterol and uptake mechanisms associated with resistance
Gene knockout to assessment of phenotype
Conclusions:
• Different resistance mechanism exists
• High diversity of resistance mechanism in non-albicans
• New identified target e.g. DIT2 may lead to future therapeutic approaches

Chapter 2

General Materials and Methods

General Materials and Methods

This chapter contains general material and methods applied in this study. Materials and Methods used specifically in individual chapters are described in the respective chapter.

2. Materials

All chemicals were purchased from Aldrich, Difco, Sigma or Fluka. The materials, reagents and solutions used are listed as followed and they were autoclaved for 20 min at 121°C when needed and stored at 4°C.

2.1. Media, Reagents and Solutions	**Composition**
2.1.1. Luria-Bertani (LB) medium and plates	tryptone 1% w/v (Difco) yeast extract 0.5% w/v (Difco) NaCl 1% w/v pH 7 agar 1.5% w/v for making solid media ampicillin 100 $mg.ml^{-1}$
2.1.2. Yeast extract peptone dextrose (YEPD)	bacto peptone 2% w/v yeast extract 1% w/v glucose 2% w/v
2.1.3. SOC medium	tryptone 2% w/v yeast extract 0.5% w/v NaCl 10 mM KCl 2.5 mM $MgCl_2$ 10 mM

	$MgSO_4$ 10 mM Glucose 20 mM
2.1.4. X-Gal stock solution (40 $mg.ml^{-1}$)	(5-bromo-4-chloro-3-indoyl-b-D-galactoside (Melford) 400 mg Dimethylformamide 10 ml
2.1.5. IPTG stock solution (100 mM)	(Isopropyl-b-D-Thiogalopyranoside, dioxin free (Melford) 23.8 mg 1 ml H_20 filter sterilize
2.1.6. Terrific medium	bacto tryptone 12 g yeast extract 24 g glycerol 0.4% 1L H_20
2.1.7. Yeast mineral medium (YM)	yeast nitrogen base without aminoacids 6.7 g glucose 2% KH phathlate 20.4 g amino acid supplement 20 $\mu g.ml^{-1}$ uracil and tryptophan 1L H_20 pH 5 filter sterilize
2.1.8. 0.1 M Sodium Phosphate, pH 7.4	13.8 g NaH_2PO_4. H_20 up to 100 ml water, filter sterilize 26.8 g Na_2HPO_4. H_20 up to 100 ml water, filter sterilize

	Mix the previous solutions 22.6 ml NaH_2PO_4 and 77.4 ml Na_2HPO_4 up to 1L water, filter sterilize
2.1.9. 10x TE	100 mM Tris, pH 7.5 10 mM EDTA, 1L H_20, filter sterilize
2.1.10. 50x TAE, pH 8	Tris.HCl 2 M sodium acetate 1M EDTA 50 mM 1L H_20, filter sterilize
2.1.11. 10x TBE pH 8.3	Tris.HCl 1 M boric acid 1M EDTA 20 mM 1L H_20
2.1.12. LiAc	1M
2.2. Antibiotics	
2.2.1. Ampicillin (sodium salt)	100 $mg.ml^{-1}$ in distilled H_20 (Melford, Suffolk, UK)
2.2.2. Carbenicillin (sodium)	100 $mg.ml^{-1}$ in distilled H_20 (Melford, Suffolk, UK)
2.2.3. Chloramphenicol	34 $mg.ml^{-1}$ in ethanol (Sigma)
2.2.4. Kanamycin	10 $mg.ml^{-1}$ in distilled H_20 (Melford, Suffolk, UK)
2.2.5. Nourseothricin-dihydrogen sulphate	200 $mg.ml^{-1}$ in distilled H_20 (Werner Bioagents, Jena, Germany)
2.3. Reagents for DNA extraction from *Candida albicans*	
2.3.1. Breaking buffer	triton X-100 2%

	SDS 1%
	Tris.HCl 10 mM
	EDTA 1 mM
	NaCl 100 mM
2.3.2. 0.4 M Na_2HPO_4	10.7 g in 100 ml distilled H_20
2.3.3. 0.2 M citric acid	4.2 g in 100 ml distilled H_20
2.3.4. EDTA (Na_2 dissodium salt)	Ethylenediamine-tetra acetic acid 15.2 g in distilled H_20
2.3.5. 200 mM Citrate phosphate buffer	58 ml of 0.4 M Na_2HPO_4
	42 ml of 0.2 M citric acid
2.3.6. Citrate phosphate buffer, Sorbitol and EDTA pH 5.6	20 mM Citrate phosphate buffer
	0.4 mM EDTA
	1.2 M sorbitol
	up to 1L distilled H_20
2.3.7. 5 M Potassium acetate	9.8 g potassium acetate in distilled H_20
2.3.8. 10% SDS	1g in 10 ml distilled H_20
	filter sterilize
2.3.9. Zymolyase 20T®	(Seikagaku Corporation, Tokyo, Japan)
2.3.10. RNA A	10 $mg.ml^{-1}$, Sigma, UK
2.3.11. Proteinase K	10 $mg.ml^{-1}$, Sigma, UK
2.3.12. DTT	1M
2.3.13. Phenol chloroform	Sigma, UK
2.3.14. DNA marker	1 kb gene ruler™ DNA ladder (MBA Fermentas; St. Leon-Rot, Germany)
2.3.15. DNA loading buffer	10x concentrate

	3 g glycerol (Fisher) and 25 mg bromophenol blue (Sigma) in 10 ml $_dH_20$
2.4. Reagents for Southern blotting	
2.4.1. 20X SSC, pH=7	NaCl 3M Sodium citrate 0.3M 1L distilled H_20
2.4.2. 10% N-lauroylsarcosine (sodium salt	10 % (w/v) in H_2O filter sterilize
2.4.3. Maleic acid buffer	maleic acid 0.1 M NaCl 0.15 M pH 7.5
2.4.4. Depurination buffer	HCl 250mM
2.4.5. Denaturation buffer	NaOH 0.5 M 1.5 M NaCl
2.4.6. Neutralization buffer	1 M Tris-HCl base pH7.5 1.5 M NaCl
2.4.7. Prehybridisation buffer	5 x SSC, 0.1 % N-laurylsarcosine, 0.02 % SDS, 1 % blocking reagent
2.4.8. Hybridization buffer	10 ml prehybridisation buffer with 15µl probe
2.4.9. 2 x Wash solution	2 x SSC 0.1 % (w/v) SDS
2.4.10. 0.5 x Wash solution	0.5 x SSC 0.1 % (w/v) SDS
2.4.11. Washing buffer	0.3 % Tween 20 in maleic acid buffer
2.4.12. Blocking buffer	1 % blocking reagent in maleic acid buffer

2.4.13. Antibody solution	anti-digoxigenin-alkaline phosphatase conjugate 1:5000 in blocking buffer
2.4.14. Detection buffer	0.1 M Tris HCl, pH 9.5, 0.1 M NaCl

2.5. Reagents for Protein studies

2.5.1. Resolving buffer (5 x concentrate)	1.88 M Tris-HCl (Melford), pH 8.8, stored at 4 °C
2.5.2. Stacking buffer (10 x concentrate)	0.6 M Tris-HCl, (Melford) pH 6.8, stored at 4 °C
2.5.3. Acrylamide stock solution	30% acrylamide (Amersham; Buckinghamshire, UK) and 0.2 % N'N'-bis-methylene-acrylamide (Amersham) stored at 4 °C
2.5.4. Resolving gel	3.7 ml of stock acrylamide solution, 2 ml of 5 x resolving buffer, 0.2 ml of 10 % SDS and water to a final volume of 10ml. The solution was mixed prior to polymerisation using 10 µl of N,N,N',N'-tetramethylethylene diamine (TEMED (Sigma)) and 100 µl of 10 % w/v Ammonium persulphate (APS) (Sigma)
2.5.5. Stacking gel	1 ml of stock acrylamide solution with 0.6 ml of 10 x stacking buffer and 0.12 ml of 10 % SDS. Water was added to a final volume of 6ml and the solution was mixed prior to the addition of 10 µl TEMED and 100 µl of APS
2.5.6. Protein Marker	SDS-PAGE Markers for molecular weights 200,000 (Sigma)

2.5.7. Prestained protein marker	Prestained SDS-PAGE standard solution for molecular weights 30,000-120,000 (Sigma).
2.5.8. Cracking buffer	120mM Tris-HCl, pH 6.8, 20 % glycerol (Fisher), 10 % SDS, 5 % β-mercaptoethanol (Sigma) and trace of bromophenol blue (Sigma), stored at -20 °C
2.5.9. Electrode tank buffer (10x concentrate)	0.25 M Tris base (Melford) and 1.92 M glycine (Fisher) in dH_2O, stored at room temperature
2.5.10. Upper tank buffer	1x electrode tank buffer and 0.1% SDS stored at room temperature
2.5.11. Lower tank buffer	0.5 % electrode tank buffer stored at room temperature
2.5.12. Coomassie Blue stain	6.25g Coomassie Brilliant Blue R250, 1.25 L methanol, 175 ml glacial acetic acid and dH_2O to a final volume of 2.5 L filtered through Watman no.1 filter paper
2.5.13. Coomassie destain	1.25 L methanol, 175 ml glacial acetic acid and water added to a final volume of 2.5 L
2.5.14. Transfer buffer	25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol and 0.025 % (w/v) SDS chilled to 4 °C prior to use
2.5.15. TBS (Tris buffered saline)	20 mM Tris-HCl pH 7.4, 500mM NaCl

and 0.05 % w/v Tween 20, stored

at room temperature

2.6. Strains used in this study

Escherichia coli		
Strains	**Genotype**	**Ref.**
2.6.1. *Escherichia coli* DH5α^{tm}	F$^-$ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E 44λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Supplied by Novagen, (Nottingham, UK)
2.6.2. *Escherichia coli* XL-1 Blue	*recA1, endA1, gyrA96, thi-1, hsdR17*	Supplied by Stratagene (La Jolla, California, USA)

***Candida* spp.**		
Strains	**Reference**	**Ref.**
2.6.5. *Candida albicans*	ATCC 90028^S	ATCC collection (USA)
	ATCC 56513^S	ATCC collection (USA)
	ATCC 28516^S	ATCC collection (USA)
	I8^R (*erg3* fluconazole-and amphotericin- resistant mutant)	(Kelly et al., 1997)

2.6.6. *Candida lusitaniae*	ATCC 200950[R]	ATCC collection (USA)
2.6.7. *Candida krusei*	ATCC 6288[R]	ATCC collection (USA)
2.6.8. *Candida dubliniensis*	CBS7987[S]	Centraalbureau voor Schimmelcultures, Baarn, The Netherlands

R= fluconazole-resistant, S= fluconazole-sensitive

2.6.1. Clinical strains

Clinical strains used *Candida parapsilosis* (G1-G10,H1, n=9), *Candida krusei* (9-C, 21-C, 46-B, 53-A, 88-C2, 123-B, 127-D, 129-B, 1-RAO, ATCC 6288), *Candida lusitaniae* (19-A1, 25-B, 75-A, 76-A, 110-A, 121-A), *Candida norvegensis* (186-A, 3-MASC), *Candida valida* (1-MBJ), *Candida glabrata* (G5 and G7). *C. albicans* isolate (1-MAB) was from the oral cavity of a 5-year old HIV1- infected child, who had acquired HIV vertically, and was monitored at the Pediatric Immunodeficiency Division, State University of Campinas, Brazil. The child started HIV antiretroviral therapy in 1999 and never received antifungal therapy. *C. dubliniensis* isolates of IFM 48184 (F6583 isolated from a Japanese patient), 48314 (CBS 7988) and 49192 (S-34 isolated from a Brazilian patient) were used as references, and isolates 73 and 390 were obtained from the Brazilian HIV-infected child during 1998 and 1999 (Sano, 2000). The *C. dubliniensis* isolates tested were 2-MLA, 3-MLA (grandmother) and 9-LPS, 3-LPS (Brazilian HIV-infected child), collected during 2000 and 2001. The Brazilian HIV-infected child received highly active antiretroviral therapy (HAART) including nelfinavir, zidovudina and 3TC. The HIV-infected child received homecare from the grandmother.

All *Candida* clinical isolates were from human immunodeficiency virus-positive patients. *Candida* stock isolates were maintained at -80°C, and were grown in yeast nitrogen base medium (YNB, Difco) 2% glucose.

Table 2.1. *C. albicans* **strains used in the *DIT2* study.**

Strains	Parent	Genotype	Reference
SC5314		Wild-type strain	(Gillum et al., 1984)
Δ1-R1-6^R	SC 5314	*dit2*Δ::*SAT1/DIT2*	This study
Δ1-R1-6C^S	Δ1-R1-6^R	*dit2*Δ::*FRT/DIT2*	This study
Δ2-S1^R	Δ1-R1-6C^S	*dit2*Δ::*FRT/dit2*Δ::*SAT1*	This study
Δ2-S3A^S	Δ2-S1^R	*dit2*Δ::*FRT/dit2*Δ::*FRT*	This study
Re-integrant	Δ2-S3A^S	*dit2*Δ::*FRT/dit2*Δ::*FRT, RP10-DIT2*	This study

Table 2.2. Plasmids used in the *DIT2* study.

Plasmid	Size (bp)	Reference
pUC19	2686	Yanich-Perron *et. al.* 1985
pGEM T-easy	3015	Yanich-Perron *et. al.* 1985
pBlue script	2961	GenBank® 523327
pSFI1	8051	(Wirsching et al., 2000)
CIp10	5198	GenBank accession A181970
CIp20	6200	This study
pSport	4109	modified (Wittbrodt et al., 2002)

Table 2.3. Primers used in this study for deletion of *DIT2* in *C. albicans*.

Primers	Sequence	Reference
DITFOR	5'-TTGGCCAAGGAATATTTCTG	This study
DITREV	3'-TGTGCTTTGATGTACCTGTG	This study
DIT2F[1]	5'-ATGCGAGCTCAGTTTGTTACCAATATGCAC	This study
DIT2R[2]	3'-ATCCGGATCCGCCATACTATGCCATTAAGG	This study
DIT2KF[3]	5'-GGCCGGTACCAGTTTGTTACCAATATGCAC	This study
DIT2XR[4]	3'-GGCCCTCGAGAATGGTGGGAAAACTATCTC	This study
DITSIIF[5]	5'-GCCGCCGCGGTTCTAGGTGAGTATAAAGTG	This study
DITSIR[6]	3'-GGCCGAGCTCGCCATACTATGCCATTAAGG	This study
SAT1	5'-GCCCGACGTCGCActcgAGCGTCAAAACTAG-3'	Reuss 2004
SAT2	5'-CTAGTGATTTCTgcAGGACCACCTTTG-3'	Reuss 2004
MAL1	5'-CATGCAAGCCAGGatCCAATAATGATTGG-3'	Reuss 2004
MAL2	5'-GTTCACTCATTGTcgacGATTATTAGTTAAACC-3'	Reuss 2004
FLP1	5'-TTCCGTTATGTGTAATCATCC	Reuss 2004
FLP2	5'-CGCTGAGTTTCGATATTGTC	Reuss 2004
FLP3	3'-GTATATGTGCCTACTAACGC	Reuss 2004
RT300	5'-GTACTTGACATGCCATACTATGC	This study
RT768	3'-AGTTTCGATAAAGAAAACAAGA	This study
TEM1R	3'-TGGTTATTCTGATCCTGTTG	This study
ACT10	5'-GCGGAATTCAGAGTCGACATT	This study

1= *SacI*; 2= *Bam*HI; 3=*Kpn*I; 4=*Xho*; 5=*Sac*II, 6=*Sac*I restriction sites are underlined

2.7. *Candida* Identification

The isolates were identified according to the standard technique (Sandven, 1990). They were cultured on corn meal agar (Difco, US), supplemented with 1% Tween 80, at 25°C for 7 days for chlamydospore formation. Germ tube formation was monitored in calf serum (Gibco BR, US) at 37°C for 2 to 4 h. Additionally, the isolates were cultured at 30°C for 48 h on chromogenic agar (CHROMagar® - France) (Beighton et al., 1995), and tested with the *Candida* Check kit® (Iatron laboratories, Inc., Japan) and ID32C® profiled (bioMerieux, Marcy l'Etoile, France). Differential growth test at 45°C was used to distinguish *Candida albicans* from *C. dubliniensis*. The isolates were maintained on potato dextrose broth containing 25% glycerol (PDA Difco, MO, USA) at -80°C.

2.7.1. Confirmation of *C. dubliniensis* by polymerase chain reactions (PCR)

The isolates were cultured on potato dextrose agar (PDA Difco, MO, USA) slants at 30°C temperature for 48 hours. Extraction of DNA was performed using a DNA extracting kit; Gen Toru Kun for yeasts (TaKaRa, Ohtsu, Japan). PCR was performed by amplifying a specific sequence of the *C. dubliniensis* cytochrome b gene (Sano, 2000, Biswas SK, 2001). The primers used were Cdub-F (5'-TTCTCTGTAAGTAATCCTACAATACAGCGT-3') and Cdub-R (5'-ACAATTGATGGAGGTGTCACCATTGGGTTT-3'). A positive result was indicated by the presence of a 305 base product after resolution by electrophoresis in 1% agarose. Comparison of DNA fingerprinting by random amplified polymorphic DNA (RAPD) patterns was also undertaken. The isolates were analyzed by RAPD patterns generated by PCR using 10 pmole of the primer R28M (5'-ATGGATCSSC). An annealing temperature of 35°C was used with Taq DNA polymerase (Biswas SK, 2001, Sano, 2000).

2.8. Propagation of the *Escherichia coli*

E. coli DH5α strain was used as a recipient for the introduction of plasmid DNA. A single colony was propagated in 5ml of LB media supplemented with 100 $\mu g.ml^{-1}$ ampicillin and grown at 37°C on an orbital shaker at 250 rpm in a 60ml sterile container (Sterilin) overnight (o/n).

2.9. Growth of *Candida* spp.

Candida spp. were grown on YEPD or YM medium. The isolates were incubated at 30°C or 37°C for 48 h. The yeast cell growth in liquid culture was estimated using a haemocytometer, to measure the concentration of cells.

2.10. Plasmid vectors used in this study

Plasmids used in this study were supplied by Promega (Corp, Southampton, UK) or were within the collection in the laboratory. Transformants containing the respective plasmids were selected on LB agar medium containing the antibiotic needed as illustrated in the map of plasmid (Fig. 2.1-5).

Plasmids	Size (bp)	Map
pGEM®-T easy vector	3015	Fig. 2.1
pUC19	2686	Fig. 2.2
pSport	4109	Fig. 2.3
pCWori+	5500	Fig. 2.4
pYEp51	7634	Fig. 2.5

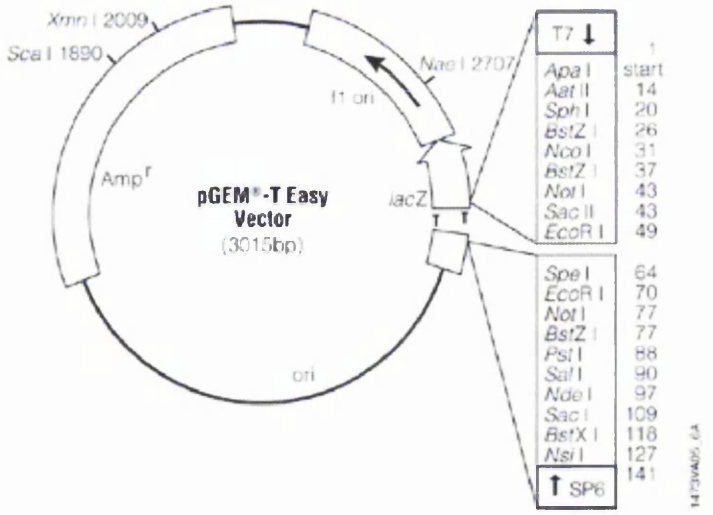


Fig. 2.1. Map of plasmid containing the restriction sites and antibiotic marker.

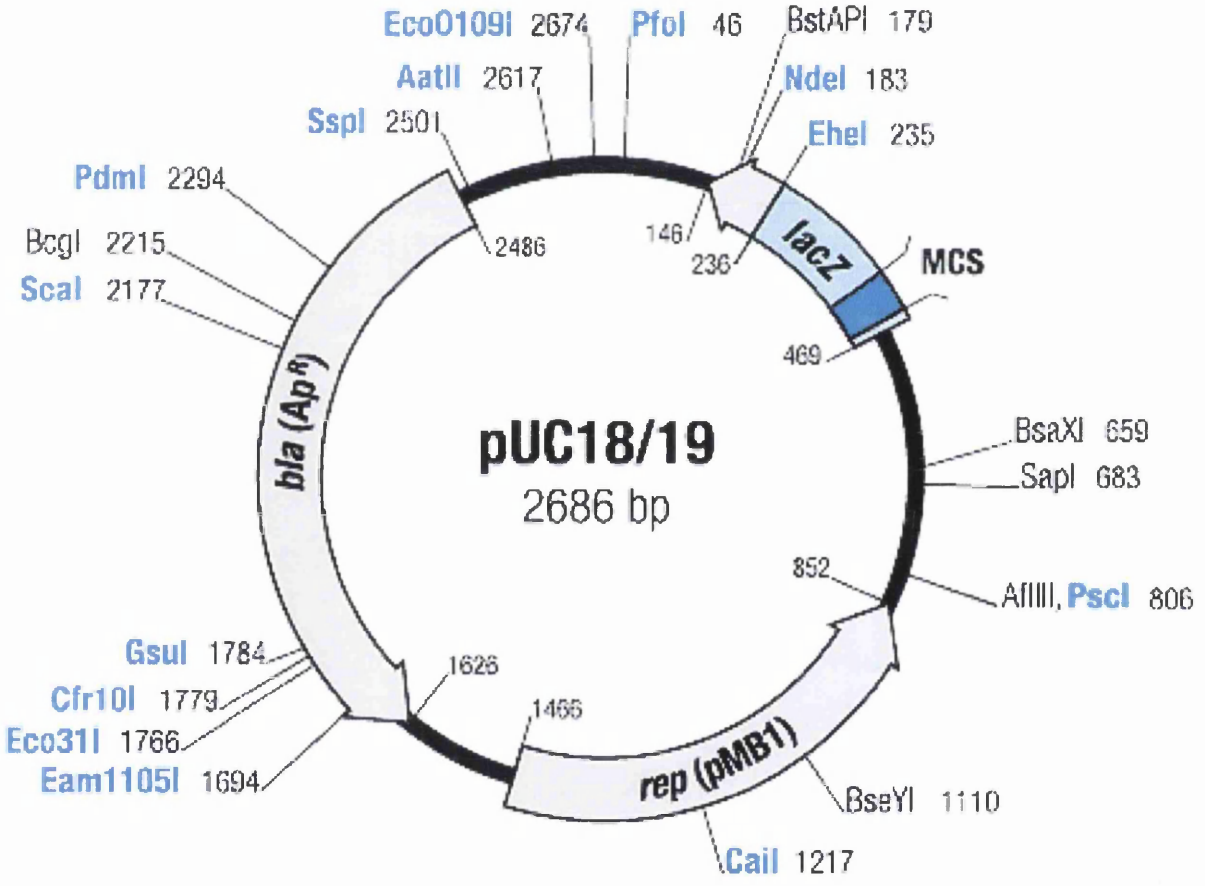


Fig. 2.2. Map of plasmid containing the restriction sites and antibiotic marker.

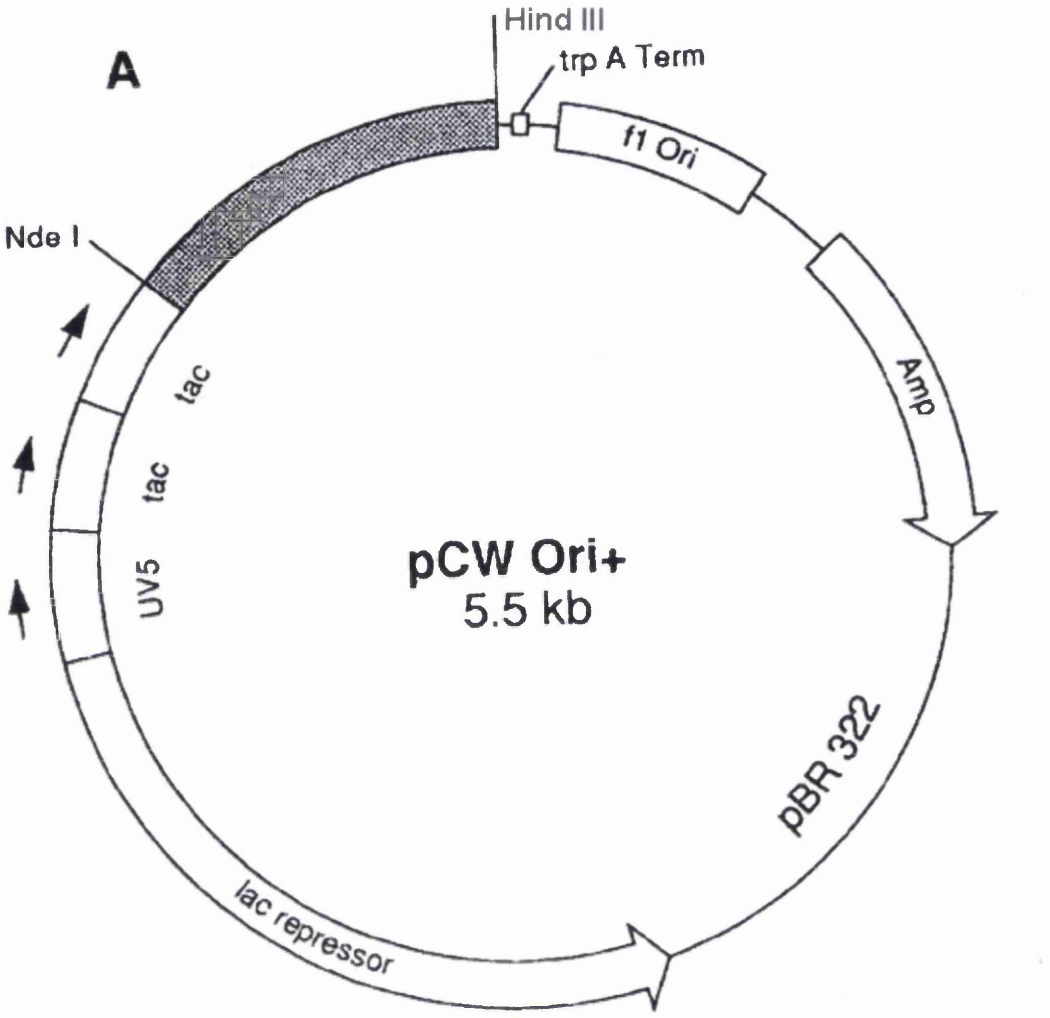


Fig. 2.3. pCW Ori$_+$ plasmid containing the *tac* promoter upstream of a multiple cloning site.

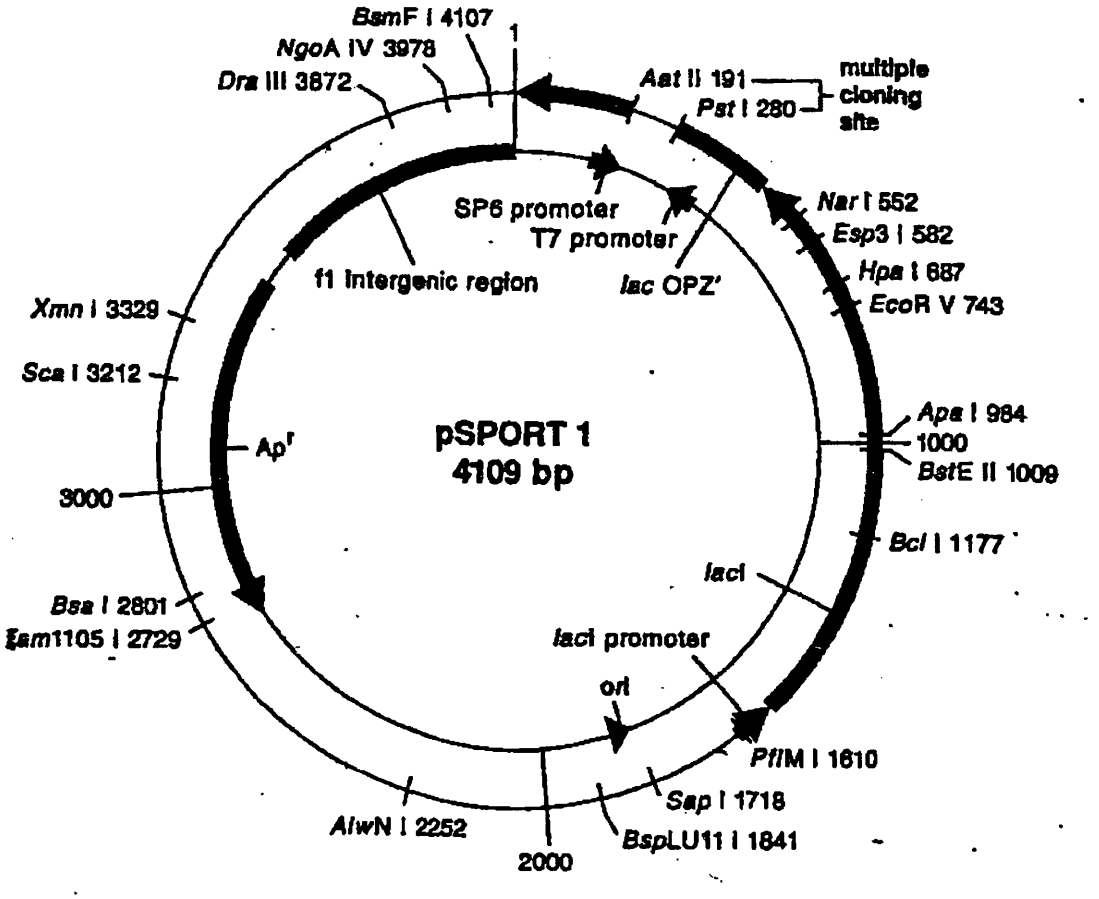


Fig. 2.4. Map of pSPORT1 containing the multiple cloning site, *lac*I promoter and antibiotic marker.

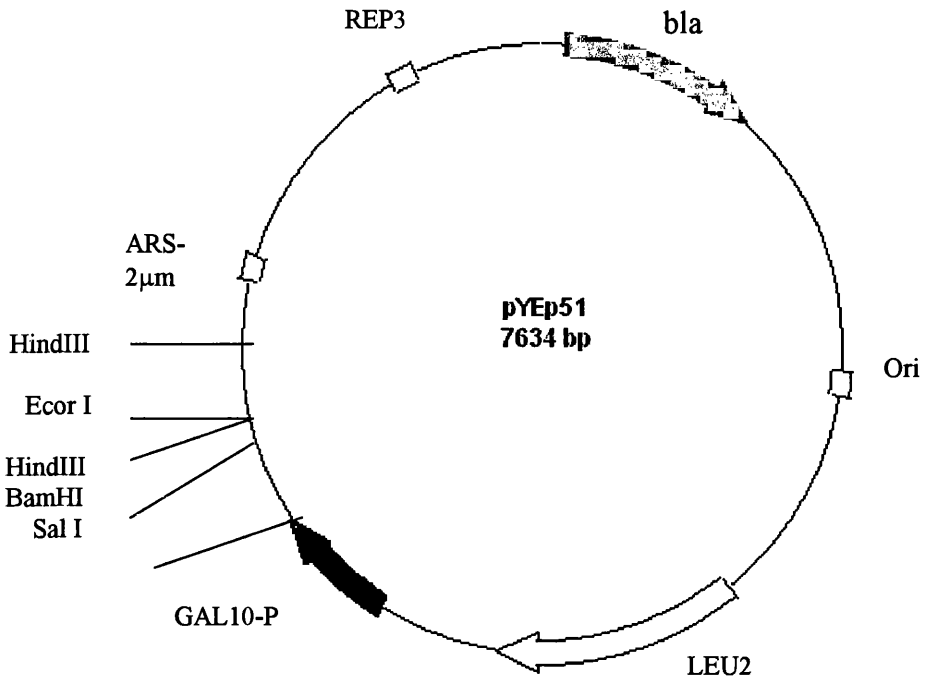


Fig. 2.5. pYEp51 yeast episomal shuttle vector containing the *GAL*10 promoter to drive heterologous expression.

2.11. Molecular biological techniques

2.11.1. PCR methodology

Polymerase Chain Reaction (PCR) was used to amplify the different genes of interest according to the techniques established by Mullis and Faloona (1987) and Erlich (1989). PCR was performed on a Quanta Biotech QB-24 DNA thermal cycler, using synthetic oligonucleotides that were purchased from Invitrogen^{TM}, (UK). The standard PCR was typically carried out in a 200µl PCR tube with 50µl reaction volume containing 200µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP) and 5units of Taq DNA polymerase A (Promega, Corp, Southampton, UK). PCR reaction consisted of 1µl of the DNA template (100ng.µl^{-1}) plasmid, 5µl of 10x buffer, 5µl $MgCl_2$ (25mM), 1µl dNTPs (10mM), 2.5µl of the forward and reverse primers (10pmol.µl^{-1}), 5µl enhancer, 1µl pfx polymerase (5units), 27µl dH_2O. The conditions were 94°C for 1 minute, then 30 cycles of 94°C for 1 minute, 55°C for 30 seconds and 72°C for 2 minutes. Occasionally little or no PCR product was detected, in this case the optimization of PCR included: 1. DNA template quality – we checked the quality and concentration of the template were checked and also a serial dilutions of DNA used to determine the optimal concentration; 2.$MgCl_2$ concentration – variation in the concentration ranged from 1.5 to 4mM; 3. Optimal annealing temperature – the temperature depends on the melting temperature of the primers. It can range from 45°C to 65°C.

2.11.2. DNA Gel Electrophoresis

A 1% (w/v) agarose gel was prepared in TAE buffer 50ml containing 2µl of 10mg.ml^{-1} stock of ethidium bromide. The gel was placed in a tank (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) and filled with TAE buffer.

0.5μg of a GeneRuler 1kb DNA marker (Roche Diagnostic Ltd, Lewes, East Sussex, UK) was also included in an additional well. Sample DNA was loaded along with a molecular weight standard mixture to aid estimation of the sizes of the sample DNA loaded. A constant voltage of 90V was applied on the gel for 30 minutes after which the fragments were visualised under ultraviolet light.

2.11.3. Gel Extraction of DNA from Agarose gels

Following agarose gel electrophoresis, DNA fragments of interest were isolated using the QIAquick gel extraction kit (Qiagen). The system is dependent on the absorption of the DNA fragment onto a silica-based membrane. Prior to this, the fragments were excised from the gel using a clean scalpel blade and the gel sizes were determined. The gels were melted at 50°C for 15 minutes. The DNA solution was then applied onto the columns. The bound DNA was washed with an ethanol based buffer and eluted with sterile distilled water.

2.11.4. PCR amplified DNA purification

Following the successful amplification of the gene of interest confirmed by agarose gel electrophoresis, the PCR mixtures were cleaned using a GenElute^{TM} PCR clean-up kit from Sigma, UK. The buffers, primers, DNA polymerase and dNTPs were then removed with an alcohol wash. The double stranded DNA was then eluted with sterile distilled water.

2.11.5. DNA Restriction Endonuclease Digestion

Restriction endonuclease digestions were carried out in 20μl reaction volume. The enzymes were obtained from Promega Corp, Southampton, UK or Roche Diagnostic Ltd, Lewes, East Sussex, UK. Typically 0.1 – 0.5 μg of DNA was used per reaction, with 1 unit of the appropriate enzyme and 2μl of the 10 x buffer to give

1x dilution of the buffer. The reaction mixtures were gently mixed, centrifuged briefly to collect the sample and incubated at the appropriate temperature overnight for optimal and complete digestion.

2.11.6. A-tailing and blunt- ending of PCR products

A-tailing of PCR products, prior to "sticky-end" ligation, is necessary when proofreading DNA polymerase enzymes are used. The method used was according to Knoche and Kephart, (1999). The method uses a non-proofreading DNA polymerase and dATP (deoxy adenosine triphosphate) to add poly A tails to the blunt ends of the fragment. The PCR product was purified by gel extraction and 6 µl was incubated with 1 µl of 15 mM $MgCl_2$ (Promega), 1 µl of 2 mM dATP (Roche), 1 µl of *Taq* polymerase (Promega) and 1 µl of 10 x Mg^{2+}-free *Taq* polymerase buffer (Promega), for 30 minutes at 70 °C.

Blunt-ending of PCR products was carried out when "blunt-ended" ligation was to be carried out. This method uses a proofreading DNA polymerase to remove extended bases from PCR-generated DNA fragments. The PCR product was purified by gel extraction and 6 µl was incubated with 1 µl of *Pfu* DNA polymerase, 1 µl of 10 x *Pfu* polymerase buffer and 2 µl of 0.25 mM dNTP, for 30 minutes at 70 °C.

2.11.7. Ethanol precipitation of DNA

DNA samples were desalted and concentrated by ethanol precipitation according to the method of Sambrook *et al.* (1989). Firstly 10 % v/v of 3 M Sodium acetate, pH 5.8, was added to the sample (DNA). Then a volume (2 x the volume of sample and sodium acetate for DNA precipitation, 100 % ethanol was added and the mixture was incubated at -80 °C for 5 minutes. The mixture was centrifuged at 13,000 rpm for 20 minutes to pellet the precipitated nucleic acid. The supernatant was

removed and the pellet washed by half filling the tube with 70 % ethanol and inverting twice. The sample was centrifuged again for 2 minutes and the supernatant removed. Residual ethanol was evaporated in a DNA speed vac and DNA resuspended in dH_20.

2.11.8. Ligation of DNA into plasmid vectors

The DNA used in ligation reactions was either purified from an agarose gel or with a PCR purification kit as described previously. About 0.1 - 0.5μg of insert and DNA fragment are used in a reaction volume of 10μl. This volume also contained 1 unit of T_4 DNA ligase and 1μl of 10 x ligase buffer (Roche Diagnostic Ltd, Lewes, East Sussex, UK). The volume was adjusted to 10μl with sterile distilled water. The reaction was incubated at 4°C overnight.

2.11.9. Preparation of competent *E. coli* cells

Transformations were undertaken using procedures largely based according to the method of Cohen *et al.* (1972). A single colony of bacteria from a fresh plate was cultured in 100ml of LB media at 37°C in an orbital shaker (250 rpm). The culture was grown to an OD_{600} of 0.4 – 0.5. The cells were aseptically transferred into a sterile ice-cold tube and placed on ice for 10 – 15 minutes. The cells were then harvested at 4000 rpm for 10 minutes at 4°C and the supernatant was decanted off. The pellets were resuspended in 10ml ice-cold 0.1M $CaCl_2$ and stored on ice for 10 minutes. Cells were then recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in 2ml of ice-cold 0.1M $CaCl_2$ for each 50ml of original culture, dispensed into aliquots of 200μl and stored at –80°C until needed.

2.11.10. Cloning

The *Escherichia coli* DH5α competent cells were transformed with various plasmids. Transformation was performed by mixing 1 µl of plasmid DNA with 50 µl of competent cells (*E. coli* DH5α) on ice. The cells were kept on ice for 20 minutes to allow uptake of the plasmid prior to subjecting the cells to heat shock by incubating in a water bath at 42°C for 45 seconds followed by incubation on ice for 2 minutes. LB (0.95 ml) was added to the cells and incubation commenced at 37°C, 225 rpm for 1 hour with the cells being recovered by centrifugation. Selection of transformants was achieved by plating out the cells on LB-agar plates containing 0.1 $mg.ml^{-1}$ sodium ampicillin or specific antibiotic followed by incubation at 37°C overnight. A control transformation was also performed using empty plasmid.

The recombinant colonies were screened by isolation of plasmid DNA from overnight cultures in conjunction with restriction enzyme digests. Colonies were grown in 10 ml volumes of LB containing 0.1 $mg.ml^{-1}$ of sodium ampicillin or specific antibiotic overnight at 37°C, 225 rpm. The plasmid DNA from 3 ml of overnight culture was isolated using a Qiagen Plasmid Purification kit. The plasmid DNA obtained was digested with the restriction endonucleases *Nde*I and *Hind*III (Roche) for 2 hours at 37°C. The digestion products were resolved electrophoretically on a 1% (w/v) agarose gel and were visualised by exposure to u.v. light in the presence of ethidium bromide.

2.11.11. Transformation of *Candida* species by electroporation

Culture of *Candida* (50 ml) at concentration of 10^{-4} cells in YPD broth and grow overnight at 30°C to an OD_{600} of 1.6-2. Spin down at 1 K for 10 min. Cells were resuspended in 8 ml distilled water and added 1 ml 10x TE and 1 ml 1 M LiAc, incubated at 30°C with shaking 150 rpm for 1 h and 250 µl of 1 M DTT was added before incubating for 30 min. Then 40 ml of distilled water was added, cells were

spun down and washed sequentially in 50 ml of ice cold water and 10 ml of ice cold 1M sorbitol. The pellet was resuspended in 50 µl 1M and kept on ice and 40 µl of the cell suspension taken for each transformation. DNA was added (0.5 to 1 µg) in a volume of 5 µl or less to cells, the suspension was mixed gently with the pipette tip and transferred mixture to 0.2 cm electroporation cuvette (Sigma-Aldrich electroporation). Electroporation was using a Bio-Rad Gene pulser at 1.6 kV, 200Ω, 25µF and then 450 µl of 1 M sorbitol was added and cells spun down 14,000 rpm for 1 min. Cells were resuspended in 1ml of YPD and shaking at 200 rpm 30°C for 4 h and plated out 50 µl aliquots of transformed cells and controls onto YPDA plates with X-Gal, IPTG and antibiotic marker. Antibiotic resistant clones grew after 24-48 h.

2.11.12. Plasmid rescue from *Candida* species

Cells were grown overnight in selective yeast nitrogen base medium at 30°C. Cells were harvested from 1.5 ml of culture in a microfuge tube. The supernatant was removed and the pellet resuspended in 200 µl of breaking buffer. Cell suspension was added to a screw capped 2 ml tube containing 0.3 g of the glass beads and 200 µl of phenol:chloroform:isoamyl alcohol (24:24:1). Cells were disrupted for 1 min in a beater/cell disrupter or alternatively vortexed thoroughly for 5 min. The homogenate was spun at 14,000 rpm for 10 min (Micro Centaur, Sanyo). The aqueous phase was removed and extracted with equal volume of chloroform:isoamyl alcohol (24:1). Centrifugation for 2-3 min followed and the aqueous phase was removed to a fresh tube. Nucleic acids were precipitated with addition of 20 µl 3 M sodium acetate pH 5 and 400 µl of ethanol at -20°C and after centrifugation at 14,000 rpm for 10 min, the pellet was washed with 70% ethanol and resuspended in 50 µl TE. 1-5 µl of DNA was used to transform in plasmid vector.

2.11.13. *Candida* chromosomal DNA preparation

50 ml YPD culture was grown overnight at 37°C in 250 ml flask. Cells were pelleted in Falcon tube were centrifuge at 2.5 K for 5 min. The supernatant was removed and cells resuspended in 5 ml 20 mM citrate phosphate buffer, 40 mM EDTA, 1.2 M sorbitol pH 5.6. 15 mg zymolyase 20T ws added and incubated at 37°C with shaking for 30-90 min. Protoplasts were harvested by centrifugation at 2.5 K for 5 min (Centrifuge Sorvall® Legend TM/T/RT Kendro). The resulting pellet was resuspended in 7.5 ml 10x TE and 0.75 ml 10% SDS added. 2.5 ml 5 M potassium acetate was then added and the suspension incubated on ice for 30 min. The lysed cells are decanted into a 50 ml centrifuge tube and subjected to a clearing spin at 8.5 K for 10 min, 4°C (Centrifuge Sorvall® Legend TM/T/RT Kendro). The supernatant was placed into a fresh Falcon tube and mixed gently with 10 ml ice cold iso-propanol, incubated on ice for 5 min and spun at 2.5 K for 5 min. The resulting pellet was dried, resuspended in 1ml TE buffer and 100 µl RNase A (10 $mg.ml^{-1}$) added before incubating for 1 h at 37°C. 100 µl proteinase K (10 $mg.ml^{-1}$) were added and incubated for 1-2 h at 37°C. Next phenol/chloroform was added and the top phase extracted three times. The DNA was precipitated with the addition of two volumes of absolute ethanol. The DNA formed a web, which was removed to a fresh eppendorf with a pipette tip. The DNA was washed in 70% ETOH for 5 min, dried and resuspended in 150 µl TE. For southern hybridisation experiments 10-20 µg of DNA was digested with restriction enzyme. The concentration of DNA samples were assessed by measuring their absorbance at 260 nm by spectrophometer (F1-Hitachi) and calculating the concentration according to following formula 1 unit of A_{260} = 50 µg DNA.

2.11.14. Deletant strain construction

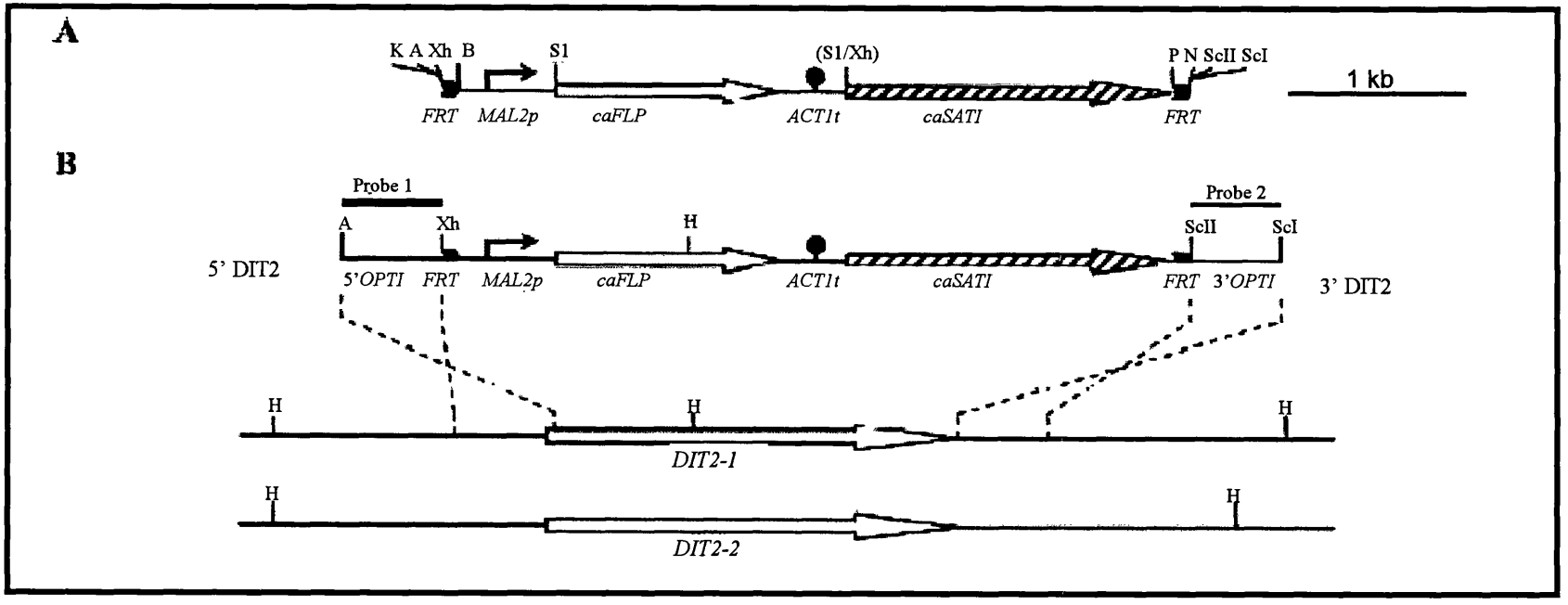
Standard molecular biology techniques for DNA manipulations and bacterial transformations were used as described in this chapter (Chapter 2.11.10). Once the complete *DIT2* gene sequence was obtained, the DIT2F and DIT2R primers were designed to amplify the entire ORF and PCR-amplified products were purified by gel extraction, digested and subcloned between sites *Sac*I and *Bam*HI in pUC19 plasmid. Subsequently a deletion was created in this construct by inverse PCR and attempts were made to ligate the caSAT1 cassette into this deletion, but these ligations failed to yield any recombinant plasmid molecules in *E. coli*. Consequently the strategy was altered, and new primers were designed to separately amplify the upstream 5' (DIT2KF and DIT2XR), and downstream 3' (DITSIIF and DITSIR) regions of the *DIT2* gene. Firstly PCR-amplified product from upstream of the gene was cloned in the pBluescript II plasmid and transformed in the DH5α^{tm} *E. coli* cells. The plasmid containing the fragment was digested with *Kpn*I and *Xho*I restriction enzymes and purified by agarose gel electrophoresis and elution using the Qiagen Qiaex II kit. Subsequently this fragment was subcloned in the *Kpn*I and *Xho*I sites of the ca*SAT1-flipper* cassette, provided by Dr. G. Moran, previously digested with *Kpn*I and *XhoI* restriction enzymes. Similarly the 3' flanking regions of the *DIT2* gene were amplified with the primers DITSIIF/DITSIIR, digested with *Sac*II and *Sac*I restriction enzymes and ligated with *Sac*II and *Sac*I digested pBluescript II. This fragment was then released from pBluescript II by digestion with *Sac*II and *Sac*I restriction enzymes and gel purification as described previously (Chapter 2.11.3). This purified fragment was subcloned into the *Sac*II and *Sac*I restriction sites of SAT1-flipper plasmid

containing the DIT2 5' upstream fragment (Fig. 2.6). Clones from this last transformation were purified and digested with *Kpn*I and *Sac*I restriction enzymes. The fragment thus generated consisted of the SAT1-flipper cassette flanked by the 5' and 3' non-coding regions of the DIT2 gene and was used subsequently for *C. albicans* strain transformation to select nourseothricin (Werner Bioagents, Jena, Germany) resistant putative gene deletion mutants.

2.11.15. Southern blot analysis

Genomic DNA obtained from strains SC5314, *dit2*-1Δ::*FRT* and *dit2*-1Δ::*FRT/dit2-2*Δ::*FRT* were digested with restriction endonuclease enzyme in large scale (10 μg) for Southern blot analysis. The Southern blotting method followed, and DNA probes used to confirm the gene disruption, were prepared by the method outlined in the Boehringer Mannhein digoxigenin (DIG) labelled DNA detection system.

Probes for the *DIT2* gene sequences were made using 15 μl (approximately 1 μg) of PCR fragments of *DIT2* (from primers DIT2KF and DIT2XR) by random primed DNA labelling. The PCR products were denatured, individually, by boiling for 10 minutes and placing immediately on ice. Once cooled, 2 μl of 10 x DIG labelled hexanucleotide mix (Boehringer Mannhein), 2 μl of 10 x dNTP mix (Boehringer Mannhein) and 1 μl of klenow fragment (final concentration 100 U/ml; Boehringer Mannhein) were added to the PCR product and incubated overnight at 37 °C. The labelling reaction was then stopped by the addition of 2 μl of 200 mM EDTA, pH 8.0. Genomic DNA samples from the three strains were isolated and digested for 4 hours with *Eco*RI (Promega). The digested genomic samples and PCR fragments were run on a 1 % agarose (BHD) gel.



Reuss 2004

Fig. 2.6. The illustration shows the scheme of gene disruption using a modified structure from Reuss (2004) of the *SAT1* cassette contained in plasmid pSFS2. *caFLP= C. albicans-adapted FLP GENE; caSAT1=nourseothricin resistance marker; MAL2=promoter; ACT1t=transcription termination sequence; FRT=34-bp FLP recombination target.*

The gel was washed in 250 mM HCl for 10 minutes to depurinate the DNA, then twice with dH_2O and twice in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 15 minute intervals. After a further wash with water, the gel was neutralised in two washes of neutralisation buffer (0.5 M Tris-HCl pH7.5, 3 M NaCl) for 15 minutes each time and then rinsed with water.

The DNA was transferred to the nitrocellulose membrane, Hybond^{TM} –N+ (Amersham) by capillary action overnight, using 10 x SSC (1.5 M NaCl, 0.15 M Sodium citrate, pH 7). The membrane was dried and the DNA crosslinked (Stratagene) to the membrane. The membrane was then incubated at 68 °C for 2 hrs in 20 ml prehybridisation buffer (5 x SSC, 0.1 % N-laurylsarcosine, 0.02 % SDS, 1 % blocking reagent). The probe was hybridised by incubating the membrane overnight in 10 ml hybridisation buffer (15 µl probe in 10 ml prehybridisation buffer).

Prior to detection the membrane was washed twice for 15 minutes with 2 x wash solution (2 x SSC, 0.1 % (w/v) SDS), and twice for 15 min at 68 °C in 0.5 x wash solution (0.5 x SSC, 0.1 % (w/v) SDS). The membrane was then equilibrated in washing buffer (0.3 % Tween 20 in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5) for 1 minute and blocked for 1 hour in blocking buffer (1 % blocking reagent in maleic acid buffer). The anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) was mixed in 30 ml of 1% blocking buffer and added to the membrane and incubating for 30 min. The membrane was washed twice for 15 min with washing buffer (0.3 % Tween 20). 80 µl of Nitroblue tetrazolium salt, 5-Bromo-4-chloro-3-indoyl phosphate mix added (NBT/BCIP; Boehringer Mannheim) in 10 ml detection buffer (0.1 M Tris HCl, pH 9.5, 0.1 M NaCl) and then incubating typically for about 5 hours. Finally the membrane was washed with water to stop the reaction.

2.11.16. Reintegration of *DIT2* gene into the homozygous *dit2* deletion mutant

To investigate the reversion of the phenotypic characteristics of the *dit2*Δ double allele mutant, primers were designed for reintegration of the *DIT*2 gene to the homozygous mutant. Primers RT300 and RT768 were designed to allow PCR amplification of the promoter region and the entire *DIT2* gene. The gene was amplified by PCR as previously described (Chapter 2.11.1) using genomic DNA template from *C. albicans* strain SC5314. The resulting PCR-amplified product was purified by gel extraction, and subsequently an A-tailing procedure for blunt-end PCR fragments (Promega) was performed. This fragment was cloned in pGEM T-easy vector for sequencing. Once the gene sequence was confirmed the clone containing the gene was digested with *Spe*I and *Xba*I and subcloned in the plasmid CIp20 containing the SAT1 nourseothricin resistance marker. The resulting digested product was digested with *Nco*I for DNA linearization within the cloned RP10 gene. The fragment was cleaned up using Qiagen Qiaex II kit. Five microliters of this product (~500 ng DNA) was used for *Candida* transformation with the homozygous *dit2*Δ mutant, the transformation was performed as described previously. Positive clones were selected from YEPD plates with 200 $\mu g.ml^{-1}$ nourseothricin after 2-3 days of incubation at 30°C. DNA from positive clones was isolated by Rapid DNA rescue for PCR screening. PCR screens were used to obtain transformants with the desired insertion of the *DIT2* gene in the chromosomal RP10 locus. Only one single allele of the gene was reintegrated. The primers TEM1R and ACT10 were used to identify recombinants with integration of the linear plasmid at the RP10 locus.

2.12. CYP protein studies

2.12.1. Primer designs and PCR conditions

In order to obtain an appropriate CaDit2p heterologous expression system in *E. coli* forward primers were designed and contained the restriction enzyme site *Nde* I. A triplet enconding alanine replaced the second amino acid (phenylalanine) in the protein sequence to improve the expression in accordance with Barnes-Waterman (Barnes et al., 1991). The reverse primer incorporated a *Sph*I restriction site as well as triplets encoding ten 3′ histidines residues to facilitate the purification of the heterologously expressed CYP protein using Ni^{2+}-NTA affinity chromatography.

The polymerase chain reaction (PCR) was used to amplify the different genes in this study in a reaction volume of 50µl. Reactions included 1µl of the DNA template (100ng.µl^{-1}) plasmid, 5µl of 10x buffer, 5µl $MgCl_2$ (25mM), 1µl dNTPs (10mM), 2.5µl of the forward and reverse primers (10pmol.µl^{-1}), 5µl enhancer, 1µl pfx polymerase (5units), 27µl dH_2O. The conditions were initially 94°C for 1 minute, then 30 cycles of 94°C for 1 minute, 55°C for 30 seconds and 72°C for 2 minutes. PCR product sizes expected for coding sequence obtained of *Dit2* gene was approximately 1.5 kb. The products were initially subcloned into pGEM-T Easy using T/A overhang, which allowed ease of verification through restriction digest analysis. After this the sequence encoding CaDit2p was released after digestion with *Nde* I and *Sph* I and ligated into a modified pSport expression vector for *E. coli (Barnes, personal communication)*. The constructs were transformed into DH5α strains for heterologous expression and the vector called pSport-Dit2.

2.12.2. CYP56 expression

The figures A-D show the DNA sequence of the *DIT2* gene locus of *C. albicans* and the corresponding amino acid sequence used in the heterologous expression studies. The yellow box in Fig. A and B indicates the DNA and amino acid sequence, respectively from the database. The green box corresponds to the gene sequence (C) and amino acid sequence (D) of the *CaDit2* gene adjusted for *E. coli* expression using the Barnes procedure (Barnes et al., 1991).

(A)

>*Dit2*_DNA

ATG TTT CAA CTA TTG AAA TAT ATC TTT ATT GGA GGA TTC ACC TAT TTA CTT
TAT TTA ATT TTG GAG ATA GTT TTC CCA CCA TTC AAT TTC CCT AGA AAT ATT
CCC ACA ATA CCA TTT TAT GTG TCG TTT TTA GGG GCT TGC ACC AAT TTG GAT
CAA GAA GAT ATT TAC AAA CTA TAT CTA CGA GAA AAA TTG GAA AAA TAT GGT
GCT GTT AAA ATG TAC TTT GCC TCG AGA TGG AAT ATT TTA ATC ACT AAA CCA
GAT TTC TTA CTT GAA ATG TTT AAA AAT GAA GAT GTA TTT GCC AAA AGT GGC
AAT CAT GTC AAG ATT CCA AAT TCG GTT TTA GCA ACT TAT ACT GGG GAT AAT
ATC ATT AGT GCT CAT GGA GAA TTA TGG AAA TTA TAC CGA GAT GTT GTT GCC
CAA AGT ATT CAA TTT CCT GAT TTG AAA CCA ATT TCC AAG AAT ACA CAA AAG
TTG TTA CGA TTT CTT GAT AAC GAA ATG GGA TCA GCA AAT CAA GCC ACT ATT
TCA GTG ACA GAT ATA TTA CAG AAA TAT TCC TTG GCC AAT GTT TGT GAA TCT
GTC CTT GGC GTT AAT TTT AAT GTA CTT GAT GAT AAA CAA TCA CTT ATG CAT
GAG AAG ATT AAA TAT GTT AAA CTG CAA ATT TTC AAT CCC ATT TTC TTA AAT
TTC CCT TAT TTT GAC AAT TTC CCC ATT CCA AGC CGA CTC AAG GCA AGA AGA
GAA GTG ATT GGG TTT CGT AAA TGG TAT GGT CAA AGC CTT ATA GAA AAG TAT
AAT TTA CAA TTG CCA AAC TCA GCA GCT ACA AAA TTG GTG GAT TCT TTG ATC
AAA GAA AAA TTG ACA GAA AAA CAA TTT TTG GAT AAT GCC ATA ATT TTA ATG
ATT GCT GGT CAT GAA AAT CCA TTA CTA TTG ATG TTA TCA TTA TTA TAT GTT
GTT TCT AAA TAT CCA CAA GTT CAA GAA TTA ATA CGC AAT GAA ACA GAA ACA
ACA AAA CCT TAC CTT CAT TCA GTT ATT TAT GAA ACT CTA AGA ATG TAT CCA
CCA TTG GGA TTA ATC ATA AAT AGA TGC ACA ACT AGA ATC ACC AAA TTG GGC
AAT ATT GTT ATC CCT AAA AAT GTT TAT TGC GGT TAT AAT AAT TTT GGT ACG
GGA AGG GAT AGA AAT GTC TGG GGA TCA GAT GCA GAT ATT TTC AAA CCA GAA
CGA TGG GGA TTG GAA ATT GAT GAA ATC AAC AAG AAA TTT ACG TTA GCC AAA
AGA TCA GCT GAA TTG CCC GCA TTT CAT GGC AGG AAA AGA GCA TGT TTA GGT
GAA AAA TAT GCA TTG TTT GAA GTT AAA CAA TTT TTA TTG GCA ATT CTA GGT
GAG TAT AAA GTG TCT CTA GAC CCA AAT TGG AAA GAA CAA TTG ACA CCT GCT
GGG CCA ATT AGC CCC TTG CGA TTG AAA TTG AAT TTT GAA AAG CTT ACA GTT
TCC TAA

(B)

>Dit2_Protein

M F Q L L K Y I F I G G F T Y L L Y L I L E I V F
P P F N F P R N I P T I P F Y V S F L G A C T N L
D Q E D I Y K L Y L R E K L E K Y G A V K M Y F A
S R W N I L I T K P D F L L E M F K N E D V F A K
S G N H V K I P N S V L A T Y T G D N I I S A H G
E L W K L Y R D V V A Q S I Q F P D L K P I S K N
T Q K L L R F L D N E M G S A N Q A T I S V T D I
L Q K Y S L A N V C E S V L G V N F N V L D D K Q
S L M H E K I K Y V K L Q I F N P I F L N F P Y F
D N F P I P S R L K A R R E V I G F R K W Y G Q S
L I E K Y N L Q L P N S A A T K L V D S L I K E K
L T E K Q F L D N A I I L M I A G H E N P L L L M
L S L L Y V V S K Y P Q V Q E L I R N E T E T T K
P Y L H S V I Y E T L R M Y P P L G L I I N R C T
T R I T K L G N I V I P K N V Y C G Y N N F G T G
R D R N V W G S D A D I F K P E R W G L E I D E I
N K K F T L A K R S A E L P A F H G R K R A C L G
E K Y A L F E V K Q F L L A I L G E Y K V S L D P
N W K E Q L T P A G P I S P L R L K L N F E K L T
V S *

(C)

>*Dit2*_DNA_Expression

ATG GCT CAA CTA TTG AAA TAT ATC TTT ATT GGA GGA TTC ACC TAT TTA CTT
TAT TTA ATT TTG GAG ATA GTT TTC CCA CCA TTC AAT TTC CCT AGA AAT ATT
CCC ACA ATA CCA TTT TAT GTG TCG TTT TTA GGG GCT TGC ACC AAT TTG GAT
CAA GAA GAT ATT TAC AAA CTA TAT CTA CGA GAA AAA TTG GAA AAA TAT GGT
GCT GTT AAA ATG TAC TTT GCC TCG AGA TGG AAT ATT TTA ATC ACT AAA CCA
GAT TTC TTA CTT GAA ATG TTT AAA AAT GAA GAT GTA TTT GCC AAA AGT GGC
AAT CAT GTC AAG ATT CCA AAT TCG GTT TTA GCA ACT TAT ACT GGG GAT AAT
ATC ATT AGT GCT CAT GGA GAA TTA TGG AAA TTA TAC CGA GAT GTT GTT GCC
CAA AGT ATT CAA TTT CCT GAT TTG AAA CCA ATT TCC AAG AAT ACA CAA AAG
TTG TTA CGA TTT CTT GAT AAC GAA ATG GGA TCA GCA AAT CAA GCC ACT ATT
TCA GTG ACA GAT ATA TTA CAG AAA TAT TCC TTG GCC AAT GTT TGT GAA TCT
GTC CTT GGC GTT AAT TTT AAT GTA CTT GAT GAT AAA CAA TCA CTT ATG CAT
GAG AAG ATT AAA TAT GTT AAA CTG CAA ATT TTC AAT CCC ATT TTC TTA AAT
TTC CCT TAT TTT GAC AAT TTC CCC ATT CCA AGC CGA CTC AAG GCA AGA AGA
GAA GTG ATT GGG TTT CGT AAA TGG TAT GGT CAA AGC CTT ATA GAA AAG TAT
AAT TTA CAA TTG CCA AAC TCA GCA GCT ACA AAA TTG GTG GAT TCT TTG ATC
AAA GAA AAA TTG ACA GAA AAA CAA TTT TTG GAT AAT GCC ATA ATT TTA ATG
ATT GCT GGT CAT GAA AAT CCA TTA CTA TTG ATG TTA TCA TTA TTA TAT GTT
GTT TCT AAA TAT CCA CAA GTT CAA GAA TTA ATA CGC AAT GAA ACA GAA ACA
ACA AAA CCT TAC CTT CAT TCA GTT ATT TAT GAA ACT CTA AGA ATG TAT CCA
CCA TTG GGA TTA ATC ATA AAT AGA TGC ACA ACT AGA ATC ACC AAA TTG GGC
AAT ATT GTT ATC CCT AAA AAT GTT TAT TGC GGT TAT AAT AAT TTT GGT ACG
GGA AGG GAT AGA AAT GTC TGG GGA TCA GAT GCA GAT ATT TTC AAA CCA GAA
CGA TGG GGA TTG GAA ATT GAT GAA ATC AAC AAG AAA TTT ACG TTA GCC AAA
AGA TCA GCT GAA TTG CCC GCA TTT CAT GGC AGG AAA AGA GCA TGT TTA GGT

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GAA AAA TAT GCA TTG TTT GAA GTT AAA CAA TTT TTA TTG GCA ATT CTA GGT
GAG TAT AAA GTG TCT CTA GAC CCA AAT TGG AAA GAA CAA TTG ACA CCT GCT
GGG CCA ATT AGC CCC TTG CGA TTG AAA TTG AAT TTT GAA AAG CTT ACA GTT
TCC CAT CAC CAT CAC CAT CAC CAT CAC CAT CAC TAG
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(D)

```
>Dit2_Protein
M A Q L L K Y I F I G G F T Y L L Y L I L E I V F
P P F N F P R N I P T I P F Y V S F L G A C T N L
D Q E D I Y K L Y L R E K L E K Y G A V K M Y F A
S R W N I L I T K P D F L L E M F K N E D V F A K
S G N H V K I P N S V L A T Y T G D N I I S A H G
E L W K L Y R D V V A Q S I Q F P D L K P I S K N
T Q K L L R F L D N E M G S A N Q A T I S V T D I
L Q K Y S L A N V C E S V L G V N F N V L D D K Q
S L M H E K I K Y V K L Q I F N P I F L N F P Y F
D N F P I P S R L K A R R E V I G F R K W Y G Q S
L I E K Y N L Q L P N S A A T K L V D S L I K E K
L T E K Q F L D N A I I L M I A G H E N P L L L M
L S L L Y V V S K Y P Q V Q E L I R N E T E T T K
P Y L H S V I Y E T L R M Y P P L G L I I N R C T
T R I T K L G N I V I P K N V Y C G Y N N F G T G
R D R N V W G S D A D I F K P E R W G L E I D E I
N K K F T L A K R S A E L P A F H G R K R A C L G
E K Y A L F E V K Q F L L A I L G E Y K V S L D P
N W K E Q L T P A G P I S P L R L K L N F E K L T
V S H H H H H H H H H H *
```

Primers designed for production of PCR derivatives of *CaDIT2* for heterologous expression in *E. coli*. Ten histidine triplets were added in the frame at the 3′end to aid the protein purification following expression.

Forward Primer (contains *NdeI* site and F2 to A2 modification)
>DIT2_F1_exp

CAT CGC <CAT ATG> GCT CAA CTA TTG AAA TAT (30-mer)

Reverse Primer (contains *SphI* site and 10-His tag)
>DIT2_R2_exp

ACT GCT <GCA TGC> CTA GTG ATG GTG ATG GTG ATG GTG ATG GTG ATG GGA AAC TGT AAG CTT TTC (45-mer)

2.12.3. Expression of CYP proteins in Terrific Broth (TB) media

Clones containing genes for expression were grown in 10 ml of LB containing 0.1 $mg.ml^{-1}$ sodium ampicillin overnight at 37°C, 225 rpm. The overnight cultures were used to inoculate 500 ml aliquots of Terrific Broth (TB) containing 0.1 $mg.ml^{-1}$ carbenicillin. The cultures were then shaken at 200 rpm, 37°C for 5 to 6 hours until an optical density of 0.6 was obtained.

After 6h of incubation α-5 amino levulinic acid hydrochloride (ALA) was added to assist maximal haem protein production. After 7h of incubation the cells were induced for protein expression by the addition of and isopropyl-β-D-thiogalactopyranoside (IPTG) to final concentrations of 1 mM, with shaking at 160 rpm, 28°C overnight. The cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C (Beckman coulter centrifuge J20XP, rotor type JLA 8.1000). The pellet was resuspended in 0.1 M potassium phosphate, pH 7.4 and the cells were broken by sonication using a Branson Digital sonifier (32x 15 second bursts on ice at power setting 40-60%). The subcellular fractions were isolated by differential centrifugation. Cell debris was removed by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was subjected to ultracentrifugation at 45,000 rpm for 1 hour at 4°C (Beckman coulter ultracentrifuge XL-100K, rotor type 52.1T). The pellet contained the membrane fraction and this was resupended in 6 ml of 50 mM potassium phosphate, pH 7.4, 20% glycerol on ice using a glass homogeniser. The supernatant after centrifugation was the cytosolic fraction, which was kept for posterior SDS-PAGE gel analysis. The concentration of cytochrome P450 present was

determined spectrophotometrically using the reduced carbon monoxide difference method of Sato (1964).

2.12.4. Cell breakage using the C5 Emulsiflex high pressure homogeniser

Typically 500ml volume of cell culture was harvested by centrifugation (Beckman coulter centrifuge J20XP, rotor type JLA 8.1000) at 5000 rpm to pellet the cells and then resuspended in a 20ml of 0.1M potassium phosphate (pH 7.4). One pass through the homogeniser at 15000-20000 psi ruptured the *E. coli* cells. The homogenate was harvested by centrifugation (Beckman coulter centrifuge J20XP, rotor type JA20) at 15,000 rpm for 10 minutes to remove the cell debris and afterwards were harvested by ultracentrifugation (Beckman coulter ultracentrifuge XL-100K, rotor type 52.1T) at 45,000 rpm at 4°C for 1hour all at 4°C to recover the microsomal pellet.

2.12.5. Cell breakage using the BL21 (DE3) pLysS cell lysis method

The BL21 (DE3) pLysS (Promega) *E. coli* strain allows the high efficiency expression of proteins under the T7 promoter. It is particularly useful because it contains the pLysS plasmid encoding the T7 lysozyme. This enzyme is bifunctional, it lowers the background expression of the target protein until IPTG is added and also digests the peptidoglycan layer of *E. coli* cell wall. This occurs upon treatment that disrupts the bacterial inner membrane and thus releases the enzyme such as by using freeze-thawing of cells. Following protein expression the cells were harvested and resuspended in 0.1M potassium phosphate buffer (pH 7.4) and frozen overnight at -80°C. The cells were then thawed at 4°C and were harvested by ultracentrifugation (Beckman coulter ultracentrifuge XL-100K, rotor type 52.1T) at 45,000 rpm at 4°C for 1 hour. Afterwards, the supernatant containing the cytosolic fraction was decanted

and the microsomal pellet was resuspended in 0.1M potassium phosphate buffer (pH 7.4) with 20% (v/v) glycerol.

2.12.6. CYP Spectrophotometric assay

Light-absorption spectra were measured using a Hitatchi U3010 scanning spectrophotometer. Cytochrome P450 concentration was estimated from reduced carbon monoxide (CO) difference spectra according to Omura and Sato (1964), using the extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance units between 450 and 490nm. The extracts containing CYP were diluted in 100mM potassium phosphate (pH 7.4) containing 1mM EDTA and 20% (v/v) glycerol to 1ml. The sample was split equally and placed into two cuvettes and the baseline was determined between 400-490nm. CO was bubbled into the sample cuvette for 45 seconds and then both cuvettes were then reduced by the addition of a small amount of sodium dithionite. The samples were again scanned between 400 and 490nm to determine changes in the absorption at 450nm.

2.12.7. Protein Solubilisation from membrane fractions

Microsomal bound heterologously expressed CYP proteins were initially disrupted with Griffiths glass tube homogenisers (Jencons-PLS, Cherrycourt Way Industrial. Estate, Bedfordshire). The microsomal suspension was then solubilised in 0.1M potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 1% (w/v) sodium cholate. The solution was gently agitated and left to stand on ice for 1 hour with occasional mixing (approximately every 5 minutes). The solution was spun at 6,000*g* (Centrifuge Sorvall® Legend TM/T/RT Kendro) for 1 minute (and the supernatant was clarified by ultracentrifugation at 45,000 rpm for 75 minutes at 4°C (Beckman coulter ultracentrifuge XL-100K, rotor type 52.1T). The supernatant was

dialysed overnight against 5 litres of 25 mM potassium phosphate buffer (pH 7.4) at 4°C. The dialysed solution was once again clarified by ultracentrifugation at 45,000 rpm for 60 minutes at 4°C (Beckman coulter ultracentrifuge XL-100K, rotor type 52.1T), and the supernatant was divided into 0.5 ml aliquots prior to freezing at -80°C. The concentration of cytochrome P450 was determined spectrophotometrically using the reduced CO method of Sato as before.

2.12.8. *Candida* CYP purification by affinity chromatography

Ni-NTA agarose (20 ml - Qiagen, Kent, UK) was equilibrated in 50 mM potassium phosphate, pH 7.4, 20% (v/v) glycerol (equilibration buffer). The matrix was washed three times with 50 ml aliquots of equilibration buffer by followed by centrifugation at 4,000 rpm. The equilibrated Ni-NTA agarose matrix was cooled to 4°C prior to mixing with the solubilised CYP51 extract. The mixture of matrix and solubilised CYP51 protein was gently stirred for 2 hours at 4°C. The matrix was recovered by centrifugation followed by resuspension in 30 ml of equilibration buffer.

The matrix suspension was packed in a C-16 column (20 cm x 1.6 cm) at a flow rate of 30 $ml.h^{-1}$ at 4°C. The column was washed with 50 ml of 50 mM potassium phosphate, pH 7.4, 20% (v/v) glycerol, 0.5 M NaCl to remove non-specifically bound protein. CYP51 protein was eluted from the column using 50 mM potassium phosphate, pH 7.4, 20% (v/v) glycerol, 0.5 M NaCl, 60 mM L-Histidine at a flow rate of 10 $ml.h^{-1}$ with 1 ml fractions being collected. The fractions collected that contained the CYP51 protein were combined and dialysed against 5 litres of 20 mM potassium phosphate, pH 7.2, 10% (v/v) glycerol overnight at 4°C.

2.12.9. Determination of CYP protein concentrations

Cytochrome P450 concentration was determined by reduced carbon monoxide difference spectra according to Omura and Sato (1964) using the extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance units between 450 and 490 nm. Protein concentration was determined by the bicinchoninic acid method (BCA) using bovine serum albumin standards. Spectral determinations were made using a UV/VIS spectrophotometer (Hitachi U-3310, San Jose, California).

2.12.10. Concentrating Protein Solutions

Prior to use on sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE), protein samples were concentrated using trichloroacetic acid (TCA). TCA was added to 3μg of protein to a final concentration of 10% (v/v). Samples were then centrifuged at 13,000 rpm for 10 minutes at room temperature. The pellet was washed with 100μl of 0.5% TCA and centrifuged again at 13,000 rpm for 10 minutes. The pellet was washed with acetone, centrifuged as before and redissolved in 15μl of Laemmli buffer containing SDS loading buffer and 10% (v/v) β-mercaptoethanol. The protein samples were heated at 95°C for 5 minutes centrifuged for 10 minutes at 13,000 rpm and the supernatant loaded onto the gels.

2.12.11. SDS - Polyacrylamide Gel electrophoresis

SDS-PAGE is based on the fact that the denatured proteins bound to the strongly anionic SDS detergent are negatively charged and migrate through the polyacrylamide gels in accordance with the sizes of their polypeptides. The discontinuous buffer system of Laemmli was the basis of the method used. The resolving gel concentration used was 11% (w/v). A constant voltage of 150V was applied for 70-80 minutes using the Bio-Rad power pack. Following the successful separation of the protein samples, the gel was stained with coomassie brilliant blue R-

250 and destained by shaking in a destaining solution containing methanol and acetic acid.

2.12.12. Spectral studies on binding of substrate to CaDit2p

Cytochrome P450 substrate binding spectra was performed according to the method of Jefcoate *et al.*, (1969). The substrates (5 mM *N*-formyl tyrosine) were dissolved in ammonium hydrogen carbonate. 2 nmoles of purified CaDit2p in 1ml of potassium phosphate buffer (pH 7.4) 20% glycerol was placed into the sample cuvettes and 1ml of buffer was placed into the reference cuvettes. The baseline was recorded between 350 and 500 nm. Substrates were added incrementally to both cuvette and a difference absorbance spectrum recorded.

The concentration of substrate in each sample after sequential addition of substrate solution was calculated as the difference in absorbance between 380 and 420 nm. The difference in absorbance between 380 and 420 nm was plotted against the concentration of substrate using the programme Profit 5.0.1 (© 1990-1996, Cherwell scientific publishing, Quantum Soft, Zurich, Switzerland). Non-linear regression using the Levenberg-Marquardt algorithm was used to fit the Michaelis-Menten equation to the data, in order to calculate the K_s values. Each substrate-binding experiment was performed in triplicate and these were used to calculate the mean K_s value for the substrate with CaDit2p. The Hill equation was also applied to determine if CaDit2p has more than one substrate binding sites at the same time.

2.12.13. Reconstitution of dityrosine (CYP56) synthase activity *in vitro*

Dityrosine synthase activity was determined using an *in vitro* reconstitution assay. This consisted of 0.1 to 1 μM of purified yeast Ncpr1p (Warrilow et al., 2002),

5 μM of expressed CaDit2p (Ni-NTA agarose purified), 10 $\mu M.ml^{-1}$ *N*-formyltyrosine, 80 nM dilaurylphosphatidylcholine and 0.1 M potassium phosphate, pH 7.4 in a total reaction volume of 1 ml. The reaction was initiated by the addition of NADPH to a concentration of 1 mM prior to gently vortexing the solution to form micelles and incubating the assay mixture at 37°C, 200 rpm for 1 hour. The reaction was terminated by the addition of 2.5 ml of 1:1 chloroform:methanol followed by vortexing for 1 minute at room temperature. The upper phase was isolated by centrifugation followed by aspiration, and the lower phase was washed with 1 ml of 10% ammonium hydroxide and the upper phase isolated. The two upper phases were pooled and centrifuged at 16,000 x g for 10 minutes prior to concentration using a SpeediVac (Heto Maxi Dry plus) under vacuum at room temperature. The components of the concentrated supernatant were separated by TLC as described in material and methods (Chapter 2.18.4). A control sample containing dityrosine generated by horseradish peroxidase was included. The separated components were examined visually under UV light and were isolated from the TLC plate by extraction with 10% ammonium hydroxide from scrapings taken from the plate. The separated components, including *N*-formyl dityrosine of both the sample and control, were identified by HPLC/MS as described in this Chapter 2.18.5.

2.13. Antifungal susceptibility test

Antifungal susceptibility tests were performed using the broth microdilution method according to the guidelines recently published by the National Committee for Clinical Laboratory Standards (NCCLS 2002).

2.13.1. Antifungal agents used in this study

Agents	Reference
Fluconazole non-labelled	Pfizer (Pfizer Pharm. UK)
[^{3}H] fluconazole	Specific radioactivity, 37kBq.mmol^{-1}, Amersham International
Amphotericin B	Bristol-Myers Squibb
Nystatin	Bristol-Myers Squibb
Voriconazole	Pfizer Pharm. Inc. UK
Miconazole	Sigma, St. Louis, Mo., USA
Itraconazole	Janssen-Kyowa Co., Ltd., UK
Ketoconazole	Janssen-Kyowa Co., Ltd., UK
Clotrimazole	Sigma, St. Louis, Mo., USA
Imidazole	Sigma, St. Louis, Mo., USA
Econazole	Sigma, St. Louis, Mo., USA
5-flucytosine	Sigma, St. Louis, Mo., USA
ritonavir	Abbott, USA
nikkomycin	Sigma, St. Louis, Mo., USA
caspofungin	Merck, USA

2.13.2. Susceptibility testing

MICs were determined by broth microdilution method of National Committee for Clinical Laboratory Standards (NCCLS 2002) (National Commitee for Clinical Laboratory Standard, 2002).

Drugs were tested for antifungal efficacy at concentrations ranging from 16 to 0.001 $\mu g.ml^{-1}$ for voriconazole, for fluconazole 64-0.001 $\mu g.ml^{-1}$, itraconazole 16-0.03 $\mu g.ml^{-1}$, clotrimazole 16-0.03 $\mu g.ml^{-1}$, amphotericin 16-0.03 $\mu g.ml^{-1}$, caspofungin 16-0.001 $\mu g.ml^{-1}$, and nikkomycin 64-0.001 $\mu g.ml^{-1}$.

The medium used was RPMI-1640 medium (Nissui Pharmaceutical Co., Japan), buffer morpholinepropanesulfonic acid (MOPS; Sigma Chemical Co.) and adjusted to pH 7.0 (Taguchi *et al.* 1995). These antifungal reagent-grade powders were dissolved in dimethyl sulphoxide solvent (DMSO) to obtain stock solutions of 12.8 $mg.ml^{-1}$. A 10-fold serial dilution was performed in RPMI-1640 medium to provide a working solution for each drug giving specific final concentration ranges as described by NCCLS guidelines (NCCLS 2002). Antifungal agents were serially diluted in 96-well microtiter plates (Falcon, Lincoln Park, N J). The isolates were subcultured onto PDA slants at 30°C for 24 h. Cell suspensions were prepared in RPMI-1640 medium and were adjusted to give a final concentration approximately 2.5×10^3 $cells.ml^{-1}$. The microdilution plates were incubated at 30°C and the MICs were determined visually at 24 and 48 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antifungal agent at which 80% inhibition of growth (MIC_{80}) compared with that of the growth control well. *C. albicans* ATCC 90028 and ATCC 90029 strains were used in each microdilution set to ensure quality

control. Breakpoints for antifungal susceptibility have been established for *Candida* isolates tested according to NCCLS.

	Polyenes	**Azoles**			**Other**
Species	AMPH B	FLCZ	ITCZ	KTC	5-FC
C. albicans	S	S	S	S	S
C. tropicalis	S	S	S	S	S
C. parapsilosis	S	S	S	S	S
C. glabrata	I	S-DD	S-DD		S
C. krusei	I	R	S-DD to R		I-R
C. lusitaniae	R	S	S-DD		R

- S = Susceptible to usual doses of this agent ($\leq 1\mu g.ml^{-1}$ amphotericin B, $\leq 8\mu g.ml^{-1}$ fluconazole, $\leq 0.125\mu g.ml^{-1}$ itraconazole, $\leq 4\mu g.ml^{-1}$ 5-flucytosine (5FC))
- S-DD = Susceptibility depends on the dose (fluconazole) or delivery (itraconazole) of the drug. Maximal tolerated doses must be used and blood levels may need to be checked.
- I = Indeterminate or Intermediate. This category reflects a general lack of certainty about the meaning of this MIC.
- R = Resistant to usual doses of this agent ($\geq 64\mu g.ml^{-1}$ fluconazole, $\geq 1\mu g.ml^{-1}$ amphotericin B and itraconazole, ($\geq 32\mu g.ml^{-1}$ 5FC)

2.13.3. Etest assay

The Etest is a susceptibility testing method which involves the placement of a plastic strip containing a defined continuous gradient of antimicrobial drug on the surface of an inoculated agar. The Etest strips had drug concentrations ranging from 0.02 to 32 $\mu g.ml^{-1}$ for itraconazole and amphotericin B and from 0.016 to 256 $\mu g.ml^{-1}$ for fluconazole. RPMI 1640 with L-glutamine but without bicarbonate was buffered with MOPS (morpholinepropanesulfonic acid) at pH 7.0, and agar-BACTO were used to prepare Etest RPMI-agar (1.5%) plates. The Etest was performed according to the manufacturer's instructions (AB BIODISK 1993). The inoculum concentration was adjusted to 2 x 10^4 $cell.ml^{-1}$. A cotton swab was used to apply the inoculum-adjusted solution onto the 150 mm diameter RPMI-agar (1.5%) surface so as to obtain even growth on the entire agar surface. The plates were allowed to dry approximately 15 min before the Etest strips were placed with a sterile forceps on the medium surface. The plates were incubated for 24-48 hours until growth was clearly seen. The MIC was read where the elliptical inhibition zone intersected the scale on the strips and compared with Etest reading and interpretation guidelines.

2.14. Accumulation of [^{3}H] fluconazole in *Candida* clinical isolates

Accumulation of [^{3}H] fluconazole in *Candida* cells was determined by a filter-based assay adapted from Sanglard *et. al.* (Sanglard et al., 1995). All experiments were repeated on three separate occasions. Overnight cultures were grown in YNB containing 2% glucose at 30°C, 200 rpm to a density of 10^9 cells per ml. The cells were centrifuged at 4000 rpm for 5 minutes and the pellet resuspended in YNB medium to the original cell density. A total of 20 µl of [^{3}H] fluconazole (0.154 kBq), with a specific activity of 37 $kBq.mmole^{-1}$, was added to 1 ml of the cell suspension.

The cells were incubated at 30°C with shaking at 200 rpm. Samples of 100 µl were withdrawn at fixed time intervals, and mixed with 0.5 ml of cold YNB medium containing 20 µM unlabelled fluconazole placed in a Spin-X nylon membrane microfiltration unit (pore size 0.45 µm - Costar, Cambridge). The cells were isolated by centrifugation at 9000 rpm for 30 s and then washed with the unlabelled fluconazole-YM medium three times. Liquid scintillant was added and the radioactivity within the cells was measured using a liquid scintillation analyzer (2500 TR-TRI carb, Packard Bioscience Company). In other experiments cells were exposed to a subinhibitory concentration of sodium azide (NaN3; 0.01 mM) (Moran et al., 1997) to establish whether [^{3}H] fluconazole accumulation was an active energy-dependent process.

2.15. Growth inhibition studies

2.15.1. Antifungal agents

Voriconazole (Pfizer Pharm. Inc. UK), fluconazole (Pfizer Pharm. Inc. UK), itraconazole, ketoconazole (Janssen-Kyowa Co., Ltd., UK), miconazole, econazole, clotrimazole (Sigma, St. Louis, Mo., USA), imidazole (Sigma, St. Louis, Mo., USA), 5-flucytosine (Sigma Chemical Co., Ltd., St. Louis, Mo., USA), amphotericin B, and nystatin (Bristol-Myers Squibb), and ritonavir (Abbott, USA) were tested at concentrations ranging from 0.125 to 64 µg.ml^{-1}.

2.15.2. Cell growth

Single colonies of *C. albicans* strains were inoculated into 10-ml aliquots of YNB (yeast nitrogen broth, Difco) medium containing 2% glucose. These were incubated at 30°C for 24 h with shaking at 250 rpm. Cells were harvested by

centrifugation at 3500 x *g* for 5 min, at 4°C, washed twice with YNB medium and resuspended in 10 ml YNB medium. Cells densities were adjusted to a final concentration of 2 x 10^3 cells.ml^{-1} in YNB medium containing 2% glucose. Preparation of antifungal drugs and dilution schemes were performed in accordance with the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Microtitre plates were inoculated with the tested strain under different drug regimes. Growth curves of isolates were determined in aerobic batch cultures at 37°C, over 48 hours using a Bioscreen C Analyser (Groeneveld et al., 2002). Growths were calculated as ΔOD per hour (ΔOD.hour^{-1}).

2.15.3. Cell wall alteration assays

Various stresses were placed on cells exposed to different agents. Drop tests were performed by spotting 10^6, 10^5, 10^4, 10^3 and 10^2 cells.ml^{-1} onto YEPD or YM agar plates supplemented with 1.5 M sorbitol, 100 μg.ml^{-1} SDS, 1 M or 1.5 M NaCl, 5 mM or 10 mM H_2O_2, 0.5 M or 1 M $CaCl_2$, 100 μg.ml^{-1} calcofluor, 100 μg.ml^{-1} congo red, 1 μg.ml^{-1} itraconazole, 10 μg.ml^{-1} amphotericin, 2 μg.ml^{-1} fluconazole, 0.5 μg.ml^{-1} nikkomycin. Other sugars were also tested in the medium besides glucose i.e. maltose and galactose, the duplicate plates were incubated 24 h at 37°C and 42°C.

2.16. Sterol extraction

Yeast cells were grown to saturation in YNB medium containing 2% glucose, shaking 200 rpm at 30°C. Cells (10^8 cells.ml^{-1}) were harvested by centrifugation for 5 min at 3000 *g*, transferred to stop glass tubes and resuspended in 5 ml of methanol, 3 ml of 60% KOH and 2 ml of 0.5% (w/v) pyrogallol dissolved in methanol. Additionally 50 μl of 1 mg.ml^{-1} cholesterol was added to the control sample. The

samples were saponified by heating at 90°C for 2 hours. Sterols were extracted using two 5 ml aliquots of hexane followed by evaporation to dryness with N_2. The isolated sterol fractions were resuspended in 100 µl of toluene and heated at 60°C for 1 h for silylation with 20 µl of BSTFA. The sterol samples were analyzed by gas chromatography/mass spectrometry using split injections at a ratio of 20:1. Sterol identification was determined by comparison of retention times and mass spectra by gas chromatography-mass spectrometry (GC/MS) (Joseph-Horne et al. 1996).

2.17. Determination of dityrosine content

Determination of dityrosine content of yeast cultures was performed (Rudge et al., 1998). Yeast cultures ($1x10^8$ cells) were collected by centrifugation, washed twice with sterile water, and resuspended in 1 ml of 10% ammonium hydroxide. Cell suspensions were maintained at -80°C overnight, thawed, and lysed by repetitive vortexing (six times for 30s each) with equal volume of glass beads (425-600 µm, acid washed, Sigma) at 4°C. Chloroform:methanol (1:1, 2.5 ml, Sigma), was added, and the mixture was mixed for 2 min at room temperature. The upper phase was collected after centrifugation, and the lower phase was washed with fresh 10% ammonium hydroxide (Sigma). The two upper phases were pooled, and 1.5 ml were prepared by centrifugation at 16000 rpm for 10 min at room temperature. Finally, 1 ml was removed, and the dityrosine content was determined by fluorescence spectrophotometry. Fluorescence was measured at room temperature with a Fluorescence Spectrophotometer F-2500 (Digilab Hitachi, Tokyo, Japan). Measurements were made in a semimicrovuvette. Intensity of emission was measured at 420 nm at excitation of 320 nm (Briza *et al.* 1986).

2.18. Synthesis of dityrosine standards

2.18.1. Reagents used in the *in vitro* dityrosine production

All reagents used were of analytical reagent grade purchased from Sigma. *N*-Formyl tyrosine solution (10 $\mu M.ml^{-1}$) were dissolved in ammonium hydrogen carbonate and were kept at 4°C. The working solution of hydrogen peroxide (H_2O_2) was 0.6%. The enzyme solution consisted of horseradish peroxidase (HRP) at concentration 3 $mg.ml^{-1}$ stock solution.

2.18.2. *In vitro* Dityrosine synthesis

Dimerization of the tyrosine employed the enzyme horseradish peroxidase. HRP, a low molecular weight peroxidase, has a heme cofactor, a Fe (III) center bound to a porphyrin ring. H_2O_2 can react with the heme to generate ferryl, which can directly or indirectly, via HRP structure, react with the tyrosine residue of the monomer to generate phenoxy radicals (Fig. 2.7). After the coupling of two phenoxy radicals and tautomerization, the complete dimer is formed (Fig. 2.7). The yield during dimerization can be suppressed by the formation of many side products. For example, trimers can form from further enzymatic coupling of a dimer and a monomer.

Dityrosine Formation

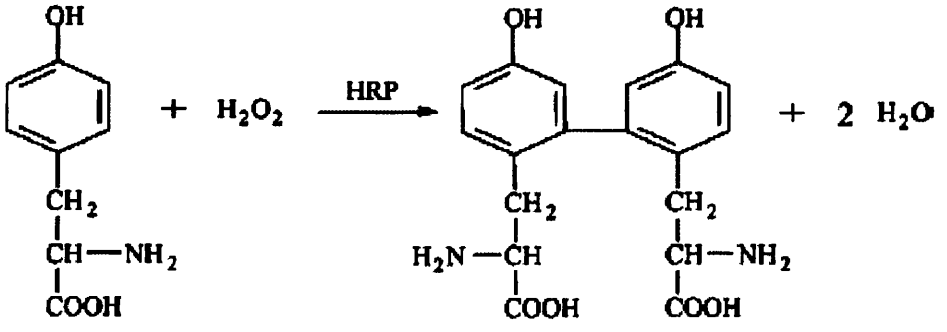


Fig. 2.7. Dimerization of the tyrosine employed the enzyme horseradish.

The dityrosine assays (Amado et al., 1984) were performed in 2 ml containing *N*-formyl tyrosine solution (2 $mg.ml^{-1}$) dissolved in 0.1 M ammonium hydrogen carbonate, and 0.3 mM H_2O_2. The reaction was started by adding HRP (10 $\mu g.ml^{-1}$), the mixture was equilibrated at room temperature for 30 min. Control samples were single L-tyrosine or *N*-formyl tyrosine, without H_2O_2 or HRP. The fluorescence excitation spectra were collected on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The excitation wavelength of dityrosine fluorescence was 320 nm and relative intensity were measured against a reagent blank. The emission of the oxidation product was 400-405 nm. Using vacuum centrifuge (Heto, Maxi Drier, UK) the products of reaction were evaporated down to dryness to concentrate the product that was stored at -80.

2.18.3. Isolation and purification of dityrosine

2.18.4. Thin-Layer Chromatography assay

TLC was used to characterize the dityrosine synthesised as described above. The concentrated samples were resuspended in 100 µl of 10% ammonium hydroxide then loaded on 20 cm long silica gel plates (silica gel 60F, Merck, Germany) (modified Method Duisburg Essen). The following solvent system was used, n-propanolol – ammonium hydroxide (70:30, v/v). Development of the TLC took 6 hours at room temperature in a glass solvent tank. The developed TLC plate was air-dried and the separated products were visualised by ultraviolet light (254 nm) and fluorescence spots were identified. The broad zone of *N*- formyl dityrosine was very well separated from trimer and tetramer of *N*- formyl dityrosine. The fluorescent zone corresponding to *N*-formyl dityrosine was scraped off and, extracted with 10% ammonium hydroxide. The extracts were evaporated to dryness and redissolved in

10% ammonium hydroxide. The fluorescence of the extract was examined using by Hitachi F-2500 fluorescence scanning spectrophotometer (Hitachi, Tokyo, Japan). The fluorescence measurements of the *N*-formyl dityrosine were performed at an excitation wavelength of 320 nm and relative intensity were measured against a reagent blank, the emission of the oxidation product was measurable at 400-405 nm.

2.18.5. High performance liquid chromatography analysis

The samples were analysed by HPLC-MS in multiple reaction monitoring mode. The HPLC system used was an Ultimate HPLC pump with FAMOS autosampler with an in-house prepared C18 (Pepmap C18 stationary phase (Dionex, UK)) HPLC column 300μm * 15cm run at a flow rate of $4\mu l.min^{-1}$. The mobile phase used was an isocratic mix of 50/50 0.1% formic acid in water / 0.1% formic acid in methanol. The HPLC column was connected directly to a LCQ XP DECA ion trap mass spectrometer (Thermo Finnigan, UK). It was used in positive ion mode with a spray voltage of 3.5kV, Sheath and Auxillary gas flows of 30% and 10% (arbitary units) respectively, a capillary temperature of 200°C and a capillary voltage of 10V. The mass spectrometer was set to study the MRM transitions for *N*-formyl dityrosine, and it gave a mass spectrum corresponding to that expected for dityrosine.

2.19. Germination test

Cell suspension at 2 x 10^3 concentration were prepared in 1 ml RPMI with 20% bovine calf serum and shaken 200 rpm at 37°C for 2-4 hours before examination for germination microscopically.

2.20. Chlamydospore formation

Strains were cultured on corn meal agar and broth (Difco, US), supplemented with 1% Tween 80 and 20 $\mu g.ml^{-1}$ methionine, at 25°C for 5-7 days or on Rice-Agar-Tween medium (RAT medium, Biomerieux) before microscopic examination of chlamydospore formation. Hyphal formation was induced using 10% calf serum (GIBCO BR, USA), or RPMI-1640 at 37°C for 2 to 4 hours. To produce anaerobic conditions during hyphal formation, Na_2S0_3 (final 50 $mg.ml^{-1}$, Sigma, St. Louis, Mo., USA) was added to RPMI or minimal medium broth as described before (Watanabe et al., 2006). Na_2S0_3 absorbs oxygen present in the medium. Airtight anaerobic containers (Oxoid) were also used. Hyphal formation was examined by Nikon optical microscope and images captured by Nikon 4500 digital camera, at 40x and 100x.

2.21. Confocal microscope analysis

Three sets of cell culture were performed to investigate the blue natural fluorescence presence in *Candida* yeast form, germination and chlamydospore forms. The germination was performed as before and the yeast form was obtained from overnight cell culture in YEPD.

Cells were cultured under specific conditions for microscopic visualization of yeast-like and hyphal growth. Aliquots of culture were transferred to sterile Eppendorf tubes and centrifuged (10,000 x *g*) for 3 min. Supernatants were discarded and each resulting pellet suspended in sufficient 10% (w/v) ammonium hydroxide to support effective image composition and reasonable distribution of experimental material. Subsequently 7.5 µl of each suspension was dispensed into wells of separate multi-spot microscope slides (Hendley, UK), a coverslip affixed and immediately

viewed with a Zeiss LSM 510 Meta Axiovert facility (Carl Zeiss Gmbh, Heidelberg, Germany). Additionally, in a separated procedure cells were stained with 2 µl calcofluor (stock 100 $\mu g.ml^{-1}$) for 5 min and washed twice with Hepes. Cells were dispensed onto microscope slides, dried at room temperature and fixed with 7 µl of acetone, finally 2.5 µl of vectashield was added to improve the fluorescence visualization. Transmission images were obtained with a 5 mW helium-neon laser, wavelength 633 nm, in turn fluorescence images were obtained with a UV 100 W lamp, moderated to 30% intensity and filter set FS01. Gain, offset and zoom functions were employed to optimize fluorescence image intensity, composition and contrast. Slides were viewed with an oil immersion Zeiss Plan-Achromat 63x, 1.4 NA objective, 512 x 512 pixel images captured and exported in TIFF format using Zeiss laser scanning microscope LSM 510 version 3.2 SP2 software.

Chapter 3

Characterization of *Candida* species: Susceptibility studies, Drug accumulation and Sterol Composition

3.1. Introduction

The incidence of fungal infections, particularly infections caused by *Candida* spp., has increased considerably during the past two decades. Among the different species causing infections, *C. albicans* has been isolated in more than 50% of clinical cases (Nguyen et al., 1996, Melo et al., 2004). Also *C. dubliniensis*, which is closely related to *C. albicans* phylogenetically, has been isolated in clinics around the world (Sullivan et al., 1995, Sano, 2000). In previous investigations, *C. dubliniensis* was isolated from the oral cavities of 27% of human immunodeficiency virus (HIV)-infected subjects and 32% of AIDS patients with oral candidosis (Coleman et al., 1997, Espinel-Ingroff et al., 1999). *C. dubliniensis* has also been recovered from the oral cavities of asymptomatic and symptomatic immunocompetent individuals, although to a much lesser extent. *C. dubliniensis* was first described in South America by Rodero *et al.* (Cuenca-Estrella et al., 2002). Transmission of genetically indistinguishable strains of *C. albicans* between HIV-infected adult partners has been reported previously (Muller et al., 1999). Little is known about the transmission of *C. dubliniensis* between children and within families (Muller et al., 1999). The majority of *C. dubliniensis* clinical isolates tested to date are susceptible to several antifungal agents (Moran et al., 1997).

Azole antifungals (eg fluconazole, itraconazole) are commonly used to treat *C. albicans* infections. Due to the repeated use of these agents, especially in HIV-positive patients with recurrent oropharyngeal candidosis, treatment failures were observed to correlate with the emergence of resistant *C. albicans* strains (Kelly et al., 1991, Sanglard and Odds, 2002, Kelly et al., 1996). The success of antifungal

treatments depends on the patient's status, the type of antifungal agent given, and the biological response of the fungal pathogen. Among the different antifungal agents available, the class of azoles has been used extensively during the past 20 years and their mode of action is through inhibition of CYP51, sterol 14α-demethylase (Erg11p).

To date, four resistance mechanisms have been suggested in the development of azole resistance in *C. albicans* with varying levels of evidence: (i) the cellular content of the azole target encoded by the *CYP51* gene can be increased; (ii) the affinity of CYP51 for azoles can be decreased by mutations in *CYP51*; (iii) the ergosterol biosynthetic pathway can be altered to suppress the effect of azoles by altering the type of sterol accumulating with treatment; and (iv) azole concentration can fail to accumulate to the same level inside the cells through altered drug efflux (Sanglard et al., 1998, Sanglard and Odds, 2002).

Among these mechanisms, increased drug efflux is the most commonly identified mechanism in clinical strains developing azole resistance, although usually comprehensive examination of all mechanisms is not undertaken in the literature. This mechanism usually involves the upregulation of multidrug transporter genes from at least two families, i.e. *CDR1* and *CDR2* (*Candida* drug resistance 1 and 2) of the ATP-binding cassette (ABC) transporter family (Prasad et al., 1995, Orozco et al., 1998, Sanglard and Odds, 2002) and *CaMDR1* (*Candida albicans* multidrug resistance 1) of the major facilitator (MF) superfamily (Franz et al., 1998, Sanglard et al., 1998, Sanglard et al., 1995). Transcriptional activation of both *CDR1* and *CaMDR1* genes as a result of different environmental stresses have also been reported (Krishnamurthy et al., 1998a, Krishnamurthy et al., 1998b, Sanglard and Odds, 2002).

The transcription of *CaMDR1*, the gene encoding CaMdr1p, is almost undetectable in azole susceptible isolates but is raised in some azole resistant isolates. In contrast, the transcription of *CDR1*, the gene encoding Cdr1p, is detectable in azole-susceptible isolates, but is increased to higher levels in some azole-resistant isolates. The upregulation of multidrug transporters in the development of antifungal resistance is also well described in the baker's yeast *Saccharomyces cerevisiae*. Like *C. albicans*, *S. cerevisiae* possesses ABC transporters or MFS transporters that are able to confer resistance to antifungal drugs (Goffeau et al., 1997).

This chapter describes work investigating the prevalence of *C. dubliniensis* infection in a Brazilian family with an HIV-infected child. Additionally, azole susceptibility including azole antifungal accumulation in various *Candida* species, sterol composition and the relationship with measurements of inhibitory potency was investigated. Some different patterns of azole resistance exhibited by various *Candida* species, investigated here, suggest that more than one mechanism of resistance may be active. Therefore a single parameter, such as intracellular drug accumulation levels, should be associated with other parameters in the investigation of fungal resistance. Further studies including molecular analysis are warranted to further investigate whether intra cellular drug accumulation of fluconazole provides supporting data on azole sensitivity.

3.2. Material and Methods

3.2.1. Materials

Chemicals were of analytical grade and are listed in the Chapter 2.13.1.

3.2.2. Strains used in this study

Fluconazole-susceptible strains used were *Candida albicans* ATCC 90028, ATCC 28516 and *Candida dubliniensis* (CBS 7889). The other strains and clinical isolates used are listed in the Chapter 2.6.9.

3.2.3 Subjects enrolled in the *C. dubliniensis* study

One HIV-infected child who acquired HIV vertically is being monitored at the Paediatric Immunodeficiency Outpatient Service, Medical School, Campinas State University. The child's parents died from HIV. The oral flora from the HIV-infected child and family members was investigated. A total of 42 oral isolates were obtained from eight family members (Table 3.1).

Table 3.1.

Characteristics of the eight family members with an HIV-infected child

Name	Kindred	Age (years)	Race	Gender
LPS	HIV child	5	white	Male
MLA	Grandmother	55	white	Female
LA	Aunt	25	white	Female
PA	Uncle	20	white	Male
CA	Uncle	21	white	Male
CHA	Cousin	2	white	Male
CAMA	Cousin	7[a]	white	Female
JA	Cousin	6	mulatto	Female

[a]months

3.3. Results and Discussion

Different *Candida* species have been investigated in terms of their antifungal susceptibility and drug accumulation within this chapter. Antifungal susceptibility exhibited by determined MIC values varied greatly among *Candida* isolates. The intracellular drug accumulation levels, which are also a balance of uptake of azoles and active efflux, varied and were species dependent. Non-*albicans* isolates, such as *C. krusei*, *C. lusitaniae* and *C. norvegensis,* exhibited a high intracellular [^{3}H] fluconazole concentration in this study (Table 3.2, Fig. 3.1). In contrast *C. albicans* and *C. dubliniensis* strains exhibited lower intracellular drug concentrations. Most of the *C. krusei*, *C. valida* and *C. norvegensis* strains were azole resistant according to MIC data. However, all *C. lusitaniae* and *C. dubliniensis* isolates were sensitive to fluconazole with MICs in the range 0.25 to 4 $\mu g.ml^{-1}$.

Table 3.2.

MIC_{80}s, intra cellular [^{3}H] fluconazole accumulation values and total percentage ergosterol level in *Candida* species.

Strain	MIC_{80}s (µg.ml^{-1})			[^{3}H] fluconazole (MD ± SD) (dpm/10^9 cells)	Ergosterol (%)
	AMPH B	FLCZ	ITCZ		
C. albicans					
ATCC 90028*	1	1	0.5	80.5 ± 12.8	93.7
ATCC 28516*	1	1	0.5	73.4 ± 6	82.2
1-MAB	8	>64	8	45 ± 4.4	76
(n=3)	1-8	1-64	0.5-16	**66 ± 8**	
C. krusei					
9C	1	16	4	169.6 ± 75.5	71
21	2	8	4	323.4 ± 38.9	85
46	0.5	32	4	221.9 ± 129.9	32
53	4	64	4	333 ± 128.1	77
88	2	64	8	288.3 ± 169.6	73.4
123B	2	32	4	201.6 ± 71.2	79
127D	2	64	4	70.8 ± 15.2	75
129B	2	64	8	168.8 ± 62.6	76.7
1-RAO	16	64	4	123 ± 33	79
ATCC 6288	0.5	32	0.5	175.5 ± 81.8	92
(n=10)	**0.5-16**	**8-64**	**4-8**	**227 ± 141.5**	
C. lusitaniae					
19-A1	0.25	0.25	0.5	182.1 ± 55.8	83.4
25-B	0.5	1	0.5	165.3 ± 61.3	78.6
75-A	1	2	2	173.9 ± 25.1	69.8
76-A	1	2	2	263.7 ± 68.2	72.9
110-A	2	4	4	180.7 ± 69.6	74
121-A	1	4	2	243.8 ± 151.3	70
(n=6)	**0.25-2**	**0.25-4**	**0.5-4**	**201.5 ± 19.5**	
C. norvegensis					
186-C	2	64	4	92.3 ± 59.4	80
3-MASC	2	32	2	162 ± 47	92.3
(n=2)	**2**	**32-64**	**2-4**	**127 ± 49.5**	
C.valida					
1-MBJ	2	64	2	**119.4 ± 41.2**	93
C. dubliniensis					
9-LPS	2	0.5	1	46 ± 11.5	45.4
2-MLA	2	0.125	1	94.6 ± 26.1	79.5
IFM 48313	2	0.125	0.125	96.2 ± 17.5	49.5
(n=3)	2	0.125-5	0.125-1	**79 ± 18.3**	

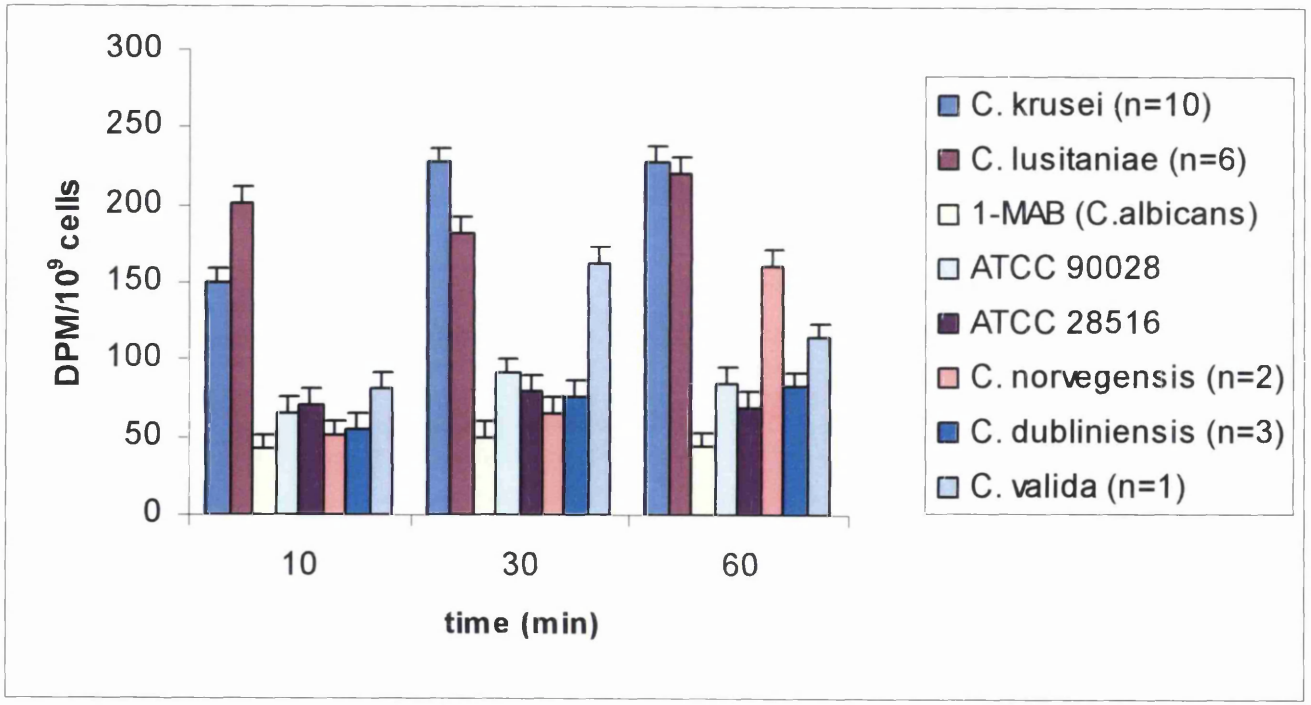


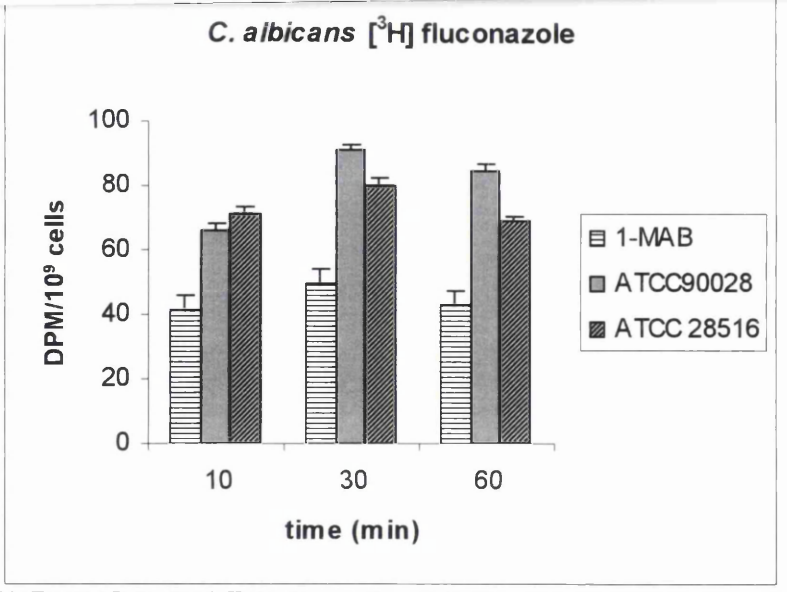
Fig. 3.1. [^{3}H] fluconazole accumulation in *Candida* clinical isolates.
***C. albicans* ATCC 90028 and ATCC 28516 are reference strains. Values for each time are the means of triplicate determinations with cells from different experiments.**

In addition these isolates have also shown drug accumulation involves an active efflux component demonstrated by the effect observed in the presence of sodium azide (an agent that blocks ATP-dependent transporters).

Three *C. albicans* strains were investigated, among them two reference strains that were fluconazole-sensitive and one *C. albicans* clinical isolate (Table 3.2). The *C. albicans* reference strains were sensitive to all drugs tested and showed 80.5 and 73.4 dpm/10^9 cells of intra cellular [^{3}H] fluconazole accumulation, respectively.

The clinical isolate (1-MAB) showed cross-resistance to amphotericin B and fluconazole (Table 3.2) with a MIC of 8 $\mu g.ml^{-1}$ and >64 $\mu g.ml^{-1}$ respectively compared to 1 $\mu g.ml^{-1}$ and 1 $\mu g.ml^{-1}$ in the sensitive isolates. The cross-resistant *C. albicans* clinical isolate was found to accumulate approximately half the amount of [^{3}H] fluconazole (44.9 ± 4.4 dpm/10^9 cells) compared with the reference strains and was shown to accumulate approximately 3.5 times more [^{3}H] fluconazole in the presence of sodium azide (Fig. 3.3). The altered patterns of fluconazole accumulation were not reduced to the same extent in strains containing mutations changing the expression of azole drug pumps as reported previously (Odds et al., 2004).

C. dubliniensis and *C. albicans* are the closest species phylogenetically among those studied here in terms of taxonomy and biological characteristics (Moran et al., 1998). All *C. dubliniensis* isolates investigated were antifungal-sensitive. Similar [^{3}H] fluconazole intracellular concentrations were found between *C. dubliniensis* (96.2 ± 17.5 dpm/10^9 cells) and *C. albicans* (80.5 ± 12.8 dpm/10^9 cells) reference strains in this study (Table 3.2). In contrast one *C. dubliniensis* clinical isolate showed half the amount of [^{3}H] fluconazole intra cellular accumulation (46 ± 11.5 dpm/10^9 cells) compared to the reference CBS strain (Fig. 3.3).



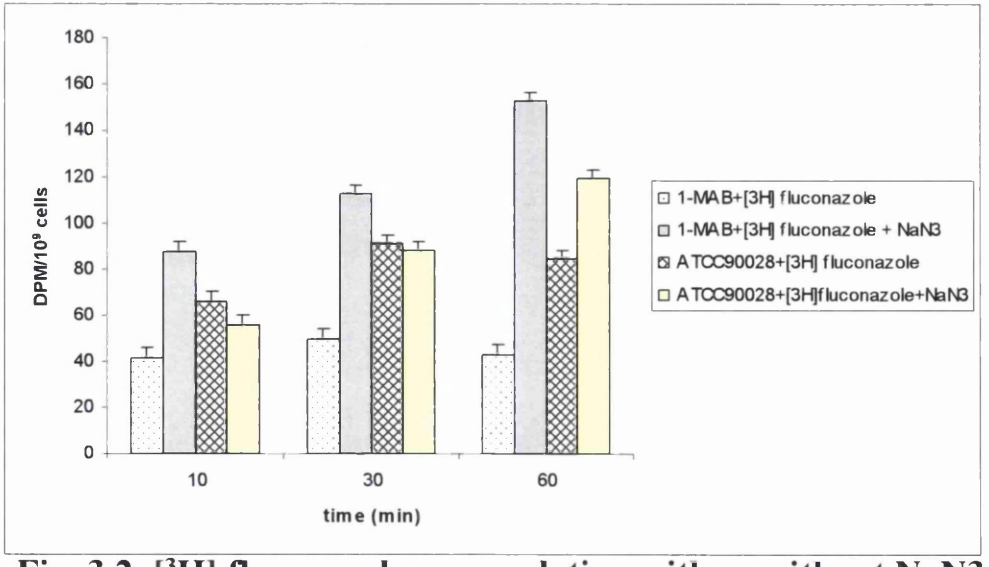
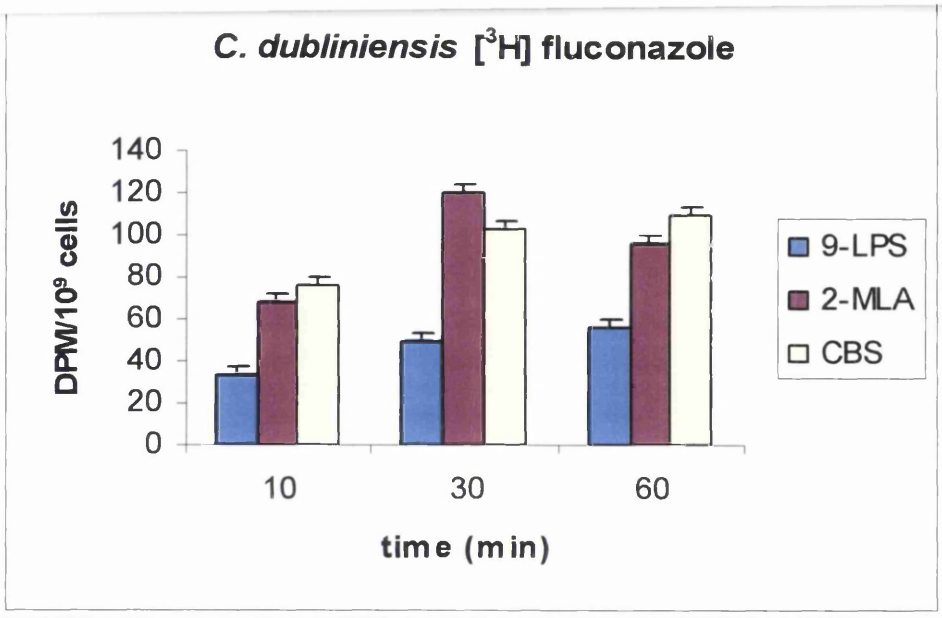


Fig. 3.2. [^{3}H] fluconazole accumulation with or without NaN3. *C. albicans* isolate (1-MAB); ATCC 90028 and ATCC 28516 (*C. albicans* reference strains).



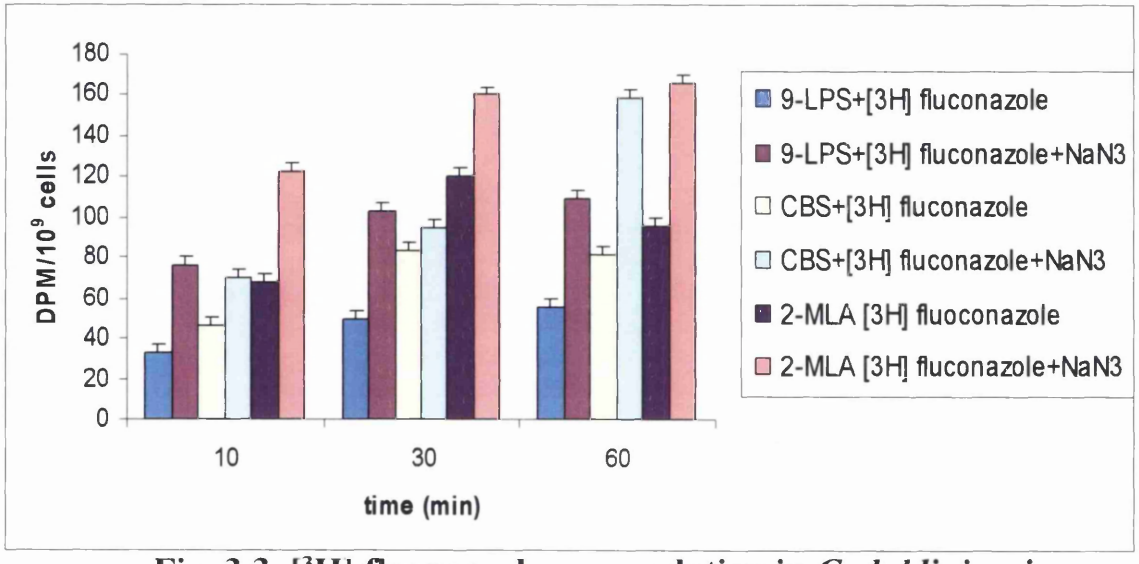


Fig. 3.3. [^{3}H] fluconazole accumulation in *C. dubliniensis* (9-LPS and 2-MLA) clinical isolates and CBS control.

Antifungal resistance described to date generally involves the emergence of naturally resistant species as in *C. krusei* or the progressive, stepwise alterations of cellular structures or functions to block the activity of an antifungal agent to which there has been extensive exposure. *C. krusei* was among the fungal species studied here. The reference strain in this study exhibited dose-dependent sensitivity to fluconazole and was sensitive to amphotericin B with a MIC of 32 $\mu g.ml^{-1}$ for fluconazole and 0.5 $\mu g.ml^{-1}$ for amphotericin B (Table 3.2). Some *C. krusei* isolates showed higher intracellular [^{3}H] fluconazole concentrations and others exhibited lower intracellular concentrations compared to the reference strain (Fig. 3.4A). Most of the clinical isolates showed MICs values comparable to the fluconazole resistance reported elsewhere. Exceptions in the susceptibility testing to antifungal agents were found in two isolates (21C and 9C), that showed MICs of 8 $\mu g.ml^{-1}$ and 16 $\mu g.ml^{-1}$ for fluconazole, respectively and hence were more sensitive. Moreover both isolates exhibited similar levels of [^{3}H] fluconazole accumulation compared to the reference strain. The majority of the isolates, with similar levels of drug accumulation compared to reference strains, were azole resistant (high MIC values). One of *C. krusei* clinical isolates (127D) studied here showed low levels of [^{3}H] fluconazole intracellular concentrations (70.8 ± 15.2 dpm/10^9 cells), 2.5-fold less compared to *C. krusei* reference strain and the MIC for fluconazole was 64 $\mu g.ml^{-1}$. This isolate was resistant to amphotericin B as well as fluconazole (Table 3.2) although efflux is not thought to play a role in amphotericin resistance.

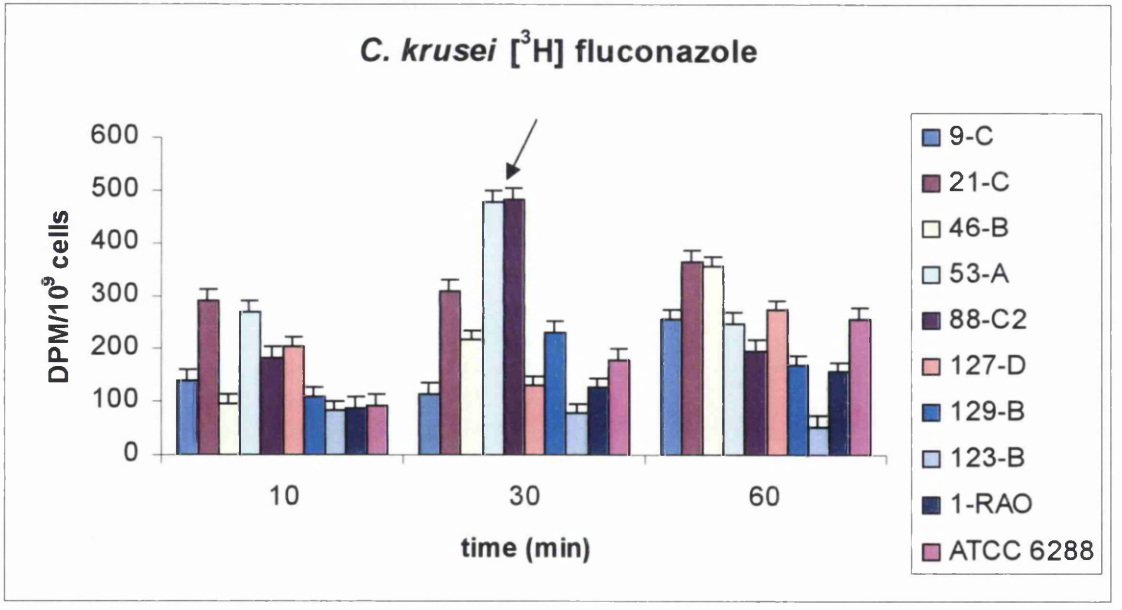


Fig.3.4A. Histogram shows [^{3}H] fluconazole accumulation in *Candida krusei* clinical isolates and ATCC 6288 reference strain.
After 30 min the 53-A and 88-C2 isolates exhibited high level (black arrow) of intra cellular [^{3}H] fluconazole accumulation compared to ATCC reference.

Amongst the strains, *C. krusei* 53-A and 88-C2 isolates exhibited a high intracellular [^{3}H] fluconazole concentration after 30 min of incubation (Fig. 3.4A) when compared to the reference strain. After 60 min both isolates showed decreased intracellular fluconazole concentrations, less than the reference strain and this could reflect differences in efflux mechanisms. Both isolates were fluconazole resistant as indicated by MIC data (Table 3.2). In addition, the 53-A isolate showed a high MIC value of 4 $\mu g.ml^{-1}$ for amphotericin B. Hence this isolate shows cross-resistance. The *C. krusei* isolates 21C and 46B after 60 min of incubation with [^{3}H] fluconazole exhibited higher intracellular drug concentrations compared to the reference strain and this was/ was not reflected in the altered MIC value to the same drug. Although some *C. krusei* isolates exhibited higher intracellular fluconazole concentrations compared with *C. krusei* reference strain, they showed higher MIC values suggesting variation in the tolerance of the intracellular fluconazole concentration. All *C. krusei* isolates were shown to accumulate approximately 1.5 times more [^{3}H] fluconazole in the presence of sodium azide (Fig. 3.4B) supporting a role for active drug efflux via the ABC transporters superfamily. In general the intracellular [^{3}H] fluconazole concentration levels in *C. krusei* isolates were higher compared to those in *C. albicans* strains (Table 3.2).

Consequently, *C. krusei* clinical isolates were fluconazole-resistant with high levels of intracellular fluconazole tolerance. This general fluconazole resistance phenotype observed elsewhere was confirmed with experimental evidence that drug efflux mechanisms may play a role. A role for other mechanisms cannot be excluded.

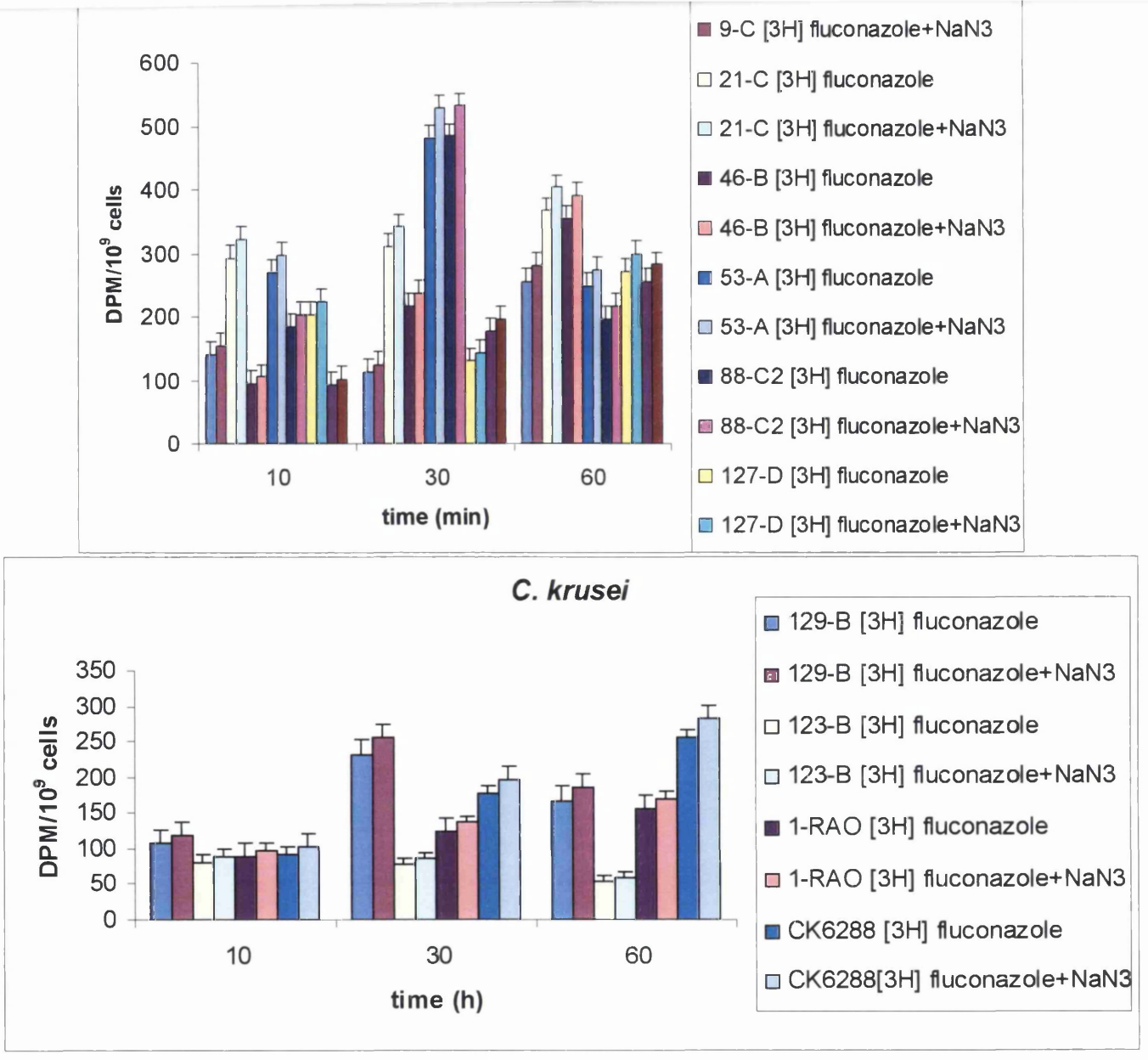
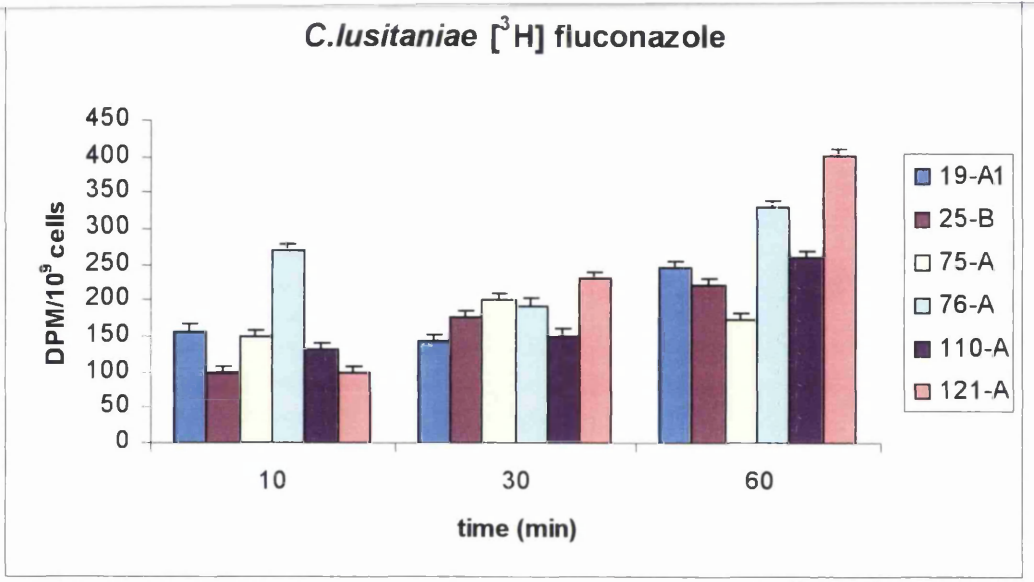


Fig. 3.4B. [^{3}H] fluconazole accumulation with or without NaN_3 in *Candida krusei* isolates.

The *C. lusitaniae* clinical isolates showed a narrow range of intracellular [^{3}H] fluconazole concentration levels (Fig. 3.5). Additionally, all *C. lusitaniae* isolates showed approximately 1.5 times more [^{3}H] fluconazole accumulation in the presence of sodium azide (Fig. 3.5). However, the intracellular [^{3}H] fluconazole concentration was higher compared to *C. albicans*, but similar to that of the *C. krusei* isolates. Unlike *C. krusei*, all *C. lusitaniae* isolates investigated were susceptible to the antifungal agents tested (Table 3.2). However, the literature has reported frequent resistance incidences for amphotericin B in *C. lusitaniae* isolates (Ghannoum and Rice, 1999, Noel et al., 2005, Peyron et al., 2002). The study here also included rare non-*albicans* species including *C. norvegensis* (n=2) and *C. valida* (n=1) which were shown to be azole-resistant.

They also showed slightly higher levels of intracellular concentrations of [^{3}H] fluconazole compared to *C. albicans* (Table 3.2). The exception was a *C. novergensis* isolate, which showed similar levels of intracellular concentrations of fluconazole to the *C. albicans* reference strain.



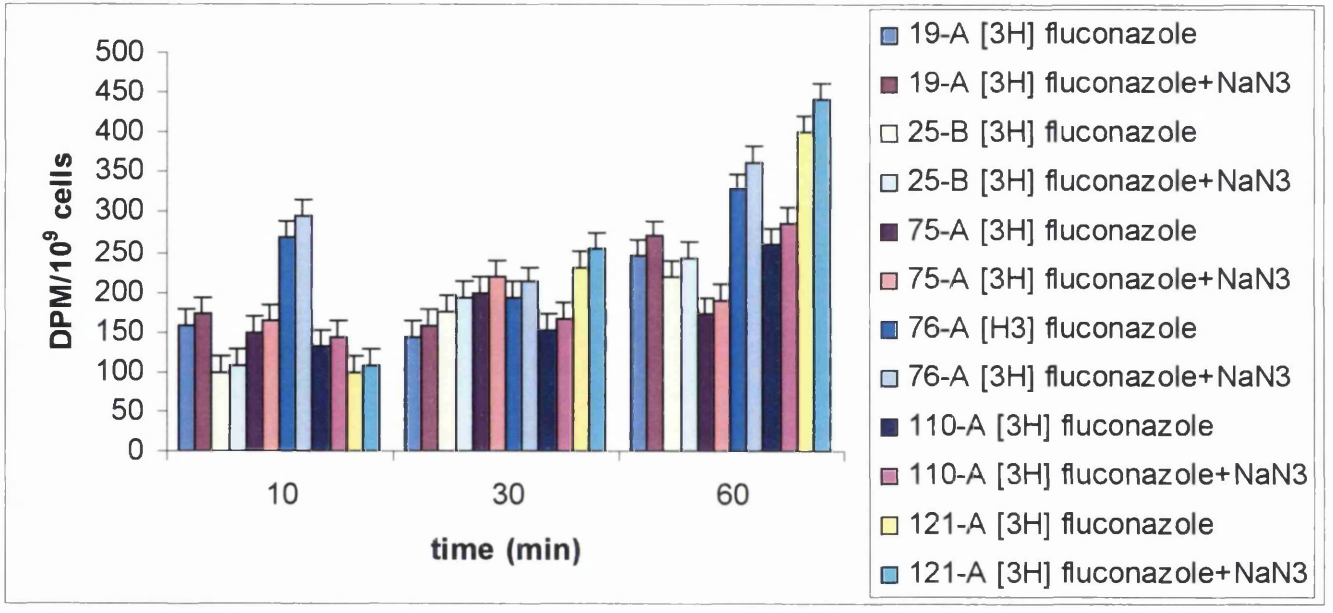


Fig. 3.5. Histogram shows results for [^{3}H] fluconazole accumulation with or without NaN_3 in *Candida lusitaniae* clinical isolates.

In the present work, the ergosterol content in *Candida* species was also investigated to reveal whether mutations in sterol biosynthesic enzymes correlated with amphotericin sensitivity and also to identify if the known azole-resistance phenotype of *Erg3* mutants was operating. The predominant sterol observed in most strains was ergosterol and the level of this sterol varied between 70-92% of the total sterol isolated with only a few exceptions (Table 3.2). The *C. krusei* reference strain ATCC 6288 produced 92% of ergosterol. Nine clinical *C. krusei* isolates were investigated and analysis of the sterol content demonstrated that ergosterol was the predominant sterol in eight of these isolates. An exception was the *C. krusei* isolate (46) which showed an ergosterol content of only 32% with the concomitant accumulation of lanosterol (16% of the total sterol content). Additionally, the MIC value for fluconazole of this isolate was 32 $\mu g.ml^{-1}$ and intracellular fluconazole concentration value was similar when compared to the *C. krusei* reference strain. On the other hand, the MICs for fluconazole varied among *C. krusei* isolates and showed 5 isolates with high MIC > 64 $\mu g.ml^{-1}$, 4 isolates with a MIC of 32 $\mu g.ml^{-1}$ and one isolate with a MIC of 8 $\mu g.ml^{-1}$. Interestingly, because isolate (46) accumulated 16% lanosterol (Table 3.2) this may indicate some defect in the CYP51 turnover of substrate in the sterol pathway.

In the remaining *C. krusei* isolates the sterol pattern was unaltered comprising predominantly ergosterol, although the MICs for fluconazole in general were very high. This may suggest that in *C. krusei* other resistance mechanisms, rather than changes in the sterol pathway, are involved. The isolate (1-RAO) revealed cross-resistance to amphotericin B with a MIC 16 $\mu g.ml^{-1}$ and fluconazole MIC > 64 $\mu g.ml^{-1}$, but surprisingly the sterol profile was unaltered with 79% of ergosterol present. The

sterol profile, with retention of ergosterol at a high percentage of total sterols present in the resistant isolate indicates no mutations of the enzymes involved in the ergosterol biosynthetic pathway. Amphotericin is believed to interact with ergosterol to mediate fungicidal effects, but no large scale sterol changes of the sort produced by polyene resistance in other studies were evident in this resistant isolate (Kelly et al., 1997, Jackson et al., 2003, Venkateswarlu et al., 1995). However, Kelly *et al.* has previously demonstrated that lack of ergosterol in *C. albicans* can be the cause of cross-resistance to amphotericin B (Kelly *et al.*, 1997).

The *C. krusei* isolates in these experiments have shown several different resistance profiles. The findings suggest that more than one antifungal resistance mechanism is active here. This organism has been described to be intrinsically resistant to fluconazole (Ghannoum et al., 1999, Marichal et al., 1995). In the literature there is substantial variability in the reported fluconazole MIC for *C. krusei* (Venkateswarlu et al., 1997, Fukuoka et al., 2003). Possibly these differences can be attributed to differences in the methodology or strain variations in the mechanism of antifungal resistance. Two mechanisms of azole resistance in *C. krusei* have been described previously. Isolates of *C. krusei* that were resistant to itraconazole exhibited reduced drug accumulation, suggesting that resistance to this drug was due to the activity of one more drug efflux pump(s) (Venkateswarlu et al., 1996). A second mechanism of azole resistance in *C. krusei* was reduced susceptibility of CYP51 to inhibition by the azole antifungal agent (Orozco et al., 1998). In this study, the CYP51 activity in cell extracts of *C. krusei* were 16 to 46-fold more resistant to inhibition by fluconazole than was CYP51 activity in cell extracts of two fluconazole-susceptible strains of *C. albicans*. Marichal *et al.* (1995) has indicated that differences in susceptibility to

itraconazole and ketoconazole are unrelated to differences in affinity for the *C. krusei* CYP51 (Marichal et al., 1995). The differences in sensitivity of *C. krusei* isolates appeared to arise from differences in the intracellular itraconazole, ketoconazole and fluconazole contents.

C. lusitaniae is an emerging opportunistic pathogen recently recognized as an important cause of nosocomial infection in severely immunocompromised patients (Young et al., 2003). It has been reported that *C. lusitaniae* strains may be intrinsically resistant to antifungal agents (Ghannoum and Rice, 1999). Resistance to amphotericin B in yeasts has been considered very rare (Rex et al., 1997, Guinet et al., 1983). Here six clinical *C. lusitaniae* isolates from HIV-infected patients were analysed and the sterol composition from the strains determined. Prior analysis of the isolates in this study has indicated that all clinical isolates were sensitive to amphotericin B (Table 3.2), similar data has been reported elsewhere (Favel et al., 2003, Pfaller et al., 2002). Analysis of the sterol profile of the clinical isolates revealed that ergosterol was the major sterol, accounting for 74% of the total sterols (Table 3.2). Peyron and collaborators have also reported that ergosterol was the predominant sterol in a *C. lusitaniae* amphotericin B-sensitive isolate (Peyron et al., 2002). That particular isolate had MIC to amphotericin B of 2 $\mu g.ml^{-1}$, which was slightly higher than those determined for the isolates tested here. In contrast, the amphotericin B-resistant strain reported by Peyron *et al.* had a different sterol composition indicating a defect in the $\Delta 8 \rightarrow 7$ isomerisation step. Amphotericin B is believed to interact with ergosterol in the plasma membrane to mediate fungicidal effects (Kelly et al., 1997, Jackson et al., 2003, Venkateswarlu et al., 1995), but no large scale sterol changes of the sort produced by polyene resistance in other studies

were evident here. The management of infections due to *C. lusitaniae* should consider the possibility of polyene resistance, but seemingly isolates can exhibit sensitive phenotypes and typical ergosterol contents.

Two *C. glabrata* isolates were studied in which the sterol composition showed 65% ergosterol (Table 3.3). Both strains revealed increased amounts of the sterol intermediates, but the accumulating sterols observed do not indicate specific mutation of the specific enzymes involved in sterol biosynthetic pathway (Table 3.3). These isolates were sensitive to antifungal agents (data not shown). *C. glabrata* has emerged as a common cause of disease seen in the outpatient setting and can potentially exhibit reduced susceptibility to azole antifungals (Bard et al., 2005, Nakayama et al., 2001, Hazen et al., 2005). Consistently, this organism has been described to be an intrinsically resistant *Candida* species similar to *C. krusei* (Nakayama et al., 2001, Melo et al., 2004). The major abundant sterol in *C. glabrata* is ergosterol (Nakayama *et al.*, 2001). This work has shown that amongst two *C. glabrata* clinical isolates the predominant sterol was also ergosterol (Table 3.3). Interestingly *C. glabrata* has ability to uptake exogenous sterol when ergosterol biosynthesis is blocked. Specifically, *C. glabrata* can use cholesterol from the human host and thus compensate the azole inhibition of ergosterol biosynthesis. The decreased susceptibility to azoles and the requirement of exogenous sterol for growth suggest that some *C. glabrata* isolates may contain defects in ergosterol biosynthesis (Bard et al., 2005).

Table 3.3. Ergosterol composition among 8 different *Candida* spp.

Candida spp.	n	Mean % Ergosterol	Range % Ergosterol
C. krusei	10	71.6	32-92
C. lusitaniae	6	74	69-83
C. parapsilosis	10	78.2	60-85
C. norvegensis	2	92.3	80-92
C. valida	1	93	93
C. dubliniensis	3	51.5	45-79
C. glabrata	2	65	60-70
C. albicans	3	84	76-94

Analysis of the sterol composition of the *C. parapsilosis* clinical isolates in this work revealed that ergosterol was the predominant sterol in all isolates examined (Table 3.4). Additionally, all isolates were susceptible to the antifungal agents tested (data not shown). Similar results for other *C. parapsilosis* strains were reported recently (Magill et al., 2006, San Miguel et al., 2005, Colombo et al., 2006). Rare non-*albicans* species also included in this study included *C. norvegensis* (n=2), and *C. valida* (n=1) which were azole-resistant. Surprisingly they revealed an unaltered sterol composition (Table 3.2, Fig. 3.6), reinforcing the high diversity of resistance mechanism in *Candida* species. This underlines the explanation for recent emergence of non-*albicans* species causing *Candida* infection diseases in the clinic.

Several mechanisms have been reported to be associated to resistance to amphotericin B and azoles in *C. albicans* (Ghannoum and Rice, 1999). In this study three clinically isolated *C. albicans* strains were investigated, among them two reference strains were fluconazole-sensitive that showed more than 82% of total ergosterol in the cell membrane. One *C. albicans* clinical isolate (1-MAB) was resistant to amphotericin and fluconazole. However, the sterol composition indicated normal level to ergosterol (Table 3.2). *C. albicans* seemingly depends solely on endogenous ergosterol synthesis and no mechanism of sterol uptake has ever been reported (Kurtz and Marrinan, 1989, Nakayama et al., 2000, Tsai et al., 2004). On the other hand Dumitri *et al.* (Dumitru et al., 2004) have reported that anaerobic growth in *C. albicans* can taken place in the absence of exogenous sterol. Ergosterol biosynthesis requires oxygen for a number of steps hence the validity of this study is open to question.

Table 3.4. Sterol composition of *Candida* spp. isolates.

STEROL	Strains									
	G1	G2	G3	G4	G5*	G6	G7*	G8	G9	G10
	(%) Sterols									
Ergosta-dienol	5.2	-	-	-	3.8	-	4.5	4.2	7.9	4.1
Ergosterol	77	83.6	81.7	83.7	60.3	85.9	70.1	79	72.4	71.4
Ergosta-7 enol	-	-	-	-	-	-	-	-	-	3.2
Obtusifoliol	-	-	-	-	2.2	-	1.3	-	-	-
Lanosterol	-	-	-	-	-	-	4.4	-	-	-
Methylfecosterol	-	-	-	-	8.8	-	-	-	-	-
Ergosta-tetra-enol	7.2	7.6	8.2	6.5	3.8	5.5	-	6.4	6.2	9.1
Dimethyl-cholesta-dienol	-	-	-	-	5.3	-	1.6	-	-	-
Ergosta-7,22 dienol	-	-	-	-	9.3	-	-	-	-	-
Others minor	10.4	8.8	10.1	9.8	21.1	8.5	10.9	10.4	13.4	12.1

G1-G10= *C. parapsilosis*; G*= *C. glabrata*

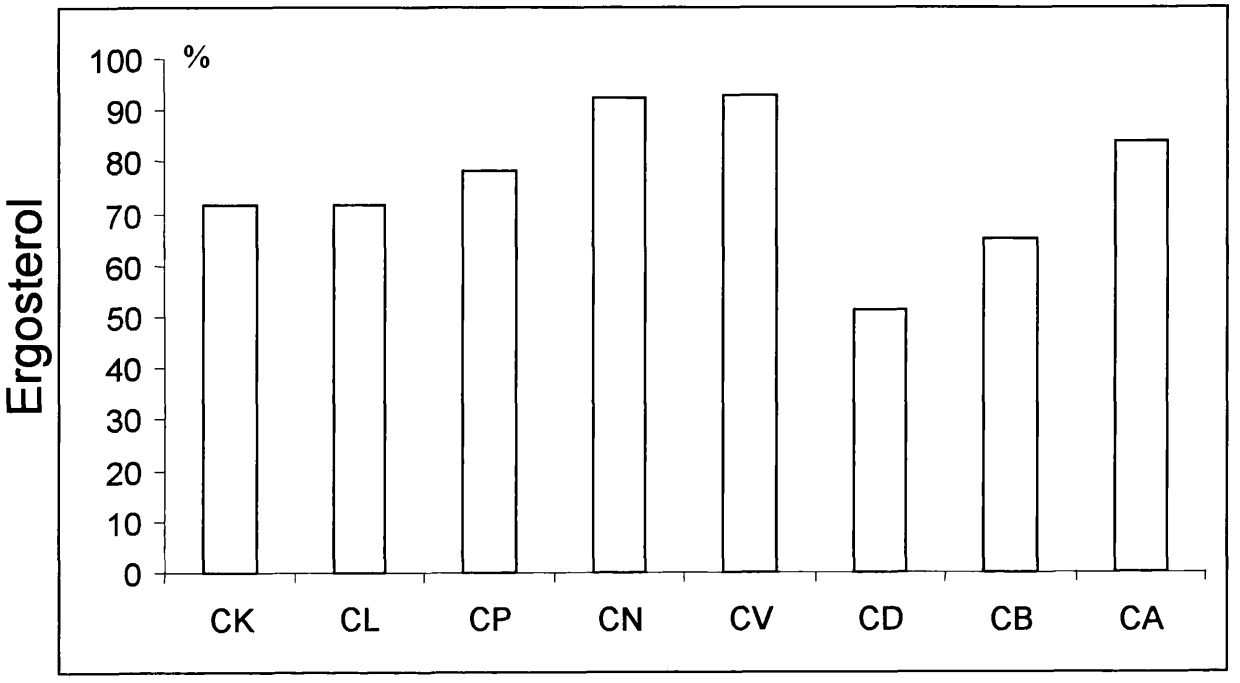


Fig. 3.6. Percentage ergosterol in *Candida* spp. CK= *C. krusei*, CL= *C. lusitaniae*, CP= *C. parapsilosis*, CN= *C. novergensis*, CV= *C. valida*, CD= *C. dubliniensis*, CG= *C. glabrata*, CA= *C. albicans*.

Recently, little attention has been paid to the clinical significance of azole-resistance in paediatric patients (Muller et al., 2000). The oral *Candida* isolate (1-MAB) from the child in this investigation showed low susceptibility to all azoles tested as judged by the MIC_{80}s obtained for fluconazole (≥ 64 $\mu g.ml^{-1}$), voriconazole (8 $\mu g.ml^{-1}$), miconazole (≥16 $\mu g.ml^{-1}$), ketoconazole (≥16 $\mu g.ml^{-1}$), and itraconazole (8 $\mu g.ml^{-1}$). The clinical isolate also showed low susceptibility to amphotericin B (8 $\mu g.ml^{-1}$) (Table 3.5). Caspofungin, a compound active against cell walls of fungi, was a potent inhibitor for all strains tested. This class of drug has shown a limited antifungal spectrum (Niimi et al., 2006). However because it is not structurally and functionally related to the polyenes or azoles drugs, caspofungin cross-resistance was expected to be rare (Hernandez et al., 2004). Therefore one approach to increasing the therapeutic index of antifungal agents is to use them in combinations for synergistic interactions.

Table 3.5.

Drug susceptibilities of the *C.albicans* isolates.

Drug	MIC_{80}s ($\mu g.ml^{-1}$)		
	1-MAB	ATCC 90028	*erg3*
FLC	64	1	≥64
VRC	8	0.5	≥64
ITC	8	0.5	≥64
KTZ	≥16	1	≥64
MCZ	≥16	1	≥16
RT	4	1	-
AMB	8	1	8
CAS	0.03	0.01	0.03

The clinical isolate, 1-MAB, showed a MIC to ritonavir of 4 $\mu g.ml^{-1}$. On the other hand the fluconazole-sensitive strain (ATCC 28516) exhibited a MIC for ritonavir of 1 $\mu g.ml^{-1}$. Thus, one could speculate that the long term use of ritonavir, as part of HIV therapy, could influence the virulence of the clinical *Candida* isolates. In spite of the recent findings of the unexpected benefit of the protease inhibitors against *C. albicans* secreted aspartyl proteases (Pozio, 2004), further experiments are required to establish the value of the HIV protease inhibitors for candidosis treatment.

In the clinical isolate (1-MAB) voriconazole and fluconazole caused 85 % and 65% growth inhibition, respectively, at a concentration of 64 $\mu g.ml^{-1}$. Amphotericin B at a concentration of 8 $\mu g.ml^{-1}$ inhibited 62% of cell growth indicating cross-resistance. 5-flucytosine was effective at inhibiting cell growth of 1-MAB at a concentration of 0.25 $\mu g.ml^{-1}$. 5-flucytosine has been successfully used in combination with amphotericin B in the treatment of several deep mycoses (Gonzalez et al., 1996). The synergistic effect obtained by this drug combination provides an alternative therapy in resistance cases (Hope et al., 2004, Melo et al., 2004, Gonzalez et al., 1996, Martin et al., 1992). Our results suggest that 5-flucytosine may be useful in the treatment of strains showing cross-resistance to polyenes and azoles although more studies are required.

Ergosterol biosynthesis defects are clearly associated with polyene resistance and *erg3* mutants associated with monogenic cross-resistance. Analysis of the sterol profile of 1-MAB revealed that ergosterol remained the predominant sterol (Table 3.2), accounting for 76% of the total sterols with 4-desmethyl sterols (17%). Varing amounts of sterol intermediates (Table 3.6) represent an intrinsic difference between

the strains unrelated to resistance and similar profiles have been observed elsewhere in sensitive strains (Venkateswarlu et al., 1995). The sterol profile with retention of ergosterol at a high percentage of total sterols in the resistant isolate indicates no mutations of the enzymes involved in the ergosterol biosynthetic pathway. Amphotericin is believed to interact with ergosterol to mediate fungicidal effects, but no large scale sterol changes of the sort produced by polyene resistance in other studies were evident in the resistant isolate (Kelly et al., 1997, Jackson et al., 2003, Venkateswarlu et al., 1995).

Table 3.6.

Sterol composition of *C. albicans* isolates.

Sterol identified	Total Sterol fraction (%)		
	1-MAB	ATCC 90028	*erg3*
Ergosterol	76	93.7	-
Ergosta-7,22 dienol	0.15	-	77
Ergosta-7-enol	0.7	-	22
4-desmethyl sterols	17.3	-	-
4-methyl sterols	5.5	-	-
Unidentified	-	6.3	1

The isolate 1-MAB was found to accumulate approximately half the concentration of [^{3}H] fluconazole (44.9 ± 4.4 dpm.10^9 cells^{-1}) than the reference strains and was found to accumulate approximately 3.5 times more [^{3}H] fluconazole in presence of sodium azide (Table 3.7). The altered patterns of fluconazole accumulation were much lower than those associated with mutations changing the expression of drug pumps described elsewhere (Odds et al., 2004).

Table 3.7.

Accumulation of [^{3}H] fluconazole (dpm.min^{-1}) in presence of NaN3 in *C. albicans* clinical isolate (1-MAB).

Strains	[^{3}H] fluconazole (MD ± SD)	[^{3}H] fluconazole + NaN_3 (MD ± SD)
1-MAB	44.9 ± 4.4	152.6 ± 32.8
ATCC 90028 (S)*	80.5 ± 12.8	87.5 ± 31.6
ATCC 28516 (S)*	73.4 ± 6	83 ± 8

*Fluconazole-sensitive

The drug efflux mechanisms of azole resistance do not give rise to amphotericin cross-resistance - hence the novelty of the findings here. Previously, an example of azole and amphotericin cross-resistance not related to a defect in sterol biosynthesis was observed in *C. neoformans* mutants isolated *in vitro* (Joseph-Horne et al., 1996) and similar mechanism(s) may be operating in the clinical *C. albicans* isolate examined here. Alternatively two independent mechanisms of resistance may be occurring although the probability for such multiple events is much lower. Elucidating the genetic cause of the resistance will require molecular analysis. Finally we conclude the presence of cross-resistance to antifungal drugs in paediatric patients, including azoles not previously used in children, does occur.

Transmission of genetically indistinguishable strains of *C. albicans* between HIV-infected adult partners has been reported previously (Muller et al., 1999). However, little is known about the transmission of the isogenic *C. dubliniensis* strain between children and within families. The aim of this study was to investigate the presence of *C. dubliniensis* among Brazilian family members. Oral flora from one Brazilian HIV-infected child and his family members was investigated. A total of 42 oral mucosa

isolates were obtained from eight family members between September 2000 and January 2002 (Table 3.1). Yeast isolates were identified by classical methods. These methods included chromogenic agar culture, chlamydospore production, germ tube formation, *Candida* Check kit®, ID32C® profile; and the temperature test was also used to distinguish *C. albicans* from *C. dubliniensis* by its differential growth at 45°C. The confirmation of *C. dubliniensis* identification was performed using molecular biotyping.

The oral flora of the family members showed high diversity with several non-*albicans* isolates identified (Fig. 3.7). *C. dubliniensis* was isolated only in the HIV-infected child and in his grandmother. The grandmother has repeatedly refused to be HIV tested. The colony forming unit (CFU) quantification was higher for *C. dubliniensis* (CFU=100) in the HIV-infected child than in his grandmother (CFU <30).

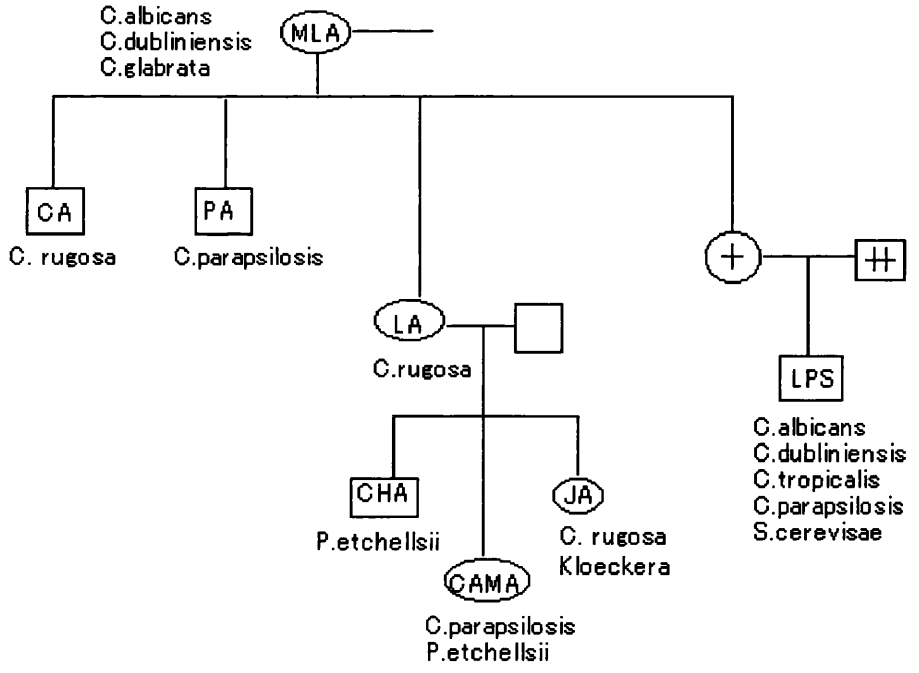


Fig. 3.7. Diagram of the species identified amongst the isolates from the family members of the HIV-infected child. ☐ LPS (HIV-infected child); + (HIV-infected mother) and ++ (HIV-infected father) both are deceased. Kindred of the HIV-infected child (MLA, CA, PA, LA, CHA, JA and CAMA).

Using classical identification methods it was not possible to identify some isolates tested. The primary culture of *Candida* isolates on CHROMagar showed a dark green colony, rough appearance and smaller size in comparison with *C. albicans* colonies. The tested isolates that were identified as *C. dubliniensis* by PCR appeared as 305 base pair bands on the gel (Fig. 3.8). The reference isolates had independent band patterns after the RAPD-PCR. Isolates 73, 390, 2-MLA, 3-MLA and 3-LPS had identical RAPD band patterns (Fig. 3.9), indicating that the clinical follow-up of *C. dubliniensis* might have the same genotype. The other Brazilian *C. dubliniensis* strain (S-34) had a different genotype. The genotypic coincidence among *C. dubliniensis* isolates from the same family members revealed that the grandmother was probably contaminated through the HIV-infected child (Fig. 3.9). Further epidemiological studies for his environment, such as neighbours, classmates and relatives might be required. Recently Milan and collaborators (Milan et al., 2001) reported that 33% of *Candida* spp. colonization with AIDS was through household contacts in contrast with 14% in the HIV-negative control (Milan et al., 2001).

Our findings also reveal that transmission through family members is possible and perhaps represents a previously under-appreciated factor in families with or without HIV infection. Moreover, the asymptomatic members who have not received antifungal therapy may also be colonized with resistant *Candida* species. The transmission of *C. dubliniensis* among siblings, parents and relatives may be through the exchange of contaminated commodties, which commonly occurs in the sharing of food, utensils, and toys.

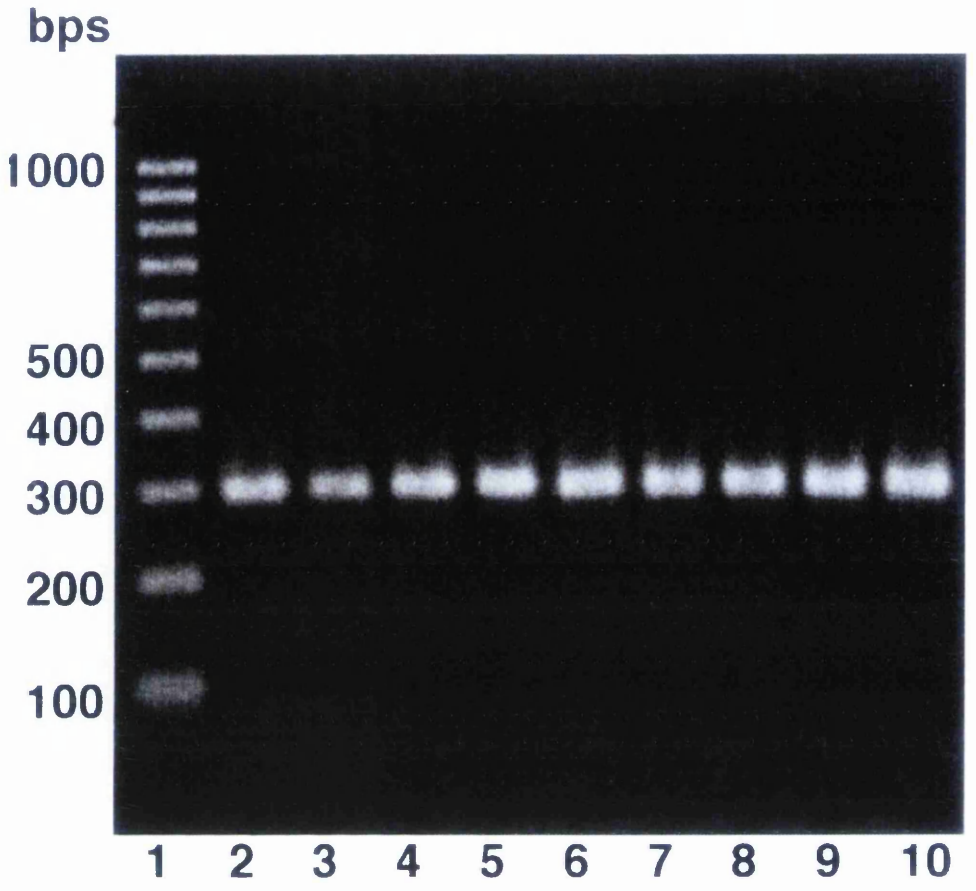


Fig. 3.8. The gel image of amplification product of the *C. dubliniensis* specific gene cytochrome *b*. Marker, 1 line; 2, IFM 48184; 3, IFM 48313; 4, IFM 48314; 5, IFM 49192; 6, 73; 7, 390; 8, 2-MLA; 9, 3-MLA; and 10, 3-LPS.

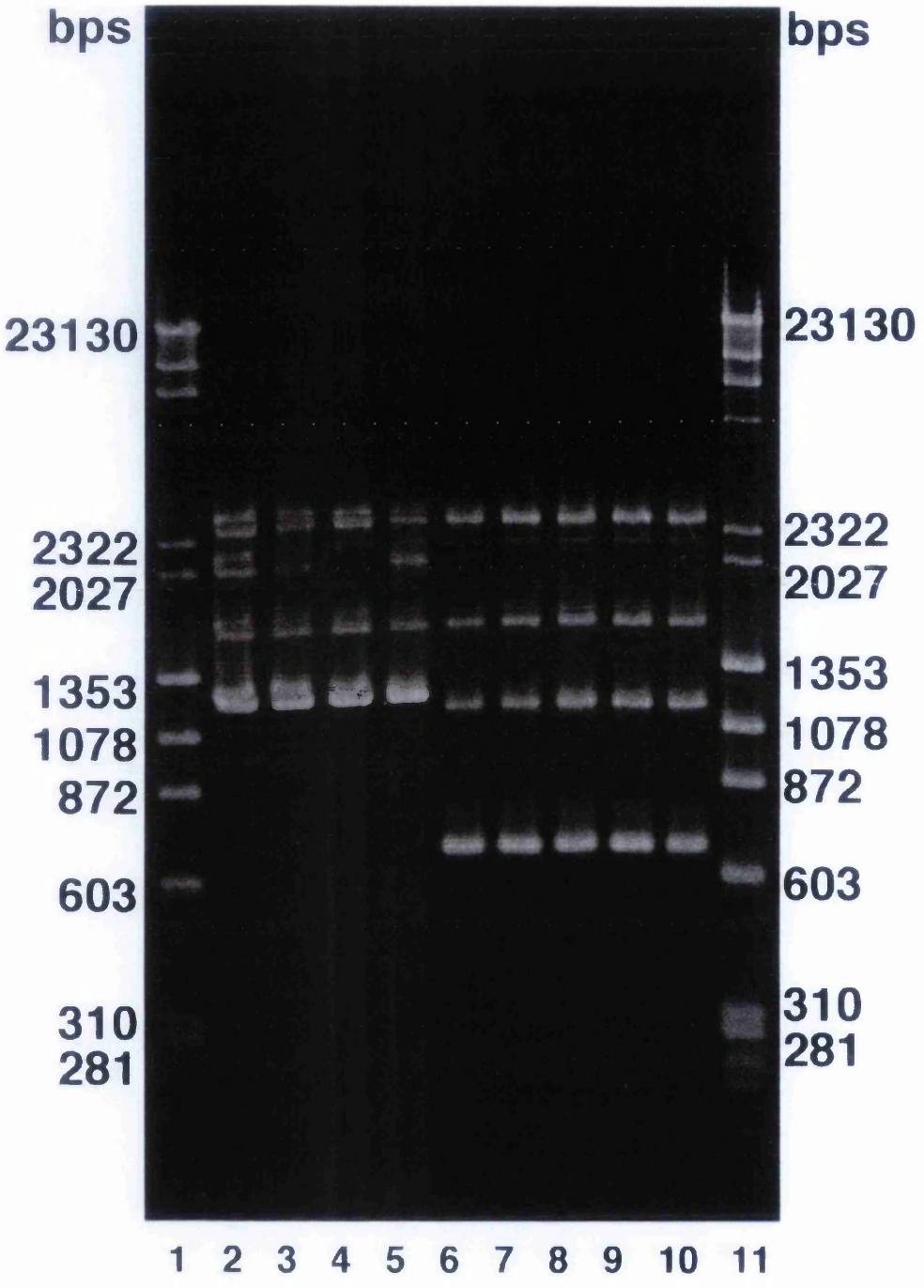


Fig. 3.9. The gel image of RAPD fingerprinting patterns of *C.dubliniensis* isolates. 1, Molecular weight marker; 2, IFM 48184; 3, IFM 48313; 4, IFM 48314; 5, IFM 49192; 6, 73; 7, 390; 8, 2-MLA; 9, 3-MLA; 10, 3-LPS; and 11, molecular weight marker.

MICs for each antifungal agent were determined and all *C. dubliniensis* isolates investigated were susceptible to the antifungal drugs tested (Table 3.8), with the exception of nystatin. *C. dubliniensis* isolates exhibited MIC values of 0.125 $\mu g.ml^{-1}$ for voriconazole. In a previous study, *C. dubliniensis* isolates from Brazil reported high susceptibility for azoles (Mariano Pde et al., 2003) and similar results have been described in the literature (Pinjon et al., 2003, Espinel-Ingroff et al., 1999, Moran et al., 1997). Non-*dubliniensis* isolates from the other members of the family were susceptible to all drugs tested (data not shown).

Table 3.8.

MICs for antifungal drugs in *C. dubliniensis* isolates

Drug	3-LPS	2-MLA	IFM 48313
	MIC (μg/ml)		
VOR	0.125	0.125	0.125
FLCZ	0.5	0.125	0.25
ITCZ	1	1	0.125
KTZ	0.03	0.03	0.03
MCZ	2	2	2
CLTZ	0.06	0.06	0.06
AMPH	2	2	2
NYS	16	4	8

Few studies have reported upon the sterol composition of *C. dubliniensis* isolates (Pinjon et al., 2003). In this study sterol profiles of *C. dubliniensis* isolates (3-LPS, 2-MLA and IF 48313) showed percentages of 45.4%, 50% and 49.5% of ergosterol of the total sterol content. This contrasts to azole-susceptible *C. dubliniensis* isolates reported in another study in which the ergosterol profile showed 65%, 56% or 60% of total sterols (Pinjon et al., 2003). *C. dubliniensis* isolates from the Brazilian HIV-infected child (3-LPS) contained several intermediate sterols (Table 3.9). These included cholesta-8,24-dienol (14%) and ergostadienol (11%). In this report *C.*

dubliniensis isolates also showed a reduced ergosterol level in comparison to *C. albicans* reference strains (ATCC 90028 and 28516). The ergosterol level has shown values above 80% to both *C. albicans* reference strains (ATCC 90028 and 28516) and these results are similar to that described previously (Kelly et al., 1997, Buurman et al., 2004). The sterol profile in this work suggests an interesting difference between *C. dubliniensis* and *C. albicans* as both species are closely related phylogenetically and further investigation of the sterol profiles in other *C. dubliniensis* isolates are required.

Table 3.9.

Sterol profile of *C. dubliniensis clinical* isolates (3-LPS, 2-MLA)

Sterol Profile	Total sterol fraction (%)				
	3-LPS	2-MLA	IFM[a] 48313	ATCC[b] 90028	ATCC[b] 28516
Cholesta-8,24-dienol	14	13.1	19.1	-	4
Ergosterol	45.4	50	49.5	93.7	80.2
Ergosta-7,22-dienol	3.4	2.8	3.7	-	0.6
Ergosta-dienol	10.9	10.4	9.6	-	3.7
Methylfecosterol	1.5	-	1.8	-	3
Obtusifoliol	4.7	5.6	6.1	-	2.5
Eburicol	0.3	-	-	-	
4,4-dimethylcholesta-dienol	6.6	5.8	-	-	
Unidentified	12.3	12.3	9.6	6.3	6

[a]*C. dubliniensis* and [b]*C. albicans* reference strains

Additionally, as these *C. dubliniensis* clinical isolates showed different ergosterol levels, we decided to investigate if the alteration in the function of efflux pumps could be detected in these isolates. Using *C. dubliniensis* isolates from HIV-infected child (3-LPS) and his grandmother (2-MLA), cells were incubated in the presence of [^{3}H] fluconazole and the intracellular concentration of this compound determined (Fig. 3.3). Interestingly, the two clinical isolates were found to differ with regard to fluconazole accumulation.

The isolate from the HIV-infected child was found to accumulate half the amount of [^{3}H] fluconazole than the isolate from grandmother and IF 48313 reference strain (Table 3.10). It is also true that in the IF 48313 reference strain, ATP-dependent pumps were operating but no effect was observed when sodium azide was added. However, the isolates from the HIV-infected child and grandmother were found to accumulate approximately 1.5 times more [^{3}H] fluconazole in the presence of sodium azide. These results indicate that the efflux of fluconazole from these two strains involved an active, energy-dependent component, as sodium azide inhibits ATP formation required for active transport.

Table 3.10. Accumulation of [^{3}H] fluconazole (dpm/min) in presence of NaN3 in clinical *C. dubliniensis* isolates

Strains	[^{3}H] fluconazole (MD ± SD)	[^{3}H] fluconazole + NaN_3 (MD ± SD)
3-LPS	46 ± 11.5	70.3 ± 20.7
2-MLA	94.6 ± 26.1	179.4 ± 64.7
IFM 48313	96.2 ± 17.5	107.7 ± 45.5

The most frequent molecular mechanism of azole resistance recently described has been the upregulation of efflux pumps (Odds et al., 2004). Reports have demonstrated azole resistance in *C. dubliniensis* isolates including its ability to rapidly develop resistance to fluconazole. This characteristic may partially explain the emergence of this species although the sensitivity of the isolates examined here and results of fluconazole accumulation studies do not point to any particular change regarding the relatively low concentration of ergosterol observed.

Until the late eighties, clinical resistance to azole antifungals was a rare phenomenon. Only a few cases of resistance to ketoconazole were found in patients with chronic mucocutaneous candidosis. The spread of AIDS and the widespread prophylactic and therapeutic use of the azole compound fluconazole resulted both in the selection and induction of resistant strains and in a shift in the nature of the infecting organisms. Most azole antifungals such as itraconazole, ketoconazole and fluconazole are active against a variety of fungal diseases. However, the concentration required to inhibit growth is dependent upon the nature of the infecting species. Physiochemical features, such as the hydrophobicity and pKa of a given azole define whether or not it will be active or cross-resistant against a given species. Fluconazole is almost inactive against *C. krusei* and *A. fumigatus*, whereas itraconazole is active against these species. Acquired or induced resistance is the most controversial type because, even within a given species, organisms may differ in their response to the same azole.

There is controversy over the cause for the increasing incidence of intrinsically resistant non-*albicans* species. The cause might be a selection of these species due to the widespread usage of fluconazole. Only a few reports have studied mechanisms of resistance in non-*albicans* species (Venkateswarlu et al., 1996, Nguyen et al., 1996, Krcmery and Barnes, 2002, Capoor et al., 2005, Loeffler and Stevens, 2003). Development of resistance to azole compounds can lead to cross-resistance to other azoles. In the present investigation the intracellular fluconazole concentration levels in *C. krusei* isolates was greater than that of *C. albicans*. In general non-*albicans* isolates exhibited higher intracellular [^{3}H] fluconazole concentrations and showed MICs with varying resistance patterns in most of the isolates in this study. One can speculate that in non-*albicans* species ,the tolerance for high levels of intracellular [^{3}H]

fluconazole concentrations is higher when compared to *C. albicans* species. However,, no clear correlation of azole drug accumulation and sensitivity in MIC have yet been determined. Such data requires to be carefully evaluated and used in special conditions such as comparison of genetically matched strains or parent/mutant comparisons.

The MIC values obtained for some *Candida* clinical isolates in this study were diverse and in some cases species specific. However, ergosterol levels in the cell membranes in most of the isolates were typical of the general expectation for fungi with only a few exceptions observed (Fig. 3.6). These exceptions contained relatively large amounts of sterol pathway intermediates instead of ergosterol. Further, the pattern of intermediate accumulation in strains was not indicative of any particular enzymatic defect. However, where it was observed, the reduced levels of ergosterol did not adversely effect the growth of the organism observed in these strains. This demonstrates the capacity of some strains to grow despite levels of ergosterol being half those present in the cell membranes of other strains/species. The mechanisms involved in controlling susceptibility to antifungal agents in any given *Candida* clinical isolate still remains unclear. Further work is required to establish the precise mechanisms that confer increased resistance to antifungal agents in the particular clinical isolates identified in this study. Studies on CYP51 sequence and sensitivity would seem a priority as drug accumulation studies did not seem to be implicated and other mutations in ergosterol biosynthesis were not detected that would warrant further study. Newer technologies such as microarray experiments may also reveal insights.

Chapter 4

ROLE OF *DIT2* IN *CANDIDA ALBICANS* GROWTH AND DRUG SUSCEPTIBILITY

4.1. Introduction

The fungal cell wall is essential for controlling cell permeability, osmotic balance, cellular shape and morphogenesis. Studies on the *Candida* sp. cell surface (Shepherd, 1987) have been of particular interest including antigenicity (Calderone and Braun, 1991, Smail and Jones, 1984), mechanisms of adherence to epithelial and endothelial cells (Calderone and Braun, 1991) and in interactions with the host immune system (Smail and Jones, 1984, Smail et al., 1988). Elucidating the mechanism(s) of cell wall biosynthesis are critical for our understanding of morphogenesis and is an important area of interest for the development of new antifungal agents (Hector, 1993).

Amongst many functions, the cell wall contributes to the tolerance of yeast cells to damaging environmental agents and stresses. Extensive studies on fungal cell wall components have tried to understand the cell wall structure, the order of formation of the cell wall and the genes involved in the its formation (Ram et al., 1998, Ruiz-Herrera et al., 2006). The cell wall is the outermost structure of yeast cells and it provides substantial mechanical strength with a dynamic plasticity, thus guaranteeing cell survival in a fluctuating environment. A compromise between rigidity and plasticity in this structure at each point of the cell life must be achieved as well as a delicate balance between synthesis and degradation.

It is generally believed pathogenic fungi such as *C. albicans* have a very similar polysaccharide structure but differ significantly in their specific protein composition, which underscores the importance of cell wall proteins for pathogenesis. However, cell wall proteomics of fungi is a highly challenging task due to the complex

biochemistry of these proteins and their glycosylated condition. The extensive post-translational modifications and potential covalent attachment of polysaccharides to the polypetide backbone of a large proportion of cell wall proteins makes it a demanding task to isolate and identify them. Regarding the genes associated with the *Candida* sp. cell wall, most of our knowledge comes from studies with *S. cerevisiae.*

Knowledge of the *S. cerevisiae* cell wall can be summarized as follows: the yeast cell wall comprises of a fibrillar network of chitin and 1,3-L-D-glucan to which mannoproteins anchor themselves, mostly through 1,6-L-D-glucan molecules. These components are synthesized into an elaborate extracellular matrix (Bulawa, 1992). This matrix constitutes an organelle that is dynamically engaged with the plasma membrane and the underlying secretory organelles (Pryer et al., 1992) along with cytoskeletal and cytoplasmic components to maintain cell integrity during growth (Cid et al., 1995). The cell surface varies in shape and composition throughout the lifecycle of the *S. cerevisiae* cell; for example, in the budding of vegetative cells, in mating projection formation, in cell fusion in haploid cell conjugation, in spore wall formation following meiosis and in the specialized cell surfaces and morphogenesis seen in pseudohyphal growth (Cid et al., 1995). In view of the complexity of the cell wall, the specific number of genes directly or indirectly involved in its biosynthesis and elaboration is expected to be large. However, a nearly complete collection of *S. cerevisiae* individual gene-deletion mutants have been identified (Ram et al., 1994, Giaever et al., 2002) and approximately 20% of these have been shown to be required for cell-wall biogenesis (Lussier et al., 1997). (February 2007, http://www.yeastgenome.org/cache/genomeSnapshot.html).

In general, the *Candida* sp. cell wall is composed mainly of mannan (linked with protein to form mannoprotein), α-glucan, β-glucan, and chitin, which are complex polymers of mannose, glucose, and *N*-acetylglucosamine, respectively. The content and distribution of these components vary with cell age, culture conditions, and morphology (Brown et al., 2000, Bogengruber et al., 1998, Ruiz-Herrera et al., 2006). The linkages between the glucan, chitin, and mannoprotein are poorly understood but presumably play a critical role in maintaining the integrity of the organism.

Different virulence factors are involved in *C. albicans* pathogenicity, but the role of the cell wall in such pathogenicity cannot be overestimated. The cell wall is the structure that: (1) first comes into contact with host cells; (2) carries important antigenic determinants of the fungus; (3) is responsible for the adherence of the pathogen; and (4) establishes a cross-talk with the host, which depends on what has been referred to as the 'glycan code', which includes modifications in the chemical composition and linkages of the cell wall polysaccharides. Microscopic observation of thin sections of fungal cells or isolated walls have revealed the existence of several layers in the wall, distinguished by their electron density in electron micrographs. Depending on the method of analysis, the wall of *C. albicans* appears to contain from four to eight layers (Poulain et al., 1978).

The spore wall in *S. cerevisiae* is a stratified extracellular matrix that is more complex than the normal vegetative cell wall (Klis et al., 2006, Smits et al., 2001). The vegetative wall consists primarily of an inner layer (closest to the plasma membrane) of β-glucans interspersed with a small amount of chitin and an outer layer of heavily mannosylated proteins (Smits et al., 1999, Klis et al., 2006). By contrast, the spore

walls are formed by the ordered synthesis of different layers: first mannan, then β-glucan, then chitosan, and finally outside of the chitosan is a layer that consists largely of cross-linked tyrosine molecules (Briza et al., 1988, Briza et al., 1990, Briza et al., 1994). This indicates that the assembly of spore walls occurs through a coordinated series of steps.

S. cerevisiae has three cytochrome P450 genes: *CYP51* (encoding lanosterol 14α-demethylase), *CYP56* (encoding *DIT2* a dityrosine forming enzyme required for spore wall biosynthesis), and *CYP61* (encoding sterol 22-desaturase). The proposed pathway of dityrosine formation in sporulating yeast cells by Briza *et. al.* (Briza et al., 1994) indicated that two N-formyltyrosine molecules are covalently cross-linked to form dityrosine, and the enzyme responsible for this enzymatic reaction is Dit2p, (also named CYP56 in the cytochrome P450 nomenclature [http://drnelson.utmem.edu/CytochromeP450.html]). In fact dityrosine is biosynthesized in a two-step process that takes place in the cytoplasm of the maturing spore. Initially, free L-tyrosine is chemically modified by Dit1p resulting in the formation of *N*-formyl tyrosine. Secondly, two molecules of *N*-formyl tyrosine are cross-linked by CYP56 to form LL-*N,N'*-bisformyl dityrosine (Briza et al., 1996, Felder et al., 2002). *NCPR1* was shown to be required for dityrosine formation by providing reducing equivalent necessary for CYP56 catalytic activity. The first description of dityrosine formation was in 1959 by Gross and Sizer (Gross and Sizer, 1959), generated by oxidation of tyrosine with hydrogen peroxide and peroxidase. Dityrosine is an intensely fluorescent compound and is proposed to result from cross-linkage of two tyrosyl radicals (Briza et al., 1986). The oxidation of tyrosine with hydrogen peroxide and peroxidase, can lead to phenolic coupling of two phenoxy

radicals of tyrosine. It was also found that dityrosine cross-links could also be formed *in vitro* by oxidation of structural and non-structural proteins (Andersen, 1964, Amado et al., 1984, Felder et al., 2002).

More recently the formation of coupled tyrosine derivatives in proteins has been associated with a variety of diseases and disorders which include Alzheimer's and Parkinson's diseases (http://www.chemistry.unimelb.edu.au/staff/chutton/research/cah.htm). Non-specific formation of cross-linked tyrosine residues has been shown to be a biological marker of oxidative stress and plays a critical role in the signalling of protein degradation and cellular damage. Dityrosines can also be obtained under a variety of conditions. Dimerization of the monomer can occur via oxidative coupling with reagents such as VOF_3. Alternatively, dimerization can occur via reductive coupling using potassium hexacyanoferrate.

After the first report in 1995 (Smail et al., 1995) of the presence of dityrosine in *C. albicans*, little was known regarding its biosynthesis. The recently completed *C. albicans* genomic sequence revealed a homologue of the *S. cerevisiae CYP56* present within this genome. Hence, it was of interest to characterise the role of this gene product both in the formation of dityrosine in *C. albicans* as well as its contribution to the life-cycle of the organism. This chapter describes experimental results undertaken to evalutate the biochemistry of the *C. albicans* CYP56 and gene knock-out experiments to decipher it physiological role. Furthermore, given that cytochromes P450 can be drug targets, such as CYP51 in azole antifungal chemotherapy, a discovered key role for CYP56 in *C. albicans* biology may proffer this enzyme as a

potential drug target for antifungal chemotherapy. Therefore, the role of the *CYP56* gene in *C. albicans* growth, drug susceptibility and virulence by undertaking gene disruption in this diploid pathogen was undertaken as well as its affinity for azole antifungal compounds being assessed.

4.2. Materials and Methods

4.2.1. Gene sequence of *C. albicans DIT2*

In the current assembly (assembly 20) of the *C. albicans* SC5314 genome sequence, the sequence of the *DIT2* gene is incomplete at the 5' end, probably due to an unfinished gap. In order to complete the annotation of *C. albicans DIT2* gene sequence, DITFOR and DITREV primers were designed to amplify the entire *DIT2* gene based on the sequence of the homologous *C. dubliniensis DIT2* gene, and are listed in Table 2.3. The gene was amplified by PCR as previously described (Chapter 2.11.1), using genomic DNA template from *C. albicans* strain SC5314. The PCR product was cloned in pGEM T-easy vector for sequencing to reveal the complete *DIT2* sequence.

4.3. Results

4.3.1 Deletant *C. albicans* homozygous *CYP56* strain construction.

The DNA sequence of the region spanning the gap in the genomic information was elucidated using PCR to generate the appropriate DNA fragment. The *CYP56* gene was then aligned against the deposited *C. albicans* genomic sequence using the computer program Clustal X. The entire *CYP56* gene sequence was found to be 1.43 kb long (Fig. 4.1 and 4.2), similar in length to the *C. dubliniensis* sequence as previously described in chapter 2.

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Since *C. albicans* is diploid, the construction of a homozygous mutant, defective in *CYP56* in both alleles, required recycling of the resistance marker after insertion into the first allele of the target gene in order to allow a second round of transformation to inactivate the remaining wild type allele (Fig. 4.2). Consequently, the *SAT1* flipping method was used, which is a highly efficient and convenient strategy for targeted gene disruption in *C. albicans* (Chapter 2, Fig. 2.6, adapted from Reuss et al. 2004).

A 'knock-out' mutant was constructed by using a cassette that contained the antibiotic resistance marker nourseothricin and a *Candida* adapted FLP gene that allowed the excision of the cassette following growth on maltose (Fig. 2.6). Two rounds of integration and excision were necessary to create the homozygous mutant. The structure of the *SAT1* flipper cassette consisted of the *MAL2* promoter, the *Candida*-adapted *FLP* gene (grey arrow), transcription termination sequence of the *Candida ACT1* gene, the main nourseothricin resistance cassette and the FLP recombination sequence.

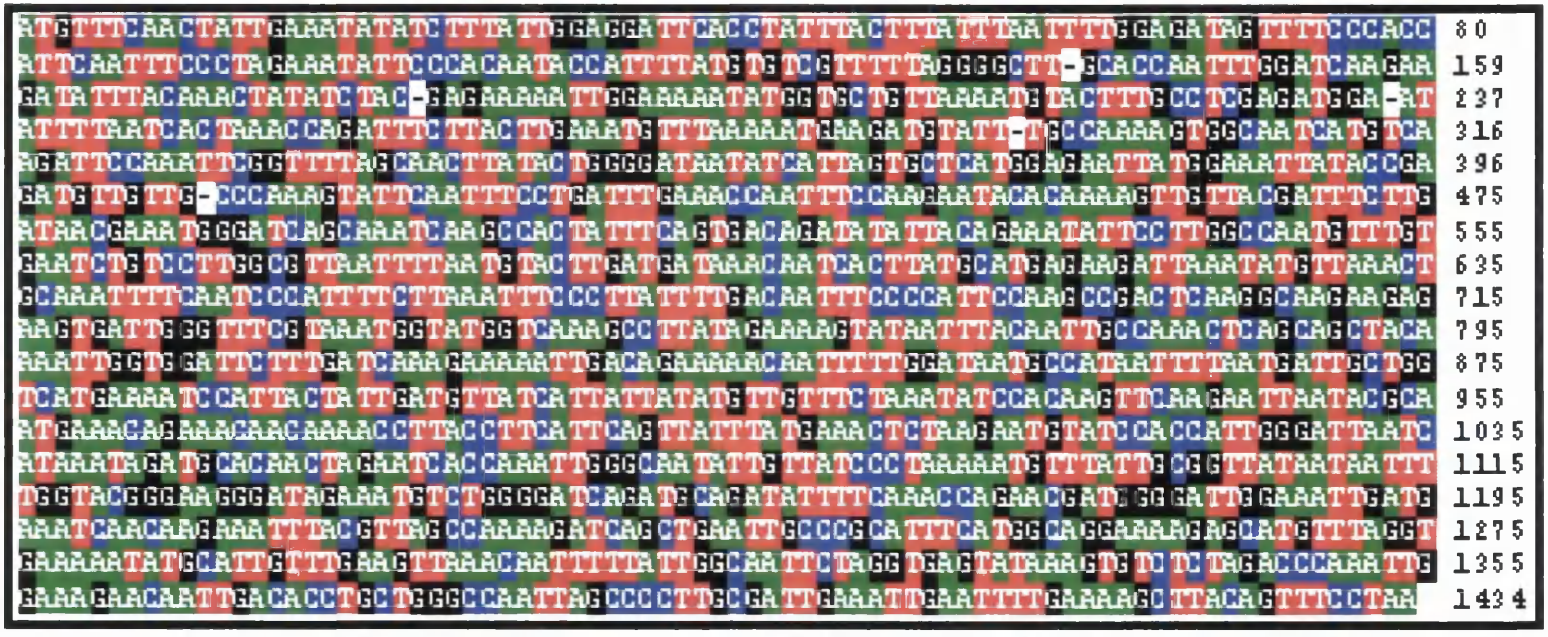


Fig. 4.1. The resulting sequence of the *DIT2* gene was aligned, using the computer program Clustal X, against the deposited *C. albicans* gene sequence which was not entirely annotated. The complete *DIT2* gene sequence was found to be approximately 1.5 kb long, similar in length to the *C. dubliniensis* sequence from which the primers for amplification were designed.

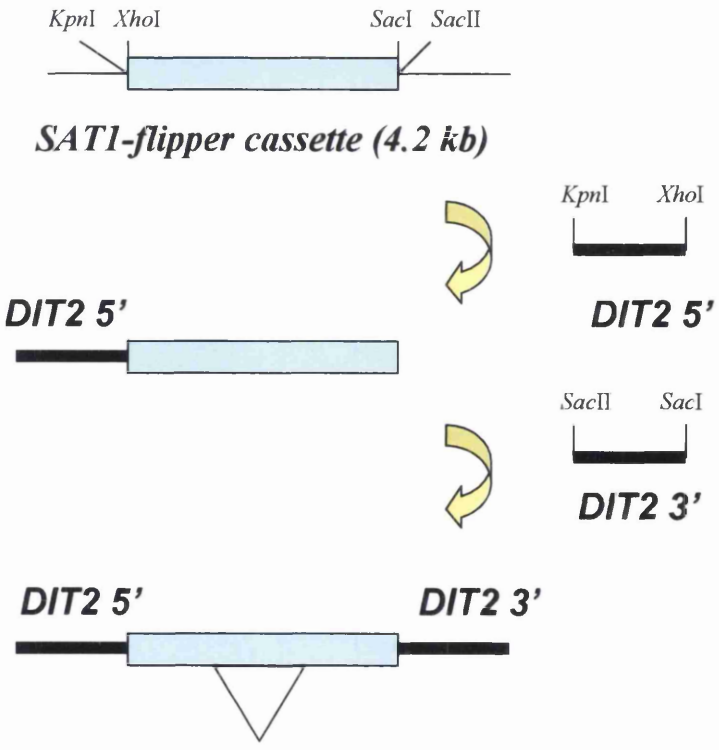


Fig. 4.2. Scheme of the strategy for cassette construction containing the *SAT1-flipper* in which 5' and 3' fragments of the *DIT2* gene were inserted. The fragments were digested using the restriction enzymes in the extreme of each fragments as illustrated in the figure.

Figure 2.6 B shows the cassette and the upstream and downstream sequence regions of *CYP56* to which probes 1 and 2 were designed. The coding region (white arrow) of the gene was replaced with the flipper cassette during recombination. The primer pairs CYP56KF/CYP56XR and DITSIIF/DITSIR were used to amplify the 5' and 3' non-coding flanking regions of the CYP56 gene using SC5314 DNA template (Fig. 4.3 A). Following cloning into pBluescript II, plasmids were then digested with *Kpn*I/*Xho*I and *Sac*II/*Sac*I restriction enzymes, respectively, to release these inserts for sequential ligation to the ca*SAT1* cassette, and the resulting product was transformed in DH5α^{tm} *E. coli* cells. The insertion of both fragments was confirmed by digestion (Fig. 4.3 B). The *C. albicans* wild-type laboratory strain SC5314 was then transformed by electroporation with a linear DNA fragment in which the ca*SAT1* cassette containing the nourseothricin antiobiotic marker was flanked by 5'and 3' CYP56 DNA (Fig. 4.4 A). Transformants were obtained after 2 days of culture on YEPD agar plates containing 200 $\mu g.ml^{-1}$ of nourseothricin (Fig. 4.4 B). Six resistant colonies were picked and used for overnight broth cultures for DNA isolation and PCR screening.

PCR amplification was performed using the primers CYP56KF which bound upstream of the CYP56 coding sequence and FLP1 which bound within the cassette. The resulting PCR confirmed that all six colonies exhibited recombination with inactivation of one copy of the *CYP56* gene and consequently insertion of ca*SAT1* cassette (Fig. 4.4 C). Thus, the transformants were further inoculated into YEPD broth without nourseothricin to allow for FLP-mediated excision of the *SAT1* flipper cassette.

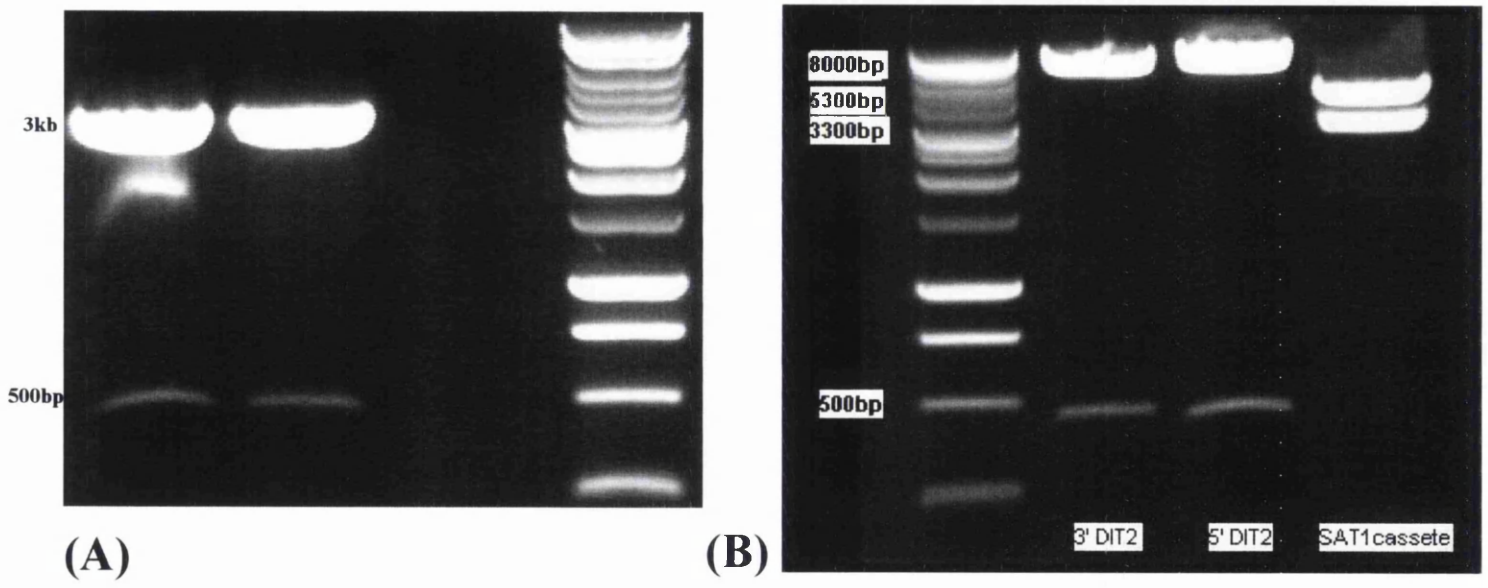


Fig. 4.3. PCR-amplification of the 5' and 3' regions of *CYP56* inserted in pGEM vector (A), and the insertion of both fragments into the SAT1-flipper cassette (B) was confirmed by digestion with *Kpn*I/*Xho*I and *Sac*II/*Sac*I restriction enzymes, respectively.

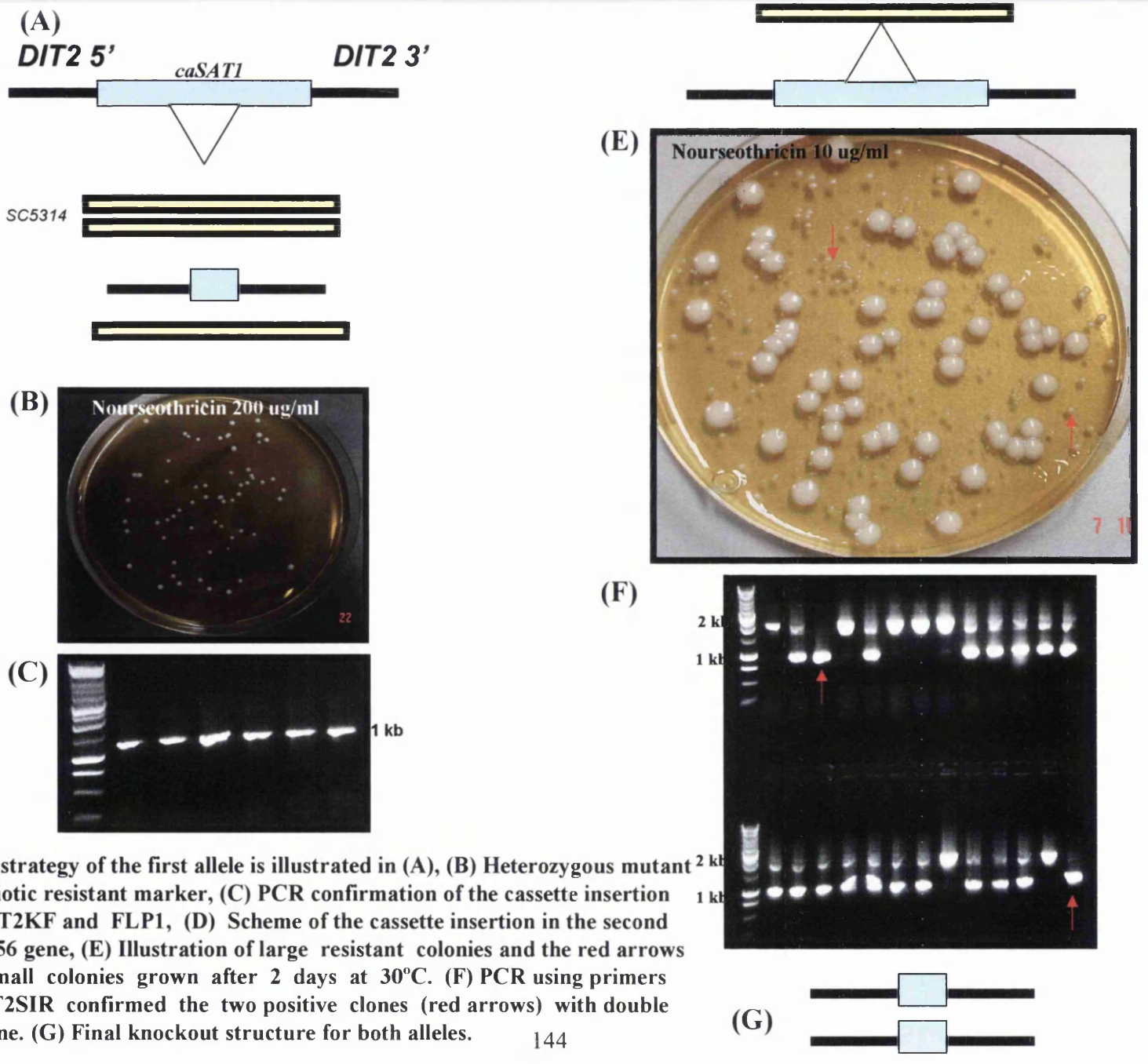
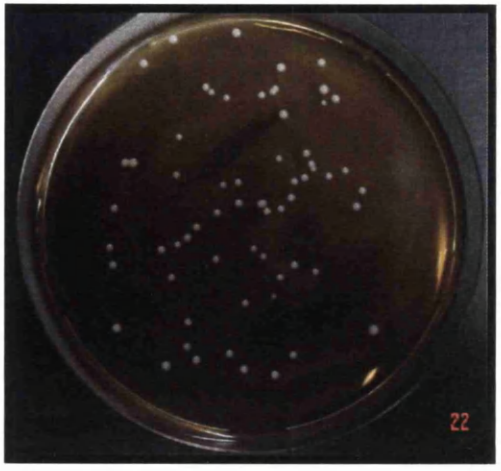


Fig. 4.4. Deletion strategy of the first allele is illustrated in (A), (B) Heterozygous mutant clones with antibiotic resistant marker, (C) PCR confirmation of the cassette insertion using primers DIT2KF and FLP1, (D) Scheme of the cassette insertion in the second allele of the CYP56 gene, (E) Illustration of large resistant colonies and the red arrows are indicating small colonies grown after 2 days at 30°C. (F) PCR using primers DIT2KF and DIT2SIR confirmed the two positive clones (red arrows) with double deletion of the gene. (G) Final knockout structure for both alleles.

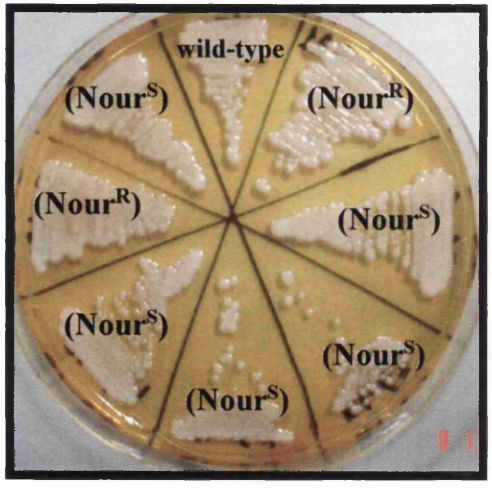
The recovery of sensitivity in derivative strains was confirmed from cultures grown on YEPD agar plates containing 100 $\mu g.ml^{-1}$ of nourseothricin (Fig. 4.4 F and 4.5). One derivative strain, Δ1-R1-6C^S, was employed to inactivate the remaining wild-type allele using the same procedure of integration and subsequent excision of the *SAT1* flipper cassette (Fig. 4.4 D), which generated Δ2-S1^R derivative strains. However, to allow for FLP-mediated excision of the *SAT1* flipper cassette, it was again necessary to grow in broth overnight. The recovery of sensitivity in Δ2-S3A^S derivative strains was confirmed after culture on YEPD agar plates containing 10 $\mu g.ml^{-1}$ of nourseothricin. Nourseothricin sensitive derivative Δ2-S3A^S strains had a smaller colony size compared with their Δ2-S1^R parental strains in the presence of this concentration of antibiotic (Fig. 4.4 E). Twenty-six colonies were screened by PCR analysis using the primers CYP56KF and CYP56SIR, and only 2 Δ2-S3A^S colonies gave a single PCR product of 1kb, indicating a homozygous mutant had been produced (Fig. 4.4 E). Correct integration was confirmed by Southern hybridization with probes from the CYP56KF and CYP56XR upstream region. Southern analysis showed that all Δ2-S3A^S had excised the *SAT1* flipper by FLP-recombination (Fig. 4.6), inactivating the *CYP56* gene. Heterozygous CYP56/*cyp56*Δ deletant mutants were compared against the double cyp56Δ/cyp56Δ mutants and the wild-type strain in phenotypic tests.

(1) Nourseothricin 200 ug.ml^{-1}



(2) Nourseothricin 10 ug.ml^{-1}

(3) Control no antibiotic



(4) Nourseothricin 100 ug.ml^{-1}

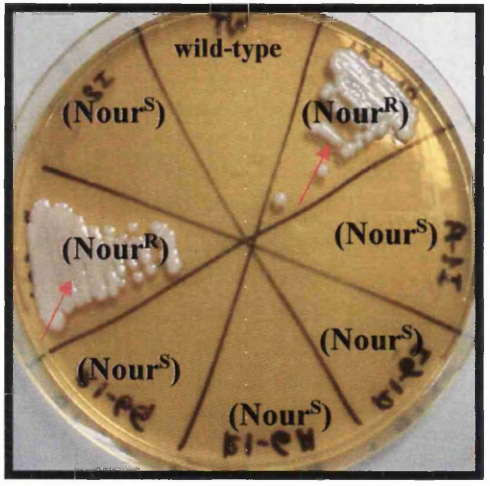


Fig. 4.5. (1) First transformants containing resistant-antibiotic marker (Nour^{R}); (2) Illustration of large antibiotic resistant colonies and small sensitive colonies grown after 2 days at 30°C after second transformation. (3) Growth of the wiid-type strain SC5314, heterozygous and homozygous mutants with (Nour^{R}) or without *SAT1* flipper (Nour^{S}) on YEPD agar plates without (3) or with 100 µg.ml^{-1} nourseothricin (4). Red arrows indicate homozygous mutants.

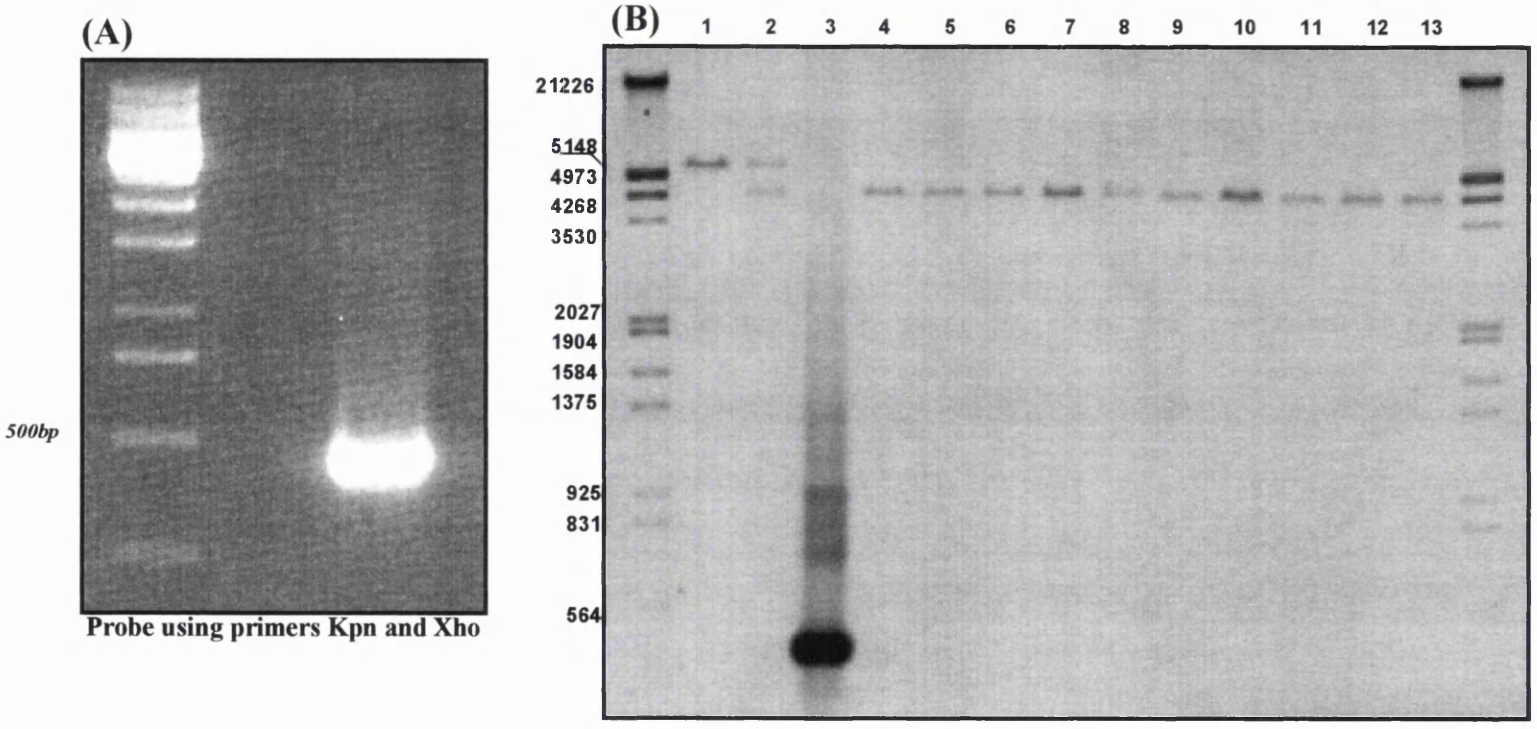


Fig. 4.6. The confirmation of the correct integration by Southern hybridization was performed using probes from the CYP56KF and DIT2XR upstream region (A). (B) Southern analysis showed that all Δ2-S3AS clones (4-13) had excised *SAT1* flipper by FLP-recombination, and knocked out the second wild-type allele, inactivating the *CYP56* gene. The upper band corresponds to the wild type-copy of *CYP56*, and is absent in lanes 4-13. The lower band corresponds to the deleted copy of *CYP56*. Lanes correspond to : (1) SC5314 (2) Heterozygous *CYP56/cyp56*Δ (3) Positive control DIT2 PCR product (4-13) Homozygous *cyp56*Δ/*cyp56*Δ mutants

4.3.2 Reintegration of wild type *CYP56* gene into the *cyp56Δ* homozygous mutant.

To confirm that observed mutant phenotypes were indeed caused by disruption of the target gene, a functional *CYP56* copy was reinserted into the homozygous *cyp56Δ* mutants. For this purpose PCR amplification of the *CYP56* gene was performed using wild-type SC5314 genomic DNA as template. Once the isolated *CYP56* gene sequence was verified as identically matching the genome sequence, *CYP56* gene was inserted into CIp20 plasmid (Fig. 4.7), which is a derivative of CIp10 described by Murad (Murad *et. al.* 2000) containing the *SAT1* nourseothricin resistance marker (G. Moran, unpublished). The linearization of the CIp20 plasmid containing the *CYP56* gene was performed by digestion with *Nco*I and transformed by electroporation into the homozygous Δ2-S3A^S derivative strain. By homologous recombination of the linearized CIp20 plasmid to the chromosomal RP10 locus (Fig. 4.7), this allowed the insertion of the *CYP56* gene into the chromosome of the Δ2-S3A^S derivative strain. The putative re-integrants were sub-cultured in YEPD broth for DNA isolation and for PCR screening to confirm their identity. Using primers CYP56KF and RT300 the insertion of the intact *CYP56* gene was verified in the Δ2-S3A^S derivative strain. Two PCR products with sizes of 2kb and 1kb corresponding to intact gene and inactivated allele, respectively were evident (Fig. 4.8). To verify the correct placement of the intact gene, clones were analysed by PCR using primers TEM1R (which binds downstream of the RP10 gene) and ACT10 (which binds within CIp20) resulting in products of 2kb as expected in a confirmatory study (Fig. 4.8).

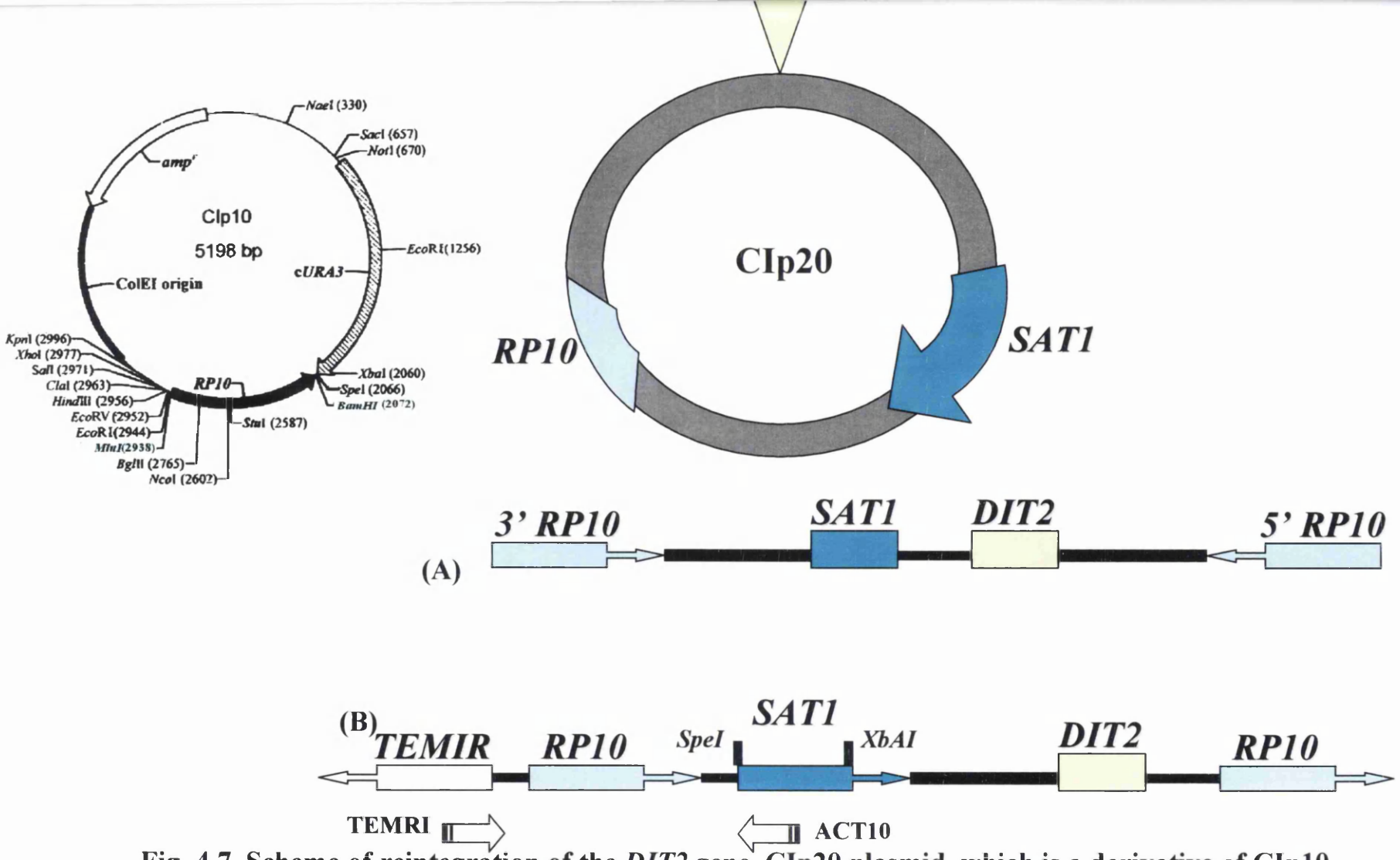


Fig. 4.7. Scheme of reintegration of the *DIT2* gene. CIp20 plasmid, which is a derivative of CIp10 described by Murad, containing the *SAT1* nourseothricin marker. (A) Linearization of the CIp20 plasmid, integrated into a copy of the *RP10* gene allowed the insertion of the *DIT2* gene into the chromosome of Δ2-S3AS derivative strain. (B) To verify the correct replacement of the intact gene, clones reintegrated were PCR amplified using primers TEM1R and ACT10.

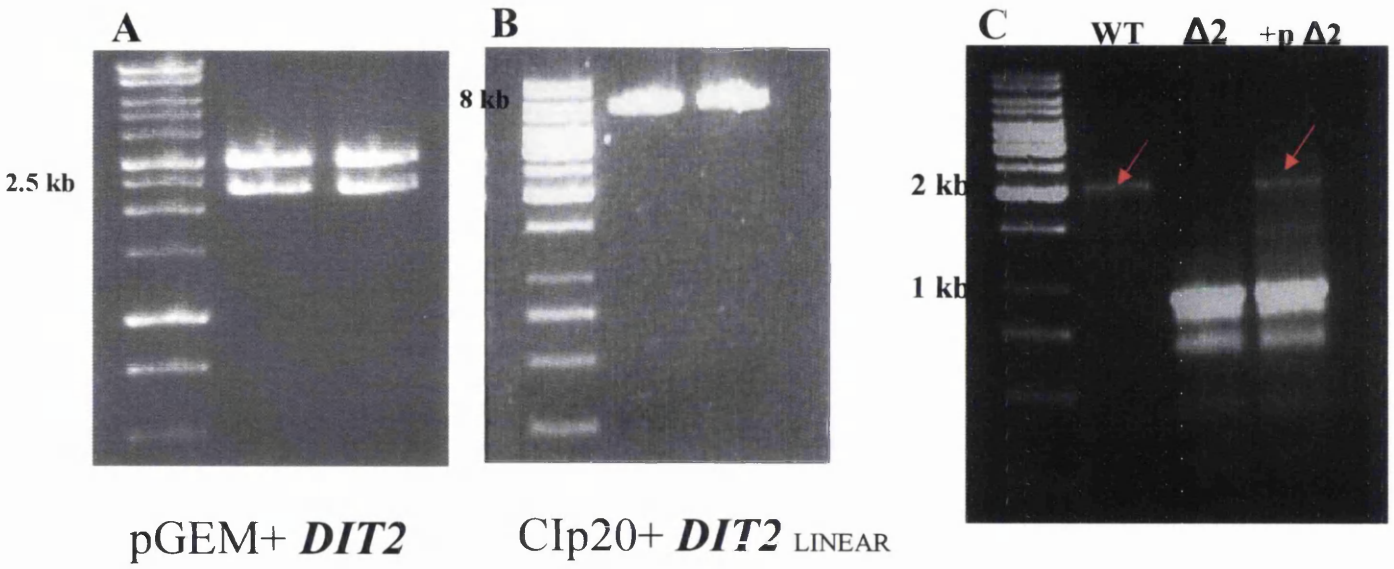


Fig. 4.8. Confirmation of reintegration of the *DIT2* gene in the homozygous mutant. (A) *DIT2* gene (2.5 kb) released from pGEM vector (3 kb) digested with *Spe*I and *Xba*I. (B) Linearization of the CIp20 plasmid containing the *DIT2* gene digested with *Nco*I enzyme. (C) PCR products obtained using primers (DIT2F and DIT2R) for the *DIT2* gene in CIp20-DIT2 transformants. The red arrow shows the presence of the full length *DIT2* gene. WT=wild-type strain, Δ2=homozygous mutant strains and + pΔ2=reintegrant.

4.3.3 Phenotypic analysis of the *cyp56Δ* homozygous mutant.

4.3.3.1 Antifungal susceptibility testing.

The MICs to various agents was determined by broth methods as illustrated in Table 4.1. The experiments revealed no differences in the MIC_{80}s for VOR, FLC, ITCZ, CLT, AMB, NYS, and CAS between homozygous *cyp56Δ* mutant and wild-type strain. Both strains were sensitive with low endpoints for all drugs tested in comparison to NCCLS guidelines. MICs were lower for some drugs when minimal medium was used in place of RPMI broth (Table 4.1). The only antifungal agent which exhibited an altered MIC towards the *cyp56Δ* mutant compared to wild-type was NIK, for which the homozygous *cyp56Δ* strain exhibited a two-fold lower MIC in both media tested.

4.3.3.2 Antifungal Etest reading.

Plates containing Etest strips for antifungal susceptibility tests were incubated for 24-48 hours in a moist incubator until growth was clearly seen. The MIC was read when the elliptical inhibition zone intersected the scale on the strips and compared with Etest reading and interpretation guidelines. The strains had insufficient growth after 24 h of incubation and consequently were read at 48 h. The homozygous *cyp56Δ* mutant and wild-type strains showed similar susceptibility and endpoint for all the drugs tested (Fig. 4.9).

Table 4.1. Susceptibility testing of the WT and homozygous mutant (D2) strains for the drugs using RPMI and YM medium

DRUGS	MIC_{80} (μg/ml)			
	RPMI		YM	
	WT	D2	WT	D2
VOR	0.007	0.007	0.015	0.015
FLCZ	0.06	0.06	0.125	0.125
AMB	1	1	2	2
CASP	0.007	0.007	0.007	0.007
NIK	2	0.5	2	0.5

VOR=voriconazole; FLCZ=fluconazole; AMB=amphotericin B; CASP=caspofungin; NIK=nikkomycin

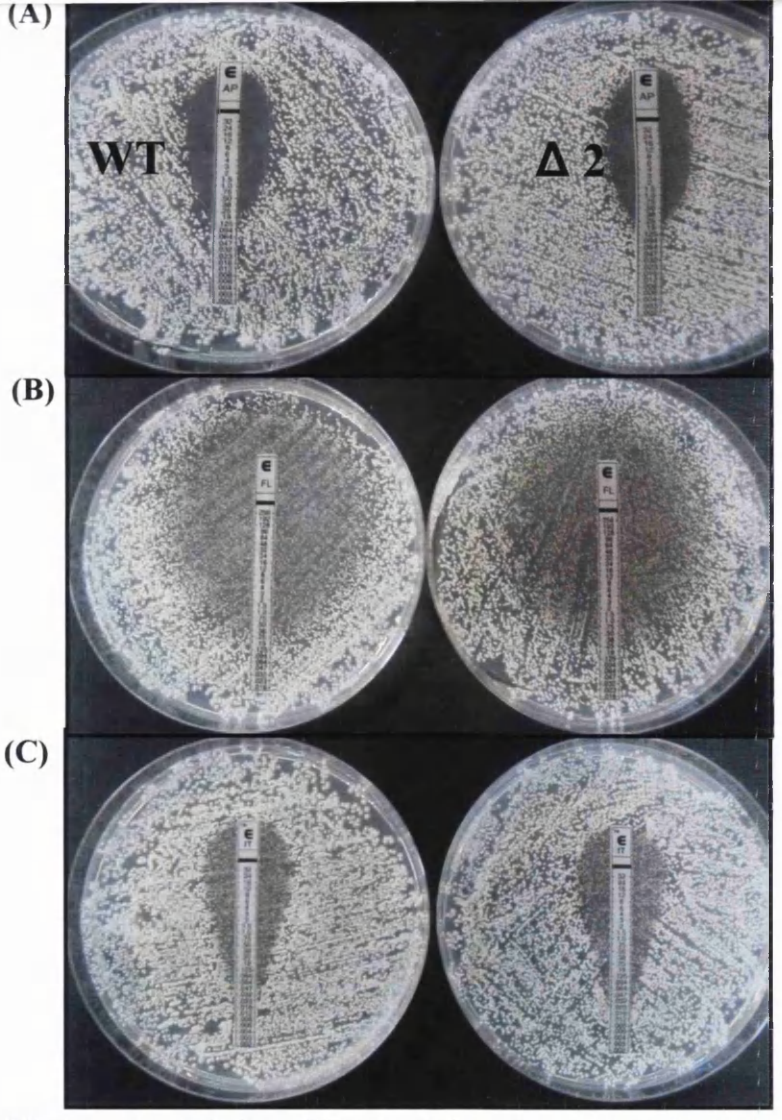


Fig. 4.9. Plates containing Etest strips were incubated for 24-48 hours. The MIC was read where the elliptical inhibition zone intersected the scale on the strips and compared with Etest reading and interpretation guidelines. The MIC for the drugs tested were 0.5 µg.ml^{-1}to amphotericin, 0.12 µg.ml^{-1} for fluconazole (B) and 0.06 µg.ml^{-1} for itraconazole (C). WT= wild-type and Δ2=*Dit2* homozygous mutant strains.

4.3.3.3 Analysis of *cyp56Δ* mutant and wild-type strain growth curves in the presence and absence of antifungal agents and cell wall alteration assays.

Although MICs demonstrated two-fold differences in susceptibility to NIK in *cyp56Δ* mutant and wild-type strains, growth curves were investigated as a more sensitive tool to examine the effects of deletion of the *CYP56* gene in laboratory media. In particular, casponfungin and nikkomycin caused slower growth of the *cyp56Δ* mutant strain in comparison to the wild-type strain in either RPMI or YM broth medium (Table 4.2). The remainder of the drugs tested showed no differences on the growth of mutant or wild-type strains (Fig. 4.10 A).

To determine the effect of deleting *CYP56* gene on the integrity of the cell wall, the null mutant was tested for sensitivity to a range of cell wall-perturbing agents and other agents whose effects have been associated with altered cell walls. Cell culture on YEPD broth supplemented with 1.5 M sorbitol, 100 $\mu g.ml^{-1}$ SDS, 1 M or 1.5 M NaCl, 5 mM or 10 mM H_2O_2, 0.5 M or 1 M $CaCl_2$, 100 $\mu g.ml^{-1}$ calcofluor, and 100 $\mu g.ml^{-1}$ congo red, at 37°C for 48 h, did not show changes in the level of sensitivity. In order to investigate the effect of glucose on cell growth, cultures of both *cyp56Δ* mutant and wild-type strains grown on minimal medium with the alternative sugar maltose or galactose at 37°C for 48 h. In this condition the homozygous mutant showed slower growth compared to wild-type (Fig. 4.10 B). In addition, after 6 h of culture the homozygous mutant started to produce hyphae continuously and the wild-type strain showed only a budding form. Cell culture in minimal broth medium, grown at 42°C for 48 h, also caused an inhibitory effect on growth of the homozygous mutant compared to wild-type.

Table 4.2. Growth curve of the *Candida* strains in the presence of cell wall inhibitors

Strains	Growth curve								
	Control			CAS			NIK		
	Lag	μ_{max}	Biomass $mg.ml^{-1}$	Lag	μ_{max}	Biomass $mg.ml^{-1}$	Lag	μ_{max}	Biomass $mg.ml^{-1}$
WT	9.26	0.11	2.70	9.20	0.09	2.1	10.15	0.06	2.25
Δ2	11.34	0.06	2.10	11.90	0.02	1.17	18.49	0.01	0.85
+pΔ2	10.87	0.10	2.33	10.50	0.06	1.52	11.32	0.04	1.85

LAG time= delay before growth; μ_{max}=specific growth rate; WT=wild-type; Δ2=homozygous mutant; +pΔ2=reintegrant; CAS=caspofungin 0.003 $ug.ml^{-1}$; NIK=nikkomycin 0.5 $ug.ml^{-1}$; Control=growth in minimal medium.

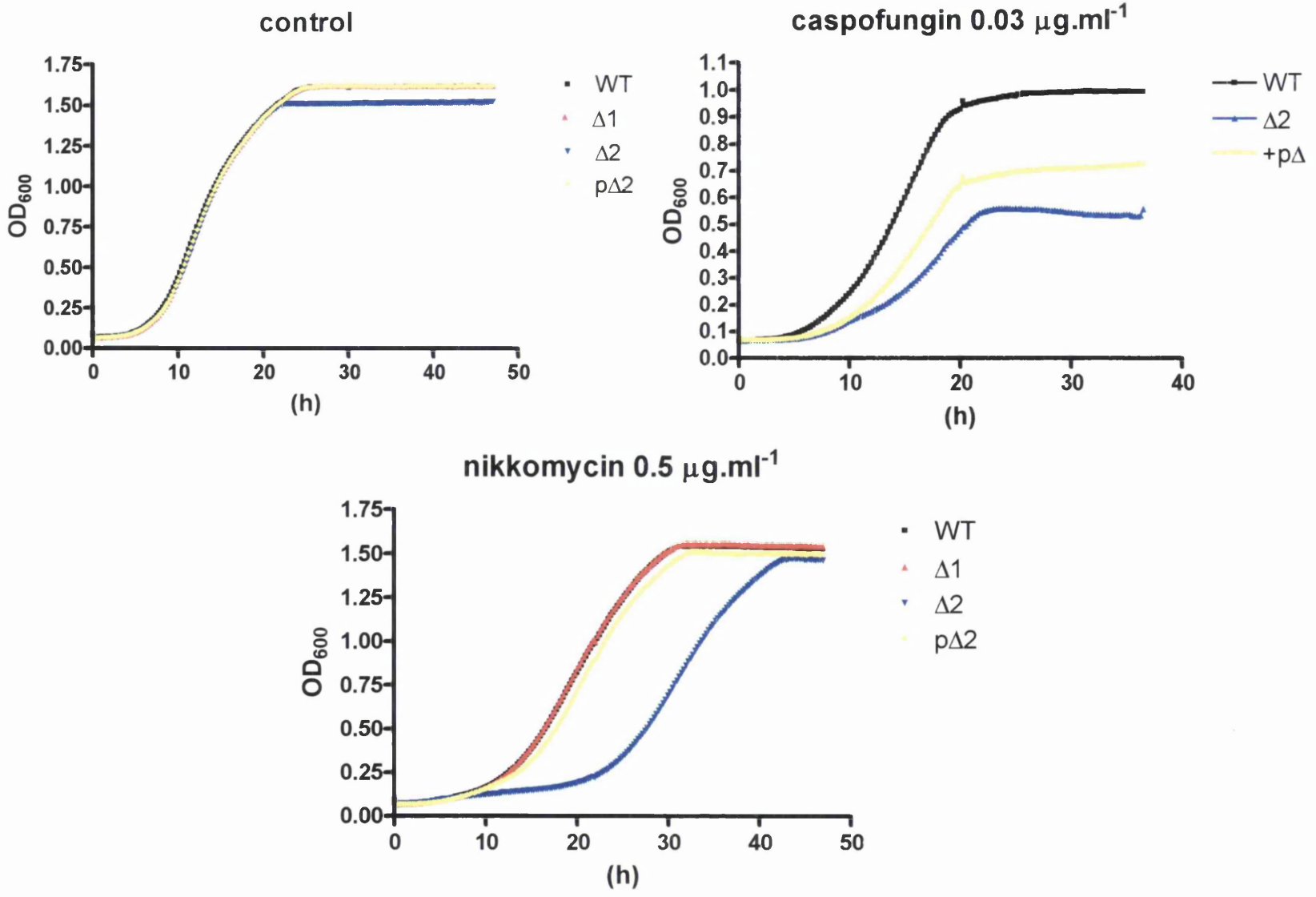


Fig. 4.10 A. Effect of inhibitors of cell wall synthesis on growth in minimal media. WT= SC5314, Δ2=homozygous *DIT2* mutant and +pΔ2= complemented *dit2* homozygote

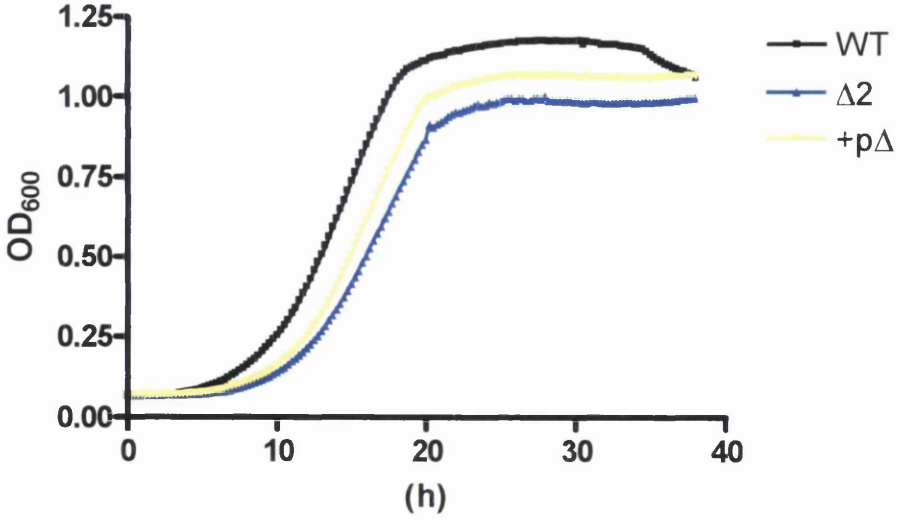


Fig. 4.10 B. Effect of maltose on the Growth curve in minimal media. WT= wild-type, Δ2=homozygous mutant and +pΔ2=reintegrant

The susceptibility of cells inoculated on YEPD or YM agar plates supplemented with metabolic inhibitors was also investigated. For these studies plates containing 1.5 M sorbitol, 100 $\mu g.ml^{-1}$ SDS, 1 M or 1.5 M NaCl, 5 mM or 10 mM H_2O_2, 0.5 M or 1 M $CaCl_2$, 100 $\mu g.ml^{-1}$ calcofluor, 100 $\mu g.ml^{-1}$ congo red, 1 $\mu g.ml^{-1}$ itraconazole, 10 $\mu g.ml^{-1}$ amphotericin, and 2 $\mu g.ml^{-1}$ fluconazole incubated at 37°C and 42°C were investigated. Cells on YEPD agar plates did not reveal any difference in their sensitivity of either *cyp56Δ* mutant or wild-type strain (Fig. 4.11 and 4.12 A). The exception was nikkomycin, which showed an inhibitory effect of 2 fold less (MIC = 0.5 $\mu g.ml^{-1}$) on *cyp56Δ* mutant compared to the wild-type strain (Fig. 4.12 B).

In contrast to YEPD medium, cells cultured on YM agar plates did exhibit a mutant phenotype. Homozygous *cyp56Δ* mutant strains showed a rough colony surface whilst the wild-type strain was smooth (Fig. 4.13). Optical microscopy confirmed the presence of hyphae in the mutant strain and only yeast cells in wild-type strain grown under these conditions (Fig. 4.14). The *cyp56Δ* homozygous mutant strain showed significantly less growth at 42°C in both YM and YEPD media compared to wild-type strain.

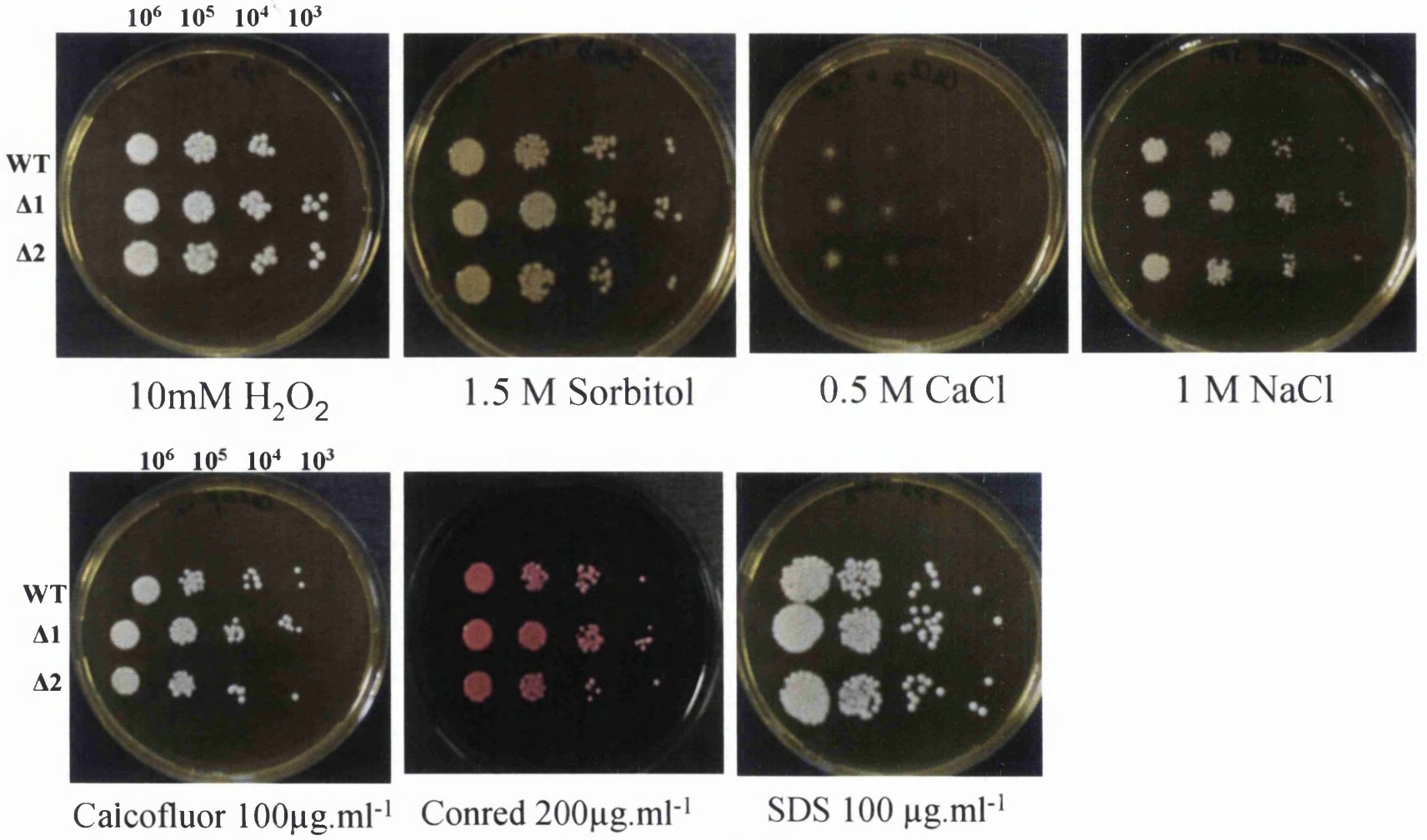


Fig. 4.11. Spot test results performed on YEPD agar supplemented with various compounds. WT= SC5314, Δ1= heterozygous *DIT2* mutant, Δ2=homozygous *DIT2* mutant strain.

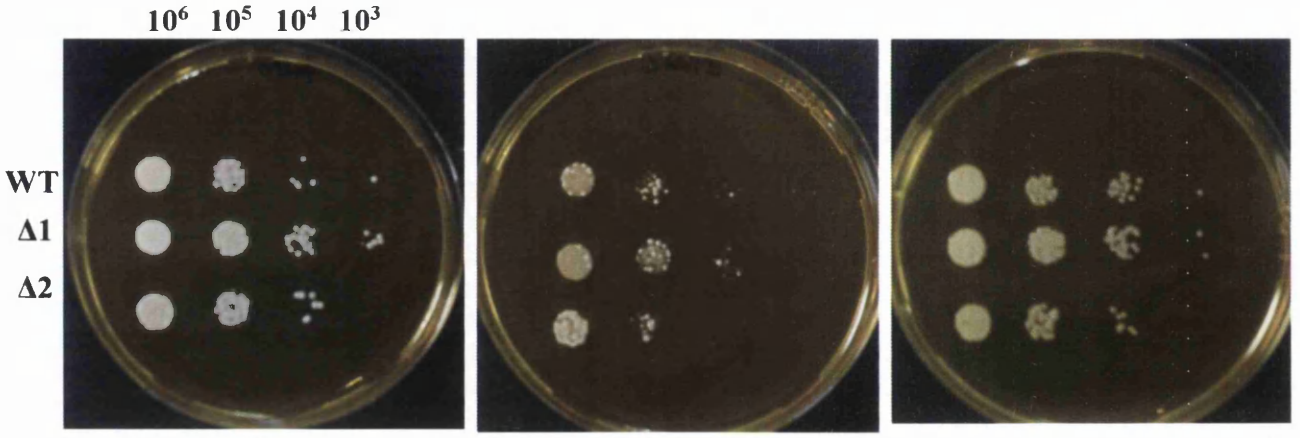


Fig. 12A. Spot test results performed on YEPD agar supplemented with antifungal drugs

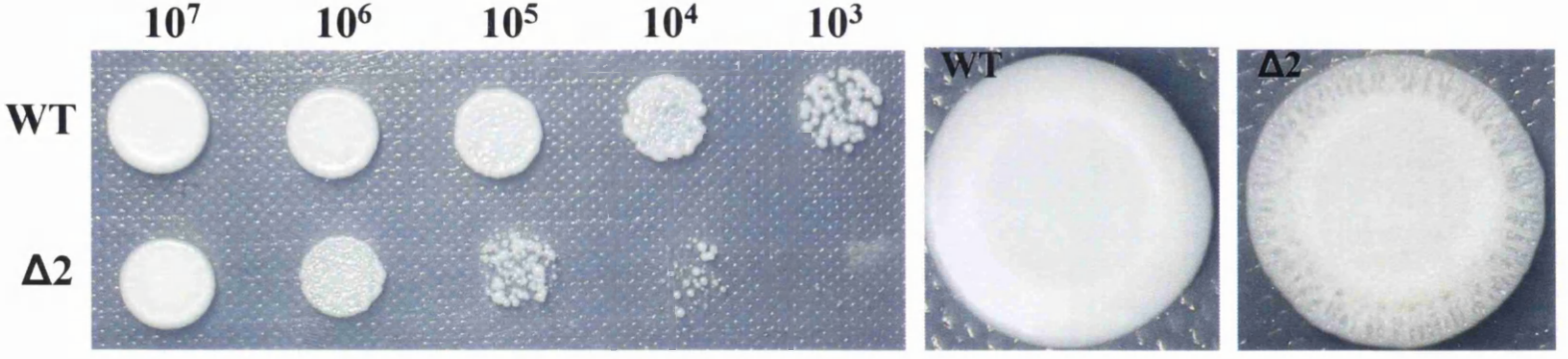


Fig. 4.12 B. Spot test onto YM agar supplemented with nikkomycin at 0.5 µg.ml^{-1}

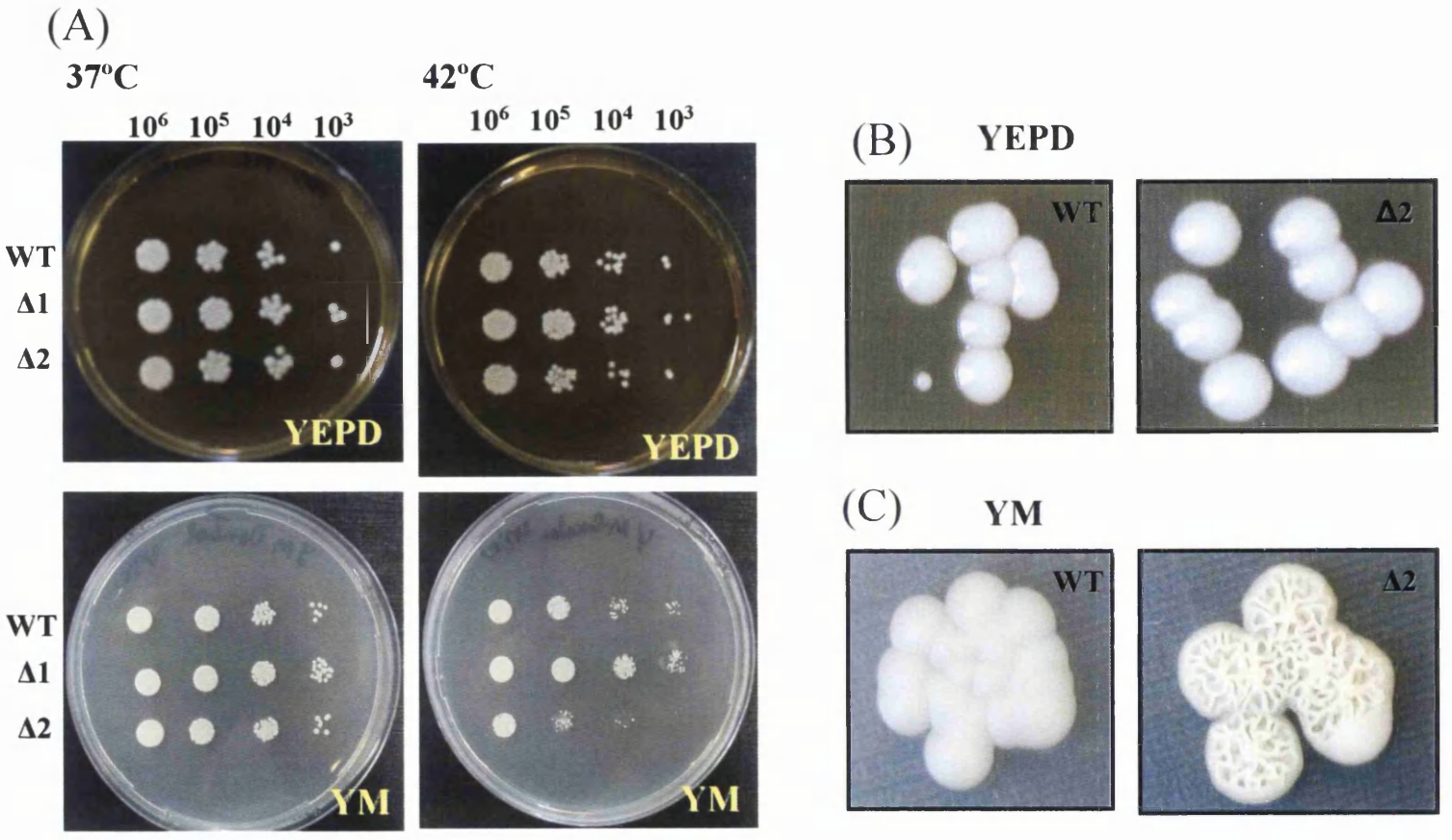
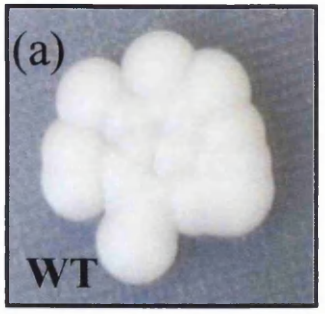
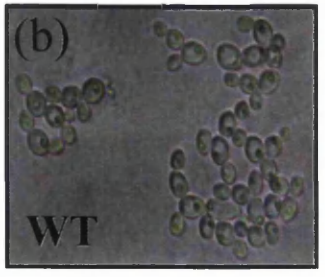
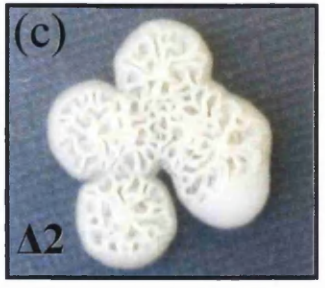
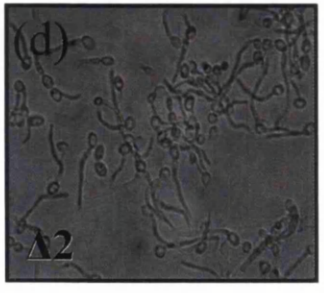


Fig. 4.13. Spot test results performed on YEPD and YM agar media, at different temperatures (A). Smooth colonies are formed on YEPD agar medium by both strains (B). On YM agar medium the wild-type strain showed smooth colonies, however the *dit2Δ* homozygous mutant strain formed wrinkled colonies. WT=SC5314, Δ1= heterozygous *dit2Δ* mutant and Δ2=homozygous *dit2Δ* mutant.









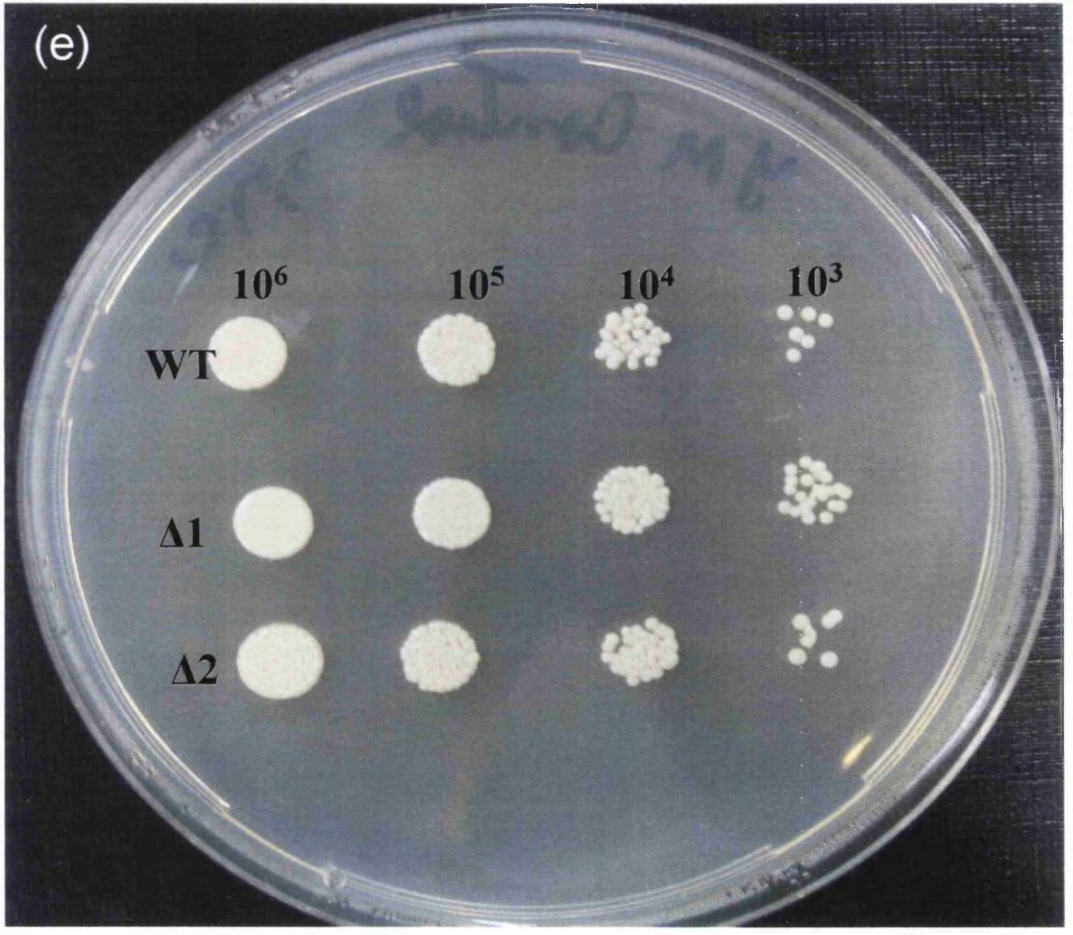


Fig. 4.14. Visualization of morphological features of strains grown on YM. (a) Smooth colonies showed by WT strain; (b) microscopic view of the yeast form showed in minimal medium broth. (c) Rough colonies displayed by *dit2Δ* heterozygous mutant and hyphal form observed microscopically in minimal medium broth (d). WT=SC5314, Δ1=heterozygous *dit2Δ* mutant and Δ2=homozygous *dit2Δ* mutant strains (e).

4.3.3.4. Chlamydospore production and hyphal formation.

In cornmeal broth supplemented with 1% (v/v) Tween 80, wild type *C. albicans* produced abundant chlamydospores after 72 h static growth at room temperature. However, the *cyp56Δ* homozygous mutant failed to produce chlamydospores in comparison to the wild-type strain (Fig. 4.15 A). This phenotype could be complemented by the reintroduction of the *CYP56* gene on the plasmid CIp20.

The complemented strain produced chlamysospores in cornmeal broth, although less abundantly and slower (72-96 h) than the wild-type strain. The chlamydospore of the wild-type strain revealed a bright fluorescent thick halo when stimulated with UV light, in contrast to the *cyp56Δ* homozygous mutant which did not produce chlamydospores (Fig. 4.15). This fluorescence was also restored in the complemented strain (Fig. 4.15 C). Although microscopic features of the germ tube formation were similar for both strains, visualization of the effect of Na_2SO_3 on hyphal formation revealed that the wild-type remained in the yeast form and the mutant was a mixture of yeast and hyphal form (Fig. 4.16).

4.3.3.5 Cell fluorescence imagining of *cyp56Δ* mutant and wild-type strains by confocal microscopy and fluorescence microscopy.

For fluorescence microscopy, cells were examined using a Nikon Eclipse E600 epifluorescence microscope. To examine autofluorescence of yeast and hyphae in the UV wavelength range, cells were examined using the Zeiss Plan-Achromat 63x, 1.4 NA objective, 512 x 512 pixel images captured and exported in TIFF format using Zeiss laser scanning microscope LSM 510 version 3.2 SP2 software. The *cyp56Δ*

homozygous mutant exhibited a reduced bluish fluorescence pattern over its surface when compared to wild-type strain (Fig. 4.17 A), possibly corresponding to chitin which shows autofluorescence. Chitin may also be stained with calcofluor (Fig. 4.17 C). When both strains were stained with calcofluor localized fluorescent septa areas were observed (Fig. 4.17 B). However, the *cyp56*Δ mutant strain showed less visual fluorescence than the wild-type strain. The chlamydospore of the wild-type strain stained with calcofluor revealed a bright fluorescent yellowish thick halo compared to the *cyp56*Δ mutant strain (Fig. 4.17 B).

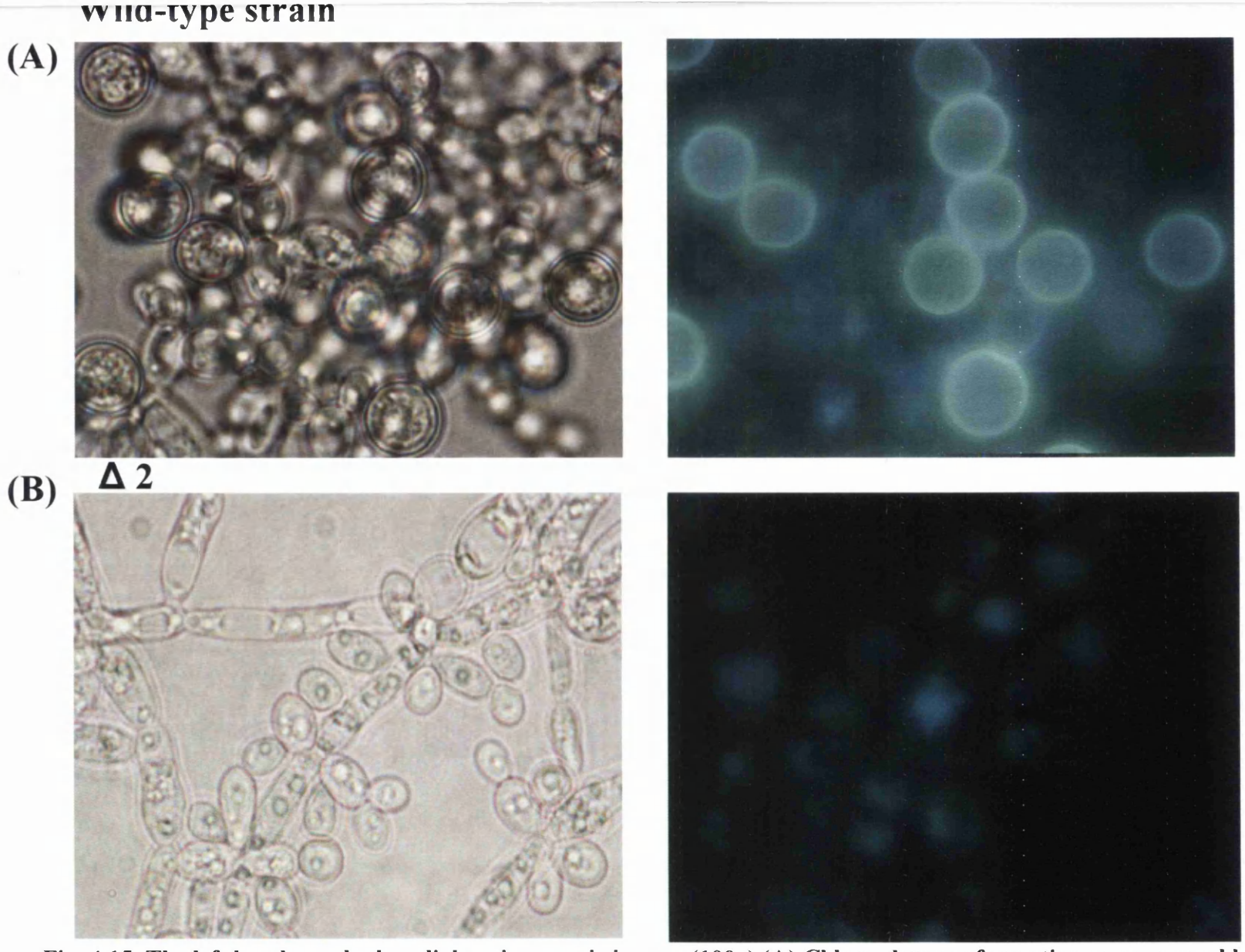


Fig. 4.15. The left hand panels show light microscopic images (100x) (A) Chlamydospore formation on corn meal broth, the *dit2Δ* mutant strain (B) did not produce chlamydospores in comparison to the WT strain. Images on the right panels were observed by fluorescence microscope. WT=SC5314, Δ2= *dit2Δ* homozygous mutant strain.

(C) **Δ 2 reintegrant strain**

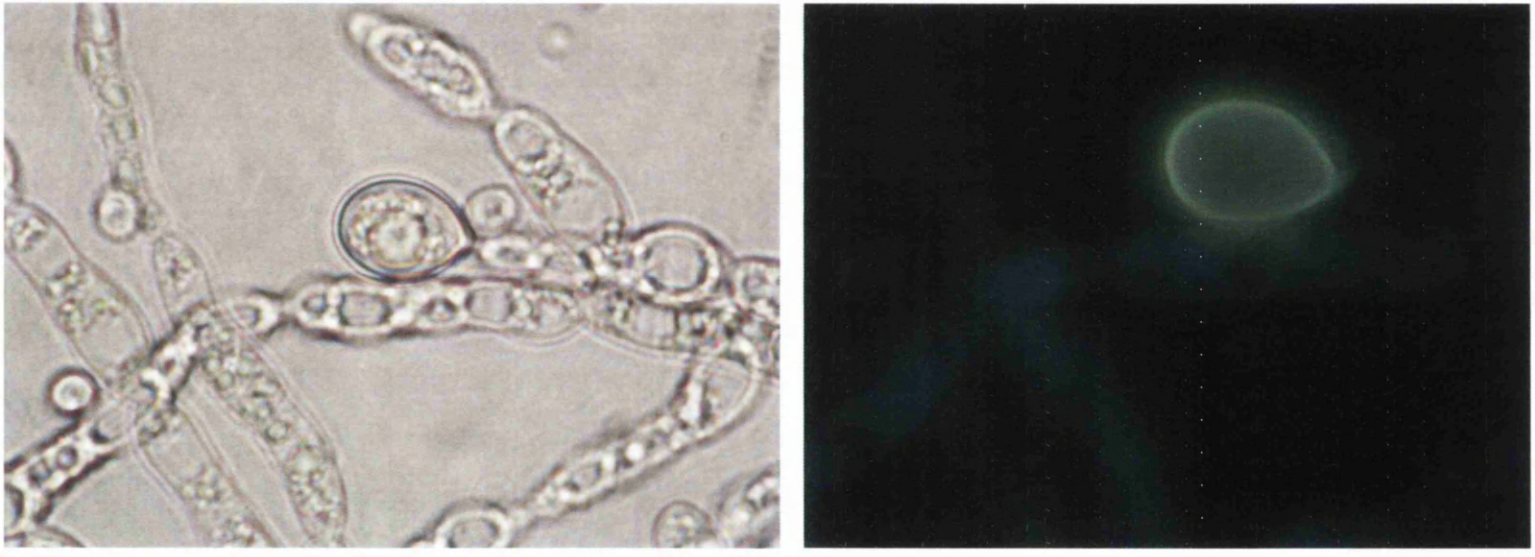


Fig. 4.15. (C) Microscopic characteristics of Chlamydospore formation on corn meal broth, the *dit2Δ* reintegrant strain produced chlamydospores similar to the WT strain. Images on the right side were observed by fluorescence microscope.

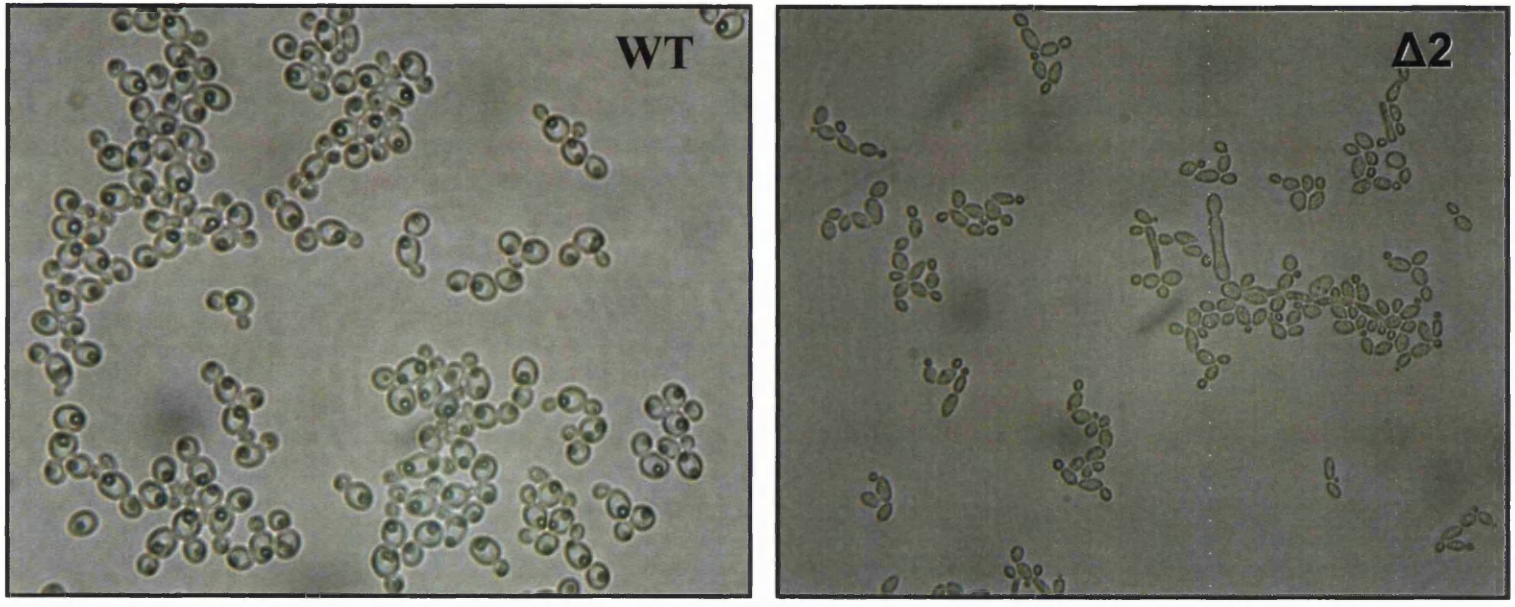


Fig. 4.16. Effect of Na_2SO_3 on hyphal growth on RPMI media after 24 hours observed by optical microscope (40x). WT showed yeast form and Δ2 exhibited mixture of hyphae and yeast form. WT= wild-type and Δ2 = homozygous mutant strains.

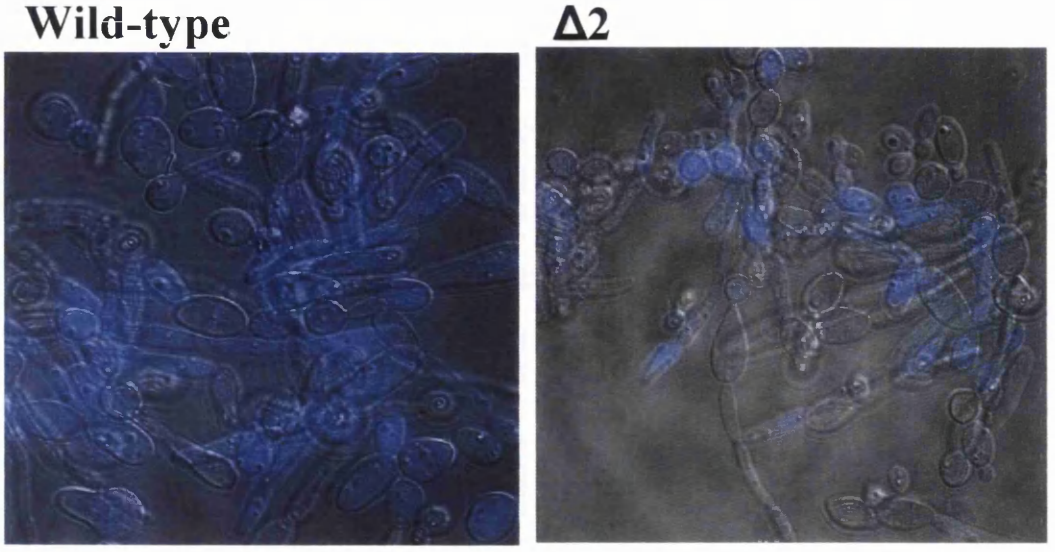


Fig. 4.17 A. Visualization of natural fluorescence by confocal microscope of germination form of *C. albicans* wild-type and *dit2Δ* knockout strain grown on YM media.

100x

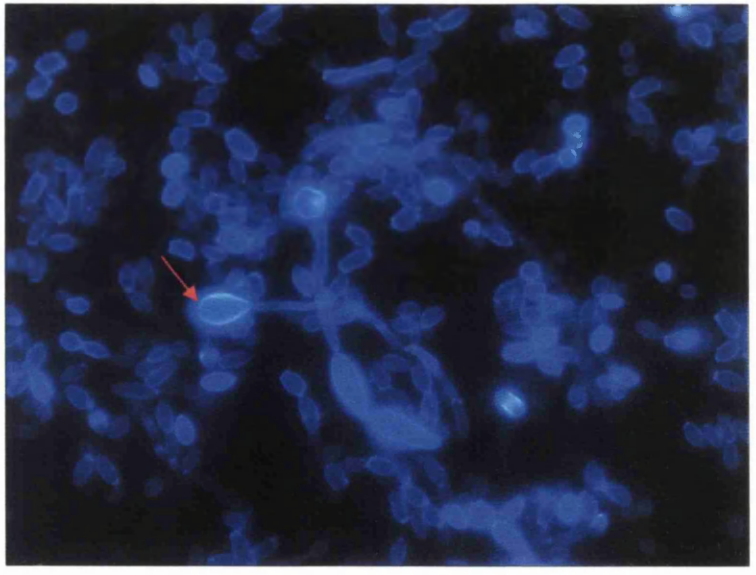
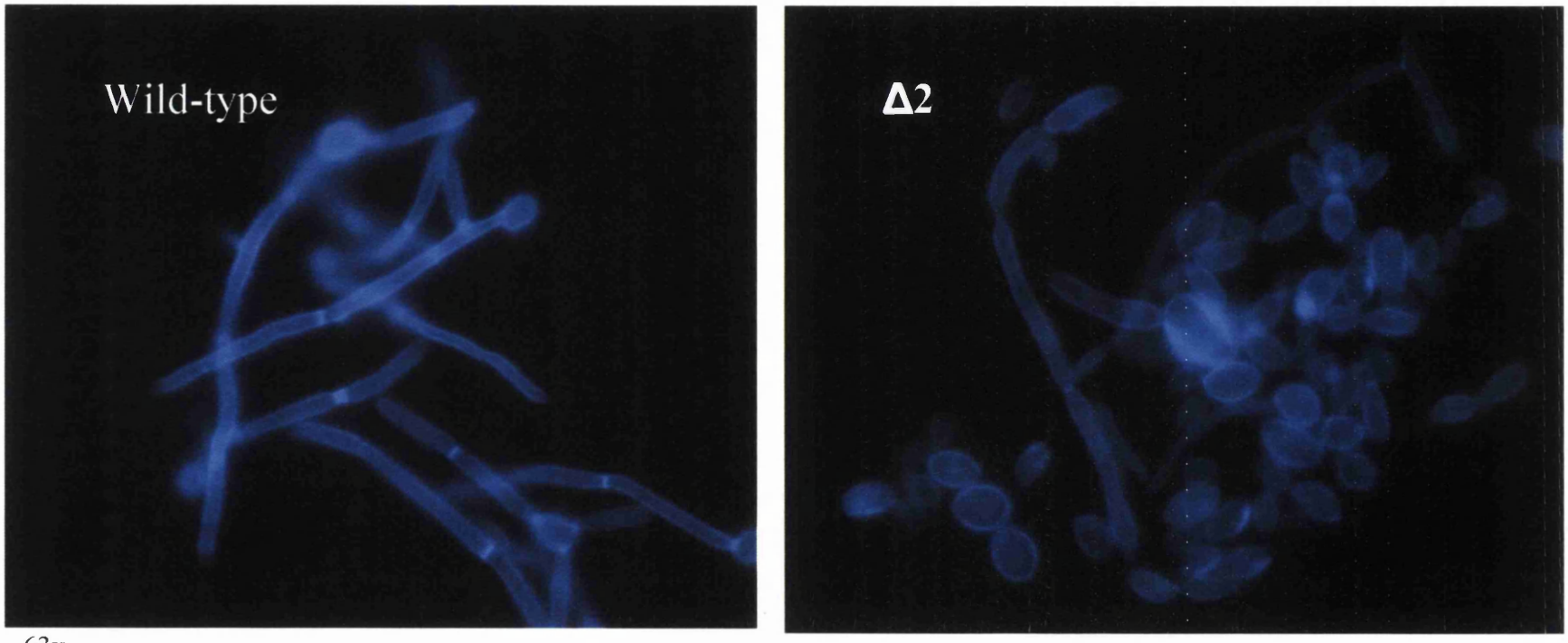


Fig. 4.17 B. Visualization by confocal microscope of chlamydospore form of *C. albicans* wild-type stained with calcofluor. Fluorescent thick halo is indicated by red arrow may correspond to dityrosine.



63x

Fig. 4.17 C. Visualization by fluorescence microscope of hyphal form of *C. albicans* wild-type and *dit2Δ* knockout strains grown on YM which were stained with calcofluor.

4.3.3.6. Sterol composition comparison of *cyp56Δ* mutant and wild-type strains.

No differences in the sterol composition between *cyp56Δ* mutant and the wild-type strain (SC5314) were identified with both strains having high level of ergosterol (Fig. 4.18). Analysis of the sterol composition for both strains in yeast-like form in this study revealed that ergosterol was the predominant sterol, accounting for 90% of the total sterols present.

4.3.3.7 Synthesis of an authentic dityrosine standard.

Dityrosine is not available commercially and a peroxidase system was used to produce a standard for experiments. The enzymatic reaction for dityrosine synthesis consisted of *N*-formyl tyrosine + H_2O_2 + HRP, which was optimized in different concentration of H_2O_2 as described in Chapter 2.18.2. The dityrosine synthesized was investigated using fluorescence spectrophometry at excitation wavelength of 320 nm and emission maximum at 400-405 nm (Fig. 4.19 A). The results were in excellent agreement with those published by Briza *et. al.* (1996). TLC was used to isolate the compounds formed as indicated by the arrows in Fig. 4.19 B corresponding to fluorescence spots visualized under UV light. The fluorescence measurements from the chemical after TLC showed the characteristic spectrum for dityrosine (Fig. 4.19 C). The dityrosine synthesized was used as a control for identification of dityrosine in cell extractions and CaCYP56p enzyme reconstitution assays.

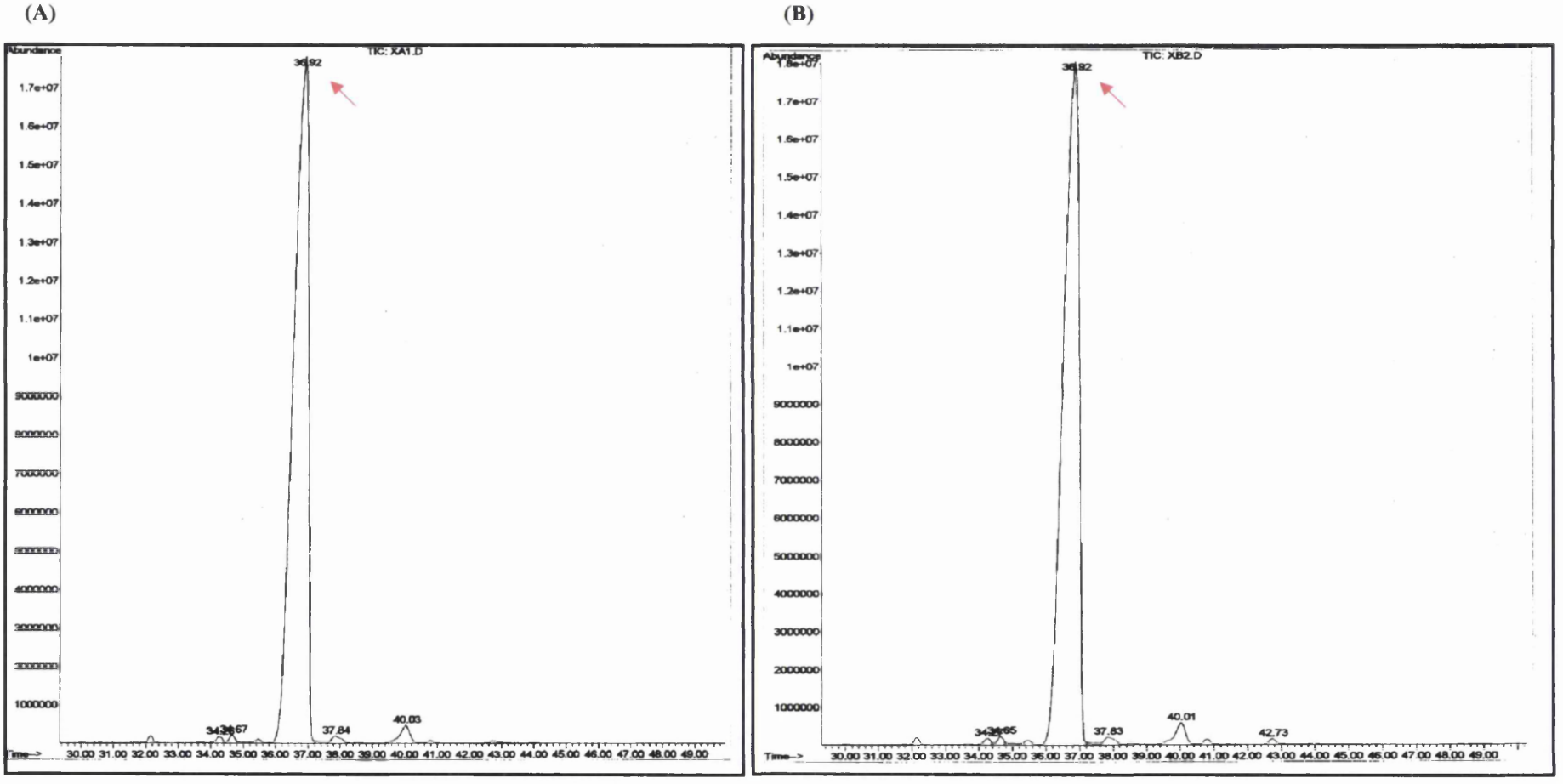


Fig. 4.18. GC analysis of sterol content in wild-type (A) and *dit2Δ* homozygous mutant (B) cells grown on YEPD. The sterol profile was similar for both strains. The higher peak in both figures indicated by the arrows represent ergosterol as the major sterol with similar retention time for both samples.

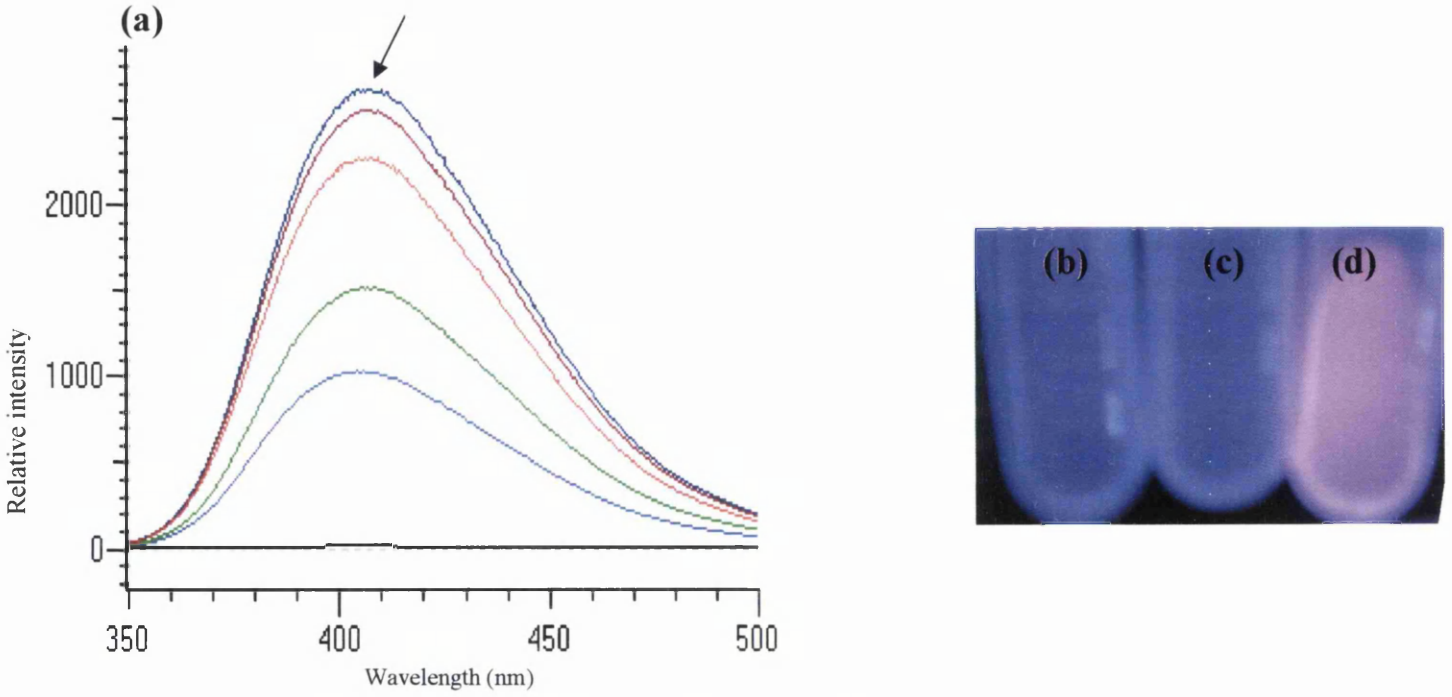


Fig. 4.19 A. The figure (a) shows the fluorescence results from enzymatic reaction of *N*-Formyl tyrosine + H_2O_2 + HRP in different concentration of H_2O_2 (different colours of peaks correspond to different H_2O_2 concentration). The fluorescence measurements were recorded at excitation wavelength of 320 nm and emission wavelength was 400-405 nm. The arrow indicates the fluorescence peak recorded at 403nm at highest concentration of peroxide. The suspensions of the enzymatic reaction before loading on TLC plates for dityrosine isolation was visualized by UV light (b-c) are controls (no H_2O_2 and no HRP), and (d) complete reaction. The controls did not show any fluorescence peak (data not shown).

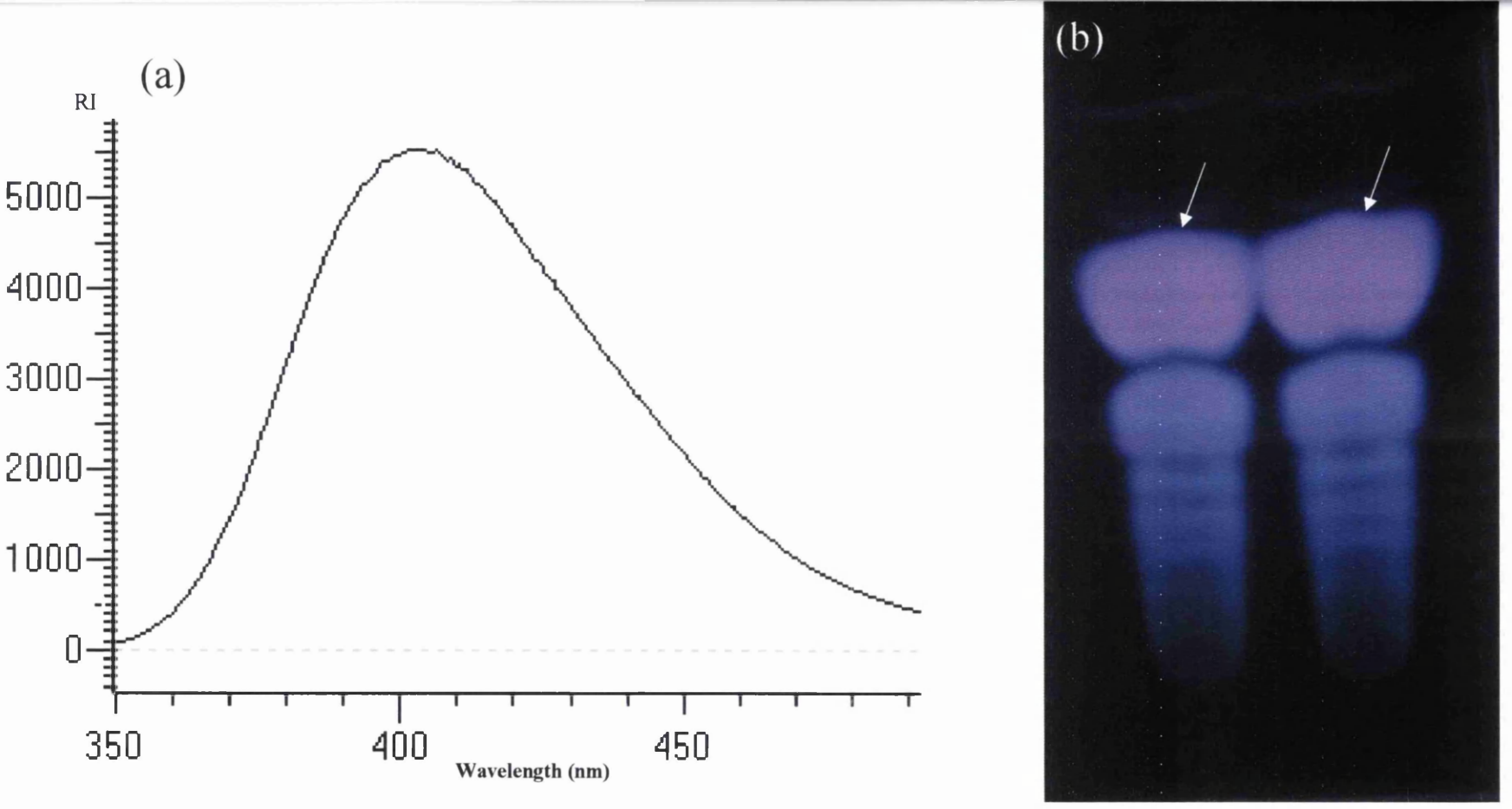


Fig. 4.19 B. (a) Dityrosine synthesis detected by fluorometric scanning with peak of 403 nm. (b) The TLC plate shows fluorescent spots corresponding to N-formyl dityrosine as indicated by the arrows.

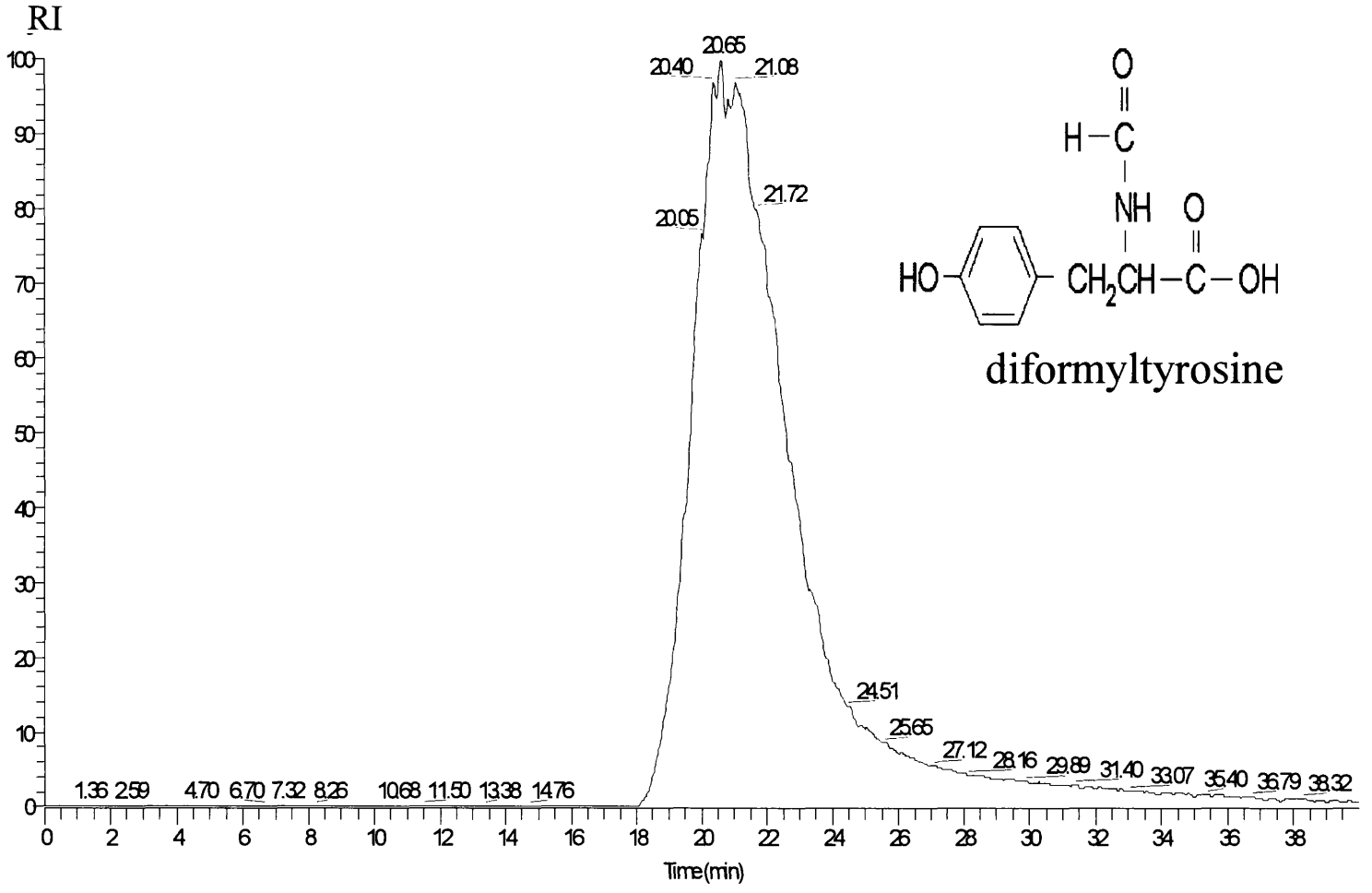


Fig. 4.19 C. The HPLC corresponds to those fluorescent spots which exhibited *m/z* 417 – *m/z* 343 for diformyltyrosine.

4.3.3.8 Extraction of Dityrosine from Cells.

Cell walls of yeast and hyphae from wild-type and homozygous mutant strains were prepared by vortexing intact organisms with glass beads, centrifugation, and extracted with chloroform:methanol. In parallel dityrosine synthesized *in vitro* was used as a control. Dityrosine was not detected in the yeast cell extracts from any strains studied by HPLC/MS. Cell walls of chlamydospore from wild-type and homozygous mutant strains are still undergoing at the time of submission of this work.

4.3.4 Biochemical analysis of recombinant *C. albicans* CYP56.

Following heterologous expression in *E. coli* and isolation of membrane fractions, recombinant *C. albicans* CYP56 was shown to be present in the microsomal fraction. Furthermore, a characteristic peak at 450 nm was obtained when the protein was reduced in the presence of carbon monoxide (CO) as demonstrated by spectrophotometry (Fig. 4.20). Control cells containing empty expression plasmid did not exhibit the peak at 450 nm in reduced carbon monoxide difference spectra. CYP56 was purified as described previously and exhibited a slight red-brownish band during Ni-NTA purification through the Ni-NTA agarose matrix. The purified protein was visualized by SDS-PAGE gel showing a molecular mass of 55 kD (Fig. 4.21).

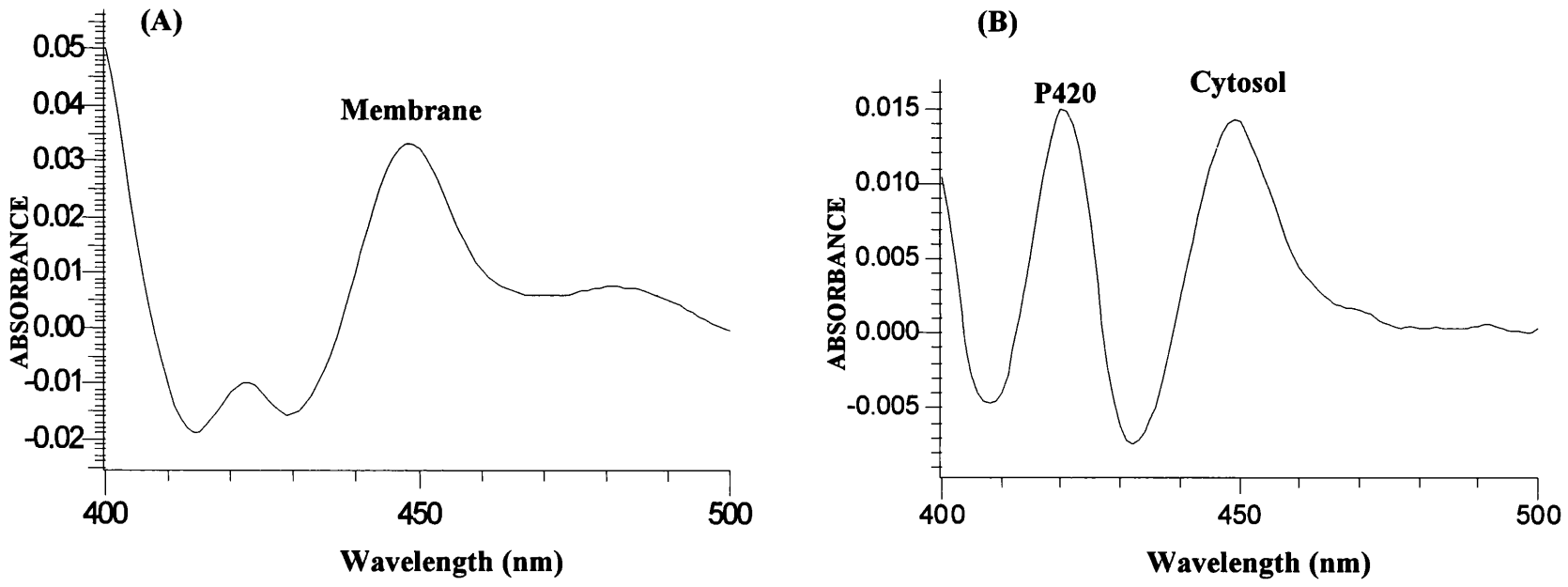


Fig. 4.20. Carbon monoxide difference spectrum of reduced *Ca*CYP56 expressed in the membrane of *E. coli* (A), overexpressed CYP56p in the cytosol of *E. coli* (B) both showing peaks of at 450 nm. In the cytosolic fraction at 420 nm peak was observed which is the result of P450 degradation.

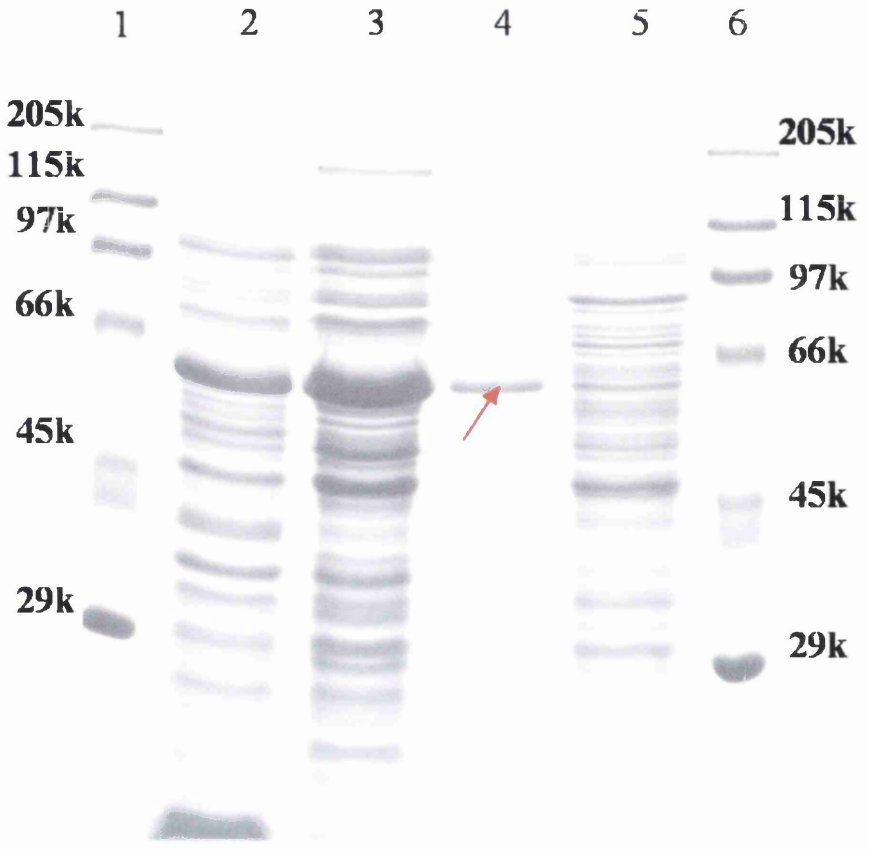


Fig. 4.21. SDS PAGE gel of CYP56 protein after heterologous expression in *E. coli*. Lanes:1 and 6= protein standards, 2= membranes, 3= cytosol, 4= purified fraction diluted 10x, 5= solubilized membrane fraction prior to purification.

4.3.5 Spectral studies on CYP56 substrate binding.

In the presence of *N*-formyl tyrosine, CYP56 produced a Type I substrate binding spectrum. The substrate binding was demonstrated using the microsomal enzyme system and *N*-formyl tyrosine as substrate. The substrate affinity of the protein was high with a calculated Ks of 26 nM (Fig. 4.22). Furthermore, binding of *N*-formyl tyrosine to CYP56 appeared to be allosteric with an observed Hill number of 1.6, suggesting the binding of two substrate molecules in the active site in CYP56.

4.3.6 Reconstitution of CYP56 activity.

In reconstituted enzymatic activity experiments, *C. albicans* CYP56 and *S. cerevisiae* CPR proteins were incorporated into DLPC micelles prior to the addition of substrate and initiation of the reaction with NADPH. TLC was employed for the identification of dityrosine bands produced in the reaction. Additionally, HPLC methods also permitted the separation of *N*- formyl dityrosine from substrate produced in this reaction. When reconstituted into DLPC micelles, CYP56 catalyzed the conversion of *N*-formyl tyrosine into *N*- formyl dityrosine (Fig. 4.23). Controls in which NADPH was omitted did not produce dityrosine. The mass spectrum of the generated authentic dityrosine had a molecular ion of *m/z* 315. The reconstitution assay revealed that CYP56 fraction converted *N*-formyl tyrosine through to the oxidation product *N*-formyl dityrosine (*m/z*=315) in the presence of CPR and NADPH. The retention time of this molecule under the HPLC conditions used was 21 min^{-1} (Fig. 4.24).

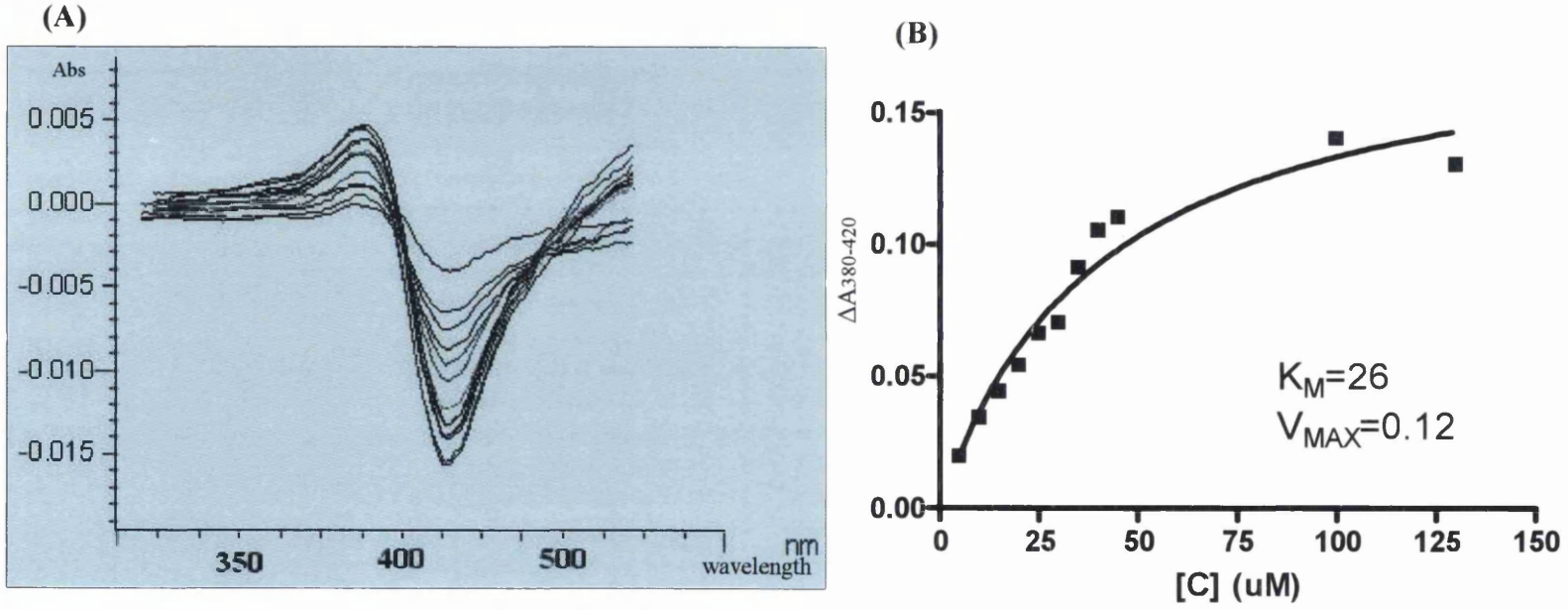


Fig. 4.22. (A) Spectra of substrate binding type I for *N*-formyltyrosine,
(B) Plot of change of absorbance against concentration of *N*-formyltyrosine.

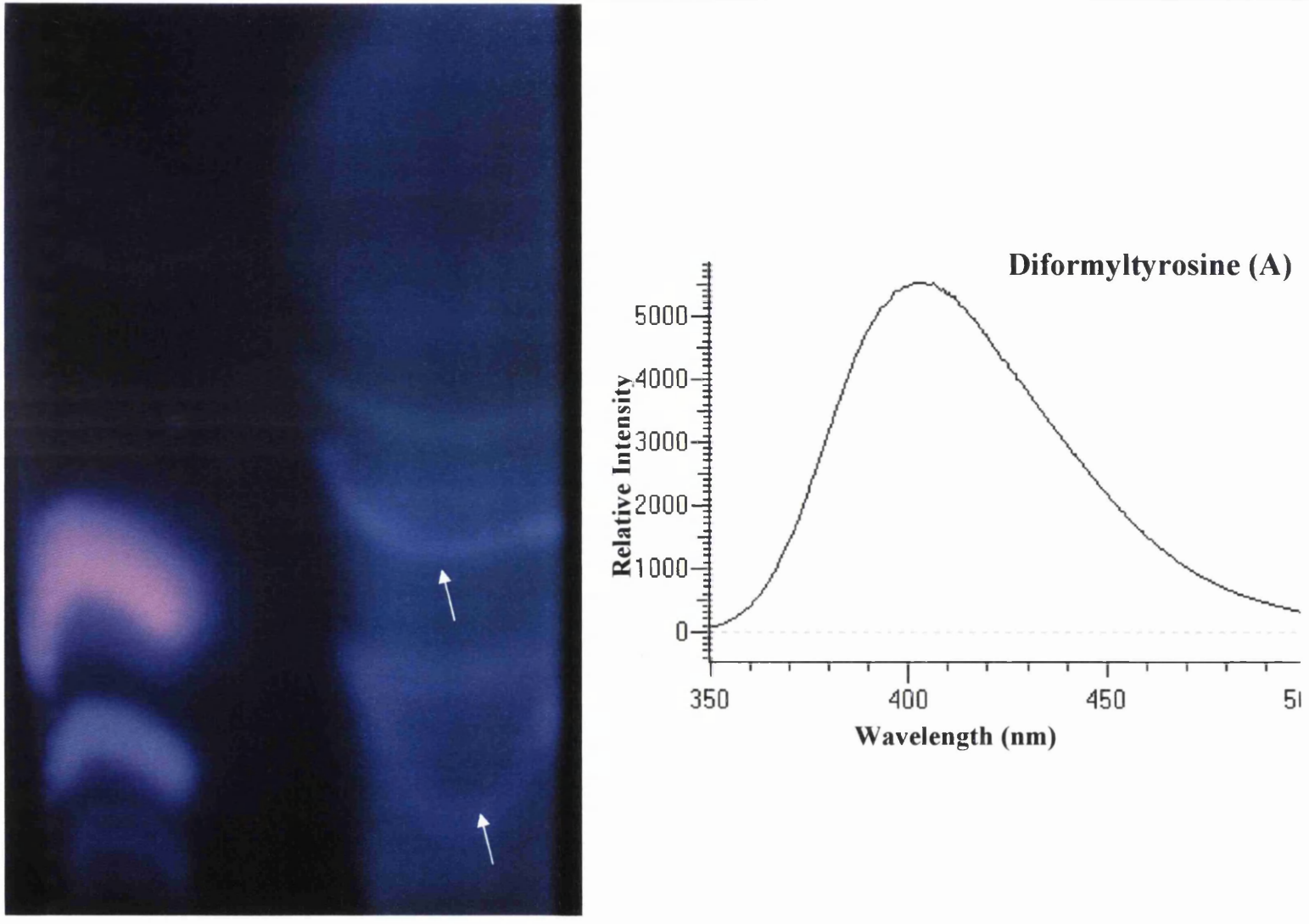


Fig. 4.23. Reconstitution of activity included of expressed CaCYP56, CPR protein, DPLC and NADPH. After the reaction the suspension was loaded on TLC plate in which the arrows indicate fluorescent bands in the correspondent position to diformyltyrosine. (A)=Diformytyrosine scanned by fluorescence spectrophotometer after excitation wavelength at 320 nm and the emission wavelength was 403 nm.

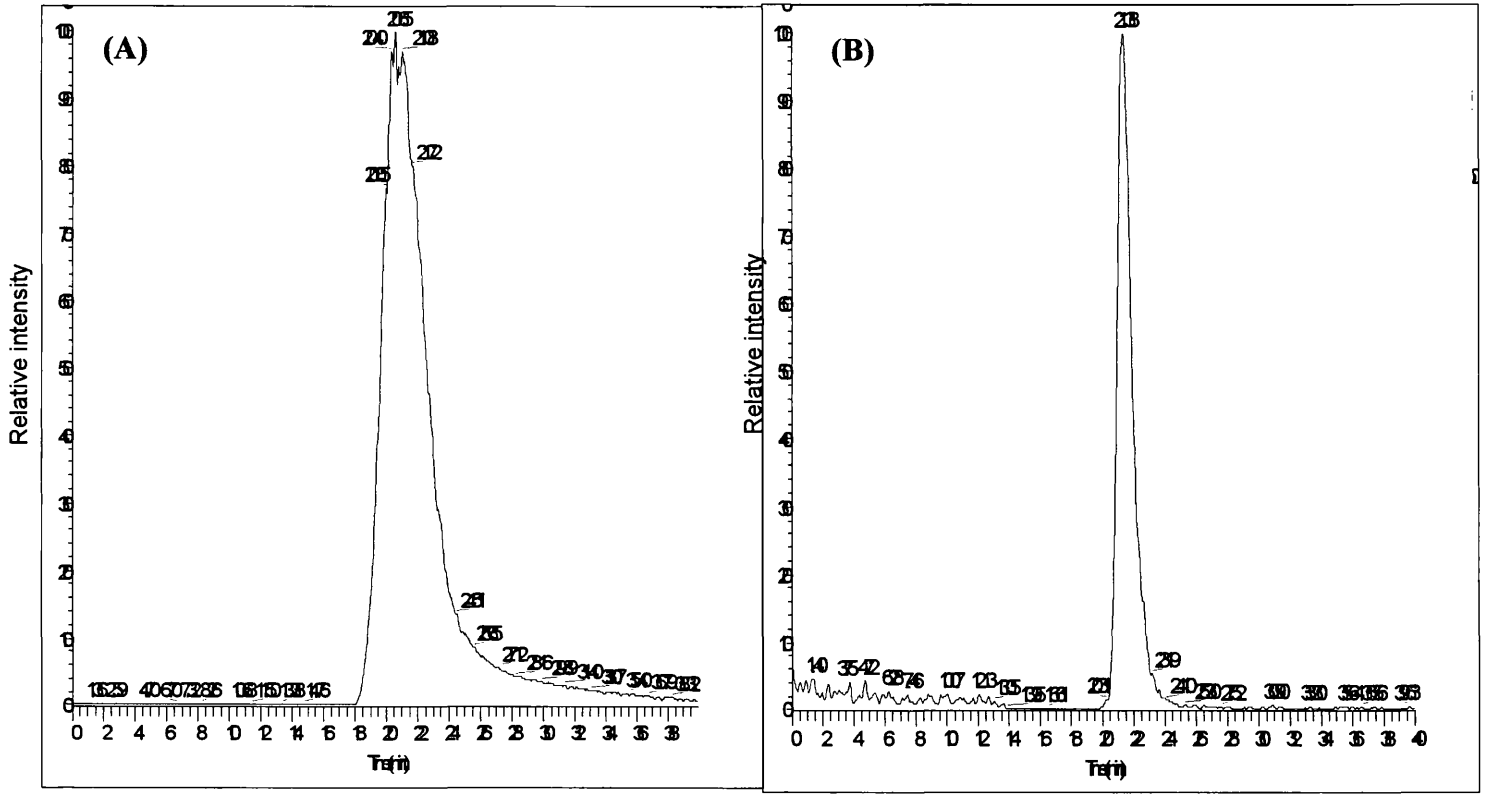


Fig. 4.24. HPLC of the extract from reconstitution of P450 activity corresponding to fluorescent TLC spots. Both were identified as diformyltyrosine, (A) Control standard and (B) Extract from P450 reconstitution.

4.3.7 Bioinformatic analysis of *C.albicans* CYP56.

The available resolved CYP crystal structures show that the general overall CYP tertiary fold is preserved during evolution from bacteria through mammals (Nelson, 1999, Kelly et al., 2001). At the same time, there are variable regions that appear to be associated with recognition and binding of structurally diverse substrates and redox partners (Podust et al., 2001). Using BLASTP 2.2.14 NCBI we have performed the alignment of CYP56 between *C. albicans and S. cerevisiae* consisting protein lengths of 477 and 489 amino acids respectively. They were found to be 46% identical as is shown in the Fig. 4.25.

The regions corresponding to I helix, K helix and the active site (involving at its base the binding of haem to a cysteine residue in red) are in the box marked with the consensus sequence (Fig. 4.25). The I Helix is thought to be central to catalytic function of CYP's (Gotoh, 1993) and is thought to play a part in proton delivery and dioxygen binding (Poulos et al., 1987). The amino acid sequences of the haem binding sites were **HGRKRAC** and **HGGRRAC** for *C. albicans and S. cerevisiae* respectively, therefore showing only two amino acids varying. However the location of a cysteine residue was conserved for both as expected for active cytochromes P450 in order to provide the fifth ligand for the haem.

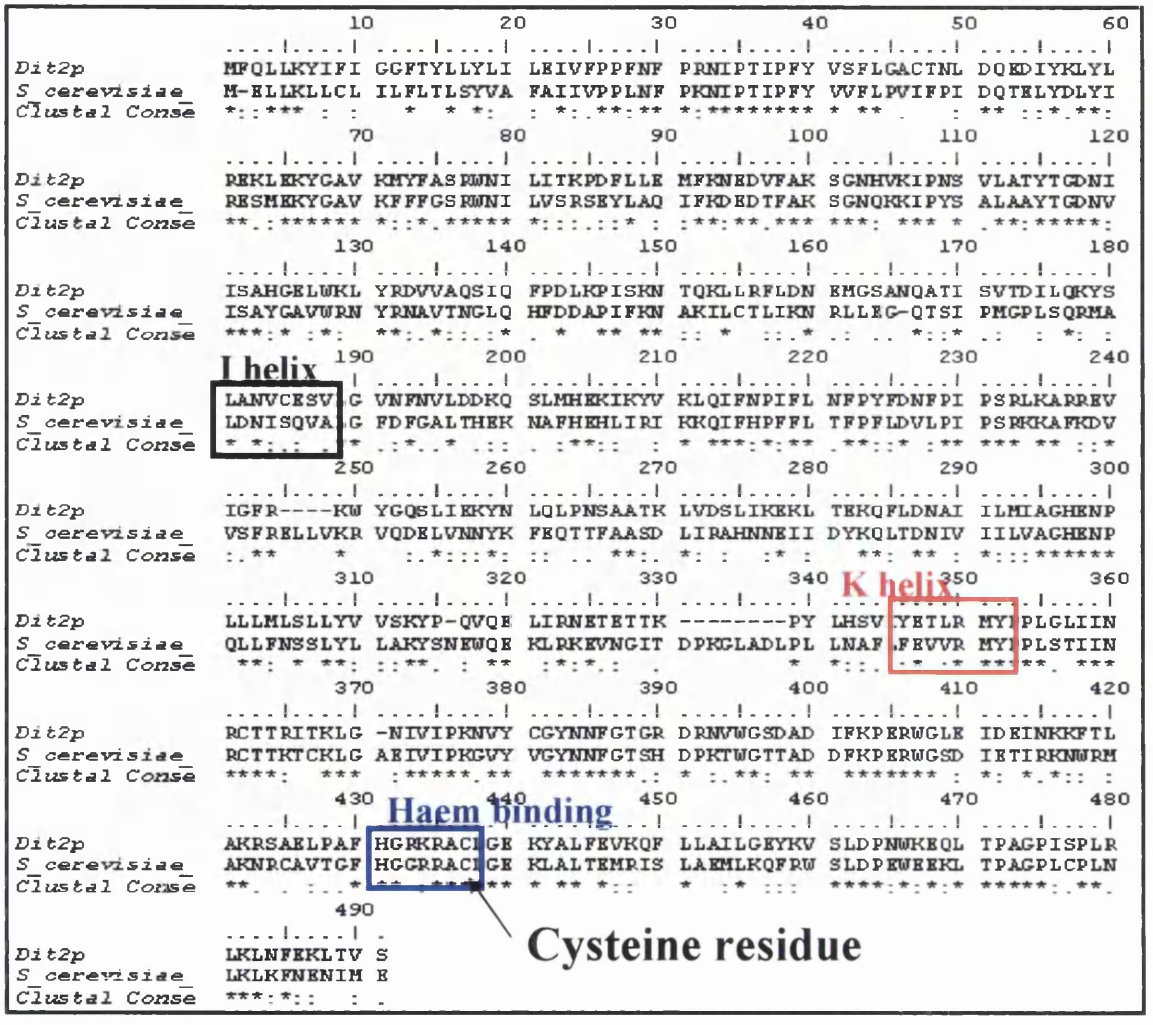


Fig. 4.25. Alignment and identity between Ca Dit2 and ScDit2. The regions corresponding to the I helix, the K helix and the haem ligand (involving the binding of haem to a cysteine residue in blue) are in the box marked with the consensus sequence. The I Helix is thought to be central to catalytic function of CYP's (Gotoh, 1993).

4.4. Discussion

After the first report of the presence of dityrosine in *C. albicans* in 1995 (Smail *et al.*, 1995), few studies have addressed the contribution of dityrosine related to the biology of this organism. This was surprising since the cell wall of *C. albicans* is of active interest as a target for new drugs and because of its role in pathogenicity and immunogenicity. The role of the novel *CYP56* gene present in *C. albicans* in growth and drug susceptibility was investigated here.

Sequencing of the *C. albicans* genome (Nantel, 2006) has allowed advances in the study of functional genomics, leading to a better understanding of the role of specific genes in the biology and virulence of this pathogenic fungi. A widely accepted and direct approach to assess the physiological function of a gene is to inactivate the gene by targeted mutagenesis. In this present study this has been achieved by using the *SAT1* flipping method (Reuss et al., 2004) which is a highly efficient way to construct a homozygous knock out mutant in the diploid *C. albicans*. The DNA sequence of *C. albicans CYP56* gene sequence was revealed and using genomic DNA template from *C. albicans* strain SC 5314 and primers designed from the full-length *C. dubliniensis CYP56* gene sequence available in the database (Dr. G. Moran, data unpublished), the gene isolated and a deletion construct produced. Subsequent deletion of the *CYP56* gene from the *C. albicans* genome allowed the characterisation phenotypically and biochemically of the homozygous *cyp56Δ* mutant against the wild-type strain, especially in terms of features associated with the cell wall, viability, natural fluorescence associated with the presence of dityrosine and sensitivity to antifungal drugs.

Susceptibility testing for the drugs tested revealed no important differences in the MICs values for *cyp56*Δ mutant and the wild-type strain. The Etest data reinforced the similarity in the MIC results for both strains. Although the MIC endpoint values have not shown remarkable difference, when the cell growth kinetics were investigated differences between homozygous *cyp56*Δ mutant and wild-type strains were observed. However, one exception was nikkomycin which showed a 2 fold lower MIC for the *cyp56*Δ homozygous mutant compared to the parent strain. The slower growth at sub-inhibitory concentrations of casponfungin and nikkomycin in the *cyp56*Δ homozygous mutant strain was clear and reproducible between experiments. Considering caspofungin is glucan synthase inhibitor and nikkomycin is a chitin synthase inhibitor, and both contribute to cell wall structure, the results from this study suggest an alteration of glucan and chitin structure in the *cyp56*Δ homozygous mutant strain. Hence, dityrosine may play a key role in cell wall arrangement or possibly have a role in the order of layer deposition in the cell wall. Chitin and glucan composition of the mutant should be investigated in future studies. It is known structural composition of the cell wall varies according with form. Ruiz-Herrera and collaborators (Ruiz-Herrera et al., 2006) have indicated in their work that hyphae showed 6% chitin composition whilst yeast forms only 2%. In the case of glucan, this was 56% in the hyphae and 60% in the yeast form.

The deletion of *CYP56* had a profound effect on chlamydospore formation in *C. albicans*. This was not surprising due to the essential nature of dityrosine in the formation of the spore wall in *S. cerevisiae*. The *cyp56*Δ mutant failed to form chlamydospores in cornmeal broth, whereas the wild-type formed abundant spores.

Interestingly, the wild-type spores had thick walls that fluoresced strongly under stimulation with UV light. This fluorescence was paler and more intense than the natural blue chitin fluorescence seen in hyphae and yeast cells in the same cultures. It is tempting to speculate that this fluorescence in chlamydospores is due to the presence of dityrosine. Although dityrosine may also be present in yeast cells, we observed little difference in the levels of fluorescence between wild-type and mutant yeast cells. This was probably due to the background levels of blue fluorescence from chitin in the cell wall. Further experiments will be required in order to determine if dityrosine is present in the yeast wall and if the *CYP56* gene is expressed during vegetative growth. The increased susceptibility of *cyp56Δ* mutant yeast cells to nikkomycin suggests some role for dityrosine in this growth form.

Yeast cells normally form smooth, white dome-shape colonies, however at low frequency, *C. albicans* can reversibly convert a variant colony shape, i.e. wrinkle, in which the colonies are composed of a mixture of yeast and filamentous cells. In our study this phenomenon was expressed in the *cyp56Δ* mutant strain grown in minimal medium agar. However this was also seen in minimal medium broth wher the *cyp56Δ* mutant strain had shown hyphal production in contrast to wild-type strain that exhibited only a yeast-like form. Here the deletion of *CYP56* gene suggested that it has switched a possible hyphal formation regulatory system or expression of genes associated with hyphal formation under the conditions of growth investigated.

The ability to switch between yeast and hyphal morphologies is often suggested to be relevant for virulence (Sudbery et al., 2004). Filamentous growth allows cells in a starving colony to extend its borders and forage for nutrients. Different responses are

exhibited by the cells when deprived of nutrients: for example, glucose depletion favours growth arrest, whereas ammonium depletion favours filamentous growth. On the other hand both wild-type and *cyp56*Δ mutant strains during the first five hours shaking in minimal medium have shown hyphal formation, but after 6 h of incubation the wild-type reverted to yeast-form and the *cyp56*Δ mutant strain produced hyphae continuously. To investigate the hyphal production of the *cyp56*Δ mutant in anaerobic conditions, Na_2SO_3 was added to RPMI medium. After 24 hours the wild-type has shown only yeast forms during growth compared to the *cyp56*Δ mutant which exhibited a mixture of yeast and hyphal form (Fig. 4.16). The *CYP56* re-integrant strain showed reversal of the phenotypes observed in the homozygous *cyp56*Δ mutant. The susceptibility of the reintegrant to nikkomycin and caspofungin was similar to the results shown by wild-type strain. A similar result was found for the hyphal production phenotype among reintegrant and wild-type cultures. The ability to produce chlamydospores was also recovered by the reintegrant strain.

Overexpression of CYP56 was achieved in *E. coli* and CYP56 was localised in the microsomal and cytosol fractions, with a characteristic Soret peak at 450 nm when the protein was reduced in the presence of carbon monoxide (Fig. 4.20). The enzymatic activity was also confirmed in catalysing C-C bond linkage for the first time *in vitro*. Finally the substrate *N*-formyl tyrosine bound to CYP56 with an affinity typical of a natural substrate for a cytochrome P450. Binding two substrate molecules per CYP56 was important as this P450 was predicted to be involved in coupled two substrate molecules together.

In conclusion, *CYP56* is not essential for growth in *C. albicans*. However, in this work the null *CYP56* double allele mutant exhibited phenotypes that implicated associated endogenous functions to cell wall structure. The increased sensitivity of the *cyp56*Δ mutant to two different cell wall inhibitors may reflect the ability of this organism to compensate for the absence of the deleted gene possibly by shifting other components of the cell wall composition. Among other phenotypes, activating the hyphal form of growth in the mutant was an unexpected phenotype. Additionally, the absence of chlamydospore production by the *cyp56*Δ mutant strain indicates that *CYP56* is essential during chlamydospore formation and implicates a possible key role for dityrosine in this specialized cell wall structure. Chlamydospores have been isolated recently from AIDS patient and have also been associated to *Candida* virulence (Nobile et al., 2003) and this could be an important characteristic of dityrosine requirement and involvement as a pathogenicity factor.

CHAPTER 5

General Discussion

Historically *C. albicans* has been the most common fungal pathogen. However, in recent years there has been a shift in the spectrum of *Candida* infections (Nguyen et al., 1996, Melo et al., 2004). Several factors might be responsible for the emergence of non-*albicans* species (Mujica et al., 2004, Mullen et al., 2003) and this has also altered the focus of studies on azole mode of action and resistance. There is an increasing population of severely immunocompromised patients who are at risk for developing infections. Particularly vunerable are those receiving chemotherapy and the increased use of antifungal agents may select for non-*albicans* spp. that exhibit decreased susceptibility to these agents. Prophylactic use of fluconazole has been associated with resistance in *C. albicans* and linked to the emergence of *C. krusei* and *C. glabrata* infections..

The failure of antifungal agents to treat certain patients with *Candida* infections is well recognized (Salavert Lleti et al., 2006, Tortorano et al., 2003). Several factors contribute to therapeutic failure including severity of the underlying illness, impaired host immune function, persistent infection or retained intravascular catheters or prosthetic devices. Antifungal resistance also involves the emergence of naturally resistant species or progressive, stepwise alterations of cell structure(s)/function(s) to avoid the activity of an antifungal agent to which there has been extensive exposure. Over the past decade, significant advances in *in vitro* antifungal susceptibility testing have been made with the development of a standardized, broth based testing methodology that demonstrates reproducibility (National Commitee for Clinical Laboratory Standard, 2002).

The rise in severe fungal infections has prompted the development of new antifungal agents with novel modes of action. Because of their eukaryotic nature, fungal cells

have a restricted set of specific targets that do not overlap with their mammalian counterparts (Miura et al., 2003). However, the cell wall is a structure that is essential for the fungus and absent from the mammalian host, and so it presents an attractive target for the development of new antifungals (Orozco et al., 1998). Various antibiotics have been reported to interfere with the formation of fungal cell walls. For example, nikkomycins are inhibitors of chitin synthesis, echinocandin interfere with glucan metabolism and tunicamycin is an inhibitor of the glycosylation of mannoproteins (Elorza et al., 1987). One potential new target studied in this project was the cytochrome P450 undertaking dityrosine formation in *C. albicans* that could be an azole-based target, but it was found to be a non-essential gene.

Additionally, part of the present study investigated different *Candida* species in terms of antifungal susceptibility and associated mechanisms of resistance. Antifungal susceptibility, demonstratable by significant increases in MIC values varied greatly among different *Candida* isolates. The intracellular drug concentration levels, which also reflect a balance of azole uptake and active efflux, also varied and were species dependent. Other *Candida* spp. such as *C. krusei*, *C. lusitaniae* and *C. novergensis* exhibited a high intracellular [^{3}H] fluconazole concentrations. However no significant correlation with MIC values or ergosterol content was found suggesting this measurement has questionable value. Tolerance to high level of concentrations of fluconazole exhibited by some non-*albicans* isolates can partially explain resistance but overall the data supported the multiplicity of antifungal resistance mechanisms.

Variation in ergosterol levels compared to the total sterol content were also observed in the strains studied. Altered ergosterol levels did not adversely effect the growth

observed in one *C. krusei* strain. This demonstrates the capacity of some strains to grow despite significantly reduced levels of ergosterol being present. In many circumstances resistance can lead to the understanding of specific mechanisms of action of different antifungal drugs. The mechanisms involved in controlling susceptibility to antifungal agents observed in many of the strains studied here still remain unclear.

The main causative agent of oropharyngeal candidosis is *C. albicans* (Odds, 1988). Infection by *C. albicans* generally involves adherence and colonization of superficial tissues (Felk et al., 2002, Kretschmar et al., 1999, Lo et al., 1997). During this process, budding yeast cells are able to transform to hyphae and penetrate deep into the tissue (Odds, 1985). *C. albicans* produces hyphal growth under a wide range of conditions, including growth in blood serum, at 37°C, and at neutral pH (Sudbery et al., 2004). Upon return to favourable conditions, the morphological program can be reversed. The control of the cytoskeleton and cell cycle are both critical elements of the transition from yeast-hyphal form and vice-versa. Understanding the biology of the different specialized cell type may be crucial in determining strategic points in terms of the development of fungicidal or fungistatic target(s) in the cell. Hyphal formation is considered to be necessary for virulence. Several antifungal susceptibility tests such as microdilution (NCCLS), agar diffusion (Hewitt, 1981) and flow cytometry are designed to work primarily with yeasts and yeast-like fungi. However, for filamentous fungi or hyphal invasion, these standard antifungal susceptibility tests do not accurately determine the effectiveness of a drug as an antifungal agent.

Understanding the processes affecting the yeast to hyphal balance was illuminated in studies on the *C. albicans CYP56* presented herein. Disruption of this gene caused growth to be altered compared to the parental strain grown under the same conditions. It is generally believed that pathogenic fungi such as *C. albicans,* have a very similar polysaccharide composition but differ significantly in their cell wall proteins, which underscores the importance of such cell wall proteins involved in pathogenesis. This may not be the case for the presence of dityrosine, which is absent in vegetative *S. cerevisiae* but may be present in *C. albicans* (the subject of ongoing work in the laboratory). The spore walls in *S. cerevisiae* are formed by the ordered synthesis of the different layers: first mannan, then β-glucan, then chitosan, and finally outside the chitosan layer is a layer that consists largely of cross-linked tyrosine molecules (Briza et al., 1988, Briza et al., 1990, Briza et al., 1994). Dityrosine was first identified in the spore wall of *S. cerevisiae* in 1986 (Briza et al., 1986, Briza et al., 1994). In contrast the first report of the presence of dityrosine in *C. albicans* was in 1995 (Smail et al., 1995). In the present study *CYP56* was investigated regarding its contribution to the biosynthesis of the cell wall in *C. albicans*. It was demonstrated that the *CYP56* null mutant was not essential for growth. However, interesting phenotypes associated with the biosynthesis of the cell wall structure and antifungal susceptibility were observed. The hypersensitivity of the *cyp56Δ* mutant to two different cell wall inhibitors suggests an accentuated vulnerability of the targets of these agents through a possible alteration in the cell wall composition. The activation of the hyphal form in the *cyp56Δ* mutant when the parent strain grew in a yeast form was an unexpected phenotype. The phenotypes described here suggest an important role for dityrosine in maintaining cell wall integrity in vegetative cell growth. Additionally, a further phenotype of the mutant indicated an essential role for *CYP56*

in the formation of chlamydospores. Chlamydospores develop from hyphal cells, so the precise basis of this fundamental alteration in cell biology requires further study, although one can speculate.

One distinct property of *C. albicans* is its ability to produce chlamydospores, an ability shared only with the closely related species *C. dubliniensis*. Chlamydospores are large, highly refractile cells with thick cell walls that form on the ends of elongated suspensor cells attached to the hyphae and, occasionally, to pseudohyphae (Odds, 1994). They are rich in RNA content (Vidotto et al., 1996) and in some cases can germinate (Sudbery et al., 2004). Under nutrient poor, oxygen-limited conditions and at low temperatures chlamydospores can be induced (Calderone and Fonzi, 2001). In cornmeal agar, which is the typical inducing medium used *in vitro*, both light and glucose inhibit chlamydospore formation (Nobile et al., 2003). Chlamydospores have been found in the lung of AIDS patients (Chabasse et al., 1988), and thus may be relevant to infection. The genetic requirements for chlamydospore formation are of interest for several reasons. Chlamydospores are the output of a developmental process. It is unclear why the *C. albicans* genome has so many genes without close homologues in other organisms but the possibility that they play a role in *C. albicans* specific processes, such as chlamydospore formation, may explain their presence. Some conserved regulatory pathways respond to well-defined signals, so identification of chlamydospore regulators may reveal the specific external signals that govern their formation. Finally, because chlamydospores form under growth conditions that are not routinely employed for *C. albicans* cultivation in the laboratory, their genetic requirements may reveal unique functional relationships, biological roles or regulatory signals that govern activity of known gene products.

The fungal cell wall is essential for maintaining the osmotic balance of the cell as well as contributing to its physical strength. The macromolecular components of the cell wall for the majority of fungi include chitin, α or β-glucan and mannoproteins (Coluccio et al., 2004, Ruiz-Herrera et al., 2006, Miura et al., 2003, Smail et al., 1995). These components may vary in composition depending on the phase of the cell cycle, environmental conditions and the developmental stage of the organism. *C. albicans* is found as a yeast form when growing saprophytically but often adopts a hyphal form when it colonises tissue. The transition from one cellular form to the other is associated with changes in the composition of the cell wall polymers (Murgui et al., 1986). The linkages between the glucan, chitin, and mannoprotein in the *C. albicans* cell wall are poorly understood but presumably play a critical role in maintaining the integrity of the organism. Although *S. cerevisiae* contains only 2-3% of chitin in its cell walls, the synthesis of this polymer seems to be essential for cell growth and survival (Selvaggini et al., 2004). Since cell wall components are crucial for fungal cell development, chitin and glucan biosynthetic steps have been attractive targets for the development of novel antifungal inhibitors (Cassone, Mason *et al.*, 1981; Maschmeyer e Ruhnke, 2004). Hence, the biosynthesis of dityrosine in the *C. albicans* cell wall may yet bring novel insights regarding the understanding of *C. albicans* cell biology and possibly identify a novel drug target. Although the production of dityrosine is not essential for growth and azole antifungal susceptibility was unaltered in the *cyp56*Δ mutant, synergistically altering the susceptibility of *C. alicansi* to other drugs could be a route to combined drug therapy.

To assess the structure/function of CYP56, overexpression of the protein was obtained in *E. coli* and CYP56 was found to be located in the membrane fraction. For fungal CYP to exert its catalytic activity it requires a source of reducing equivalent usually provided by P450-reductase (CPR). CPR is a microsomal-bound flavoprotein which transfers electrons from NADPH to the CYP molecule allowing the molecular scission of atmospheric dioxygen. Accordingly, reconstitution of CYP56 required its insertion into a lipid micelle together with a CPR, in this work *S. cerevisiae* CPR. When reconstituted into DLPC micelles, CYP56 catalyzed the dimerisation of *N*-formyltyrosine in the biosynthetic reaction proposed by Briza (Briza et al., 1996). Here we demonstrated *in vitro* for the first time the proposed pathway of dityrosine biosynthesis in *Candida albicans.*

CYP enzymes constitute a superfamily of haem-thiolate containing proteins and are involved in metabolism of both exogenous and endogenous compounds. The alignment of *C. albicans and S. cerevisiae* CYP56 revealed 46% homology. *C. albicans* is closely related to *S. cerevisiae* and it was of interest that it has *CYP56* as well. *S. pombe* is more distantly related, perhaps branching from yeast 500-1000 million years ago. Both CYP51 and CYP61 are found in all three species, but CYP56 is present only in *S. cerevisiae* and *C. albicans.* It seems probable that CYP56 evolved from CYP51 or CYP61 and that the ancestral yeast may have had only the two CYPs and not three. However it is also possible that *S. pombe* has lost *DIT2* during ascomycete evolution.

Drug resistance in *Candida* spp. including *C. albicans* and other fungi has given an important impetus to the detailed investigation of specific mechanisms of resistance

to assist in selecting the appropriate antifungal agent to treat refractory fungal infection(s). The availability of molecular genetic tools has led to a rapid enrichment in our understanding of the mechanisms by which antifungal resistance emerges and spreads. This will also be an important route for the development of novel, potent and safer agents in the future. Once the diversity of resistance mechanisms is established, the identification of new resistant determinants in pathogenic fungi may also give the opportunity to define new targets for the design of alternative and effective antifungal agents as outlined in the present thesis.

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