Chapter 1. Introduction

1. Importance of *Candida albicans*

Candida albicans is an opportunistic commensal. Virtually all of us carry it in our gastrointestinal and genitourinary tracts and to a lesser extent, on our skin. A crucial feature of this microorganism is its versatility; this goes beyond the fact that it is both a pathogen and a commensal to possess such ability to survive at and infect several anatomically distinct sites, each with its own specific set of environmental pressures (Calderone *et al.*, 2002).

When the function of the immune system is weak (for example, as a result of cancer chemotherapy, HIV infection or in neonates) or when the competing florae are eliminated (for example, after antibiotic treatment), *C. albicans* colonizes and invades host tissues. Furthermore, if the organism gains access to the blood stream (a condition known as candidaemia) by invasion of host tissues or by contamination of indwelling catheters, the infection can progress to the growth of fungal masses in the kidney, heart, or brain.

Candida species are the fourth most common cause of hospital-acquired bloodstream infections in the United States (Edmond *et al.*, 1999) and *C. albicans* is the most common one among them. These infections are associated with attributable mortality rates that have ranged from 38% between 1983 and 1986 (Wey *et al.*, 1989) to 49% in the same institutions between 1997 and 2001 (Gudlaugsson *et al.*, 2003). In Taiwan, *Candida* species are among the leading causes of nosocomial

bloodstream infection at a major teaching hospital (Chen *et al.*, 1997). Nosocomial *Candida* infection is an independent poor prognostic factor for critically ill patients (Chen *et al.*, 2001) and nosocomial candidaemia is associated with a high mortality rate of 59.3% (Hung *et al.*, 1996).

2. Resistance of *C. albicans* to antifungal agents

Antifungal drugs that are available for the treatment of *Candida* infections include the polyene, such as amphotericin B, and the azoles, such as fluconazole. Among the polyenes, which interact with ergosterol in fungal membranes and catastrophically alter their membrane permeability, amphotericin B is the most widely used agent for deep-seated mycoses. Among the triazoles, which specifically inhibit fungal ergosterol biosynthesis, fluconazole, itraconazole, and ketoconazole are used to treat various types of life-threatening fungal diseases, and three novel triazoles, namely, the posaconazole, ravuconazole, and voriconazole, are in development for clinical uses. Caspofungin, anidulofungin, and micafungin represent the echinocandin antifungals, specific inhibitors to the β-glucan polymers in the fungal cell walls (Sanglard *et al.*, 2002).

Due to the frequent use of antifungal drugs, especially the azoles, the incidence of drug resistant *Candida* species isolates has increased dramatically. In the 1990s, many human immunodeficiency virus (HIV)-infected patients received long-term, low-level azole antifungal therapy, which resulted in the development of azole-resistant isolates of *C. albicans* (White *et al.*, 1998). One study documented azole resistance in up to one-third of the oral *C. albicans* isolates from HIV-positive patients

(Law *et al.*, 1994). In recent years, resistance to antifungal drugs has been documented in other patient populations such as bone marrow transplant recipients (Holmberg *et al.*, 1999; Marr *et al.*, 1998; Marr *et al.*, 1997; Mori *et al.*, 1997; Nolte *et al.*, 1997). In a clinical setting, there are many reasons why a fungal infection does not respond to antifungal drugs, including the immune status of the patient, the characteristics of the drug, and the susceptibility of the fungus to the drug (White *et al.*, 1998; Holmberg *et al.*, 1999; Yang *et al.*, 2001).

3. Resistance of *C. albicans* to azoles

Among the different categories of antifungal drugs, the development of azole resistance is the most relevant medical problem. Treatment failures have been observed following the extensive use of fluconazole for the management of *Candida* infections. Azoles are against C14α-demethylase in the ergosterol pathway directly. The resulting ergosterol depletion and accumulation of 14α-methylsterols (e.g. lanosterol and 14α-methyl-3-6-diol) interfere with the functions of ergosterol as the predominant cellular membrane component (Kelly *et al.*, 1997).

Extensive biochemical studies highlighted a significant diversity in the mechanisms conferring resistance to azoles. To date, four resistance mechanisms have been identified in the development of azole resistance in *C. albicans*: (i) the cellular content of the azole target encoded by the *ERG11* gene can be increased; (ii) the affinity of Erg11p to azoles can be decreased by mutations in *ERG11*; (iii) the ergosterol biosynthetic pathway can be altered; and (iv) azoles can fail to accumulate inside the

cells (Sanglard *et al.*, 1998). Among these mechanisms, the last is the most commonly observed one in clinical strains developing azole resistance. The vast majority of findings point to the increased levels of active efflux of drugs being the prime mechanism of this type of resistance. One major action mode is the association with the up-regulation of genes encoding the efflux pumps. For instance, increased mRNA levels of *Candida CDRs* are members of the ATP binding cassette (ABC) transporter superfamily, and *MDR1*, a major facilitator with a much narrower substrate spectrum including fluconazole, have been associated with azole resistance. To date, at least seven *CDR* genes have been identified in *C. albicans* (Lyons *et al.*, 2000), but only *CDR1* and *CDR2* are so far associated with azole resistance (Henry *et al.*, 1999). Nuclear run-on assays show that *CDR1* and *CDR2* mRNAs are transcriptionally overexpressed in the resistant isolates, suggesting that the antifungal drug resistance is associated with the promoter and *trans*-acting factors of the *CDR1* and *CDR2* genes (Lyons *et al.*, 2000; Lopez-Ribot *et al.*, 1998; Marr *et al.*, 1998).

4. *Candida* drug resistance gene: *CDR1*

In recent years, much effort has been devoted to understand the regulatory mechanisms of multidrug transporter genes in *C. albicans*. It has been shown that *CDR1* was induced in response to agents such as miconazole, fluconazole, nystatin, vinblastine or steroid hormones (Krishnamurthy *et al.*, 1998). The *C. albicans CDR1* gene is a homologue of the *S. cerevisiae PDR5*, which encodes a multi-drug efflux pump. *CDR1* is the gene most often associated with energy-dependent drug

efflux in fluconazole-resistant clinical isolates and it is commonly over-expressed in the resistant isolates (Lopez-Ribot *et al.*, 1998; Perea *et al.*, 2001; Sanglard *et al.*, 1995). In general, the *CDR1* gene is not normally expressed at high levels in susceptible isolates (Lyons *et al.*, 2000). This is well established with the reports from fluconazole-resistant clinical isolates of *C. albicans* where the enhanced expression of Cdr1 protein has been shown to help the pathogen to efflux this therapeutic azole and hence to facilitate its own survival (Sanglard *et al.*, 1997; Sanglard *et al.*, 1995; White 1997).

The Cdr1 protein (~170kDa) contains two highly hydrophobic transmembrane domains (TMD) and two cytoplasmically localized nucleotide binding domains (NBD). Each TMD comprises six transmembrane segments (TMS), which are envisaged to confer substrate specificity to Cdr1p. The NBDs of ABCTs (ATP binding cassette transporters) bind ATP through an ABC that consists of several conserved peptide motifs, including two Walker domains, a Signature domain and a Center domain. The ATP that is bound to the ABC is used as a source of energy for the ABCT, although the mechanism by which the ATP energy causes transport of the substrate molecule is unknown.

5. Regulation of the *CDR1* gene

The understanding of regulation of fungal drug resistance mostly came from the much well-studied yeast *S. cerevisiae*, in which several drug extrusion pumps were isolated, such as the *PDR5* (pleiotropic drug resistance), the homologue of *CDR1* in *C. albicans.* A transcriptional repressor of *PDR5* has recently been identified, designated *RDR1*

(repressor of drug resistance), which binds to PDRE (pleiotropic drug response element), a *cis*-acting regulatory element also shared by the master regulators *PDR1* and *PDR3* (Hellauer *et al.*, 2002). Pdr1p and Pdr3p can form both homo- and heterodimers *in vivo*, with the dimerization domain located in the N-terminal half of both proteins close to the DNA binding site. Pdr1p and Pdr3p are Cys6-Zn(II) transcription factors binding the PDRE sites present in the *PDR5* promoter (Katzmann *et al.*, 1996). Pdr1p is the main regulator of Pdr5p, controlling resistance to hundreds of xenobiotics (Hnatova *et al.*, 2003). Although Pdr3p recognizes all three PDREs in the *PDR5* promoter *in vitro* (Katzmann *et al.*, 1994), its deletion shows less influence on Pdr5p expression. A Δ*pdr1* Δ*pdr3* double deletion completely abrogates *PDR5* expression (Hallström *et al.*, 1998; Mahé *et al.*, 1996). Recent genome-wide approaches have revealed that *PDR1*, *PDR3*, *YRR1*, and *PDR8* are four zinc-finger transcription factors which regulate the *PDR* genes of *S. cerevisiae*. Taken together, it appears that *PDR* genes of *S. cerevisiae* are regulated by multiple factors, involving cross-talk between several regulatory networks (Le *et al.*, 2002; Hikkel *et al.*, 2003).

It has also been shown that *CDR1* was induced by agents such as miconazole, fluconazole, nystatin, vinblastine or steroid hormones (Krishnamurthy *et al.*, 1998). Using a promoter deletion analysis, four upstream-activating and four upstream-repressing domains were identified along the entire *CDR1* promoter. Mutational analysis demonstrated that while the proximal promoter (-345/+1) contains all the regulatory domains required for its induction by various other stresses, the miconazole response is mediated via the distal sequence (-857/-1147)

harboring an AP-1 site (Puri *et al*., 1999). DNase I footprinting and 5'-deletion analysis revealed four *cis*-acting regulatory elements (W1, W2, W3 and W4), whose mutation led to the modulation of basal promoter reporter activity to varying degrees. The mutation of one such element (W1) led to a maximum activation of *Renilla* luciferase reporter activity and was designated a negative regulatory element (NRE). A purified ~55-kDa nuclear protein specifically interacted with the NRE (Gaur *et al*., 2004).

Sanglard and his group have identified a common drug/steroid-responsive element (DRE), which is located at -397 to -376 bp and -303 to -280 bp (from the transcription start point) in the *CDR1* promoter (de Micheli *et al*., 2002). The DRE identified in their work does not match any other DNA-binding elements existing in current databases. The structure repeat with CGG triplet motifs is, however, typical of binding sites of transcription factors with zinc finger motifs such as Gal4p.

Recently, in a transcription profiling study, Murad *et al*. (2001) have demonstrated that *CDR1* is repressed more than 3-fold by each of the Mig1p, Nrg1p and Tup1p repressor factors. However, no direct evidence for the interaction of these factors with the *CDR1* promoter has yet been established (Murad *et al*., 2001). So far attempts to identify the transcription factors involved in drug resistance of *C. albicans* have largely been unsuccessful. Talibi *et al*. (1999), by functional complementation of a *pdr1* and *pdr3* mutation in *S. cerevisiae*, have identified a few transcription factors from *C. albicans* designated Fcrps (Fcr proteins). Interestingly, over-expression of Fcrps in *S. cerevisiae*

resulted in increased resistance to fluconazole, whereas in *C. albicans* the disruption of Fcrp resulted in increased resistance to fluconazole and other antifungal compounds (Talibi *et al.*, 1999). Thus, Fcrp could acts as a negative regulator of drug resistance in *C. albicans*.

The regulation of *CDR1* involves both the promoter region (*cis*) and other genes acting in *trans*. The study here focuses on *trans*-acting regulatory factors of *CDR1*.

6. Previous work in the laboratory

Previous work in the laboratory (Shih, 2001) has construct *CDR1* promoter-*lacZ* (*CDR1*p-*lacZ*) fusion plasmid, including the *CDR1* promoters of SC5314, Ym990348, and Ym990361 strains. SC5314 is a laboratory strain; Ym990348 and Ym990361 are clinical strains. Those plasmids were then transformed into *S. cerevisiae* to monitor the activities of *CDR1* promoter. Using the *S. cerevisiae* harboring the SC5314 *CDR1* p-*lacZ* integrated in the chromosome as the host, a *C. albicans* library was introduced into the recombinant strains and the *trans*-regulatory factors were isolated when the library clones can cause a difference in the level of β-gal activities. Two *trans*-regulatory factors, *REP1* and *REP2*, were isolated and they increased the *CDR1*p-*lacZ* activity (Shih, 2001).

7. The purpose of the study

Although two regulatory factors have been isolated in the previous study, the basal activity of *CDR1* promoter of SC5314 is relatively too high. It was difficult to screen for genes with less activities. In addition,

some regulatory genes may express only in specific induction environment, such as at in the presence of drugs, serum, and/or steroid hormone etc. Hence, other *trans*- factors are still yet to be discovered. Hence, to screen for new *trans*-regulatory factors of *CDR1*, I used the *CDR1* promoter from the Ym00348 strain. The *CDR1* promoter activity of Ym00348 is relatively low compared to that of SC5314. In addition, the screening was performed in the presence of drugs for this study. The candidate genes were then analyzed for their functions by mutagenesis using the homolgous recombination technique. The information obtained may help to understand more about the regulatory mechanism of drug resistance in *C. albicans*.

Chapter 2. Materials and Methods

1. Materials

1.1 Strains

1.11 *Escherichia coli* (DH5 α)

1.12 *Saccharomyces cerevisiae*

Strains	Genotype	Reference
10560-2B	*his3::hisG leu2::hisG ura3-52*	Fink laboratory collection
CSY9	*his3::hisG leu2::hisG ura3-52* [348(2)*CDR1*p-*lacZ*/ YEP363]	this study
CSY29	*his3::hisG leu2::hisG ura3-52* [348(2)*CDR1*p-*lacZ*/ YEP363, pRS 426(1)]	this study
CSY34	*his3::hisG leu2::hisG ura3-52* [348(2)*CDR1*p-*lacZ*/ YEP363, *REP2*/pRS 426(3)]	this study
YSHI10	*his3::hisG leu2::hisG ura3-52* SC5314*CDR1p-lacZ::ade3*	Yang laboratory collection; Shih, 2001

CSY396	*his3::hisG leu2::hisG ura3-52* *SC5314CDR1p-lacZ::ade3* [pRS 426]	this study
CSY397	*his3::hisG leu2::hisG ura3-52* *SC5314CDR1p-lacZ::ade3* [*REP2*/pRS 426]	this study

1.13 *Candida albicans*

The *REP3* and *REP6* heterozygous and homozygous strain

Strains	Genotype	Reference
SC5314	Wild type	Gillum *et al.*, 1984
BWP17	*arg4/arg4 his1/his1 ura3/ura3*	Wilson *et al.*,1999
BWP17/tetR	*arg4/arg4 his1/his1 ura3/ura3* *ENO1/ENO1*-tetR-ScHAP4AD-3×HA-Ca*HIS1*	Lo. laboratory collection
CSC21, 22	*rep3*::*ARG4/REP3* [parental strain:BWP17]	this study
CSC32, 34	*rep3*::*ARG4/REP3* [parental strain:BWP17/tetR]	this study
CSC80, 81	*rep3*::*ARG4/rep3*::*URA3* [parental strain: CSC21]	this study
CSC9, 101	*rep3*::*ARG4/rep3*::*URA3* [parental strain: CSC22]	this study

CSC106	*rep3*::*ARG4*/*rep3*::*URA3* [parental strain: CSC32]	this study
CSC111, 119	*rep3*::*ARG4*/*rep3*::*URA3* [parental strain: CSC34]	this study
CSC3, 45	*rep6*::*ARG4*/*REP6* [parental strain:BWP17]	this study
CSC12, 13	*rep6*::*ARG4*/*REP6* [parental strain:BWP17/tetR]	this study

1.2 Plasmids

Plasmid	**Description**	**Reference**
PRS 426	*URA3* 2 μm plasmid	Sikorski *et al.*, 1989
YEP363	*LEU2* 2 μm plasmid	Myers *et al.*, 1986
*REP*2/ pRS 426	3.5 kb *Sac* I-*Xho* I fragment containing *REP2* in pRS 426	Lo. laboratory collection
pSHI16 (348*CDR1*p-*lacZ*/YEP363)	1.2 kb *Xma* I-*Hin*d III fragment containing the clinical Ym990348 *CDR1* promoters in YEP363	Yang laboratory collection; Shih, 2001
pSHI12	1.2 kb *Xma* I-*Hin*d III	Yang

(5314*CDR1*p-*lacZ*/YIP363)	fragment containing the clinical Ym990348 *CDR1* promoters in YIP363	laboratory collection; Shih, 2001

1.2 Primers

Primers used for *REP3* gene disruption and confirmation

Name	**Sequence(5' to 3')**	**location**
HJL449	5'- **TAGACAGGCT GAAAATTTCG ACCAAGAGGA TGATGCAGAT AAACACATTG ATCAGGAGTT GAGACA**ACGCCAAGCTCGGA ATTAAC	*C. albicans REP3* (56705-56770): +119~+54 (boldface)
HJL450	5'- **AACGAAACTC GGAGCTTTAT CATCGTCCGC AAGCCACATT CGAGCCAGAT TATGTTTCAC AAATA**ACTATAGGGCGAATT GGAGC	*C. albicans REP3*: (55483-55547): +1341~+1277 (boldface)
HJL451	5'- **ACTACCAATC ACGATGCTAC CAAACTGATC AACACCATTA AACGAGAGCA TGAAGAACAT GCTGG**AACGACGGCCAGTGA ATTGT	*C. albicans REP3*: (56606-56670): +218~+154 (boldface)
HJL452	5'- **CAGAGAGTACACGGAAATCA**	*C. albicans*

	GAATGCCTCT CCTTTGGCTC GATGAATTGA AAAGATATGA GACTGAGGGA ACAAAAGCTG GGTAC	*REP3*: (55664-55728): +1160~+1096 (boldface)
HJL457	5’- CCACTCGAG**G GAAGTGAAAC CCTACCT**	*C. albicans REP3*: (57271-57254): -448~-431 (boldface)
HJL458	5’- CCACCCGGG**C TCGGAGCTTT ATCATCG**	*C. albicans REP3*: (55490-55507): +1333~+1316 (boldface)
HJL241	5'- TCAATGGATC AGTGGCAC	*C. albicans ARG4* (pRS-*ARG4*△ *Spe* I 3339~3357):+ 61~+79
HJL133	5’- ACCAGTAGCA CAGCGATT	*C. albicans URA3* (pDDB57: AF173953

		sequence3796~3813):+51~+68

Primers used for *REP6* gene disruption and confirmation

HJL431	5'- **AACCCACCAG CACATATACA CACACCCATT TTCTTATCCA ACTATAAACA TACATCCAAT CCGCC** ACGC CAAGCTCGGA ATTAAC	*C. albicans REP6*: (41324-41388): -234~-298 (boldface)
HJL432	5'- **TCACTGTATT TGGCATACTG AATGCTGACC AAACCATGAG TGACATGATC GGAATGGACA TGATG** ACTA TAGGGCGAAT TGGAGC	*C. albicans REP6*: (39751-39815): +1340~+1276 (boldface)
HJL433	5'- **GAACAAAACC CTCGCAATCC CCAGGTTTAC CAGAACTATC ATTTCATCCA GCAACAGCAA CATTT** AACG ACGGCCAGTG AATTGT	*C. albicans REP6*: (40830-40904): +261~+186 (boldface)
HJL434	5'- **GGACACTCTT TAACTGGACA AATAAAAGTT CTTGACAGTA AGTACCGTTGCAAGCCATGC**	*C. albicans REP6*: (39856-39920):

	CCTTC AGGG AACAAAAGCT GGGTAC	+1234~+1170 (boldface)
HJL412	5'- GGCGGAGCTC **TGAGTCGAAT GCCACGACTA**	*C. albicans REP6*: (38961-39558): +2130~+1533 (boldface)
HJL414	5'- **GTATCACCAC ATACCCACCT** ACGCCAAGCT CGGAATTAAC	*C. albicans REP6*: (41132-41113): -42~-23 (boldface)
HJL418	5'- **CCATGCTACTTTATTTATCA GC**	*C. albicans REP6*: (42444-42465): -1354~-1375 (boldface)
HJL241	5'- TCAATGGATCAGTGGCAC	*C. albicans ARG4* (pRS-*ARG4*△ *Spe* I 3339~3357):+ 61~+79
HJL133	5'- ACCAGTAGCA CAGCGATT	*C. albicans*

		URA3 (pDDB57: AF173953 sequence3796~3813):+51~+68

1.4 *C. albicans* genomic DNA library (Liu *et al.*, 1994)

C. albicans genomic DNA was partially digested with *Sau3* A. DNA fragments larger than 4kb were purified, filled in with dATP and dGTP and ligated into a *S. cerevisiae URA3*/2 μ plasmid that had been digested with *Sal*I and filled in with dCTP and dTTP. Therefore, genomic DNA fragments were inserted at *Sau* I site, and the *Sal* I site was destroyed during the construction.

1.5 Chemicals and reagents

1.51 Difco laboratories

Bacto agar, LB agar, yeast nitrogen base w/o amino acid, LB broth, YPD broth

1.52 Invitrogen

Agarose

1.53 Bio-Rad

Coomassie Brilliant Blue (G-250), TAE, TBE

1.54 Sigma Chemical Co.

Adenine sulfate, Ammonium acetate, Dithiothreitol (DTT), ethidium bromide, miconazole, Glassbeads (425~600 μm), Lithium acetate (CH_3COOLi), Phenylmethylsulfonyl-fluoride (PMSF),

PolyethyleneGlycol$_{3350}$ (PEG$_{3350}$), L-leucine, phenylmethylsulfonyl fluoride (PMSF), Sodium citrate, Uracil, Urdine, Histidine

1.55 E Merck. Germany

Disodium ethylenediamine-tetraacetate (EDTA), Disodium hydrogen phosphate dihydrate (Na_2HPO_4), Dodecyl sulfate sodium sat (SDS), Ethanol, Glucose, chloroform, Dimethyl sulfoxide (DMSO), Ethanol, Glycerol, Magnesium chloride hexahydrare, N,N-dimethylformamide, Isopropanol, Magnesium chloride hexahydrate ($MgCl_2$-$7H_2O$), Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), Potassium chloride, Sodium chloride, β-Mercapto-ethanol (2-ME), Potassium chloride (KCl), Sodium acetate trihydrate ($NaHCO_3 \cdot 3H_2O$), Sodium carbonate (Na_2CO_3), Sodium citrate dihydrate, Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydroxide (NaOH), Tris (hydroxymethyl) aminomethane hydrogen chloride (Tris-HCl), Sodium chloride (NaCl)

1.6 Buffers

1.61 TE buffer

10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)

1.62 10X TAE buffer

0.4 M Tris, 0.4 M glacial acetic acid, 10m M EDTA

1.63 10X TBE buffer

1.0M Tris, 0.9M Boric acid, 10mM EDTA

1.64 10X PCR buffer

100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ (pH 8.3)

1.65 Breaking buffer

0.1M Tris-HCl, 20% glycerol (v/v), 1mM Dithiothreitol

1.66 Z buffer

Na_2HPO_4 $7H_2O$ 16.1g, NaH_2PO_4 H_2O 5.5g, KCl 0.75g, $MgSO_4$ $7H_2O$ 0.246g, β-mercaptoethonol 2.7 ml added dd H_2O to 1000ml pH 7.0

1.7 Medium

1.71 LB (Luria-Bertni) broth

1% tryptone, 0.5% yeast extract, 1% NaCl

1.72 LB / ampicillin broth

1% tryptone, 0.5% yeast extract, 1% NaCl, 50 μg/ml ampicillin

1.73 LB / ampicillin agar

1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50 μg/ml ampicillin

1.74 YPD/ Uridine broth

2% Bacto-peptone, 1% yeast extract, 2% dextrose, 80 mg/l Uridine

1.75 YPD/Uridine agar

2% Bacto-peptone, 1% yeast extract, 2% dextrose, 2% agar, 80 mg/l Uridine

1.76 SD (Synthetic Dextrose)/ Uracil/ Histidine broth

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 20 mg/l Uracil, 20 mg/l Histidine-HCl

1.77 SD agar

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 2% agar, 20 mg/l Uracil, 20 mg/l Histidine-HCl

1.78 SD/ Histidine broth

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 20 mg/l Histidine-HCl

1.79 SD/ Histidine agar

0.67% Bacto-yeast nitrogen base w/o amino acid, 2% dextrose, 2% agar, 20 mg/l Histidine-HCl

1.791 Auxotrophic supplements concentraction:

L-Histidine-HCl (Sigma H-9511): 20 mg/l

L-Arginine-HCl (Sigma A-5131): 20 mg/l

L-Uracil (Sigma U-0750): 20 mg/l

L-Adenine (Sigma A-9126): 20 mg/l

Uridine (Sigma U-3003): 80 mg/l

1.8 Filters: (for β-gal colony lift assay)

1.81 S&S (Schleicher & Schuell) #576 filters (90mm)

REF. NO.: 311409

LOT.: BG0470-1

1.82 S&S (Schleicher & Schuell) #593ilters (90mm)

REF. NO.: 10314509

LOT.: CJ1016-1

1.83 Whatman # 3 filters (150mm)

CAT. NO.: 1003 150

1.84 Amersham pharmacia biotech filters (137mm)

CAT. NO.: RPN137E

1.9 Enzymes:

1.91 NEB

*Bsa*B I, *Eco*R I, *Eco*R V, *Hin*d III, *Msc* I, *Sal* I, *Spe* I, *Xma* I, $Vent_R$ DNA polymerase, CIP

1.92 Amersham Biosciences

rTaq DNA polymerase

1.93 GIBCO BRL

*Bam*H I, *Cla* I, *Hpa* I, *Kpn* I, *Pst* I，*Sma* I, *Xba* I, *Xho* I, T4 ligase，

Xba I，*Xho* I

1.10 Equipments

Peltier Thermal Cycler PTC-225 (MJ Research)

Orbital shaking incubator OSI500 (TKS)

AlaImager 2000 (Molecular devices)

Vortex-2 genie Si (Scientific Industry)

Dry bath incubator (Violet Bioscience Inc.)

Brushless microcentrifuge Denville 260D (Scientific Industry)

Power pac 300 (Bio-Rad)

Elektroporator Gene pulser II (Bio-Rad)

Spectra max plus (Molecular devices)

Quick spin (Violet Bioscience Inc.)

Thelco laboratory incubator (Precision Scientific Inc.)

High speed refrigerated centrifuges J2-MC (Beckman)

2 Methods

2.1 *In vitro* assay of β-galactosidase activity (Colony-Lift Filter Assay) with drug induced

Fresh colonies (*S. cerevisiae* strain) were grown at 30 ℃ in selective agar plate for 2-3 days. Colonies on the agar plates were then replicated onto filters (filters on new agar plates) and were incubated for overnight. The final concentration of drug (Miconazole) for the assay is 100 μg/ml. Stock solution of drug was prepared at the concentration of 100 mg/ml in DMSO and then was diluted to 1000 times in selective medium. Sterile filter was pre-soaked in 100 μg/ml Miconazole medium and another filter with colonies was placed on it. These filters were incubated at 30℃ for one hour. After one hour, the filters with colonies were then transfer to a pool of liquid nitrogen for 1 min and thawed at room temperature for another 1 min. This freeze/thaw treatment is to permeabilize the cells. The filters were carefully place on the pre-soaked filters which is in Z buffer/2ME (β-mercaptoethonol)/X-gal stock (10 ml/14 μl/84 μl) solution. Trapping air bubbles under or between the filters should be avoided. The filters were checked periodically for the appearance of blue color.

2.2 Yeast transformation with library DNA or plasmid DNA (LiOAC method)

Yeast were grown in 10 ml of YPD or selective medium overnight at 30 ℃ (OD_{600} > 1.0). The overnight culture was transferred into fresh 15 ml of YPD or selective medium, and the cell density was at OD_{600} of

0.4. Then they were incubated at 30 ℃ till OD_{600} of 0.9 (it takes about 4-5 hours). Cells were spun for 5 minutes at 3000 rpm and the supernatant was poured off. The pellet was resuspended with 15 ml of 0.1 M TE (pH 7.5). Cells were spun for 5 minutes at 3000 rpm and the supernatant was poured off before the pellet was suspended gently with 4 ml of 0.1 M TE/ LioAc (pH 7.5). Cells were spun for 5 minutes at 3000 rpm and the supernatant was poured off before the pellet was suspended gently with 1 ml of 0.1 M TE/ LioAc (pH 7.5). Cells were sat at room temperature for 10 minutes. 4 μl of boiled Salmon Sperm DNA (10 mg/ml) was added as carrier (boil DNA for 1 minutes and put it on ice right away), and mixed with 1-3 μg of library DNA or plasmid DNA (to be transformed). Then cells were mixed with DNA gently. 0.7 ml of 50% PEG_{3350} /0.1 M of LiOAC/TE was added and mixed gently. The cells were incubated at 30 ℃ on wheel for 30 minutes before they were heat shocked at 42 ℃ for 20 minutes in water bath. The cells were then chilled on ice for 1-2 minutes. Cells were then spun for 5 minutes at 3000 rpm and the supernatant was poured off. The pellet was suspended gently with 1 ml of 0.1 M TE (pH 7.5). Then, the cells were spun for 5 minutes at 3000 rpm, and the supernatant was poured off (remained medium approximately 100 μl) before the cells were plated on selective plates with suspension.

2.3 Yeast plasmid DNA extraction

Yeast cells were inoculated in 10 ml of SD broth adding

appropriate selective nutrition and incubated for 2 days at 30 ℃ with vigorous shaking (~150 rpm). Cells were spun for 5 minutes at 3000 rpm, the supernatant was poured off and resuspend in 5 ml of dd H_2O. The yeast solution was transferred to 15 ml centrifuge tube. Cells were spun for 5 minutes at 3000 rpm, and the supernatant was poured off. All traces of supernatant are removed by inverting the open centrifuge tube on hand towel. Cells were resuspend in 200 μl Breaking buffer, acid-washed glass beads were added until just below the liquid level. Cells were vortexed at highest speed for 1 min, rest 1 min, and repeat 4 times at cold room. 200 μl of TE was added and the mixture was vortexed for 10 seconds. Cells were spun for 5 minutes at 3000 rpm at 4℃, and the clouded supernatant was transferred to 1.5 ml centrifuge tube. Equal volume (~200 μl) of phenol/ chloroform / isoamylalcohol (25:24:1) was added before the mixture was vortexed for 60 seconds. The solution was centrifuged for 1 minutes at 13000 rpm, and the aqueous layer was transferred carefully to new tubes. Phenol extraction steps were repeated until the aqueous layer is clear. The modified QIAEX II (desalting and concentrating DNA from solution) protocol was proceed to purify the yeast plasmid.

2.4 *E coli* transformation with electroporaton

Frozen cells were thawed on ice. 80 μl of cells were mixed with 2 μl of transforming DNA in a chilled 1.5 ml of microcentrifuge tube. The Bio-Rad Gene Pulse was setted at 25 μF and 2.45 kV. Pulse controller was set to 200Ω.The cell/DNA mixture were transferred to a chilled 0.2

cm electroporation cuvette and the suspension were shacked to the bottom of the cuvette. The moisture on the outside of the cuvette was wiped off with a tissue and the cuvette was put in the slide, which was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. Two red buttons on the machine were pushed on the left at the same time (pulse once and heard the beep). The cuvette was removed from the chamber and 1 ml of SOC broth was added immediately before the resuspension of the cells. The cells were transferred to a 1.5 ml centrifuge tube and incubated at 37 ℃ for 1 hour with sharking. Cells were spun for 5 minutes at 3000 rpm and the supernatant was poured off (remained medium approximately 100 μl) before the cells were plated on LB /ampicillin plates after suspension.

2.5 *In vitro* assay of β-galactosidase activity (Liquid Culture Assay)

The levels of β-galactosidase of candidates can be measured quantitatively by the liquid culture assay. The candidates were cultured in 3 ml of SD broth adding appropriate selective nutrition broth and incubated for overnight at 30℃ with vigorous shaking (~150 rpm). The overnight culture was diluted in 15 ml selective nutrition broth (in 50 ml Corning centrifuge tube) to ~OD_{600} of 0.2 and incubated about 6-7 hours (~OD_{600} of 0.8) at 30℃ with vigorous shaking. The yeast cells were harvested by centrifugation at 3000 rpm for 5 minutes at 4℃. The cell pellets were suspended with 5 ml of ddH_2O and transferred to 15 ml centrifuge tube and the yeast cells were harvested. The experiment can be

arrested at this step by stock the cell pellet in -20℃. The pellets were resuspended in 250 μl of breaking buffer with 12.5 μl of 40 mM PMSF and placed on ice. The acid-washed glass beads were added to just below the liquid level, the suspension was vortexed at maximum speed for 60 sec and rest for 60 sec at cold room (4℃). The vortex procedures were repeated for 4 times, then 300 μl of breaking buffer was added. The cell extract was centrifuged at 3000 rpm for 10 minutes at 4℃ after 10 sec mixed by vortexing. Supernatant was transferred to a new cold eppendorf, and centrifuged at 3000 rpm for 10 minutes at 4 ℃. The supernatant was transferred to another new cold eppendorf. The β-galactosidase assay and Bradford protein quantitative assay were prepared to process.

A total of 10 μl (X μl) of yeast extract was added to a 12 × 75 mm disposable glass tube contained 990 μl (1000-X μl) of cold Z buffer (with β-mercaptoethonol 2.7 ml/l). This mixture was preincubated in preequilibrated 28 ℃water bath for 5 minutes. Then, 200 μl of ONPG (Orthonitropheny-D-galactoside , 4 mg/ml in Z buffer) as enzyme substrate was added to start the reaction as time 0 min. When a light yellow color has been developed, stopped the reaction by adding 500 μl of 1 M Na_2CO_3 and noted the time. Determine the OD_{420} value of the supernatant.

Bio-Rad Protein Assay Dye (catalog no. 500-0006) was used to determine the protein concentration of the cell extract. Based on the Bradford dye-binding procedure (3), determined OD_{595} of the supernatant, compared to a standard curve in which 500 μg/ml BSA (bovine serum

albumin) was serial 2-fold diluted to 62.5 μg/ml as the protein standard.

Calculated β-galactosidase activity units (moles of ONPG cleaved per min per mg protein) with the following equation:

$$\frac{OD420 \times 378}{\text{Time (minutes)} \times \text{vol. Extract (mls)} \times \text{protein (mg/ml)}}$$

2.6 Etest— for antifungal susceptibility testing of yeasts

Agar plates during Etest for *S. cerevisiae* is YNB (w/o amino acid) + 2% Dextrose + necessary auxotrophic supplements + 2% agar (pH 5.8); for *C. albicans* is RPMI + 2% glucose + MOPS + necessary auxotrophic supplements + 2% agar for all agents or YNB (w/o amino acid) + 2% Dextrose + necessary auxotrophic supplements + 2% agar (pH 5.8). Homogenized well colonies were precultured in SD plate or YPD plate with necessary auxotrophic supplements for 72 hours. The cells were suspended in 2.5 ml saline (0.85% NaCl) to 0.5 *McFarland* turbidity for *S. cervisiae* and *C. albicans.* An agar plate was use with a depth of 4.0±0.5 mm.

A sterile, non-toxic swab (not too tightly spun) was dipped into the inoculum suspension and the swab was rolled against the side of the tube. The entire agar surface was swabbed evenly and in three directions. Excess moisture was allowed to absorb for about 5-10 minutes so that the surface was completely dry before applying the Etest strips. A template was used to position 5 strips on 150 mm plate. A sterile forceps were used to pick strips and place them on the agar surface. The strips were

placed with the concentration maximum towards the periphery of the plate. Any large air bubbles were removed underneath the strip by gently pressing the strip with the forceps, moving from the lowest concentration upwards. Small bubbles under the strip would not affect results. *S. cervisiae* is incubated at 30 ℃ for 48-72 hours and *C. albicans* is incubated at 35 ℃ for 24-48 hours.

Rreagents : 100 units per pack of the following agents are available:

Agent	Code	MIC range (μg/ml)
Amphotericin B	AP	0.002 - 32
Flucytosine	FC	0.002 - 32
Fluconazole	FL	0.016 - 256
Itraconazole	IT	0.002 - 32
Ketoconazole	KE	0.002 - 32

After the required period of incubation whereby growth becomes distinctly visible, the MIC value was read at the point of intersection between the zone edge and the Etest strip if the end point was clear. When different growth-inhibition patterns were seen, the illustrations in the Etest antifungal reading guide can be used to correctly select the MIC end point. When growth occurs along the entire strip i.e. no inhibition ellipse is seen, the MIC should be reported as > than the highest value on the MIC scale. When the inhibition ellipse is below the strip i.e. the zone edge does not intersect the strip, the MIC should be reported as < than the lowest value on the MIC scale.

For flucytosine, the MIC was read at almost complete inhibition of growth (95%) and for amphotericin B, at the point of complete

inhibition (100%). For azoles, the MIC was read at the first point of significant inhibition or marked decrease in growth density. Use the principle of 80% inhibition to visually select the end point.

2.7 Cloning two open reading frames of C13

C13 has two major open reading frames—C13a and C13b. Each ORF was cloned to vector containing no insert sequence. C13 was digested with *Bsa*B I and *Msc* I, and self-ligated. This plasmid only contains C13b. C13 was digested with *Bsa*B I and *Eco*R V and subcloned the 2 kb of C13a fragment into *Sma* I site of pRS426 plasmid. This plasimd only contains C13a.

2.8 *C. albicans* gene disruption with PCR products

(*C. albicans* transformation)

C. albicans cells were inoculated in a 2 ml YPD+uridine overnight (about 18~20 hours) culture in a 15 ml tube and incubated on a shaker at 30 ℃. 270 μl of overnight culture was transferred into 27 ml of YPD+ uridine (OD_{600} of about 0.1). The 27 ml culture was incubated at 30 ℃ with shaking (about 150 rpm) for 6 hours (OD_{600} of about 1.0- 0.7). Cells were harvested in 50 ml centrifuge tubes at 3000 rpm for 10 minutes at room temperature. Cell pellet was washed with 10 ml of sterile ddH_2O, washed with 5 ml of sterile 0.1M TE buffer (pH 7.5), and washed with 2 ml of sterile 0.1M LiOAc/TE buffer. The cell pellet was suspended in 250 μl of 0.1 M LiOAc/TE buffer and let it sit in room temperature for 10 minutes. DNA samples were setted up [PCR product (about 2~10 μg in

vol. ≤ 13 μl TE buffer, pH 8.0) mix with 10 μl of 10 mg/ml Salmon Sperm DNA (boil sperm DNA for 2 minutes with PCR machine and put it on ice right away); control test: equal volume. TE buffer (vol. ≤ 13 μl) mix with 10 μl of 10 mg/ml Salmon Sperm DNA] in eppendorf tubes while preparing cell. 100 μl aliquots of cell were dispensed into the prepared DNA sample and let it sit at 30 ℃ incubator for 30 minutes. 700 μl of 0.1 M PEG/LiOAc/TE buffer was added and mixed in each tube. Cells and the buffer were mixed by slow speed vortex (about speed 5) or inversion and incubated on Hybrid rotator or wheel with slow speed at 30 ℃ for 16 hours.

The cells were heat shocked at 42℃ for 60 minutes and were put on ice right away for about 2 minutes. Cells were spun at 3000 rpm for 3 minutes, poured off supernatant and the cell pellets were washed with 1 ml of 1 X TE buffer (pH 7.5). Cells were suspended with the remainder buffer (about 100 μl) and spread on selective SD plates. Cells on selective plates were incubated at 30℃ for 3~4 days.

2.9 Construction *REP3/rep3* heterozyous and *rep3/rep3* homozygous strain

For constructing the heterozygous and homozygous mutant strain of *REP3*, a 2.2 kb *ARG4* fragment was amplified by PCR from the pRS-*ARG4*Δ *Spe* I plasmid DNA with primers HJL 449 and HJL 450, and an 1.8 kb *URA3* fragment was amplified by PCR from pDDB57 plasmid DNA with primers HJL 451 and HJL 452.

For construct the heterozygous and homozygous mutant strain of *REP6*, a 2.1 kb *ARG* fragment was amplified by PCR from the pRS-*ARG4*Δ *Spe* I plasmid DNA with primers HJL 431 and HJL 432, and an 1.8kb URA fragment was amplified by PCR from pDDB57 plasmid DNA with primers HJL 433 and HJL 434.

2.91 PCR amplification

Typical PCR condition of *ARG4* fragment is 2 μl of mini-prep 100 X dilution template DNA (plasmid pRSArg4Δ Spe I) (final concetraction of template DNA is 100 ng/ml), 2 μl of a 5 μ M stock of each primer, 5 μl of 10 X PCR buffer, 0.5 μl of 25 μM deoxynucleoside triphosphates (dNTPs), 1 μl of 25 mMMgCl_2, 37 μl of water, and 0.5 μl of *Taq* DNA (1 U/ml).

The PCR mixture was incubated at 94 ℃ for 5 minutes followed by 30 cycles of 94 ℃ for 1min, 58 ℃ for 1 min, and 72 ℃ for 3 minutes. After a final extension at 72 ℃ for 10 minutes, the reaction mixture was stored at 4 ℃ before further use.

Typical PCR condition of *URA3* fragment is 2 μl of mini-prep 100X dilution template DNA (plasmid pDDB57-*Ura3*) (final concetraction of template DNA is 100 ng/ml), 2 μl of a 5 μM stock of each primer, 10 μl of 10 X PCR buffer, 1 μl of 25 μM deoxynucleoside triphosphates (dNTPs), 2 μl of 25 mM $MgCl_2$, 81 μl of water, and 1 μl of *Taq* DNA(1 U/ml).

The PCR mixture was incubated at 94 ℃ for 5 minutes followed

by 30 cycles of 94 ℃ for 1 min, 58 ℃ for 2 minutes, and 72 ℃ for 3 minutes. After a final extension at 72 ℃ for 8 minutes, the reaction mixture was stored at 4 ℃ before further use.

Generally, 0.5 μl of the reaction mixture was examined on an agarose gel to confirm the presence of a product of expected size. Before transformation, the PCR products were purified using MinElute PCR purification Kit (QIAGEN).

2.92 *C. albicans* transformation

For transformations with PCR products, 5-10 μg of the PCR mixture was used per transformation. For disruption of *REP3* or *REP6*, *ARG4* fragments were used for constructing heterozygous strains at first transformation. *URA3* fragments were used for constructing homozygous strains at second transformation.

2.10 Genomic DNA extraction

An isolated single colony was picked and inoculated at 6 ml YPD broth in 15 ml centrifuge tube. Tubes were incubated on a shaker (~180 rpm) at 30 ℃ for 24~48 hours. Cells were spun at 3000 rpm for 10 minutes, the supernatant was poured off and the cell pellets were washed with 5 ml ddH_2O. Cells were spun at 3000 rpm for 10 minutes, the supernatant was poured off and the cell pellets were resuspended with 500 μl breaking buffer by vortex then acid-washed glass beads (~ 400 μl) and 500 μl of phenol/choroform/isoamyl alcohol (25:24:1) were added.

The mixtures were vortex at highest speed for 10 minutes at room temperature. 500 μl TE was added and the mixtures were vortexed for few seconds. Cells were spun at 3000 rpm for 15 minutes at RT and were transfered aqueous layer to a new 15 ml centrifuge tube. ~850 μl of (equal volume) phenol/choroform/isoamyl alcohol (25:24:1) were added and the mixtures were vortex for 60 seconds. Phenol extract steps were repeated 3 times, then aqueous layer was transferred to 2 new 1.5 ml eppendorf (~400 μl /tube).

1 ml (2.5 X volume) of cold 100 % ETOH was added and the solution was mixed by inversion. The solution were spun at 13000 rpm for 10 minutes at 4 ℃, the supernatant was discarded carefully. The DNA pellet was dried in the air about 10~15 minutes. Total DNA pellets were resuspended in 0.4 ml of TE buffer with 3 μl of 10 mg/ml RNase A. They were mixed and incubated for ~15 minutes at 37 ℃. 40 μl of 3 M ammonium acetate (pH 5.2) and 1 ml of cold 100 % ETOH were added and mixed by inversion. They were spun at 13000 rpm for 10 minutes at 4 ℃ and the supernatant was discarded carefully. 1ml of cold 70 % ETOH was added to wash DNA pellet. They were spun at 13000 rpm for 5 minutes at 4 ℃. The supernatant was removed carefully. The DNA pellet was dried in the air about 5 minutes. DNA was resuspended in 100~200 μl of dd H_2O (or TE buffer)

Chapter 3. Results

1. Selecting the appropriate stain and condition for library screening

1.1 Activity of SC5314 and clinical strain *CDR1p-lacZ*

Filter assay with *lacZ* activities as the reporter for library screening require appropriate differentiation ability between the positive and negative result. If the *lacZ* activity is higher, the blue color of the colony will be darker. When screening the activators of *CDR1*, the basal level of the activity of the *CDR1p-lacZ* must be low enough. The basal activity of the *CDR1* promoter of SC5314 is too high for this purpose. To find a proper *CDR1* promoter whose basal activity is proper for the library screening, I compared the *lacZ* activity of *CDR1p-lacZ* of two Taiwan clinical strains Ym990348, Ym990361, and the laboratory strain SC5314 using filter assays.

Figure 1 shows the results of the filter assays. The *CDR1* promoter of Ym990348 has the lowest activity. So the Ym990348 *CDR1* promoter is the most appropriate one for my screening under the experimental condition.

2. *C. albicans* library screening

2.1. Rationale and design of library screening and result

2.1.1 Drug induction during screening

Because *CDR1* is a multi-drug transporter protein that can contribute to drug resistance, *trans*-regulatory factors of *CDR1* may be

activated by drug treatment. Miconazole is a potent inducer of *CDR1* expression, hence, it was used in my experiments at the concentration of 100 μg/ml.

2.1.2 Screening

At first, *C. albicans* genomic DNA library was transformed into *S. cerevisiae* containing Ym990348 *CDR*1p-*lacZ* plasmid (348 *CDR1*p-*lacZ*). Transformants with darker blue were collected and assessed by comparison with the negative and positive control strains. The negative control strain contains the 348 *CDR*1p-*lacZ* plasmid and the pRS426 plasmid with no insert on it. The positive control strain contains the 348 *CDR*1p-*lacZ* plasmid and the pRS426 plasmid with *REP2* gene cloned on it. Expression of *REP2* gene is known to increase the activity of *CDR1*p-*lacZ* (Shih, 2001). All transformants with blue color darker than that of the negative control were collected. Totally, 184,000 transformants were screened and there were 310 transformants collected for further analysis.

To verify that the increased activity is due to the presence of the library plasmids in the cell instead of other reasons such as chromosomal mutations, plasmid loss procedure for the library plasmids in these cells was carried out. If the cells losing the library plasmids become as white as the negative control, then this suggested that the increased activity is due to the presence of the library plasmids in the cells. There were 19 transformants whose *lacZ* activity was lost after the plasmids in the cells were removed.

Next I re-transformed the library plasmid back into fresh cells of

the original strain, which contained 348 *CDR1*p-*lacZ* plasmid. If the library plasmid is responsible for the increase of the activity of 348 *CDR1*p-*lacZ*, the cells will become darker again. There were 19 candidates having positive results that can be further tested in the following steps.

2.2 Assessing the candidates

2.2.1 Classification of the candidate plasmids

To amplify the candidate plasmids, those plasmids were extracted from the cells expressing higher *lacZ* activities and then were transformed to *E. coli*.

To assess whether those candidate plasmids have insert of *Candida* genomic sequence or not, the candidate plasmids were digested by *Bam*H I and *Kpn* I for electrophoresis. Comparing the restriction fragments to that of vector alone, 10 of the 19 plasmids had inserts (Figure 2). The sequences of the termini of the inserts were then subjected to sequence comparison with the Stanford *C. albicans* genome database (http://www-sequence.stanford.edu/group/candida/). After the elimination of the candidate plasmids which containing the same ORFs, there were 5 different candidate plasmids in all. These plasmids were named C13, C19, C82, C92, and C8.1. The insert sequences of C13, C19, C82,and C92 were located, according to the Stanford genome center, in contig6-2308, contig6-2505, contig6-2197,and contig6-2488, respectively. Figure 3 is the flow chart of screening procedure and the result.

The ORF of C8.1 is *REP2*, having been isolated in the laboratory previously (Shih, 2001). *REP2* is known to increase the activity of

*CDR1*p-*lacZ*. The inserts of C82 and C92 both have only one complete ORF. The inserts of C13 and C19 both have two complete ORFs. Table 1 is the relevant information and study of these ORFs.

2.2.2 β-gal activites of the candidates with filter assay

Figure 4 and 5 are the results of filter assay for the 5 candidates. Figure 4 is the result for comparing the original transform stains and the plasmid loss strains. The activity of β-gal decreased when plasmids were lost (color from blue to white). The activity of some candidate strains was even as low as that of the negative control (the same strain but containing only library vector without insert). Figure 5 is the comparison of the original and the re-transformed strains. The β-gal activity of the cells increased again when the individual candidate plasmids were re-transformed into the cells. With these results, it was confirmed that the increased activity of *CDR1*p-*lacZ* was due to the plasmids containing the inserts.

2.3 Results of β-gal activities with quantitative liquid assay

To further quantify the activity, liquid assay was used for measuring the activity of β-gal.

The activity of 348*CDR1*p-*lacZ* was too low for the liquid assay. Hence, candidate plasmids were transformed to *S. cerevisiae* cells containing SC5314 *CDR1*p-*lacZ*, which had a higher basal activity than that of the 348 *CDR1*p-*lacZ*. Figure 6 is the result of the liquid assay for 5314*CDR1*p-*lacZ* with the 5 candidate plasmids. The activity of the β-gal in the cells with any one of the 5 candidate plasmids was higher than cells with the vector alone. Comparing with the negative control strain (strain

containing only vector), the increased activities of C8.1, C13, C19, C82, and C92 were 9-, 4-, 5.5-, 7-, and 6-fold, respectively.

2.3 Results of drug susceptibility with Etest

C13, C19, C82, and C92 increase the activity of *CDR1*p-*lacZ*, and *CDR1* is related with drug resistance. Thus, over-expression of these candidate genes may increase the drug resistance ability of the cells. Since the candidate plasmids are based on 2 μ, a high copy plasmid, the candidate genes can be considered over-expressed. Figure 7 is the result of the drug susceptibility with Etest. The *S. cerevisiae* cells with C13 were more resistant to ketoconazole and intraconazole than cells with vector alone (Figure 7). But the cells with C92 were less resistant to azole drugs than those with vector alone. Because the test was with *S. cerevisiae*, it is plausible that there will be different with *C. albicans*. To verify the gene function and relation with drug resistance in *C. albicans*, all the ORFs in the insert sequences of the candidate plasmids were subjected to elimination procedure to define which and whether a certain ORF is responsible for the change of the *CDR1* promoter activities. Since the results of Etest showed that C13 was the most resistant and the activity of 348*CDR1*p-*lacZ* in C92 was higher than that of all the others, C13 and C92 were chosen for further studying.

3 . Determining which ORF affects the activity of *CDR1*p-*lacZ*

3.1 C13

C13 has two ORFs (13a and 13b), so it is necessary to determine which ORF affects the activity of *CDR1*p-*lacZ* (Figure 8). I cloned the

insert of C13 to the expression vector pRS426 and the 13a sequence was deleted by restriction of the C13 sequence with *Bsa*B I and *Msc* I enzymes to remove the 13a sequence. The restriction product containing the 13b ORF and vector backbone were re-ligated and transformed into *E. coli*. The plasmid was named C13b. The C13 was digested by *Bas*B I and *Eco*R V. The DNA fragment containing the 13a ORF was then cloned to pRS426 vector and the plasmid was named C13a. C13a and C13b were then separately transformed to the cells of *S. cerevisiae* 2B/int5314*CDR1*p-*lacZ* strain to determine the β-gal activities. Figure 9 and 10 are the results of filter and liquid assay of cells with C13a or C13b. The control strain containing vector alone (pRS426) showed an activity of 1 unit. The cells containing C13a showed an activity of 3.5 and 3 units while the cells containing C13b showed 1 unit. The cells containing the original C13 showed 4.5 units. The activity of *CDR1*p-*lacZ* in cells with C13a was as high as the *CDR1*p-*lacZ* in cells with C13, while the activity with C13b was as low as the one with vector alone. This means that C13a, not C13b is the ORF responsible for increasing the activity of *CDR1*p-*lacZ*. The gene containing the ORF of C13a was named *REP3*, standing for Regulator of Efflux Pump 3.

3.2 C92

C92 has only one complete ORF. According to the result of plasmid loss test, this ORF really affected the activity of *CDR1*p-*lacZ* and the gene was named *REP6*.

4. Constructing homozygous and heterozygous mutant strains of *REP3* and *REP6*

All the previous results were in *S. cerevisiae*, but the genes I have studied were from *C. albicans*. Hence, the functions of those candidate genes have to be determined in *C. albicans* by gene disruption.

The strategy of gene disruption is to transform a parental strain with a marker-containing PCR product that is capable of homologous integration into the sites of *REP3* or *REP6* alleles to disrupt the functions (Figure 11). To create the homologous region to *REP3* or *REP6* for recombination, synthetic primers with specific sequences were used (detail in material and method) to produce PCR products that contain short regions of homology to the genes in the vicinity of the terminal sequences of the target genes (65 bps in each terminus) (Figure 11). BWP17 and BWP17 *tetR-His* are the parental strains used for gene disruption and markers *ARG4* and *URA3* are used for heterozygous and homozygous knock out.

4.1 Constructing and confirming the heterozygous mutant strain of REP3

To construct heterozygous mutant strain of *REP3*, the *ARG4* PCR product produced by primers HJL449 and HJL450 was transformed into BWP17 and BWP17 *tetR-His* strains. Thirteen and eleven transformants were obtained for BWP17 and BWP17 *tetR-His* heterozygous mutant strains, respectively. In order to confirm the correct location of the *ARG4* fragment, PCR was used to analyze the construct as showed in Figure 12(a). The genomic DNA of heterozygous mutant strain was extracted and used as the template in the PCR. If the *ARG4* fragment was located at

the correct position, then a 1.3 kb fragment would be produced when using primers HJL 241 and HJL 457 in PCR. Figure 12(b) shows the result of the PCR. The 1.3 kb fragments observed in the products from these *REP3/rep3* heterozygous mutant mutants.

4.2 Constructing and confirming the homozygous knockout strain of *REP3*

To construct homozygous mutant strain of *REP3*, the *URA3* PCR product produced by primers HJL 451 and HJL 452 were transformed into BWP17 and BWP17 *tetR-His REP3/rep3* heterozygous mutant strains. In order to confirm whether the location of the *URA3* fragment is correct, PCR analysis was employed and the location of the primers for the analysis were shown in Figure 13(a). The genomic DNA of the homozygous mutant strain was used as the template in the PCR. If the *URA3* fragment was located at the correct position, a 1.2 kb fragment would be produced when using primers HJL133 and HJL458 in PCR. Figure 13(b) shows the result of the PCR. A 1.2kb fragments were observed in the products from these *rep3/rep3* homozygous mutants.

4.3 Constructing and confirming the heterozygous mutant strain of *REP6*

To construct heterozygous mutant strain of *REP6*, the *ARG4* PCR product produced by primers HJL431 and HJL432 was transformed into BWP17 and BWP17 *tetR-His* strains. Two and fourteen transformants were obtained for BWP17 and BWP17 *tetR-His* heterozygous mutant strains, respectively. The locations of the primers designed to confirm the correct location of the *ARG4* fragment are shown in Figure 14(a). The

genomic DNA of the heterozygous mutant strain was used as the template in the PCR. If the *ARG4* fragment was located at the correct position, a 1.7 kb fragment would be produced when using primers HJL 241 and HJL 418 in the PCR and a 2.8 kb fragment would be produced when using primers HJL 412 and HJL 414. Figure 14(b) shows the result of the PCR. Both the 1.7 and 2.8 kb fragments were produced as predicted from these *REP6/rep6* heterozygous mutants.

5. Phenotypic characterization

5.1 Etest results of *rep3/rep3* homozygous strain

Over-expressing *REP3* can increase the activity of *CDR1*p-*lacZ* in *S. cerevisiae*. But the relationship between *REP3* and *CDR1* in *C. albicans* is unknown. If *REP3* can also increase the expression of *CDR1* in *C. albicans*, the *rep3/rep3* homozygous null mutant strain may decrease the expression of *CDR1*. Since *CDR1* is involved in the resistance to azoles, *rep3/rep3* homozygous null mutant strain shall be more susceptible to azole drugs than wild type strain (SC5314) is.

The antifungal agents used in the test were azoles and polyenes. Four difference azoles, fluconazole, itraconazole, ketoconazole, voriconazole, and one polyenes, amphotericin B, were tested. Amphotericin B was used as the control antifungal agent because the resistance mechanism of *CDR1* has less relationship with this agent. *CDR1* is especially involved in the resistance to azoles, so azoles were used for the test. For azoles, the MIC was read at the first point of significant inhibition or marked decrease in growth density. Use the principle of 80% inhibition to visually select the end point of significant

inhibition. For amphotericin B, the MIC was read at the point of complete inhibition (100 %). Figure 15 shows the results of Etest for the *rep3/rep3* homozygous null mutant strain. The MIC value of each agent has no significant difference when comparing the mutant and the wild type strains. But the growth inhibition patterns have significant differences between the mutant and the wild type strains in some azoles. In fluconazole, itraconazole, and voriconazole, the wild type strain (SC5314) has lawn of micro-colonies within a discernable ellipse but *rep3/rep3* homozygous mutant strain has clear end point (few micro-colonies within an ellipse). In ketoconazole and amphotericin B, there is no difference in the growth inhibition patterns when comparing the mutant and the wild type strains. In amphotericin B, both the mutant and the wild type strains have sharp end points while in ketoconazole, both the mutant and the wild type strains have lawn of micro-colonies within a discernable ellipse.

Chapter 4. Discussion

Among the different categories of antifungal drugs, the development of azole resistance is the most relevant medical problem. Drug resistance is often associated with the upregulation of genes encoding efflux pumps. Increased mRNA levels of *Candida* drug resistance gene family (*CDR*), which are members of the ATP binding cassette (ABC) transporter superfamily, and *MDR1*, a major facilitator with a much narrower substrate spectrum including fluconazole, have been associated with azole resistance. In recent years, much effort has been devoted to understanding the regulatory mechanisms of multi-drug transporter genes, such as *CDR1*, in *Candida albicans*. But little is known about the *trans*-regulatory factors of *CDR1*. In this study, with these series experiments, the *trans*-regulatory factors of *CDR1* have been identified and their functions were investigated. But their relationship with drug resistance and the mechanism within must be further examined.

1. Library screening in miconazole induction

Although two regulatory factors have been isolated in the laboratory in previous study (Shih, 2001), the basal activity of SC5314 *CDR1*p-*lacZ* is relatively too high for identification of more positive regulatory genes. Hence, I used the Ym990348 *CDR1*p-*lacZ* (in 2 μ plasmid) for the screening, in which the *CDR1* promoter activity is relatively low and hence it would be easier for screening activator of

CDR1 by β-gal filter assay (Figure 1). There were many candidates after the library screening (Figure 3), which reveals that the screening system of Ym990348 *CDR1*-promoter is well-suited for this purpose. In addition the screening was performed under the presence of 100 μg/ml miconazole. Because *CDR1* is a multi-drug transporter protein that can contribute to drug resistance, *trans*-regulatory factors of *CDR1* may be activated by drug treatments. But the β-gal activity from the recombinant *CDR1* promoter showed no distinct difference with or without miconazole induction (Figure 16). This is maybe because the difference caused by drug induction was measured by β-gal filter assay, the difference was not obvious in the filter assay. Or the expression of the candidate gene (*trans*-regulatory factors of *CDR1*) is not regulated by miconazole. Or the system is performed in *S. cerevisiae*, while the regulation of *CDR1* promoter may have large difference between *S. cerevisiae* and *C. albicans*.

2. The effect of candidate plasmids to SC5314*CDR1*p-*lacZ* and Ym990348*CDR1*p-*lacZ*

For comparing the effect of candidate plasmids in SC5314 *CDR1* gene, the 5 candidate plasmids were transformed to *S. cerevisiae* with the SC5314*CDR1*p–*lacZ* plasmid integrated in the chromosome. SC5314 *CDR1*p-*lacZ*. One of the candidate plasmid, C92, induced the largest differences in both SC5314 and Ym990348. The increasing amount of C92 activity is 16-fold in Ym990348 *CDR1*p-*lacZ* and 6-fold in SC5314 *CDR1*p-*lacZ*. The difference in the amount of increase may due to the difference in the *CDR1* promoter sequences of

Ym990348 and SC5314. If the C92 gene products interact with promoters directly, the difference in promoters may have affected the binding affinity of the C92 gene products. The difference of promoters between SC5314 and Ym990348 strain is listed in Table 2. The exact interaction between C92 and *CDR1* promoter is still unknown and requires further investigation. Alternatively, there may be mutations in other genomic genes of the YM990348 that maintain a lower level expressing of *CDR1* genes in the cells. Hence, when being induced, the relative amount of the increased expression level is always much higher.

3. Regulation and function of *REP3* and *REP6*

After series experiments of screening and sequence analysis, four new candidate ORFs were isolated and their ability to increase the activity of *CDR1* promoter have been confirmed. The ORFs were named *REP3*, *REP4*, *REP5*, *REP6* (*REP*: Regulator of Efflux Pump). Among the four ORFs, two (*REP3*, *REP6*) of them have the C2H2 type zinc finger domain, which is the structure of nucleic acid-binding protein. A protein containing the zinc finger domains often acts as a transcription factor. *REP3* and *REP6* maybe act as the transcription regulation factors of *CDR1* (direct) or the upstream genes of *CDR1* (indirect).

REP3 contains six C2H2 type zinc finger domains, which is a characteristic of a transcriptional factor. The C2H2 zinc finger is one of the classical zinc finger domains, whose two conserved cysteines and histidines coordinate to interact with a zinc ion. These “fingers” have been found to bind to about 5 base pairs of nucleic acid containing short

runs of guanine residues (Marco *et al.*, 2003). An example of this type of zinc finger protein is Sp1, of which the accepted consensus binding sequence usually defined by the asymmetric hexanucleotide core GGGCGG (Marco *et al.*, 2003).

The promoter deletion analysis of *CDR1* promoters identified DREI (drug-responsive element I) with the consensus sequence 5'-ACGG(A/T)TATCGGATATTTTTTT- 3'. It contains two direct repeats (underlined). Several experiments confirmed this DRE to be an important regulatory element in the presence of oestradiol (Karnani *et al.*, 2004). The validation of the function of the different DRE elements in the background of an azole-resistant strain constitutes an additional argument suggesting that, in such strains, as yet unidentified factors are capable of interacting in *trans* with these elements (Michelle *et al.*, 2002). The structure repeat of DRE with CGG triplet motifs is, however, typical of binding sites of transcription factors with zinc finger motifs. Rep3 is also a protein containing the C2H2 type zinc finger motif, DRE may be one of the binding site for Rep3 protein. But the interaction between Rep3p and *CDR1* promoter has to be further verified.

The PDRE (pleiotropic drug-responsive element) identified in the promoter of the ABC transporter gene *PDR5* of *S. cerevisiae* has the consensus sequence 5'-TCCG/aC/tGG/cA/ g-3' (DeRisi *et al.*, 2000). It also contains the inverted repeat of GGC. *PDR5* is the functional homologue of the *CDR1* in *C. albicans* and PDRE is the target sequence of the transcription factors Pdr1p and Pdr3p (Katzmann *et al.*, 1996). Both Pdr1p and Pdr3p are Zn(II)2Cys6 zinc finger transcription factors, sharing common structural motifs and an overall amino acid identity of

36%. The expression of Pdr3p is activated by Pdr1p (Delahodde *et al.*, 1995). Pdr1p and Pdr3p can form both homo- and heterodimers *in vivo*, with the dimerization domain located in the N-terminal half of both proteins close to the DNA binding site. Furthermore, protein–protein interactions with other regulatory factors may also play a role in target gene regulations. Indeed, binding partners of Pdr1p and Pdr3p were identified. Ngg1p binds to the C-terminal domain of Pdr1p and decreases its transactivation activity (Martens *et al.*, 1996). And Pdr3p was post-translationally regulated by positive regulatory signals from the mitochondria and Rtg1p (Hallstrom *et al*., 2000). So the regulation of *CDR1* in *C. albicans* may be involved in complex transcription factors and interaction of other regulatory factors, similar to that of the *PDR5* in *S. cerevisiae*. The relationship between candidates identified in this study can be further investigated to understand the regulation network of *CDR1*.

The Etest result of *REP3* is in Figure 15. Etest is a method used for testing the susceptibility of drugs of yeasts. It is based on a combination of the concepts of dilution and diffusion tests. Like dilution methods, Etest directly quantifies antifungal susceptibility in terms of discrete MIC values. As Etest consists of a predefined and continuous concentration gradient, the MIC values obtained can be more precise than values from conventional procedures based on discontinuous 2-fold serial dilutions. Although processed like the disc diffusion test, the stable concentration gradient in Etest, differentiates the two methods clearly. Unlike disc diffusion, MICs obtained by Etest are unaffected by drug properties such as molecular weight, aqueous solubility and diffusion characteristics or

by varying growth rates of different yeasts.

The MIC values of the agents tested showed no significant difference between the mutant and the wild type strains in this study. But the growth of colony has obvious differences in certain azoles. In fuconazole, itraconazole, and voriconazole, the wild type strain (SC5314) has lawn of microcolonies within a discernable ellipse but the *rep3/rep3* homozygous null mutant strain has clear end point. Fluconazole, itraconazole, and voriconazole are triazoles. The *rep3/rep3* homozygous mutant appears to be more susceptible to these three azoles. *CDR1* is known to be involved in efflux of fluconazole, ketoconazole, itraconazole, and miconazole (Nakamura *et al.*, 2001) and cells lacking Cdr1p becomes hypersensitive to fluconazole, itraconazole, and ketoconazole (Sanglard *et al.*, 1996). The *rep3/rep3* homozygous mutant may decrease the expression of *CDR1* and lead the cells to become more sensitive to azoles. It suggests that *REP3* affects the drug susceptibility in *C. albicans* through regulating the expression of *CDR1*. However, the interaction between *REP3* and *CDR1* is not necessary direct.

Chapter 5. Future work

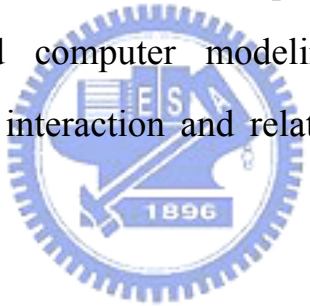
Trans-acting regulatory factors play important roles in the regulation of *CDR1*expression. The result of this study provides evidence that there are several genes involved in the *trans*-regulation of *CDR1*. But there are still more that have to be investigated in the future.

1. The integration constructs of the homozygous knockout strains of the candidate genes have to be confirmed by Southern blot. The vicinity location of the *ARG* or *URA* marker can be determined by PCR but not the genomic structure. Southern blot can be used for confirming whether the target gene is disrupted at the proper chromosomal location and whether the copy number of the marker is correct.

2. The *REP3* homozygous null mutant strain is more sensitive to some of the azoles. This result has to be further confirmed by phenotype rescuing (complementation). The wild type *REP3* gene needs to be complemented back into the homozygous knockout mutant. If the change of phenotype is due to the disruption of *REP3*, the wild type phenotype will be restored when the wild type *REP3* gene is knock-in to the original location.

3. The effect of over-expressing the candidate genes in *C. albicans* can be investigated. The phenotype may be opposite to the homozygous knockout strain, or, there will be other phenotypes that may reveal more biological function of the gene. For instance, the morphology test (germ tube assay) can be performed on the over-expression strains.

4. Structural information and domain analysis by bioinformatics can be performed. There is information from other researches that may provide new insights. For instance, the secondary or tertiary structure and specific functional domains of the protein can be found out by data comparison and computer modeling, which may provide information about the interaction and relationship of each candidate genes.



5. Gel shift assay of the candidates and the *CDR1* promoter can be performed to determine whether there are direct or indirect interactions between the candidate gene products and the *CDR1* promoter sequence. If the candidates and the *CDR1* promoter interact directly, the promoter sequence of *CDR1* can then be analyzed to find out the putative *cis*-acting elements interacting with these *trans*-acting factors. This is especially useful in the case of *REP3* and *REP6* because they are both zinc finger binding proteins. Or *CDR1* promoter shall be analyzed by bioinformatics to find out more possible binding sites.

6. The *trans*-regulation of *CDR1* expression is a network or complex cascade according to the previous data (Shih, 2001) and this study, therefore the interactions between the candidates can be investigated more. It can be compared with the regulation network of *PDR* in *S. cerevisiae*. By observing the change of phenotype and by analyzing sequence of each candidate, the relationship between the candidates and *CDR1* may be further revealed. And the DNA binding domain and interaction domain with other proteins may be found by sequence analysis of the candidate genes. The effect on drug susceptibility by the candidate genes can also be investigated. It will be useful in re-constructing the regulation cascade of *CDR1* and for the putative drug targets in the future.

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