中文摘要

先前實驗室(Lo, 2002)在麵包酵母菌系統下利用刪減序列分析以及定點突變方式指出對白色念珠菌 *MDR1* promoter 的 M12 (-736 ~-734)位置突變後可使活性明顯下降。為了找出其他在 *MDR1* promoter 的 *cis*-element 序列，我將不同臨床菌株帶有不同序列的 *MDR1* promoter 與 *Lac Z* 的 open reading frame 建構成重組基因。藉由測量β-galactosidase 活性，找到了-640、-617 以及-259 的序列可能會調節 *MDR1* promoter 的活性。為了確認這些點以及 M12 對 *MDR1* promoter 活性的重要性，我將含有這些突變點的 *MDR1* promoter 接上水母冷光酵素基因並送回白色念珠菌的基因體內，測量冷光活性。正如預期的，*MDR1* promoter 在 4-NQO 藥物的誘導下，活性增加約二十倍。平均看來這些含有突變的 *MDR1* promoters 比野生株的活性高，但這個結果並不明顯。出乎意料的，在 *cph1/cph1* homozygous 菌株內，*MDR1* promoter 的活性增加將近十倍，此資料顯示 Cph1 蛋白質在白色念珠菌內可能扮演負向調控因子角色。

Abstract

Previously research in the laboratory (Lo, 2002) has used deletion analysis and mutagenesis method to demonstrate that the activity of the promoter of *Candida albicans MDR1* gene was greatly decreased when it contains a mutation at the sequence named "M12"(-734 to -736；A of translation initiation site ATG as +1) in *S. cerevisiae*. To identify other potential *cis*-acting regulatory elements on the *MDR1* promoter, I have constructed *MDR1* promoter-*lacZ* (*MDR1p-lac Z*) fusion plasmids of which *MDR1* promoters were from different clinical *C. albicans* strains. The –640, -617, and –259 bp were identified as potential sites for regulating the activity of the *MDR1* promoter by comparing the activity of β-galactosidase of different *MDR1p-lac Z* fusions. The importance of these sites and the M12 site for the activity of the *MDR1* promoter in *C. albicans* were further investigated by integrating *MDR1* promoter-*Renilla* luciferase gene (*MDR1p-RLUC*) fusion of which the *MDR1* promoters contained different mutations into *C. albicans*. As expected, the activity of the *MDR1* promoter has increased approximately 20-fold under the 4-nitroquinoline oxide (4-NQO) induction. By average, the *MDR1* promoters with mutations have higher activities than the wild type *MDR1* promoter under 4-NQO induction. Nevertheless, the increase is not dramatically. Surprisingly, the activity of *MDR1p-RLUC* was increased approximately 10-fold in the *cph1/cph1* homozygous mutant strain. This data suggests that the Cph1 acts as a negative regulator of the *MDR1* promoter in *C. albicans*.

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努力了兩年，終於到了離畢業所要完成的最後一步了；這一路上走來，多虧有許多貴人的相助，才能完成我的碩士論文。

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1. Introduction

1.1 *Candida* infection

The incidence of nosocomial fungal infection has increased over the past several decades. From January 1980 to April 1990, 27,200 fungal isolates causing nosocomial infections were reported from 180 hospitals and *Candida* species accounted for 19,621 (72.1%) of these isolates (Jarvis, 1995) in USA. 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 **(**Hung *et al.*, 1996). In Taiwan, the overall nosocomial fungal infections is also increased gradually and the nosocomial candidaemia increased rapidly during 1981–1996 (Appendix 1). *Candida albicans* was the most common species (50.4%) among them (Chen *et al.*, 2003) (Appendix 2). These species can cause a wide range of human diseases ranging from superficial mucosal infections, such as vulvovaginal (VVC) and oropharyngeal candidosis (OPC), to life-threatening invasive infections. In the majority of cases OPC and systemic infections occur only in individuals who are severely ill and/or immunocompromised (Sullivan *et al.*, 2004). Nonetheless, *Candida* species have become more important as the bloodstream infection isolates increased from 1.0% in 1981-1986 to 16.2% in 1999 (Hsueh *et al.*, 2002).

Candida albicans is an opportunistic fungal pathogen that is found in the normal gastrointestinal flora of most healthy humans. However, in immunocompromised patients, blood-stream infections often cause death, despite the use of anti-fungal therapies (Berman *et al.*, 2002). In the United States, *Candida* is the fourth most common cause of blood stream infection and accounts for about 10% of all blood stream infections (Edmond *et al.*, 1999). Majority of those isolates are *C. albicans* (Chen *et al.*, 2003).

1.2 *Candida albicans*

Candida albicans is an obligately diploid, apparently asexual fungus with a nuclear genome of 16 million base pairs, 33% larger than that of *Saccharomyces cerevisiae*. *C. albicans* can undergo morphological conversion between yeast and filamentous (including pseudohyphal and hyphal form) forms depending upon various environmental conditions (Hwang *et al.*, 2003).

As a research system, *C. albicans* also offers a range of molecular-genetic tools (De Backer, 2000), a complete genome sequence (Tzung, 2001), and a sufficiently close phylogenetic relationship to the model yeast system, *S. cerevisiae*, that many genes and pathways have highly similar counterparts in both yeasts. Unfortunately, *C. albicans* is not amenable to conventional genetic analysis. This disadvantage can be overcome with complementary studies using *S. cerevisiae* as a genetic stand-in. *S. cerevisiae* stands on its own as a pathosystem based on recent reports of its clinical isolation from a variety of body sites and patient groups, with subsequent successful infection using these isolates in animal models (Goldstein *et al.*, 2001). To prevent the difficulties of carrying out genetic studies directly in *C. albicans*, many *C. albicans* genes have been identified and/or analyzed using *S. cerevisiae* as a 'surrogate'. For example, many *C. albicans* genes were cloned by their ability to complement a mutation in *S. cerevisiae*. This approach is not as important as it once was, because homologues can be identified on the basis of their sequence similarity, as a result of the *C. albicans* genome sequencing project. Nonetheless, if a gene does function in *S. cerevisiae*, then the effects of mutant alleles can now be tested in *S. cerevisiae* before the more laborious process of testing them in *C. albicans* (Devasahayam *et al.*, 2002).

Genetic manipulations of *C. albicans* have been fraught with difficulties from the lack of a useful sexual cycle and a lack of molecular tools. Today, reverse genetic approaches, in which genes are first identified by their sequences and then both genomic copies are sequentially deleted or mutated, are commonly used. Another significant challenge is posed by the unconventional *C. albicans* codon usage — *C. albicans* translates the CUG codon as serine, rather than the 'universal' leucine (Santos *et al.*, 1995). For this reason, many heterologous markers do not function in *C. albicans* unless the CUG codons are first modified. However, many *C. albicans* genes are at least partially functional in *S. cerevisiae*, which facilitated their identification by complementation studies.

In the past few years, several crucial tools have greatly enhanced our ability to manipulate *C. albicans* genetically (Ernst *et al.*, 2002). Methods for transformation were modified from protocols for transformation of *S. cerevisiae*. A PCR-mediated transformation system similar to that used in *S. cerevisiae* (Wach, 1996) has been developed for use in *C. albicans* (Wilson *et al.*, 1999；Wilson *et al.*, 2000), obviating the need to clone a gene before disrupting it. Strain BWP17, which is triple auxotrophic (*ura3*, *his1* and *arg4*) has made the generation of double mutants simpler by allowing sequential transformation steps without the need to regenerate a single selectable marker.

The *C. albicans* sequencing project carried out at the Stanford Genome Center is based on shotgun sequencing and assembly, and the total assembly now comprises 14.9 Mb of sequences. A preliminary estimate suggests that there are ~8000 open reading frames (ORFs); this gene density is in good agreement with the results from *S. cerevisiae*, in which the genome project has identified 6000 ORFs in the 12-Mb genome.

Genome sequencing has uncovered many *C. albicans* ORFs that have obvious *S. cerevisiae* homologues. Among them are many of the putative homologues of *S. cerevisiae* genes that are required for sexual differentiation and meiosis (Tzung *et al.*, 2001). *C. albicans* also contains many genes that have no obvious *S. cerevisiae* homologues, some of which are most similar to genes from other fungi, but others that encode novel gene products (Scherer, 2002). A striking observation on the *C. albicans* genome is that the proportion of ORF products nonhomologous to known proteins is larger than that in *S. cerevisiae*. That is, the genome of *C. albicans* seems to contain a significantly high proportion of genes specific to this organism, which could be related to its versatile way of living. A relatively larger number of proteins are assigned to functional categories related to interactions with the environment and morphogenesis in *C. albicans* than in *S. cerevisiae*, compared with proteins involved in metabolism, proliferation or subcellular compartmentalization (Herrero *et al.*, 2003).

Homologues of genes that are common to all fungi, especially those that are essential for fungal growth, might be good candidates for broad-spectrum anti-fungal targets. *C. albicans* genes that lack human homologues are considered especially promising in this respect, because they are less likely to cause the negative side effects that are associated with most anti-fungal therapies.

1.3 Drug resistance

1.3.1 The importance of the study of drug resistance

The ability of microorganisms to become resistant to the therapies used against them has long been recognized, and resistance rates for many isolates, although variable across locations, are rising rapidly (Appelbaum, 1992; Reacher *et al.*, 2000). Resistance, reduces the

effectiveness of antimicrobial therapies and increases morbidity, mortality and health care expenditure (Smith *et al.*, 1996; Coast *et al.*, 1996). Although resistance is essentially the result of individual decisions concerning antimicrobial treatment made by doctors and/or patients, its impact is global, affecting all regions of the world and unrestricted by national boundaries. To 'solve' the problem of resistance, there are two broad strategies: (1) develop new antimicrobial and/or alternative treatments so that resistance is no longer important and/or (2) implement policies to fight resistance (Coast *et al.*, 2003). The study of drug resistance belongs to the later strategy. If we can understand the drug resistant genes and mechanisms for the purpose of develop treatments to counter them, then the problem may be improved and brought under control.

1.3.2 Methods for measurement of antifungal resistance

Drug resistance can be measured as the minimum inhibitory concentration (MIC) that curtails the growth of the fungus under standardized in vitro test conditions (Reyes *et al.*, 2000). The MICs of common antifungal drugs are listed below (Rex *et al.*, 2001). S is susceptible, R is resistant, and S-DD is susceptible-dose dependent.

Antifungal drugs	MIC(μg/ml)
Fluconazole	S：< 8 μg/ml S-DD：> 8 μg/ml and ≤ 32 μg/ml; R：> 64 μg/ml
Itraconazole	S：≤ 0.125 μg/ml S-DD：> 0.25 μg/ml and ≤ 0.5 μg/ml

	R：＞ 1.0 μg/ml
Ketoconazole	R：＞ 0.125 μg/ml
Flucytosine	S：≤ 4 μg/ml S-DD：＞ 4 μg/ml and ≤ 16 μg/ml R：＞ 16 μg/ml
Amphotericin B	R：＞ 0.5 μg/ml

1.4 Molecular mechanisms of antifungal agent resistance(Appendix 3)

1.4.1 Reduction of drug import

The first-line mechanism for drug resistance is a defect in drug import. Defects in drug imports are common mechanisms of drug resistance. However, it is important to emphasize the distinction between the import of a drug into a cell and the gradual accumulation of the drug in the cell, which is the result of a balance between import into the cell and efflux of the drug from the cell. Drug import may also be affected by the sterol composition of the plasma membrane. To prevent drugs from entering, cells can alter the composition of the membrane. Several studies have demonstrated that when the ergosterol component of the membrane is eliminated or reduced in favor of other sterol components such as 14a-methyl sterols, there are concomitant permeability changes in the plasma membrane and a lack of fluidity (Vanden *et al.*, 1987). These changes may lower the capacity of azole drugs to enter the cell. However, direct correlation between the cytoplasmic composition and drug resistance has not been established. Hence, how fugal cells develop a mechanism to decrease the accumulation of drugs by preventing drug entry is still unknown (Yang *et al.*, 2001).

1.4.2 Alteration in intracellular of drug processing

It is important to note that alterations in drug processing (degradation or modification) are important drug resistance mechanisms in a variety of bacterial and eukaryotic systems (Borst, 1991).

1.4.3 Molecular alterations of the target enzymes

The target enzyme of the azole drugs is lanosterol demethylase. The gene encoding this protein is currently designated *ERG11* in all fungal species, although it has previously been referred to as *ERG16* and *CYP51A1* in *C. albicans*. Several genetic alterations have been identified that are associated with the *ERG11* gene of *C. albicans*, including point mutations in the coding region, overexpression of the gene, gene amplification (which leads to overexpression), and gene conversion or mitotic recombination (White *et al.*, 1998). For example, several investigators compared the sequence of the *ERG11* gene of fluconazole-resistant *C. albicans* strains with the published *ERG11* sequence and that of fluconazole-susceptible strains. As compared with the published *ERG11* sequence, alternations F105L, E266D, K287R, G448E, G450E, G464S and V488I were found only in fluconazole-resistant isolates but not in the susceptible isolates (Loffler *et al.*, 1997).

1.4.4 Modifications of the ergosterol biosynthetic pathway

Another common mechanism of drug resistance is the modification of the target enzyme and/or other enzymes in the same biochemical pathway. For azole drugs, that pathway is the ergosterol biosynthetic pathway. There are three different known mechanisms to develop a resistant phenotype through the ergosterol biosynthesis pathway: alternation of the target to prevent the drug from binding, increase the expression of target gene, and modification of other enzymes

in the same pathway so the cell can bypass the drug-targeted enzymes (Yang *et al.*, 2001). Inhibition of sterol 14a-demethylase (14DM) by fluconazole not only results in ergosterol depletion but also in the accumulation of the methylated sterol 14a-methylergosta-8, 24 (28)-dien-3h, 6adiol, which inhibits cell growth (Kelly *et al.*, 1997). Alterations in the sterol biosynthesis pathway that avoid the accumulation of this growth inhibiting sterol in the presence of fluconazole can cause fluconazole resistance. Inactivation of D5, 6 desaturase (ERG3), an enzyme that acts at an earlier step than 14DM in the ergosterol biosynthesis pathway, results in altered sterole composition of the membrane (high fecosterol content) and fluconazole-resistance. (Kelly *et al.*, 1997; Nolte *et al.*, 1997). Recently, it was shown that deletion of the *ERG3* gene in *C. albicans* resulted in reduced susceptibility to fluconazole, providing direct genetic evidence that alteration of the sterol biosynthesis pathway can cause fluconazole resistance (Joachim, 2002).

1.4.5 Decreased accumulation of drug

An important mechanism of fluconazole resistance is the reduction of intracellular accumulation of the drug. In recent years, it became evident that fluconazole is actively transported out of the cells in an energy-dependent manner and that an enhanced drug efflux is caused by the overexpression of genes encoding membrane transport proteins. The highly homologous genes *CDR1* and *CDR2* (*Candida* drug resistance) encode ATP-binding cassette (ABC) transporters, which use adenosine triphosphate (ATP) as the energy source, whereas the *MDR1* (multidrug resistance) and *FLU1* (fluconazole-resistance) genes encode major facilitators, which use the proton gradient across the membrane as the driving force for transport (Morschhauser 2002).

In recent years, several studies have investigated the accumulation

of drugs in cells of which the drug MIC is high. Using radioactively labeled drugs such as fluconazole, these studies have demonstrated that resistant isolates frequently accumulate fewer drugs than do matched sensitive isolates (Albertson *et al.*, 1996; Lamb *et al.*, 1997; Sanglard *et al.*, 1995; Venkateswarlu *et al.*, 1995).

1.5 *MDR1*

1.5.1 Introduction of *MDR1*

The *MDR1* gene (originally termed *BEN* ^{r}) had been cloned by its ability to confer resistance to benomyl and methotrexate upon *S. cerevisiae* transformants (Fling *et al.*, 1991), and its overexpression in *S. cerevisiae* was later shown to mediate resistance to cycloheximide, benztriazoles, 4-nitroquinoline-*N*-oxide (4-NQO), and sulfometuron methyl (Ben-Yaacov *et al.*, 1994). This gene was shown to be related to *S. cerevisiae ATR1*, which encodes a transmembrane protein conferring resistance to aminotriazole (Kanazawa *et al.*, 1988) and 4-NQO (Gompel-Klein *et al.*, 1989) and imparts resistance to these agents to susceptible strains of *S. cerevisiae* (Ben-Yaacov *et al.*, 1994)

This sequence contained an open reading frame encoding a polypeptide of 564 amino acids with one copy mapping to chromosome 6 of *C. albicans* (Ben-Yaacov *et al.*, 1994). The protein is composed primarily of 12 to 14 transmembrane segments (Marger *et al.*, 1993 ; Paulsen *et al.*, 1996) and belongs to the major facilitator (MF) superfamily which uses the proton motive force of the membrane as a source of energy. In general, the MF works by antiport; that is, protons are pumped into the cell and substrate molecules are pumped out.

1.5.2 The regulation of *MDR1*

The overexpression of *MDR1* has been linked to azole resistance in *C. albicans* (White *et al.*, 1998). The transcription of *MDR1* is at least 9.3-fold greater in a fluconazole-resistant isolate than in a fluconazole-sensitive isolate (Lyons *et al.*, 2000). In many fluconazole-resistant clinical *C. albicans* isolates, *MDR1* is constitutively overexpressed, indicating that mutations must have occurred in these strains that abolish the normal regulation of *MDR1*. Such mutations could either occur in the promoter region of the genes themselves or involve *trans*-regulatory factors. In some study, the activation of the *MDR1* gene in fluconazole-resistant isolates has been caused by mutations in *trans*-regulatory factors. (Morschhauser, 2002).

Clinical *C. albicans* isolates which overexpressed *MDR1* were also more resistant to other drugs in addition to fluconazole, e.g. 4-NQO, cerulenin, and brefeldin A, as compared with matched isolates that did not detectably express *MDR1* in vitro. The increased resistance was abolished when the *MDR1* gene was deleted from the genome of these isolates, providing genetic evidence that *MDR1* overexpression in clinical *C. albicans* isolates indeed confers resistance to various, structurally unrelated drugs (Wirsching *et al.*, 2001). *MDR1* appears to be the sole mediator of 4-NQO resistance (Wirsching *et al.*, 2000)

Alarco *et al* (1997) found that the deletion of *CAP1* in FR2 (fluconazole-resistant) strain resulted in *MDR1* up-regulation, indicating that Cap1 behaved as a negative transcriptional regulator of *MDR1* in this strain. This finding was unexpected, in light of the previous demonstration that Cap1 (and Yap1) behaved as a positive transcriptional regulator of *FLR1* in *S. cerevisiae* (Alarco *et al.*, 1997) and that deletion of *CAP1* did not result in *MDR1* upregulation in *C. albicans* CAI4. It is possible that in FR2, the genetic alteration leading to *MDR1*

overexpression also modifies the activity of Cap1, which, in this mutated context, would function as a transcriptional repressor rather than as an activator.

1.5.3 The study of *MDR1 cis*-element

The study of *MDR1 cis*-element is few. Lo in the laboratory used deletion constructs of the 1242 bp *MDR1* promoter and cloned in the expression vector YEP363 (2002). The constructs were further transformed to *S. cerevisiae* 2B strain. β-galactosidase liquid assay was performed to identify the –763 ~-725 region for further analysis by the site-directed mutagenesis (Appendix 4). When M12 (-736 ~-734) was deleted, the activity was decreased 5-fold compared with the wild type 1.2 kb promoter. There is a potential negative regulatory site in M12 and the data needed to be demonstrated in *C. albicans*.

1.6 *Saccharomyces cerevisiae* as model system to study *Candida*

C. albicans system is always difficult to study by the following reasons (Edwards, 1990):

1. Only diploid exist-- Most mutations are recessive and are difficult to study.
2. No known sexual cycle-- Lack of molecular tools for genetic analysis.
3. Transformation efficiency is low-- A natural plasmid similar to the *S. cerevisiae* 2 μ plasmid has not yet been found in *C. albicans* (De Backer *et al*., 2000), so *C. albicans* can not used as the recipient strain to perform library screening.

Saccharomyces cerevisiae is a genetically conformable yeast which is closely related to *C. albicans*. As a consequence, *S. cerevisiae* is the commonly used model yeast in fungal molecular research, including DNA sequence analysis, mechanism of action of and resistance to

antifungal drugs, and the investigation of factors of pathogenicity, such as adhesion. *S. cerevisiae* is easiler to study than *C. albicans*, because of the following features (Sherman, 1997) :

1. Like mammalian cells, it is eukaryotes.
2. It is unicellular (much simple).
3. It can be haploid and/or diploid (easy to control).
4. It has known genetic map (well to studied).
5. Its genome is stable.
6. It is suitable to do the gene recombination (multiple choice of plasmid and selection marker, transformation efficiency high>10^4, easily succeed in transformation).

Although *C. albicans* genes are usually expressed in *S. cerevisiae* without difficulty (>117 *S. cerevisiae* mutations have been functionally complemented by using *C. albicans* homologs), the converse is not normally true (Kirsch *et al.*, 1990). So the data must be further demonstrated in *C. albicans*.

1.7 *Renilla* luciferase as the reporter for studying *C. albicans*

Reporter genes which code for bioluminescent gene products, like the luciferases, have provided a very rapid method for analyzing the regulation of gene expression (Bronstein *et al.*, 1994) and a highly sensitive method for single-cell analysis (Wick, 1989). Recently, some studies used the firefly luciferase gene (*FLUC*) fused in frame with the phase-regulated *WH11* gene of *C. albicans* as a reporter to functionally characterize the 59 upstream regulatory regions of *WH11* (Srikantha *et al.*, 1995), but the analyses were restricted to Northern (RNA) blots because they were unable to identify a translation product of the firefly luciferase, either through enzyme activity or as a *FLUC*-related peptide in Western blots. The lack of a detectable translation product was most likely due to

a non-conventional codon strategy adopted by *C. albicans* and related species (Ohama *et al.*, 1993; Santos *et al.*, 1990; Santos *et al.*, 1993). Recently, it was demonstrated that the conventional leucine isoacceptor tRNA for CUG from *S. cerevisiae* is toxic to *C. albicans* (Leuker, 1994). Furthermore, direct determination of the amino acid sequences of peptides derived from three aspartyl proteinases of *C. albicans* confirmed the presence of serine instead of leucine at nucleotide positions containing the CUG codon (Wick, 1989). *FLUC* contains nine in-frame CUG codons, making it highly unlikely that a functional luciferase could be expressed in *C. albicans*.

The sea pansy *Renilla reniformis* also encodes a luciferase gene, *RLUC*, which contains no CUG codons in its ORF. Hence, it is suitable to be the luciferase reportor gene in *C. albicans*. It has been demonstrated that fusion of the *Renilla* luciferase ORF to the promoters of *C. albicans* can produce a functional protein in *C. albicans* and can be used to analyze the strength and developmental regulation of *C. albicans* promoters (Srikantha *et al.*, 1996). The bioluminescent reactions were listed in the below (Dual-Glo™ luciferase assay system technical manual, TM058 promega)：

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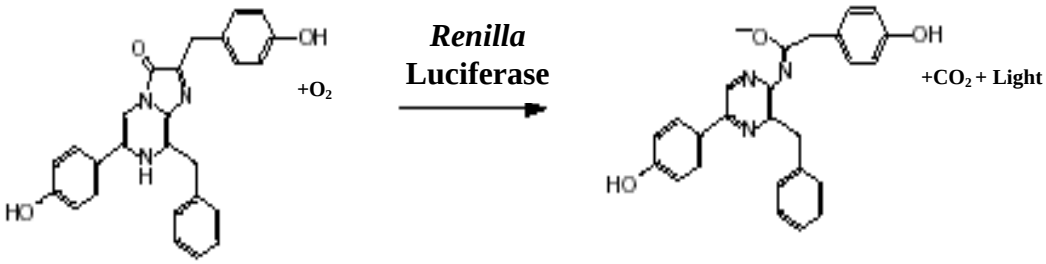

Coelenterazine **Coeleteramide**

2. Materials and Methods

2.1 Materials

2.1.1 Strains

2.1.1.1 *Escherichia coli*：DH5α , XL1-Blue

2.1.1.2 *Sacchromyces cerevisiae* ：

Strains, Genotypes, and Description	Source
2B：*MATα his3::hisG leu2::hisG ura3-52*	Fink laboratory collection
The deletion strains of *MDR1* promoters *MATα his3::hisG leu2::hisG ura3-52* (YEP363)	Yang laboratory collection (Lo, HL thesis, 2002)
YYY-1 ~YYY-153 *REP1*/pRS426; *REP2*/pRS426; pRS426 in deletion strains of *MDR1* promoters：*MATα his3::hisG leu2::hisG ura3-52* (YEP363, B2803)	this study
The clinical *MDR1* promoters /YEP363/2B *MATα his3::hisG leu2::hisG ura3-52* (YEP363)	this study

2.1.1.3 *Candida albicans*：

Strains, Genotypes, and Description	Source or Reference
CAI-4 : *ura3::1 imm434/ura3::1 imm434*	Fonzi *et al.*, 1993
JKC18：*ura::1 imm434/ura3::1 imm434 cph1:: hisG/cph1::*	Liu *et al.*,

hisG	1994
YLO-167 ~168；YLO-171 ~ 180 The mutations of *MDR1* promoters were integrated to CAI-4 : *ura3::1 imm434/ura3::1 imm434 MDR1p::RLUC*	this study
YLO-169 ~170；YLO-181 ~ 182 The mutations of *MDR1* promoters were integrated to JKC18 *ura::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG MDR1p::RLUC*	this study

2.1.2 Plasmids

Plasmid	Description	Source or Reference
pRS 426	*URA3* 2 μm plasmid	Sikorski *et al.*, 1989
YEP363	*LEU2* 2 μm plasmid	Myers *et al.*, 1986
LOB-60	pGEM-*URA3-RLUC-WH113*	Lo laboratory collection
***REP1*/pRS 426**	1.9 kb Sac I-Xho I fragment containing *REP1* in pRS 426	Lo laboratory collection
***REP2*/ pRS 426**	3.5 kb Sac I-Xho I fragment containing *REP2* in pRS 426	Lo laboratory collection
C1-9/YEP363	1.2 kb *Bam*H I-*Hin*d III fragment containing the clinical (1-9) *MDR1* promoters in YEP363	this study

LOB-70	*MDR1p*S5314-*RLUC* in LOB-60	this study
LOB-71	*MDR1p* with M12-*RLUC* in LOB-60	this study
LOB-72	*MDR1p* with 640-*RLUC* in LOB-60	this study
LOB-73	*MDR1p* with 617-*RLUC* in LOB-60	this study
LOB-74	*MDR1p* with 640/617-*RLUC* in LOB-60	this study
LOB-75	*MDR1p* with 259 -*RLUC* in LOB-60	this study

2.1.3 Primers (Merck)

2.1.3.1 The primers for cloning *MDR1* promoter

Name	sequence	location
HJL30	5’-AACCCAAGCTTGCATTGTGAAGTTCTATGT-3’	**+ 4 to -15**
HJL31	5’-CGCGGATCCGGCTTGCTAAACATTATCA-3’	**-1242 to -1224**
HJL 378	5'-GGATCCAGAGAATCGATAAAAG-3'	**-2690 to -2669**
HJL 379	5'-ATGTTTAAAGTATTTGATCGCCAC-3'	**-867 to -890**
HJL 380	5'-TACTTTAAACATTAGATTAGATACC-3'	**-878 to -854**
HJL 381	5'-AAGCCCCGGGTGTGAAGTTCTATG-3'	**+ 10 to -14**

2.1.3.2 The primers for sequencing *MDR1* promoter

Name	sequence	location
HJL 370	5’-CACCATGTTAATCATGTTTCCG-3’	**-1116 to –1137**
HJL 397	5’-ACCATCAGTCAACCCACC-3’	**-2156 to –2139**
HJL 398	5’-CACAGCCGTGAATCTTAG-3’	**-976 to –959**

2.1.3.3 The primers for PCR on *Candida* clones

Name	sequence	location
HJL 372	5’-ACCAGATTTGCCTGATTTGC –3’	***RLUC*: +264 to +245**
HJL 399	5’-AGAACCAATAGCCGTCTTCATC-3’	**-2779 to -2758**
HJL 453	5’-TCTTTAGCTTCTTCTGGATGGG-3’	**+ 94 to +74**

2.1.3.4 The primers for performing the site-directed mutagenesis on *MDR1* promoter

Name	sequence	location
HJL 405	5'-CAGTTCTTACAACCTTCA**T**GTAACCTTGCAATCCTGTC-3'	**-658 to -621**
HJL 406	5'-GACAGGATTGCAAGGTTAC**A**TGAAGGTTGTAAGAACTG-3'	**-621 to -658**
HJL 407	5'-AACCTTGCAATCCTGTCCCA**T**TATGCCAAATAAAAGAAAAAAGC-3'	**-637 to -594**
HJL 408	5'-GCTTTTTTCTTTTATTTGGCATA**A**TGGGACAGGATTGCAAGGTT-3'	**-595 to -637**
HJL 419	5’-GGGAAAAATACCGAGAATGA**A**ACAACCTAAGATTTTGCACTC-3’	**-279 to -238**
HJL 420	5’-GAGTGCAAAATCTTAGGTTGT**T**TCATTCTCGGTATTTTTCCC-3’	**-238 to -279**

2.1.4 chemical, enzyme and reagents

2.1.4.1 Difco :

Bacto Agar , LB Agar , Bacto-yeast extract , yeast nitrogen base w/o amino acid, LB broth, YPD broth, bactortryptone, Ampicillin

2.1.4.2 Invitrogen :

Agarose

2.1.4.3 Amersham Biosciences :

rTaq DNA polymerase , $MgCl_2$

2.1.4.4 Bio-Rad :

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

2.1.4.5 Sigma :

Ammonium acetate, Dithiothreitol (DTT), ethidium bromide, Glassbeads (425~600 μm), Lithium acetate (CH_3COOLi), Phenylmethylsulfonyl-fluoride (PMSF), $PolyethyleneGlycol_{3350}$ (PEG_{3350}), phenylmethylsulfonyl fluoride (PMSF), 4-NQO, ONPG (Orthonitropheny-β-D-galactoside), Uracil, Histidine, Uridine

2.1.4.6 Merck

Disodium ethylenediamine-tetraacetate (EDTA), Disodium hydrogen phosphate dihydrate (Na_2HPO_4), Ethanol, Dextrose, 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, dodecyl sulfate sodium sat (SDS), 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 dehydrate, Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydroxide (NaOH), Tris (hydroxymethyl) aminomethane hydrogen chloride (Tris-HCl), Triton X-100, QuickChange Site Directed Mutagenesis kit

2.1.4.7 NEB :

Blp I, *Cla* I, *Xma* I, CIP, Vent

2.1.4.8 GIBCO :

*Bam*H I, *Dra* I, *Hin*d III, T4-ligase

2.1.4.9 Promega :

ligase and ligase reagents, Dual-Glo™ luciferase assay system

2.1.5 buffers

2.1.5.1 TE buffer

100mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)

2.1.5.2 10X TBE buffer

1.0 M Tris, 0.9 M Boric acid, 0.01 M EDTA

2.1.5.3 10X TAE buffer

0.4 M Tris, 0.4 M glacial acetic acid, 0.01 M EDTA

2.1.5.4 10X PCR buffer

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

2.1.5.5 Breaking buffer

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

2.1.5.6 Z buffer

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

2.1.6 medium

2.1.6.1 LB (Luria-Bertni) broth

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

2.1.6.2 LB / ampicillin broth

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

2.1.6.3 LB / ampicillin agar

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

2.1.6.4 YPD / Uridine broth

2 % YPD broth, 1 % yeast extract, 2 % dextrose, 80 mg/l Uridine

2.1.6.5 YPD / Uridine agar

2 %YPD broth, 1 % yeast extract, 2 % dextrose, 2 % agar, 80 mg/l Uridine

2.1.6.6 SD broth

0.67 % Bacto-yeast nitrogen base w/o amino acid, 2 % dextrose

2.1.6.7 SD agar

0.67 % Bacto-yeast nitrogen base w/o amino acid, 2 % dextrose, 2% agar

2.1.6.8 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

2.1.6.9 SD / Uracil/ Histidine agar

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

2.1.6.10 SD/ Histidine broth

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

2.1.6.11 SD/ Histidine agar

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

2.1.7 equipments

Peltier Thermal Cycler (PTC-255)

Orbital shaking incubator

AlaImager 2000

Vortex-2, genie

Dry bath incubator

Brushless microcentrifuge

Power pac 300 (Bio-Rad)

Gene pulser II (Bio-Rad)

Spectra max plus

Stirrer / Hot plate corning

Hybrid shake and stack

Quick spin

Thelco laboratory incubator

Medical freezer

Speedy autoclave (vertical type)

Top COUNT ^{TM} Microplate Scintillatioon & Luminescence counter (PACKWARD)

2.2 Methods

2.2.1 Transformation of *REP1*, *REP2* into different *MDR1* promoter deletion strains

A single colony of the *MDR1* promoter deletion strains was inoculated in 3 ml of SD /－Uracil /－Histidine broth and grew overnight at 30℃ with vigorous shaking (~200 rpm). The overnight culture was transferred into 15 ml of SD /－Uracil /－Histidine broth and was adjusted to a concentration of OD_{600} = 0.3. The cultures were then incubated with shaking at 30℃, 200 rpm until the OD_{600} is between 0.6 and 0.8 (about 5-6 hours). The cells were pelleted by centrifugation at 3000 rpm for 5 minutes at room temperature and then resuspended in 4 ml of 1×LioAC/TE Buffer. The cells were then pelleted by centrifugation at 3000 rpm for 5 minutes at room temperature. The pellet was resuspended in 1 ml of 1×LioAC/TE Buffer and let stand for 10 minutes at room temperature. While waiting, salmon sperm DNA were boiled for 2 minutes and chilled on ice immediately. 4 μl of sperm DNA (10 μg/μl) and 0.5 μg (*REP1*, *REP2* or 2803 control vector) of target DNA were added to a new 1.5 ml eppendorf before 100 μl competent cell were transferred into the eppendorf. 700 μl of 1×LioAC/TE/40% PEG was added and mixed gently. The mixture was incubated at 30℃ on wheel for 30 minutes and then heat shocked at 42℃ for 15 minutes in water bath prior to chilling on ice immediately. The cells were pelleted for 5 minutes at 3000 rpm at room temperature. 1ml of 1×TE was added to each eppendorf and suspension. The cells were pelleted for 5 minutes at 3000 rpm at room temperature. 100 μl of supernatant was used to resuspend cells. Then the cells were plated on SD/－His selective plates and incubated at 30℃ for 3 days. Three colonies were picked from each plate

for the β-galactosidase liquid assay.

2.2.2 β-galactosidase liquid assay

The transformants were cultured in 3 ml of SD/+His broth and incubated for overnight at 30℃ with vigorous shaking (~200 rpm). The starter culture was diluted into 15 ml of SD/+His broth (in 50 ml *Corning* centrifuge tube) to OD_{600} = 0.2 and incubated for about OD_{600} = 0.8 at 30 ℃ with vigorous shaking. The yeast cells were harvested by centrifugation at 3000 rpm for 5 minutes at 4℃. The cell pellet was suspended with 5 ml of ddH_2O and transferred to 15 ml centrifuge tube. The yeast cells were harvested. The experiment can be arrested at this step by storing the cell pellet in -20℃. The pellet was resuspended in 250 μl of Breaking buffer with 12.5 μl of 40 mM PMSF and placed the tube on ice. The acid-washed glass beads were added to just below the liquid level, and the suspension was vortexed at maximum speed for 60 seconds and rest for 60 seconds at cold room (4℃). The vortex procedure was repeated 4 times, then 300 μl of Breaking buffer was added. The cell extract was centrifuged at 3000 rpm for 5 minutes at 4℃ after a 10 seconds mixing by vortexing. The supernatant was transferred to a new cold eppendorf and then centrifuged at 3000 rpm for 5 minutes at 4℃. The supernatant was transferred to a new cold eppendorf, for the following β-galactosidase assay and Bradford protein quantitative assay.

A total of 10 μl (x μl) of yeast extract was added to a 12 × 75 mm disposable glass tube containing 990 μl (1000-x μl) of cold Z buffer (with β̃-mercaptoethonol 2.7 ml/L). This mixture was pre-incubated in pre-equilibrated 28℃ water bath for 5 minutes. Then, 200 μl of ONPG (Orthonitropheny-β̃-D-galactoside, 4 mg/ml in Z buffer) as enzyme

substrate was added to start the reaction as time 0 minute. When a light yellow color has been developed, the reaction was stopped by adding 500 μl of 1M Na_2CO_3 and noted the time.

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), the OD_{595} of the supernatant was determined, by comparing to a standard curve in which 500 μg/ml of BSA (bovine serum albumin) in serial 2-fold dilution to a final concentration of 62.5 μg/ml was the protein standard.

The β-galactosidase activity units were calculated (moles of ONPG cleaved per minute per mg protein) with the following equation：

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

2.2.3 Cloning and expression of the *MDR1* promoters from clinical strains (see Fiigure 2-1)

The *MDR1* promoters were constructed by polymerase chain reaction (PCR) amplification with HJL 30 and 31 primers and genomic DNA of clinical strains as templates. The PCR was proceeded with *Taq* polymerase plus Vent (9 units：1 unit) and the PCR products were digested at the 5'-end with restriction enzyme *Bam*H I and the 3'-end with restriction enzyme *Hin*d III. The restricted fragment was then cloned into the *Bam*H I/*Hin*d III sites of the YEP363 vector. Then the constructed plasmids were transformed to the *S. cerevisiae* 2B strain. The β-galactosidase liquid assay was performed as described previously see section 2.2.2. The cloning procedure was detailed as following.

2.2.3.1. PCR reaction

Primers HJL 30 and 31 which contain the restriction enzyme sites were added to the PCR mixture. Genomic DNAs of nine clinical strains were the templates for the PCR. The reaction to synthesize the product was listed as indicated below：

5 μl of 10 × reaction buffer

X μl (5-50 ng) of dsDNA template

0.5 μl (50 μM) of forward primer (HJL 31)

0.5 μl (50 μM) of reverse primer (HJL 30)

0.5 μl (25 mM) of $MgCl_2$

0.5 μl (25 mM)) of dNTP mix

0.5 μl of *Taq* plus Vent (9units：1unit)

ddH2O to a final volume of 50 μl

The condition of PCR reactions were performed with the following program：

Segment	**Cycles**	**Temperature**	**Time**
1	1	94℃	5 minutes
2	30	94℃ (denature)	1 minute
		53℃ (annealing)	1 minute
		72℃ (extention)	90 seconds
3	1	72℃ (extention)	10 minutes

2.2.3.2. Ligation

The YEP363 vector and the PCR products of the interested fragments were digested with *Bam*H I and *Hin*d III. After purification, the vector was subjected to the CIP (calf intestine phosphotase) treatment. The purpose was to remove the phosphate group at the 5'end to avoid self-ligation. The detail of the treatment was listed as following.

10 μl of 10 × reaction buffer

50 μl of YEP363 vector

1 μl of CIP

39 μl of ddH2O (100 μl of final volume)

After treatment at 37℃ for 30 minutes, 1 μl of CIP was added and the mixture was treated for 37℃ for 30 minutes again. Then the sample was incubated at 65℃ for 20 minutes to stop the reaction.

The molar ratio of the vector and insert were about 1：3 in the ligation reaction. The amount of the PCR fragment used for insert was calculated with the following equation：

$$\frac{\text{ng vector} \times \text{kb insert}}{\text{kb size of vector}} \quad \text{molar ratio of} \quad \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The ligation reaction was incubated at 16℃ overnight and the contents were listed as indicated below：

1 μl of 10 × reaction buffer

X μl of YEP363 vector

X μl of insert

1U of T4 DNA ligase

ddH2O to a final volume of 10 μl

2.2.3.3. *E. coli* electroporation

Frozen cells were thawed on ice. 80 μl of cells were mixed with 2 μl of transforming DNA and the control (the vector treated with CIP) in a chilled 1.5 ml of microcentrifuge tube. The BioRAD gene Pulse was set 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 added to the bottom of the cuvette. The Outside surface of the cuvette was wiped with a tissue to remove moisture before, the cuvette was put in the slide. Then, the slide 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 to resuspended the cells. The cells were then transferred to the original 1.5 ml centrifuge tube and incubated at 37ºC for 1 hour with sharking. Cells were spun for 5 minutes at 3000 rpm, and the supernatant was poured off (retained approximately 100 μl of medium). After resuspension, the cells were plated on LB -ampicillin plates. The plates were incubated at 37℃ for at least 16 hours. The plasmid constructs from the transformants were checked by *Bam*H I and *Hin*d III and sequencing.

2.2.4 Construction of the *MDR1* promoters into the *Renilla* luciferase vector (see Figure 2-2)

The purpose of this procedure was to construct recombinant DNAs containing the promoter of *MDR1* either from the SC5314 wild type and the M12 (Lo, 2002) and the ORF of luciferase into vector, pGEM-*URA3*. The construct with wild type sequence was later used as the template to perform the site-directed mutagenesis (see section 2.2.5).

The 1.2 kb *MDR1* promoter and upstream fragment was cloned into the luciferase vector to create a recombinant luciferase gene under the control of *MDR1* promoter. The 2.7 kb fragment *MDR1* promoter and upstream fragment was divided into a 1.8 and 0.9 kb fragments to facilitate the cloning. First, I used the HJL378 (contained the *Cla* I site) and 379 (contained the *Dra* I site) primers to performed the PCR to obtain the 1.8 kb product (*MDR1* promoter from -2690 to -890). Then I used the HJL380 (contained the *Dra* I site) and HJL381 (contained the *Xma* I site) primers to performed the PCR to obtain the 0.9 kb product (*MDR1* promoter from -878 to +10). The 1.8 kb and 0.9 kb DNA fragments of the PCR products and the luciferase vector were restriction digested by *Cla* I and *Dra* I, *Dra* I and *Xma* I, and *Cla* I and *Xma* I respectively. After treating the vector by CIP, these two restriction fragments and vector are used for three-parts ligation. The successful constructs were assessed by restriction mapping with *Cla* I and *Xma* I.

2.2.5 Site-directed mutagenesis (Appendix 5)

2.2.5.1 Primer design

The mutagenic oligonucleotide primers for this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers :

1. Both the mutagenic primers must contain the desired mutation and are complementary to the same sequence on opposite strands of the plasmid.
2. Primers should be between 25 and 45 bases in length, and the melting temperature (Tm) of the primers should greater than or equal to 78 ℃.

The following formula is commonly used for estimating the Tm of primers :

$$Tm = 81.5 + 0.41 (\% GC) - 675/N$$

3. The desired mutation should be in the middle of the primer with ~10-15 bases of correct sequence on both sides.
4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.
5. Primers need not to be 5' phophorylated but must be purified by polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency.

In this study, HJL 405 and HJL406 primers were used to synthesize the –640 site mutant；HJL 407 and 408 primers were used to synthesize the –617 site mutant；HJL 406 and 407 primers were used to synthesize the –640/–617 site mutant；HJL 419 and 420 primers were used to synthesize the –259 site mutant.

2.2.5.2 PCR reaction

The reaction to synthesize the product which contained the desired mutation was listed below：

5 μl of 10 × reaction buffer

5-50 ng of dsDNA template

125 ng of oligonucleotide primer＃1

125 ng of oligonucleotide primer＃2

1 μl (125ng) of dNTP mix

ddH2O to a final volume of 50 μl

Then added

1 μl of P*fuTurbo* DNA polymerase (2.5 U/μl)

The condition of PCR reactions were performed with the following program：

Segment	Cycles	Temperature	Time
1	1	95℃	30 seconds
2	18	95℃ (denature)	30 seconds
		55℃ (annealing)	1 minute
		68℃ (extention)	20 minutes

The mutant at –617 site was hard to synthesize, so I used the "denatured DNA" method to treat the template DNA (detail in Molecular Cloning chapter 13:22). Besides, the program for the terminal cycle of the PCR was 94℃, 1 minute ; 55℃, 1 minute ; 72℃, 10 minutes.

2.2.5.3. Digesting procedure

The purpose of this step was to remove the original DNA and to make sure the interested DNA was transformed into *E. coli.* The *Dpn* I endonuclease (target sequence：5'-Gm^{6}ATC-3') is specific for methylated and hemimethylated DNA and was used to digest the parental DNA template and to select for mutation-containing synthesized DNA (Nelson *et al*., 1992). 1 μl of the *Dpn* I restriction enzyme (10 U/μl) was added to each amplification reaction and gently inverted several times. The reaction mixtures were incubated at 37℃ for 1 hour to digest the parental supercoiled dsDNA.

2.2.5.4. Transforming into XL1-Blue supercompetent cells

The XL1-Blue super-competent cells were thawed on ice. For each control and sample reaction to be transformed, 50 μl of the super-competent cells were needed. 1 μl of the *Dpn* I-teated DNA was transferred to 50 μl of the super-competent cells. The transformation

reactions were swirled to mix gently and then were incubated on ice for 30 minutes. The transformation reactions were heat pulsed for 45 seconds at 42℃ and then placed the reactions on ice for 2 minutes. 0.5 ml of LB broth was preheated to 42℃ and added to the reaction mixture. Then the transformation reactions were incubated at 37℃ for 1 hour with shaking at 225-250 rpm. The appropriate volume of each transformation reaction was plated on LB plate containing ampicillin at 37℃ overnight. The plasmids from the transformants were subjected to sequencing to confirm that the mutation-containing DNA was transformed successfully.

2.2.6 *Blp* I digestion and *C. albicans* transformation (see Figure 2-3)

The constructs were restriction digested into linear form and were transformed to *C. albicans*. The *Blp* I was the restriction enzyme used to linearlize the 2.7kb of *MDR1* promoters at the -1340 site.

An isolated single colony was picked and inoculated into 3 ml of YPD plus uridine (80 μg/ml) and incubated for about 18~20 hours. 270 μl (1:100 dilution) of overnight culture was transferred into 27 ml of YPD plus uridine (in 125-ml flask). The solution should be mixed well and 1 ml of the solution was used to determine whether the OD_{600} was about 0.1. The remaining 27 ml culture were incubated at 30℃ with shaking (about 200 rpm) for 6 hours until the OD_{600} was about 1.0- 0.7. The cells were pelleted in 50 ml centrifuge tubes at 3000 rpm at room temperature for 10 minutes. The cell pellets were first washed with 10 ml of sterile ddH_2O and then with 5 ml of sterile 1X TE buffer (pH 7.5). The cell pellets were then washed with 2 ml of sterile 1X LiOAc/TE buffer. Finally, the cells were suspended in 250 μl of 1X LiOAc/TE buffer and sit in room temperature for 10 minutes.

The *MDR1* promoter-*Renilla* luciferase genes (*MDR1p-RLUC*)

were linearlized by *Blp* I and used as templates for PCR. About 2~10 μg of template DNA (in volume ≤ 13 μl ddH2O) were mixed with 10 μl of 10 mg/ml Salmon Sperm DNA (the Salmon Sperm DNA has been boiled for 2 minutes with PCR machine and put it on ice right away). For the control test, equal volume of DNA (≤ 13 μl) were mixed with 10 μl of 10 mg/ml Salmon Sperm DNA in eppendorf tubes, then 100 μl aliquots of cell suspension was dispensed into the tube. After sitting in 30℃ for 30 minutes, 700 μl of 1X PEG/LiOAc/TE buffer was added to each tube. Cells and the buffer were mixed by inversion and incubated on wheel at slow speed at 30℃ for 16 hours. The cells were then heat shocked at 42 ℃ for 60 minutes and the tubes were chilled on ice right away for about 2 minutes. Cells were spun at 3000 rpm for 3 minutes and the supernatant was poured off. The pellets were washed with 1 ml of 1 X TE buffer (pH 7.5). Much of supernatant was poured off. The cells were suspended with the remaining buffer (about 100 μl) and spread on SD plates. The plates were incubated at 30℃ for 3~4 days.

2.2.7 Isolation of the genomic DNA

An isolated single colony was picked and inoculated into 6 ml of YPD broth in 15 ml centrifuge tube. Tubes were incubated on a shaker (~200 rpm) at 30℃ for 24 ~ 48 hours. Cells were spun at 3000 rpm for 10 minutes and the supernatant was discarded before the cell pellets were washed with 5 ml of ddH_2O. Cells were then spun at 3000 rpm for 10 minutes and the supernatant was discarded before the cell pellets were resuspended with 500 μl of breaking buffer by vortexing. Then the acid-washed glass beads (≒ 400 μl) and 500 μl phenol/choroform/isoamyl alcohol (25:24:1) were added to the tubes. The

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

1 ml (2.5 X volume) of cold 100% ethanol 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 for 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 3M ammonium acetate(pH 5.2) and 1ml of cold 100% ethanol were added and mixed by inversion. They were spun at 13000 rpm for 10 minutes at 4 ℃ and the supernatant was discarded carefully. 1 ml of cold 70% ethanol was added to wash the DNA pellet. The tubes were spun at 13000 rpm for 5 minutes at 4℃ and the supernatant was removed carefully. The DNA pellet was air dried for about 5 minutes. DNA was resuspended in 100~200 μl of dd H_2O (or TE buffer)

2.2.8 In vitro assay of *RLUC* activity

A single colony was cultured in 3 ml of SD broth and incubated for overnight at 30℃ with vigorous shaking (~200 rpm). The starter culture was diluted into 20 ml of SD broth from OD_{600}=0.2 and incubated until

OD_{600} researched 0.5-0.6 (about 4 hours) at 30℃ with vigorous shaking. The culture was separated into two 50 ml Corning centrifuge tube. One tube is exposed to 4-NQO (0.5 μg/ml, dissolved in DMSO) and another is exposed to the equal volume of DMSO. The culture was incubated for another 30 minutes with shaking 200 rpm (OD is about 0.7-0.8). The cells were harvested by centrifugation at 3000 rpm for 5 minutes at 4℃. The cell pellet was suspended with 5 ml of ddH_2O and transferred to 15 ml centrifuge tube. The cells were harvested by centrifugation. The experiment can be arrested at this step by stock the cell pellet in -20℃. The pellet was resuspended in 750 μl of *RLUC* buffer (TM055, Promega) and chilled on ice. The acid-washed glass beads were added to just below the liquid level. The suspension was vortexed at maximum speed for 60 seconds and rested for 60 seconds at cold room (4℃). The vortex procedure was repeated 4 times, then the cell extract was centrifuged at 3000 rpm for 5 minutes at 4℃. The supernatant was transferred to a new cold eppendorf, centrifuged at 3000 rpm for 5 minutes at 4℃. The supernatant was transferred to a new cold eppendorf for the processes of the luciferase activity assay and Bradford protein quantitative assay.

The Dual-Glo™ luciferase assay system was used to detect the luciferase activity. First, The Dual-Glo™ Stop＆Glo substrate was diluted 1：100 into an appropriate volume of Dual-Glo™ Stop＆Glo buffer to create the Dual-Glo™ Stop＆Glo reagent. First of cell, 100 μl of each cell extracts was kept in a well of 96-well plates on ice. Then 75μl of these cell extracts were transferred by a multi-channel pipette to black 96-well plates containing 75 μl of the reaction reagent in each well. The samples were mixed gently at room temperature. After waiting for 10 minutes at room temperature, the luminescence of the sample was measured.

3. Result

3.1 Sequences at –640, -617, and -259 of *MDR1* promoter may be potential regulatory sites.

Previously, researcher in the laboratory has used deletion analysis and mutagenesis method to demonstrate that the activity of *MDR1* promoter was decreased greatly when it contains a mutation at the M12 (–736 TGA to CCC) site in *S. cerevisiae* (Lo, 2002). However, the activity associated with M12 was not demonstrated in *C. albicans* in this study.

In order to identify for more information about potential regulatory sites of the *MDR1* promoters, the genomic DNAs of several different clinical strains were used as templates for cloning their *MDR1* promoters into expression vector YEP363 and in-frame with a *lacZ* ORF. The constructs were then transformed into the *S. cerevisiae* 2B strain to determine their β -galactosidase activities (Figure 2-1). The background values of *MDR1* expression of nine clinical strains were listed in Table 3.1. The β -galactosidase liquid assay showed that the relative activity of β-gal with *MDR1* promoters from the C3 and C8 strains increased remarkably (Figure 3-1). Further analysis of the sequences showed that C3 and C8 were completely identical. To determine whether there is sequence variation in the *MDR1* promoter that may be responsible for the higher level of β -galactosidase activity, I compared C3, C8, and several other clinical strains to find out major differences in the promoter sequences. There are differences at -872, -829, -640, -617, -259, -66 and –21 (Figure 3-3). Deletion analysis has showed that the promoter activity changed when the -644 ~ -563 region was removed (Figure 3-4), within which the –640 and –617 sites resided. In addition to the spontaneous variation, PCR technique was also applied to increase the

variation on the promoter sequence. C1 and C8 are used as templates for PCR-mediated mutagenesis procedure and the PCR products were cloned into the expression vector to be in frame with *lacZ*. Three clones from each template were picked randomly and named C1-3, C1-4, C1-5 and C8-2, C8-3, C8-4, respectively. I further transformed these constructs to *S. cerevisiae* 2B individually for β -galactosidase liquid assay. The levels of the relative activity can be separated into two groups. One was about 2-2.5-fold higher than that of the wild type (C1-4, C8, C8-2) and the other was similar to that of the wild type (C1, C1-3, C1-5, C8-3, C8-4) (Figure 3-2). The –259 site has an "A" in C1-4, C8, C8-2 (the higher activity) while there is a "C" for the rest. Interestingly, there is an important transcriptional binding site at the –259 site, a potential PRE site (pheromone response element). The PRE site (ATGA**A**ACA) in *S. cerevisiae* is the binding site for Ste12, whose homologue in *C. albicans* is Cph1 (Liu *et al*., 1994).

To understand whether the PRE site at –259 of *MDR1* promoter could interact with Ste12 or the homologue of Ste12 (Cph1), I put the 2 μ plasmid containing *STE12* and *CPH1* separately into *S. cerevisiae* containing C1-4 and C8-2, both of which contained the "perfect" PRE site on the -259 of *MDR1* promoter. The data shows that the activity of *MDR1* promoter could not be affected by overexpression either *STE12* or *CPH1* in *S. cerevisiae*. However, the activity of the –259 site was still subjected to be further studied in *C. albicans*.

By the above mentioned results, –640, -617, and –259 were considered to be located at potential regulatory sites in *MDR1* promoter.

3.2 Rep1, Rep2 decrease the activity of the *MDR1* promoter with mutation at the –644~-563 region in *S. cerevisiae.*

To further search for other *cis*-regulatory elements and to

understand whether there are cross regulation in the two known drug resistance molecular pumps, *MDR1* and *CDR1*, two *trans*-regulatory factors of *CDR1*, Rep1 and Rep2, obtained in the laboratory by screening a *Candida* library in *S. cerevisiae* (Shih, 2001) are transformed into those *MDR1* promoter deletion strains (Lo, 2002) in *S. cerevisiae*. Since the strains carry *MDR1* promoter-*lacZ* (*MDR1*p-*lacZ)* fusion, the expressions of the promoter mutants can be measured by the β-galactosidase liquid assay. The results are showed in Figure 3-4 and Figure 3-5. The strain containing *MDR1* promoter and pRS426 vector alone had three times higher activity compared to the strain with mutation at –644~ -563 region (Figure 3-4). Rep1 in wild type up-regulates the *MDR1* promoter strain. However, when the –644~-563 region of the promoter was removed, the up-regulation disappeared. The regulation of Rep2 in wild type was not very manifest, and Rep2 in –644~-563 region down-regulates the *MDR1* promoter strain. The data indicate the –644~-563 region of *MDR1* promoter has at least one potential regulatory site which regulated by *trans*-factors either directly or indirectly. There is no known *cis*-element for the Rep *trans*-factors of *C. albicans* at this region.

3.3 The *MDR1p-RLUC* with different mutants were constructed by site-directed mutagenesis.

Wild type and the *MDR1* promoter with M12, -640, -617, -640/ -617, and -259 mutants were fused in frame with *Renilla* luciferase vector (LOB-60). The 2.7 kb of *MDR1* promoter of wild type SC5314 and the *MDR1* promoter with M12 were constructed to LOB-60 by *Cla* I and *Xma* I (Figure 2-2). The sequence of wild type *MDR1* promoter was listed in Appendix 6. The wild type *MDR1p-RLUC* fusion construct was used as template for the site-directed mutagenesis. The mutations were the -640, -617, -640/ -617 double mutation, and -259 mutations. The -640 mutation

was an “A” to “T” change；the -617 mutant was a “C” to “T“ and the -259 mutant was a “C” to “T“. The -640/-617 mutants were performed by two primers with different mutants and the products contained both mutations. The forward primer contained –640 mutation and the reverse primer contained –617 mutation.

After performing the site-directed mutagenesis, the *MDR1p-RLUC* mutants were transformed to XL1-blue cells. About $>10^2$ transformants per plate was obtained. A successful clone which contained the interested mutant could be obtained by selecting four colonies in average. The wild type *MDR1p-RLUC* and the *MDR1p-RLUC* with different mutants were further transformed to *C. albicans* genome.

3.4 Construction of *MDR1* promoter integrated *Candida* strains.

MDR1 promoter with wild type and mutation sequences were fused in frame with the *Renilla* luciferase vector and transformed into *C. albicans* strain CAI-4 (*ura3/ura3*). In addition, the construct that contained the –259 mutant, which contained the potential PRE site was transformed to the *cph1/cph1* homozygous mutant strain (JKC18).

The constructs integration were assessed by PCR method to check for the correctness of the insertion location and whether the insertion is to one or both alleles. If the mutations were introduced correctly, then a 3-kb fragment would appear in the PCR. Several correct transformants were obtained (Figure 3-6). Then I selected these transformants to check whether the DNA has inserted into one or both alleles. If the homology recombination happened only to one allele, a 2.8-kb fragment would also appear in the PCR (Figure 3-7). Hence, the homology recombination performed correctly and three different correct transformants were obtained.

3.5 The activity of wild type *MDR1* promoter can be induced by 0.5 μg/ml of 4-NQO.

It has been reported that the expression of *MDR1* was induced by 10 μg/ml of 4-NQO for 30 minutes according the Northern blot data (Gupta *et al*., 1998). Hence, one reason that there is no significant difference in the different mutant promoter activities is simply due to lack of induction. The culture was grew from OD_{600} is about 0.2 until OD_{600} researched 0.5-0.6. The culture was then separated into two tubes. One tube was exposed to 10 μg/ml of 4-NQO (dissolved in DMSO) and the other one was exposed to the equal volume of DMSO. The promoter activity and the growth of cells were inhibited in the presence of 10 μg/ml of 4-NQO for 30 minutes. The data of several clones were represented in Table 3.2~Table 3.5.

The effect of 4-NQO at a serious concentrations was examined (Figure 3-8 and 3-9). The tested range of drug concentration was from 8 μg/ml and was diluted in 2-fold series till 0.5 μg/ml. When 4-NQO was added to mid-log-phase-grown cells at 0.5 μg/ml for 30 minutes before harvesting the cells, the growth condition was closed to the control (only DMSO treatment) in Figure 3-8. The activity of the wild type *MDR1* promoter was able to be induced approximately 25-fold in this experiment (Figure 3-9).

As the expected, The *MDR1* promoter can be induced by 4-NQO. In my study, the expression of the wild type *MDR1* promoter was induced about 20-fold by 0.5 μg/ml of 4-NQO for 30 minutes. The result is the average of five independent experiments (Figure 3-10).

3.6 The integrated *MDR1* promoter with mutations at -640, -617, -259 and M12 showed no difference in the activity comparing to that of the wild type in culture without drug.

Wild type and the *MDR1* promoter containing the different mutations were fused in frame with the *Renilla* luciferase vector and transformed into *C. albicans* strain CAI-4 (*ura3/ura3*). In addition, the construct that contained the –259 mutant, which contained the potential PRE site was transformed to the *cph1/cph1* homozygous mutant strain (JKC18).

The result in Figure 3-11 showed the numbers of relative activity. The activity of the wild type strain containing wild type *MDR1* promoter was defined as 1 to standardize that of other strains containing different mutations of the *MDR1* promoter. In CAI-4 strain, M12 showed an activity of 0.9 unit, -640 showed an activity of 0.85 unit, -617 showed an activity of 1.05 units, -640/–617 double mutants showed an activity of 1.01 units and PRE showed an activity of 1.17 units. In JKC18 strain, pre showed an activity of 1.51 units and PRE showed an activity of 2.02 units. All experimental data were the average of five independent experiments.

Hence, in *C. albicans*, there is no significant difference in the luciferase activity under control of those mutant promoters compared to that of the wild type (Figure 3-11).

3.7 The integrated *MDR1* promoter with different mutations showed different activities under the 4-NQO induction.

The activities of the wild type and the different mutant *MDR1* promoter were determined under the 4-NQO induction. The result showed the data of relative activity (Figure 3-12). The activity of the wild type strain (CAI-4) containing the wild type *MDR1* promoter was defined as 1 to standardize that of others strains which contained different

mutations. In CAI-4 strain, M12 showed an activity of 3.3 units, -640 showed an activity of 3.2 units, -617 showed an activity of 2.8 units, -640/–617 double mutants showed an activity of 4.6 units, and -259 showed an activity of 3.7 units. In JKC18 (*cph1/cph1*) strain, pre showed an activity of 9.2 units and PRE showed an activity of 11 units. All experimental data were the average of five independent experiments.

The integrated *MDR1* promoter with the M12 mutation in *C. albicans* showed the opposite result as to the expressing vector YEP363 containing *MDR1* promoter with M12 mutation in *S. cerevisiae*. Other integrated mutant *MDR1* promoters in CAI-4 strains showed similar results as to that in *S. cerevisiae*.

Surprisingly, mutations on CPH1 increase the activity of wild type *MDR1* promoter. The data suggests the Cph1 acts as a negative regulator of *MDR1* promoter (Figure 3-12).

4. Discussion

4.1 The study of *cis*-element of *MDR1* promoter.

Previously, Lo (2002) in the laboratory has used promoter deletion analysis in the *MDR1* promoter to narrowed down regions affecting the β-gal activities of the *MDR1p-lacZ* fusion gene in *S. cerevisiae*. He has further performed three-nucleotide scanning mutagenesis to search for potential *cis*-acting regulatory elements. M12 (-736 ~-734) site was identified by this method (Appendix 4). However, the experiment was performed in *S. cerevasiae*. Hence, his results require confirmation in *C. albicans.* In recent studies, the deletion analysis method was popular for the study of *cis*-acting regulatory elements (Puri N, 1999；Lachke, 2003；Karnani, 2004).

In this study, I used another approach to study the *cis*-acting regulatory elements of *MDR1* promoter. Several clinical strains with variations in the sequences of their *MDR1* promoters were obtained and their DNA sequences were used for comparison and also as materials for PCR-mediated variations to search for more potential *cis*-acting sites. The functions of those sites were studied in *C. albicans* and this successful example provides another approach to study the *cis*-acting regulatory elements of the *MDR1* promoter.

The study of *cis*-acting regulatory elements of *MDR1* promoter is important. First, it can be a model system and knowledge obtained from the research can be applied to others drug resistance genes. Second, if others *trans*-acting regulatory factors were identified in the future, the potential *cis*-elements obtained may be the targets. Third, the regulatory site of *cis*-element can provide a direction for the development of drug targets.

4.2 The integrated *MDR1* promoter with mutations showed no difference in the activity compared to that of the wild type in culture without drug.

S. cerevisiae is often used as a model to study *C. albicans*. S. *cerevisiae* is simple, convenient and easy to manipulate (Sherman, 1997). *C. albicans* systems have many limitation for using it to study, including the low transformation efficiency, no known sexual cycle (Edwards, 1990), and no known stable plasmid. There are many successful examples using *S. cerevisiae* to study *C. albicans* . The isolation of the *MDR1* gene is an example that the gene of *C. albicans* screened with genomic *C. albicans* libraries which resistant to benomyl or methotrexate in *S. cerevisiae* (Fling *et al*., 1991). On the other hand, although the *S. cerevisiae* and the *C. albicans* are similar, there are still differences between them. *C. albicans* is a major fungal pathogen, but *S. cerevisiae* is not. Other differences include the codon usage, growth condition, cellular morphology, and regulation of genes. *Candida* research performed in *S. cerevisiae* must further be demonstrated in *C. albicans*.

I used the *Renilla reniformis* luciferase as the reporter system. The luciferase can be detected at low levels of expression and was suitable for the purpose of integration study in which only one or two copies of genes are integrated and present in the genome. There are others advantage, such as there is no *C. albicans* homologue and no CUG codons (Srikantha *et al*., 1996). However, the data in *C. albicans* showed no significant difference between these *MDR1* promoter mutants and wild type (Figure 3-11). I speculate that the reasons are as the following：1. The screening procedure was in *S. cerevisiae*, and they may not have the same regulation on *MDR1*. For example, the condition for inducing *MDR1* promoter activities may be different. 2. The original screening used the 2 μ vector (YEP363) high copy number plasmid, but the

lucifrease activity assay was in *C. albicans* with only one integrated copy. The 2 μ vector (YEP363) high copy number plasmid might have amplified the difference to a level that can be detected.

To understand which reason is true, I tested the activity of these mutants under the condition with drug induction.

4.3 The activity of *MDR1* promoter can be induced by 0.5 μg/ml of 4-NQO.

MDR1 appears to be the sole mediator of 4-NQO resistance (Wirsching *et al*., 2000). The expression of *MDR1*, or of its alleles, in *C. albicans* cells was enhanced by 10μg/ml of 4-NQO for 30 minutes (Gupta *et al*., 1998). But the growth and the total protein were inhibited by 10 μg/ml of 4-NQO in this study (Table 3.2-3.5). In the paper of Gupta *et al* (1998), there are several differences between these strains. The tested strain was the *C. albicans* ATCC 10261 strain and the cells grew in YPD broth and were collected for Northern blot analysis. In this study, the CAI-4 stain was used for the luciferase assay and the cells grew in SD broth. As it is shown in Figure 3-8 and 3-9 that 4-NQO of 0.5 μg/ml concentration was optimal to induce the *MDR1* promoter of CAI-4 strain growing in SD broth.

4.4 The integrated *MDR1* promoter with different mutations showed the different phenotype under the 4-NQO induction.

The clinical *C. albicans* isolates of which *MDR1* gene is overexpression are often from patients under long-term drug treatment. The regulation of *MDR1* may be involved in drug induction. Hence, many promoter analysis studies are under drug induction (Puri, 1999). In this study, the integrated *MDR1* promoter with different mutations

showed different activities when induced with the 4-NQO (Figure 3-12). In CAI-4 strain, M12 showed an activity of 3.3 unit, -640 showed an activity of 3.2 unit, -617 showed an activity of 2.8 unit, -640/–617 double mutants showed an activity of 4.6 unit, and -259 showed an activity of 3.7 unit. In JKC18 (*cph1/cph1*) strain, pre showed an activity of 9.2 unit and PRE showed an activity of 11 unit. However, the standard deviations of the experimential data were very large. To determine whether the differences were real, statistic methods were applied. The statistic method used in analyzing this data is one sample T test with one-sided-hypothesis. The analysis data was listed in Table 4.1. The luciferase activities of different integrated *MDR1p-RLUC* mutants were demonstrated to be at least 1.5-fold higher than that of wild type *MDR1p-RLUC* with the P value < 0.05.

The integrated *MDR1* promoter with M12 mutant showeds different results between the *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, the expressing vector YEP363 containing *MDR1* promoter with M12 mutation showed only 25% activity when compared to the 1.2 kb wild type promoter (Lo, 2002). However, the M12 mutation increased the activity of the *MDR1* promoter at least 1.5-fold in *C. albicans* (Table 4.1). This kind of difference between *S. cerevisiae* and *C. albicans* has been reported in the past. For example：the overexpression of *FCR1* in the *S. cerevisiae pdr1*/*pdr3* strain led to an increased expression of the *PDR5* ABC transporter, suggesting that Fcr1p could act as a positive regulator in *S. cerevisiae.* In contrast, the deletion of the *FCR1* alleles in *C. albicans* led to an increased resistance to fluconazole, suggesting that Fcr1p could act as a negative regulator in *C. albicans* (Talibi *et al*., 1999).

The integrated *MDR1* promoter with either -640 or -617 mutants was demonstrated to be at least 1.5-2-fold higher than that of the wild type promoter (Table 4.1). The result of -640/-617 double mutant was

predicted to show a >2-fold increase in activity when compared to that of the wild type strain. However, the result of -640/-617 double mutant has only showed an activity 2-fold higher than that of the wild type, suggesting -640/-617 mutant is under the same regulation.

4.5 The Cph1 acts as a negative regulator of the *MDR1* promoter.

There is a potential PRE site in the –259 of *MDR1* promoter and this site has been demonstrated to bind Cph1 in vitro (Malathi, 1994). The mutation of PRE (TGA**A**ACG to ACT**T**ACG) reduced gradually expression of the reporter (Madhani *et al.*, 1997). In this study, I changed the sequence of the –259 site (which contained the unperfect site of PRE in the study of Madhani *et al.*, 1997) to become perfect match and the promoter activities increased at least 2-fold than that of wild type. The activity of the integrated *MDR1* wild type promoter in *cph1/cph1* double mutant strain was demonstrated to be at least 5.5- fold increased than that of the wild type in CAI-4 (Table 4.1). This data suggest the *CPH1* acts as a negative regulator of *MDR1* promoter. On the other hand, the integrated *MDR1* promoter with either PRE or pre site in *cph1* double mutant strain showed similar expression levels (Figure 3-12 and Table 4.1), suggesting that *CPH1* regulates the *MDR1* promoter indirectly. The regulation model of *CPH1* is illustrated in Figure 4-1.

5. Future work

5.1 More investigation on the *MDR1* promoter activities.

The different integrated mutant *MDR1* promoters have demonstrated differences in their activities (Figure 3-12). However, the standard deviation was very large, and the investigation will require more carefully performed experiments.

5.2 Using Southern blot to assess these integrated constructs.

These integrated constructs which contained the different mutations in *MDR1* promoter were integrated to the correct site and only inserted one allele according to the PCR outcomes (Figure 3-6 and 3-7). But the PCR results could not tell whether the constructs have inserted two or more copies to the same allele. The Southern blot analysis will be needed to determine the copy number.

5.3 Application of the agar dilution or Etest to determine susceptibility of *MDR1*-related drugs in *cph1/cph1*.

In *cph1/cph1*, the activity of *MDR1* promoter was increased gradually than that in wild type CAI-4 strain (Figure 3-12 and Table 4.1). The data suggest that Cph1 is a negative regulator to *MDR1* in this study. These data must be demonstrated by other experiments, like agar dilution or Etest. Because the *MDR1* overexpression has resulted in the increase of resistance, the *cph1/cph1* strain can be predicted to become more resistant, which can determined with agar dilution or Etest. The drug for the agar dilution or Etest shall be *MDR1*-related, like fluconazole, 4-NQO, etc.

5.4 Indentification of the Cph1 potential regulatory site in *MDR1* promoter.

If the 5.2 experiment is confirmed, then it will be useful for finding the Cph1 regulatory site in *MDR1* promoter. The deletion constructs of *MDR1* promoter (Lo, 2002) can be transformed to *cph1/cph1* strain and the β-galactosidase assay will be proceeded. The activity of *cph1/cph1* strain containing different length of *MDR1* promoter will also be useful to screen the regulatory sites of Cph1 in *MDR1* promoter.

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Table 3.1 The drug susceptibility of nine clinical strains.

	Strain number	F80/24	F50/48	MIC (μg/ml)
S (susceptible)	1	0.125	0.125	$\leq$ 8
	2	0.125	0.125	
	3	2048	4	
	4	8	4	
SDD (susceptible-dose dependent)	5	16	16	8 -32
	6	16	16	
R (resistantant)	7	1024	64	> 64
	8	256	256	
	9	2048	512	
		MDR1 overexpression		

*F80/24 indicates that the MIC 80 is determined after treatment with fluconazole for 24 hours.
*F50/48 indicates that the MIC 50 is determined after treatment with fluconazole for 48 hours.
*MIC means the minimal concentration of inhibition.

Table 3.2. The cell growths of different mutation strains in the presence or absence of 4-NQO

strain	No drug	4-NQO
WT	0.816	0.712
M640	0.783	0.692
M617	0.779	0.683
M640/617	0.775	0.669

The cultures were grew from OD_{600}=0.2 until OD_{600} researched 0.5-0.6. The culture was then separated into two tubes. One tube was exposed to 4-NQO (which was dissolved in DMSO, 10 μg/ml) and another was exposed to DMSO. The culture was incubated for another 30 minutes with shaking at 200 rpm (OD_{600} is about 0.7-0.8) before the cells were collected. The numbers were determined by the optical density at 600 nm (OD_{600}). WT represents the SC5314 wild type *MDR1* promoter integrated to CAI-4. M640 represents the *MDR1* promoter with –640 mutation integrated to CAI-4. M640/617, double mutation on CAI-4.

Table 3.3 The luminescent activity in different strains in the presence or absence of 4-NQO

strain	No drug	4-NQO
WT	1610	299
M640	2392	279
M617	2610	299
M640/617	2197	407

The numbers represent the luminescent activity of *Renilla* luciferase. The cells were collected as in Table 3.2. WT represents the SC5314 wild type *MDR1* promoter integrated to CAI-4. M640 represents the *MDR1* promoter with –640 mutation integrated to CAI-4. M640/617 is the double mutant.

Table 3.4 The total luciferase protein in different strains

strain	No drug (μg/ml)	4-NQO (μg/ml)
WT	156	53
M640	165	35
M617	142	31
M640/617	189	58

The numbers represent the total protein of *Renilla* luciferase. The cells were collected as in Table 3.2. WT represents the SC5314 wild type *MDR1* promoter integrated to CAI-4. M640 represents the *MDR1* promoter with –640 mutation integrated to CAI-4. M640/617 is the double mutant.

Table 3.5 The specific activity was calculated.

strain	No drug	4-NQO
WT-3	619	342
M640-3	546	273
M617-1	1101	573
M640/617-1	696	420

The numbers represent the specific activity (luminescent activity/ total protein) of *Renilla* luciferase. The cells were collected as in Table 3.2. WT represents the SC5314 wild type *MDR1* promoter integrated to CAI-4. M640 represents the *MDR1* promoter with –640 mutation integrated to CAI-4. M640/617 is the double mutant.

Table 4.1 The luciferase activity of integrated *MDR1p-RLUC* of different mutants

Strain	Relative activity (WT was defined as 1 unit)	P value
M12/ CAI-4	1.5	0.039
M640/ CAI-4	2	0.024
M617/ CAI-4	1.5	0.031
M640/ 617/ CAI-4	2	0.02
PRE/ CAI-4	2	0.043
pre/ JKC18	5.5	0.035
PRE/ JKC18	5.5	0.05

Table 4.1 The luciferase activity of integrated *MDR1p-RLUC* of different mutants. The one sample T test with one-sided-hypothesis is used for the calculation of the P value. M12, the mutation of *MDR1* promoter is at –736 ~-734, M640 represents the *MDR1* promoter with –640 mutation integrated to CAI-4. M640/617 is the double mutant. PRE represents the *MDR1* promoter with "perfect" PRE site at the –259. pre represents mutation of PRE (ATGAAACA to ATGACACA) on the –259 of *MDR1* promoter with PRE site at the –259. CAI-4 and JKC18 are the strains in which the mutation constructs integrated.

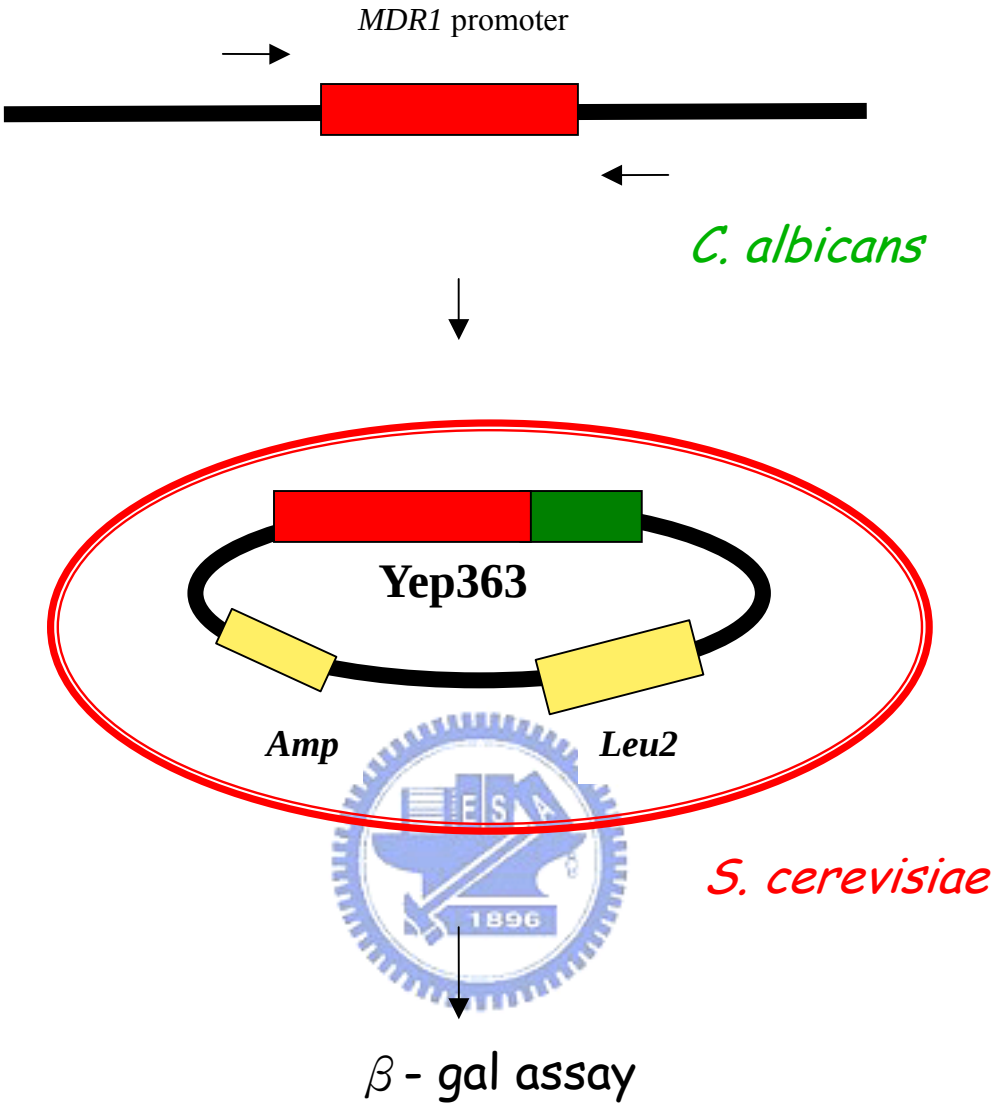


Figure 2-1 Cloning and expression of the *MDR1* promoters from clinical strains

The red block was represented the *MDR1* promoter and the green block was represented the *lacZ* gene. The genomic DNAs of several different clinical strains were used as templates for cloning their *MDR1* promoters into expression vector YEP363 and in-frame with a *lacZ* ORF. The constructs were then transformed into the *S. cerevisiae* 2B strain to determine their β -galactosidase activities.

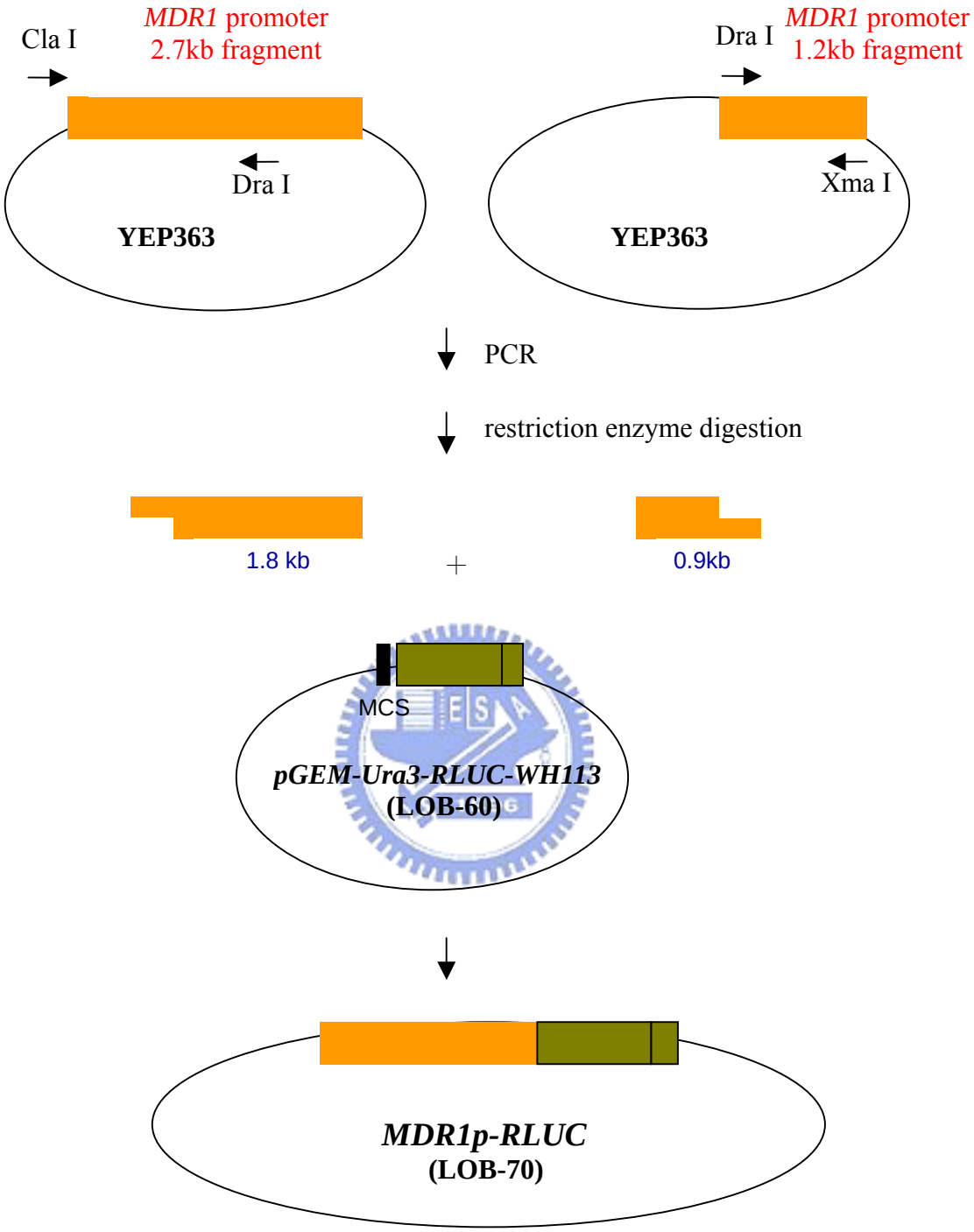


Figure 2-2. Construction of the *MDR1* promoters into the *RLUC* gene vector

The orange blocks represent *MDR1* promoter and green blocks represent the *RLUC* sequence plus *WH113* (terminator).

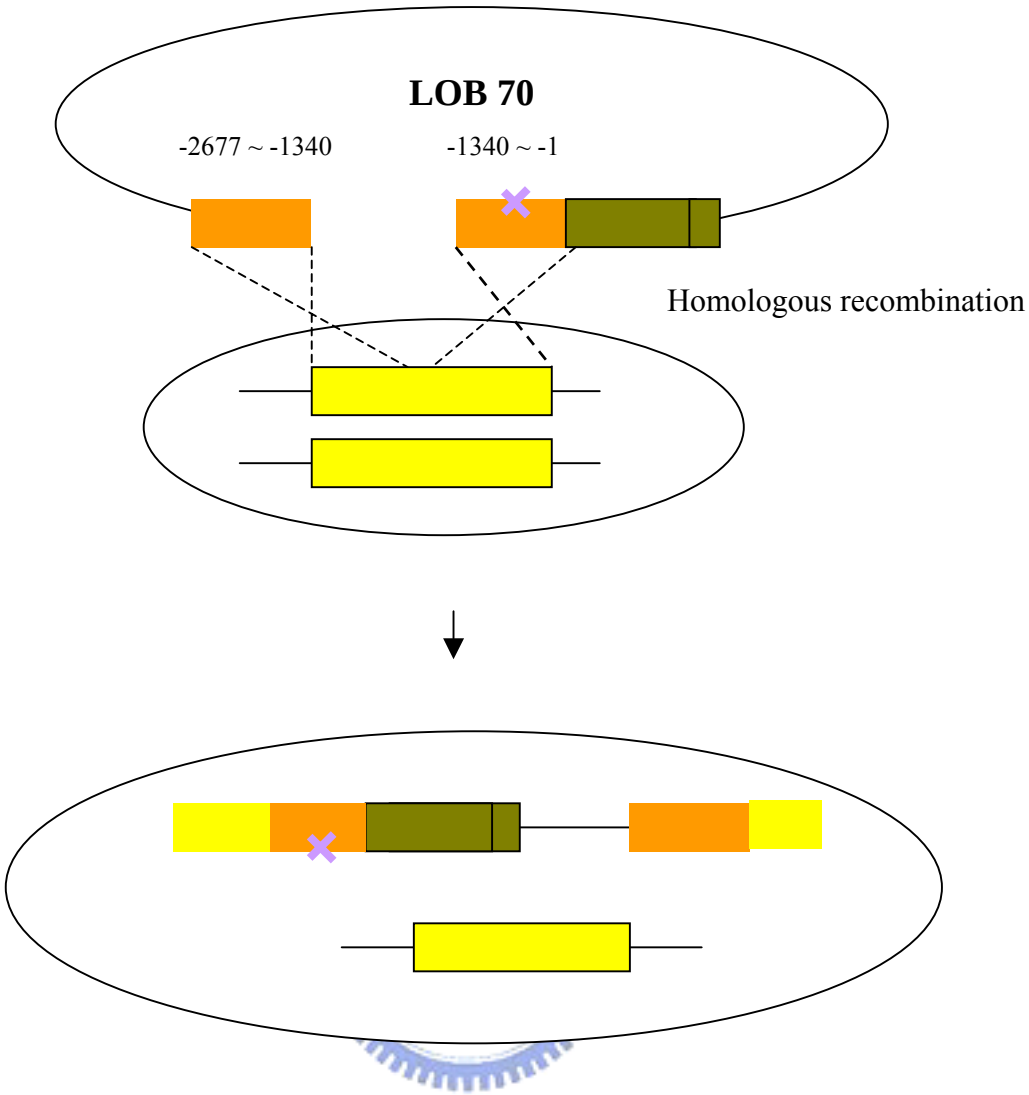


Figure 2-3. The integration of the *MDR1p-RLUC* construct into *C. albicans* genome with homologous recombination.
The orange blocks represent the *MDR1* promoter with different mutants (purple cross). The green blocks represent the *Rellina* luciferase sequence plus *WH113*. The yellow blocks represent the wild type *MDR1* promoter in *C. albicans* genome. The LOB 70 constructs were restriction digested into linear form by *Blp* I (at –1340 site) and the sites of two parts of *MDR1* promoters are –2677 to –1340 and –1340 to –1. Then the linear form of LOB 70 constructs were transformed to *C. albicans*. In *C. albicans*, the linear form DNA which contained the *MDR1* promoters with different mutants were inserted to the wild type *MDR1* promoter loci by homologous recombination. The *MDR1* promoters with different mutants were determined by luciferase assay.

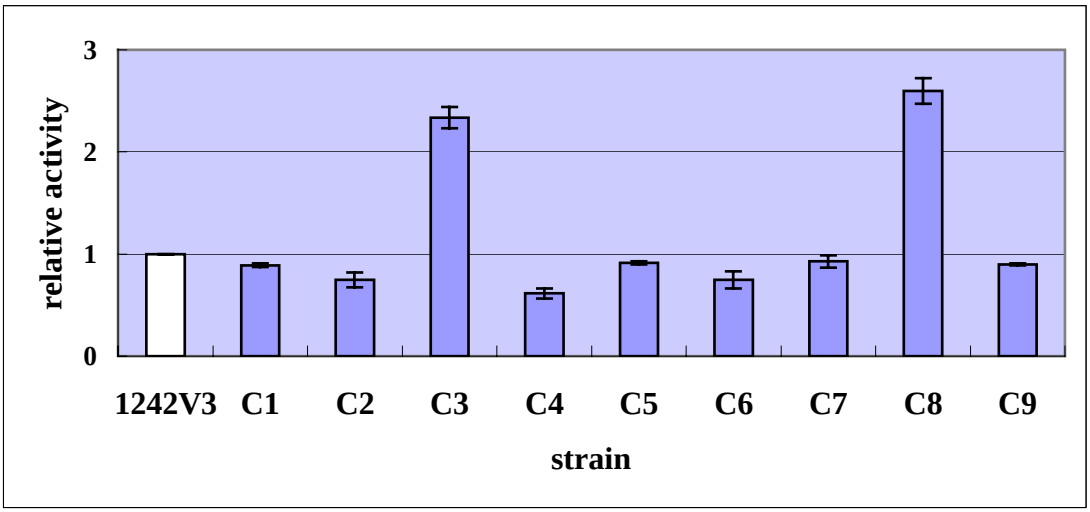


Figure 3-1. The relative β -galactosidase **activity of clinical *MDR1* promoters in 2B.**

The activity of the 1242V3 is defined as 1 unit to standardize the data of other strains.1242V3 carries the 1.2 kb wild type of *MDR1* promoter of *C. albicans* SC5314. C1 through C9 represent the numbers of clinical strains of which the backgrounds were listed in Table 1.

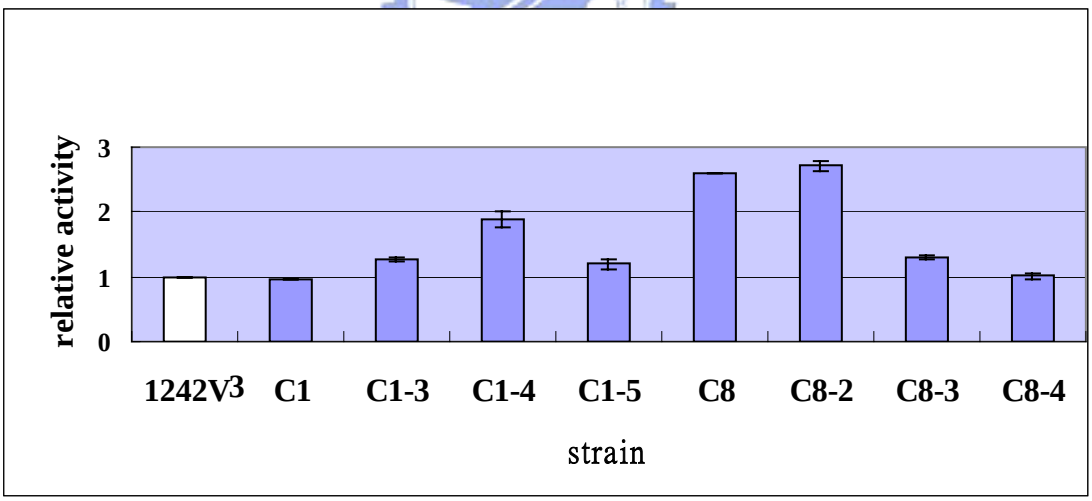
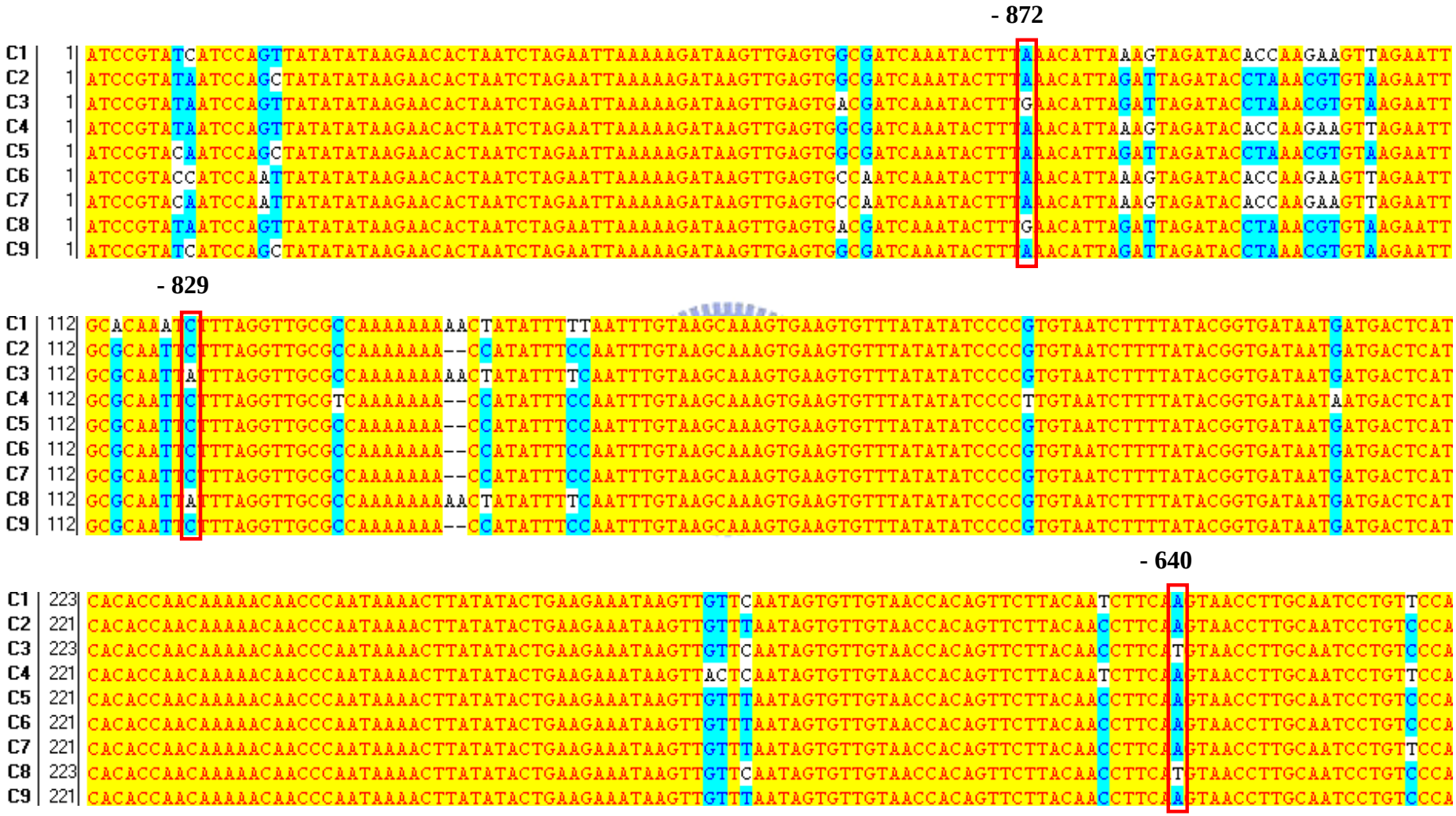
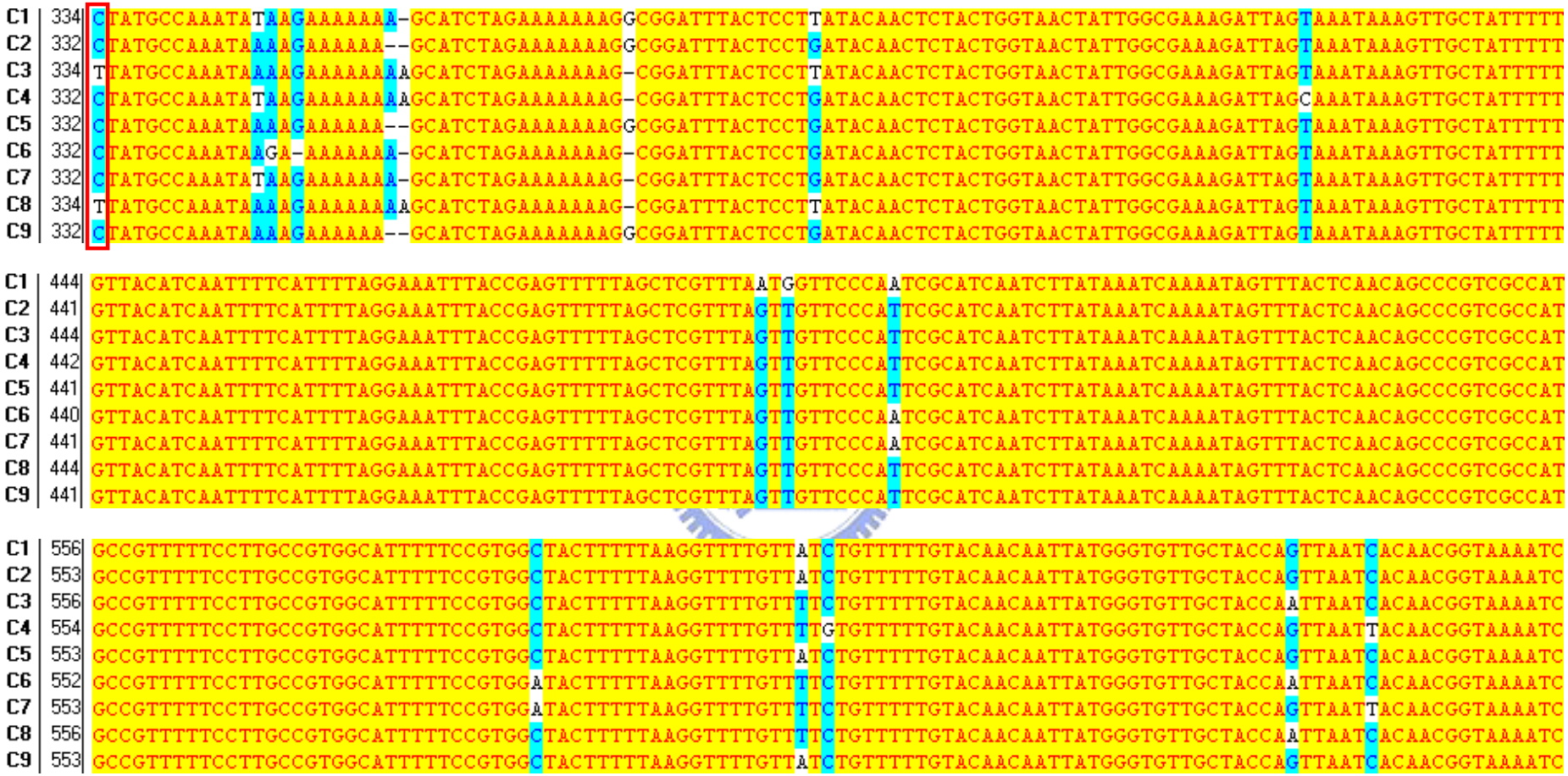


Figure 3-2. The relative β -galactosidase **activity of various *MDR1* promoters of clinical 1 and 8 strains.**

The activity of 1242V3 is defined as 1 unit to standardize that of other strains.1242V3 carries the 1.2 kb wild type of *MDR1* promoter of *C. albicans* SC5314. 3 different sequences caused by PCR-mediated variations from C1 and C8 were chosen for this study and were named C1-3, C1-4, C1-5 (from C1); C8-2, C8-3, C8-4 (fromC8).


- 872
- 829
- 640

- 617


C1 334 CTATGCCAAATATAAGAAAAAAA-GCATCTAGAAAAAAAGGCGGATTTACTCCTTATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C2 332 CTATGCCAAATAAAAGAAAAAA--GCATCTAGAAAAAAAGGCGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C3 334 TTATGCCAAATAAAAGAAAAAAAAGCATCTAGAAAAAAAG-CGGATTTACTCCTTATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C4 332 CTATGCCAAATATAAGAAAAAAAAGCATCTAGAAAAAAAG-CGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGCAAATAAAGTTGCTATTTTT
C5 332 CTATGCCAAATAAAAGAAAAAA--GCATCTAGAAAAAAAGGCGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C6 332 CTATGCCAAATAAGA-AAAAAAA-GCATCTAGAAAAAAAG-CGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C7 332 CTATGCCAAATATAAGAAAAAAA-GCATCTAGAAAAAAAG-CGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C8 334 TTATGCCAAATAAAAGAAAAAAAAGCATCTAGAAAAAAAG-CGGATTTACTCCTTATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C9 332 CTATGCCAAATAAAAGAAAAAA--GCATCTAGAAAAAAAGGCGGATTTACTCCTGATACAACTCTACTGGTAACTATTGGCGAAAGATTAGTAAATAAAGTTGCTATTTTT
C1 444 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAATGGTTCCCAATCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C2 441 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C3 444 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C4 442 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C5 441 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C6 440 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCAATCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C7 441 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCAATCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C8 444 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C9 441 GTTACATCAATTTTCATTTTAGGAAATTTACCGAGTTTTTAGCTCGTTTAGTTGTTCCCATTCGCATCAATCTTATAAATCAAAATAGTTTACTCAACAGCCCGTCGCCAT
C1 556 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTATCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATCACAACGGTAAAATC
C2 553 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTATCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATCACAACGGTAAAATC
C3 556 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTTTCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAATTAATCACAACGGTAAAATC
C4 554 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTTTGTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATTACAACGGTAAAATC
C5 553 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTATCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATCACAACGGTAAAATC
C6 552 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGATACTTTTTAAGGTTTTGTTTTCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAATTAATCACAACGGTAAAATC
C7 553 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGATACTTTTTAAGGTTTTGTTTTCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATTACAACGGTAAAATC
C8 556 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTTTCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAATTAATCACAACGGTAAAATC
C9 553 GCCGTTTTTCCTTGCCGTGGCATTTTTCCGTGGCTACTTTTTAAGGTTTTGTTATCTGTTTTTGTACAACAATTATGGGTGTTGCTACCAGTTAATCACAACGGTAAAATC

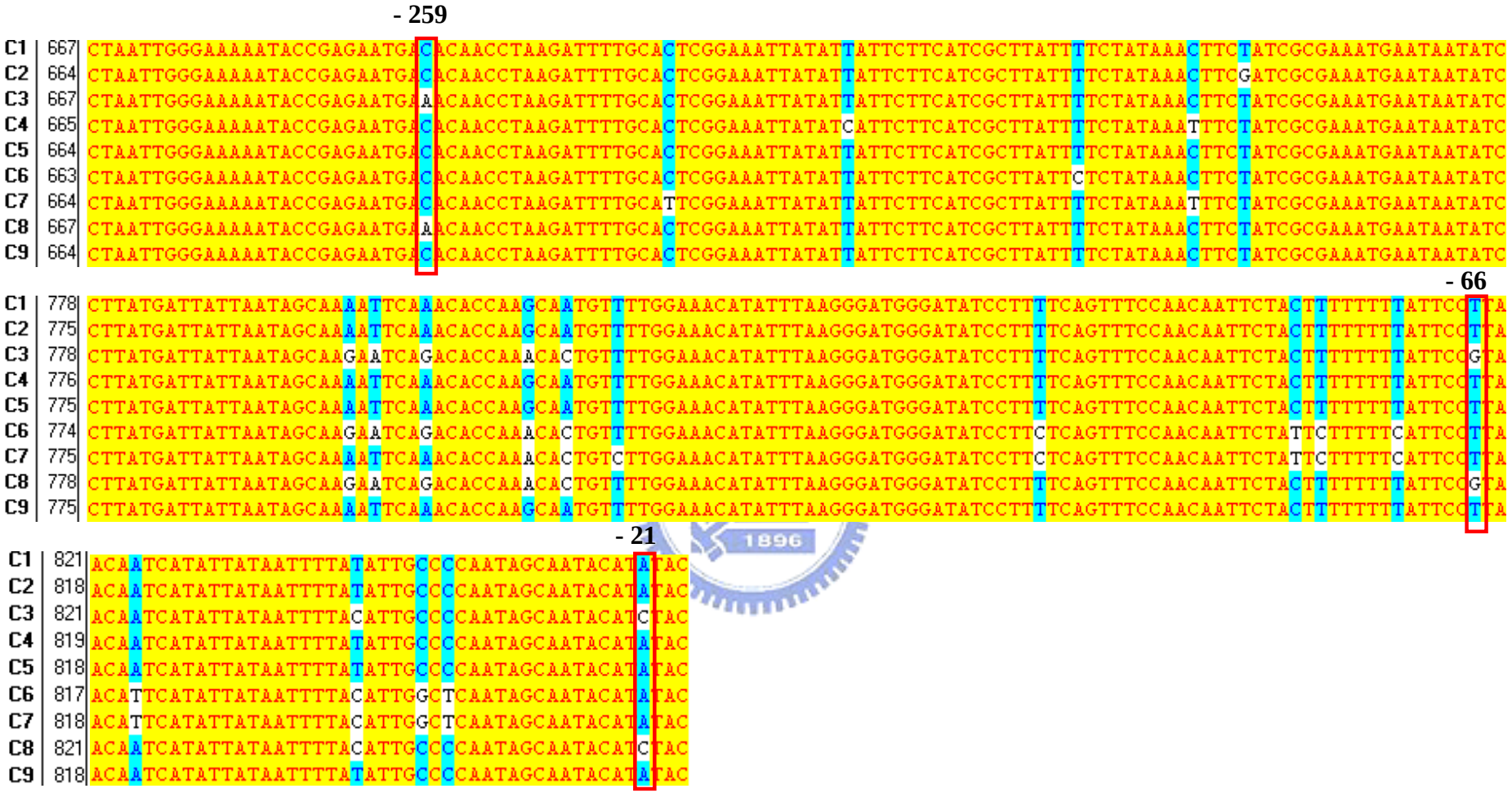


Figure 3-3 Alignment of nucleotide sequences of *MDR1* promoter (-948 ~ -18) from clinical strains C1 to C9.
The red blocks are the sites of which the sequences from C3 and C8 are different from other strains.

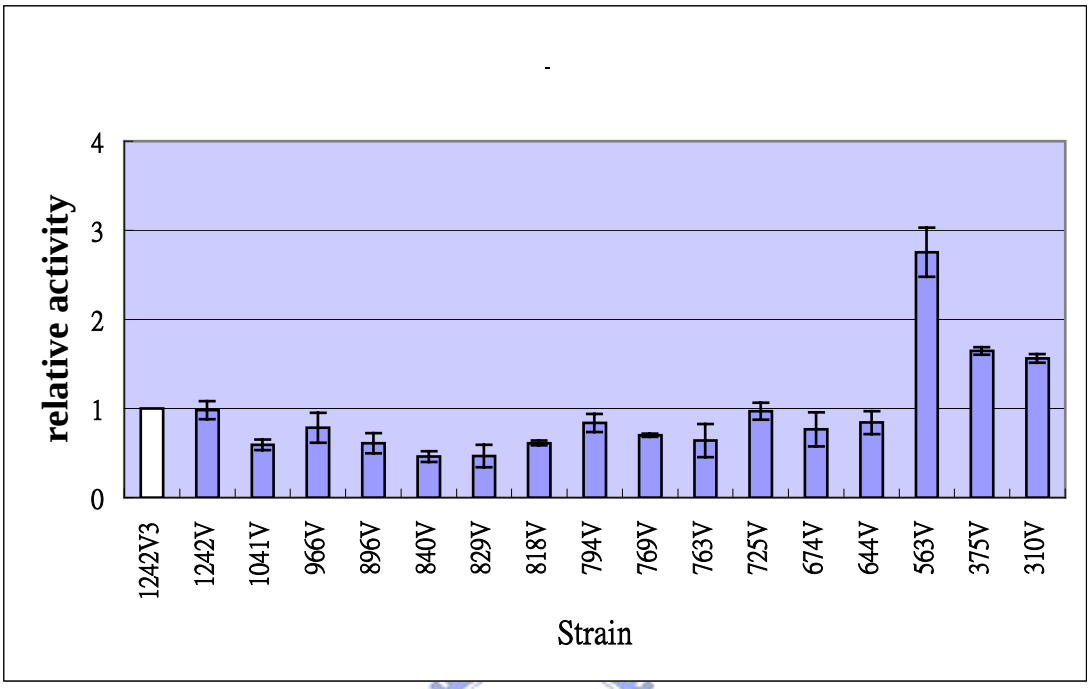


Figure 3-4. The deletion analysis of *MDR1* promoter.
The β -galactosidase activity from each promoter is shown in relative percentage using 1242V3 as 1. The numbers in the names of the strains represent the length of the *MDR1* promoter in the recombinant construct carried within the cells. 1242V3 carries the 1.2 kb wild type of *MDR1* promoter of *C. albicans* SC5314. 1041V carries the 1.0 kb promoter fragment, and 996V carries the 0.99 kb promoter fragment etc. The A of the initiation codon ATG is +1.

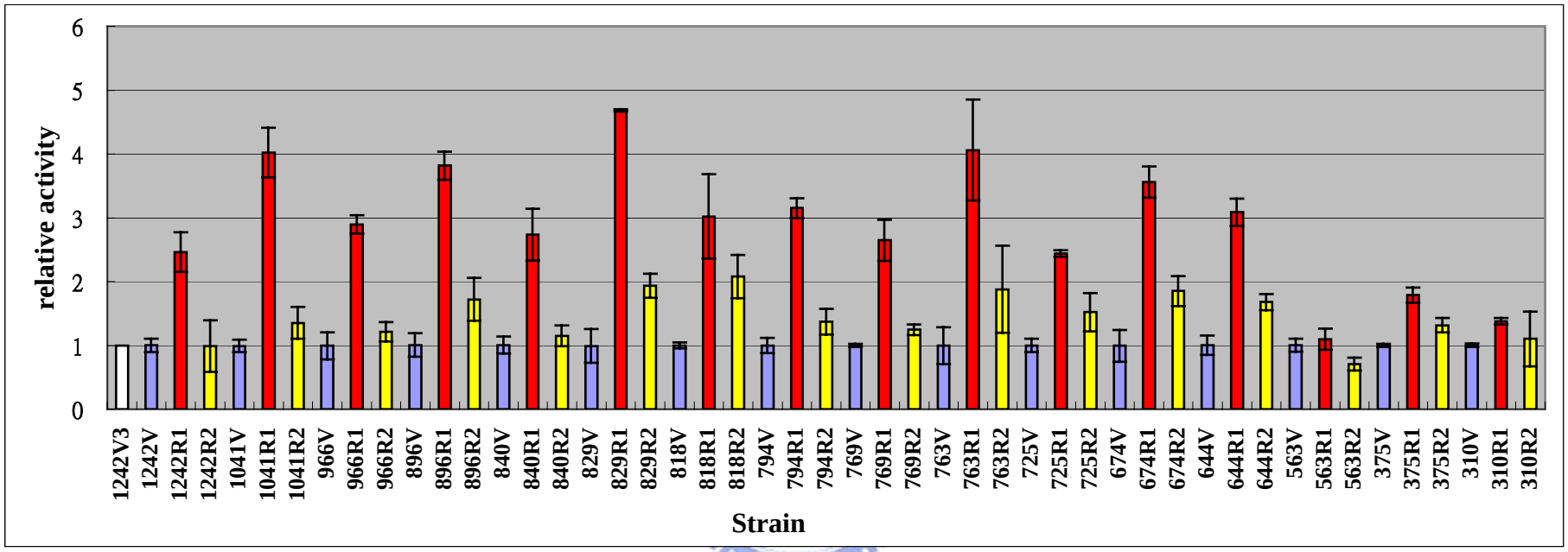


Figure 3-5. Relative activities of Rep1/pRS 426, Rep2/pRS 426 and pRS 426 in the deletion strains of *MDR1* promoter.

R1 represents the Rep1/pRS 426; R2 represents the Rep2/pRS 426; V represents the pRS 426 vector alone. The blue bars represent the pRS 426 vector alone in *MDR1* promoter deletion strains. The red bars represent the Rep1/pRS 426 in *MDR1* promoter deletion strains. The yellow bars represent the Rep2/pRS 426 in *MDR1* promoter deletion strains. 1242V3 is the strain containing the YEP363 plasmid with wild type 1.2 kb SC5314*MDR1* promoter and the pRS 426 vector. 1041R1 is the strain containing the YEP363 plasmid with wild type 1.0 kb SC5314*MDR1* promoter and the Rep1/pRS 426. etc. The 1242V3 is defined as 1 unit to standardize other strains.

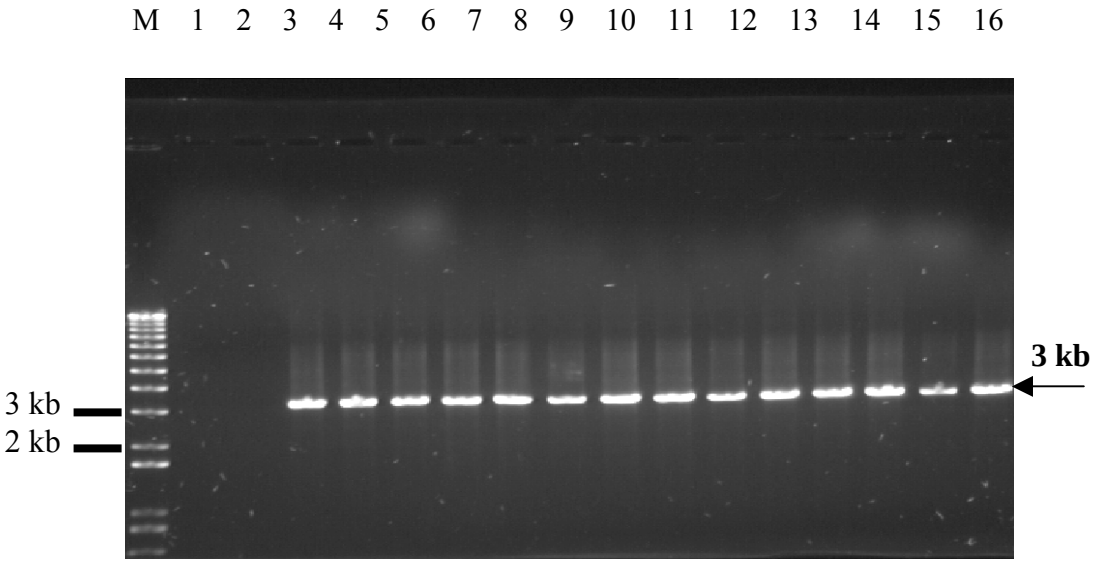
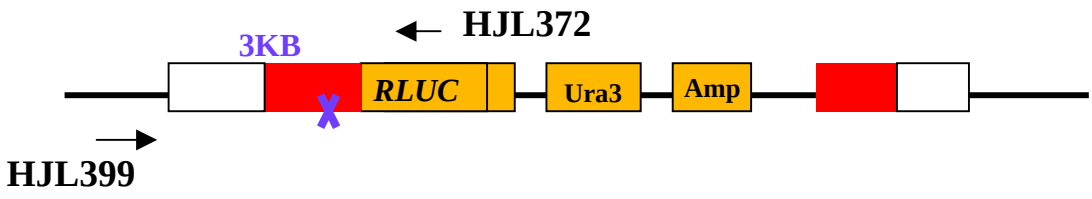


Figure 3-6. PCR method to determine whether the clones were integrated to the correct site.
Lane 1 and Lane 2 represent the negative control of CAI-4 and JKC18. Lane3 to Lane 16 were PCR products from experimental samples. If the *MDR1p-RLUC* fragments were located at correct position, a 3 kb fragment would be produced when using primers HJL 399 and HJL 372 in the PCR (below). M; DNA size marker, the size of the marker fragments are labeled by the side.



HJL 399 : *MDR1* promoter -2779 ~ -2758
HJL 372 : *RLUC* +264 ~ +245

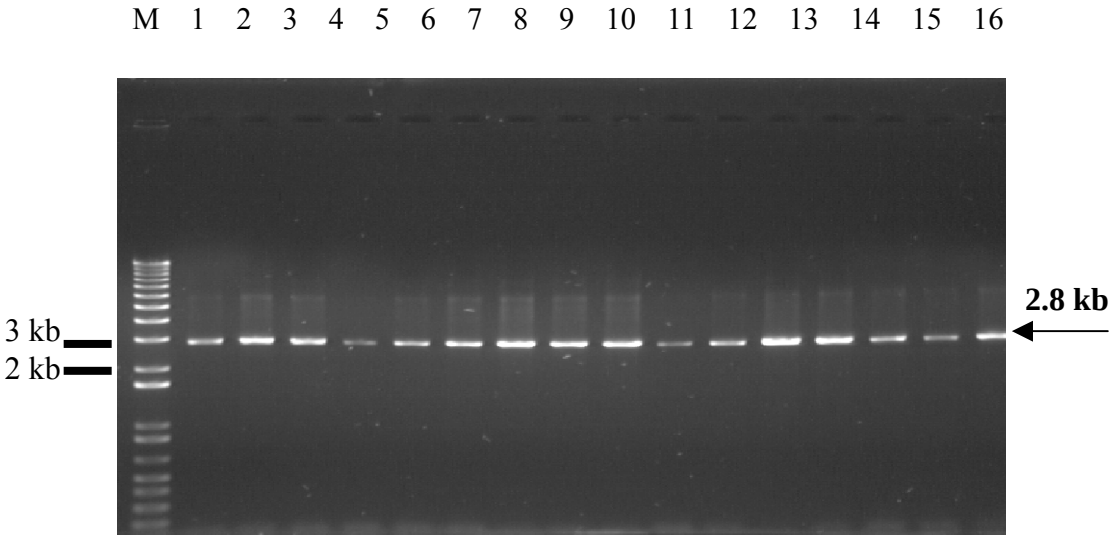
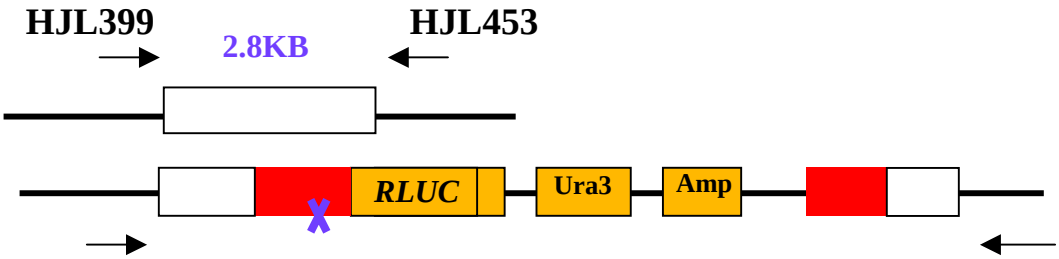


Figure 3-7. PCR method to determine whether the clones were integrated to one allele in the chromosome of *C. albicans*.
Lane 1 and Lane 2 represent the positive control of CAI-4 and JKC18. Lane3 to Lane 16 were PCR products of experimental samples. If the DNA was integrated only to one allele, a 2.8 kb product would be produced when using primers HJL 399 and HJL 453 in the PCR (below).



HJL 399 : *MDR1* promoter -2779 ~ -2758
HJL 453 : *MDR1* promoter + 94 ~ +74

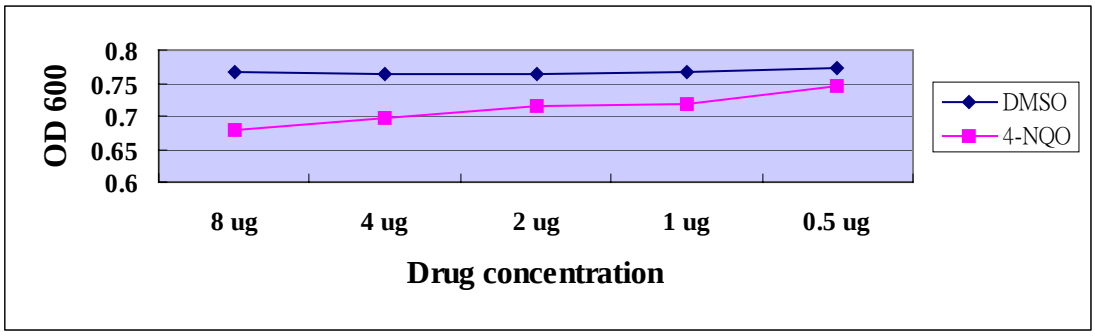


Figure 3-8. The growth curve of wild type *MDR1* promoter under different concentrations of 4-NQO.
The wild type *MDR1p-RLUC* was integrated to *Candida* genome. The range of drug concentration are as indicated below the panel. The purple points indicate cells treated by 4-NQO dissolved in DMSO. The blue points indicate cells only treated by the DMSO.

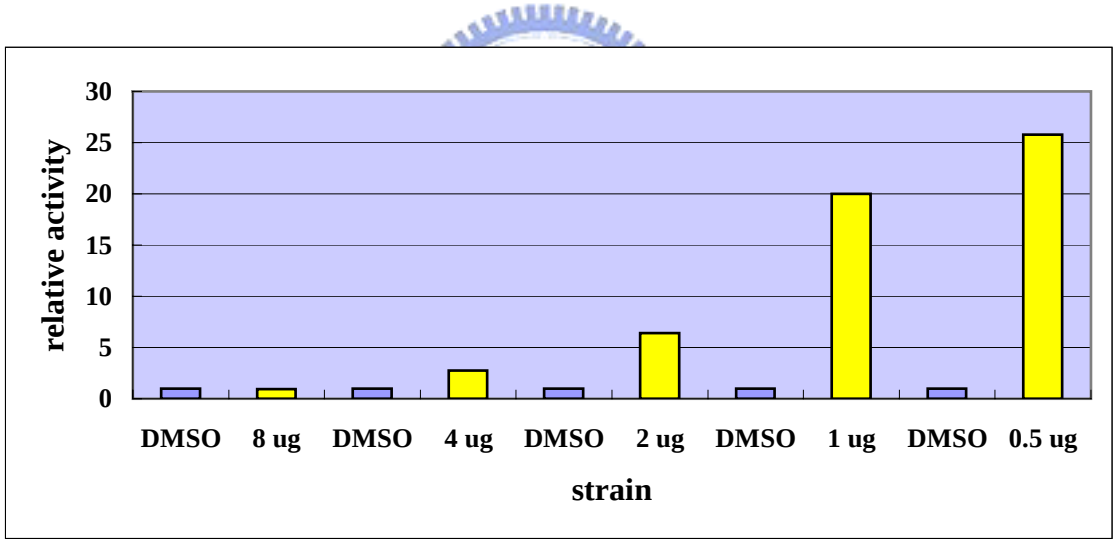


Figure 3-9. The *Renilla* luciferase relative activity of wild type *MDR1* promoter under different concentrations of 4-NQO.
The wild type *MDR1p-RLUC* was integrated to *Candida* genome. The range of drug concentration are as indicated below the panel. The yellow bars represent the activity from cells treated by 4-NQO dissolved in DMSO. The blue bars represent activity from cells only treated by the DMSO. The activity from the DMSO control is defined as 1 unit to standardize other strains which were treated with 4-NQO in DMSO.

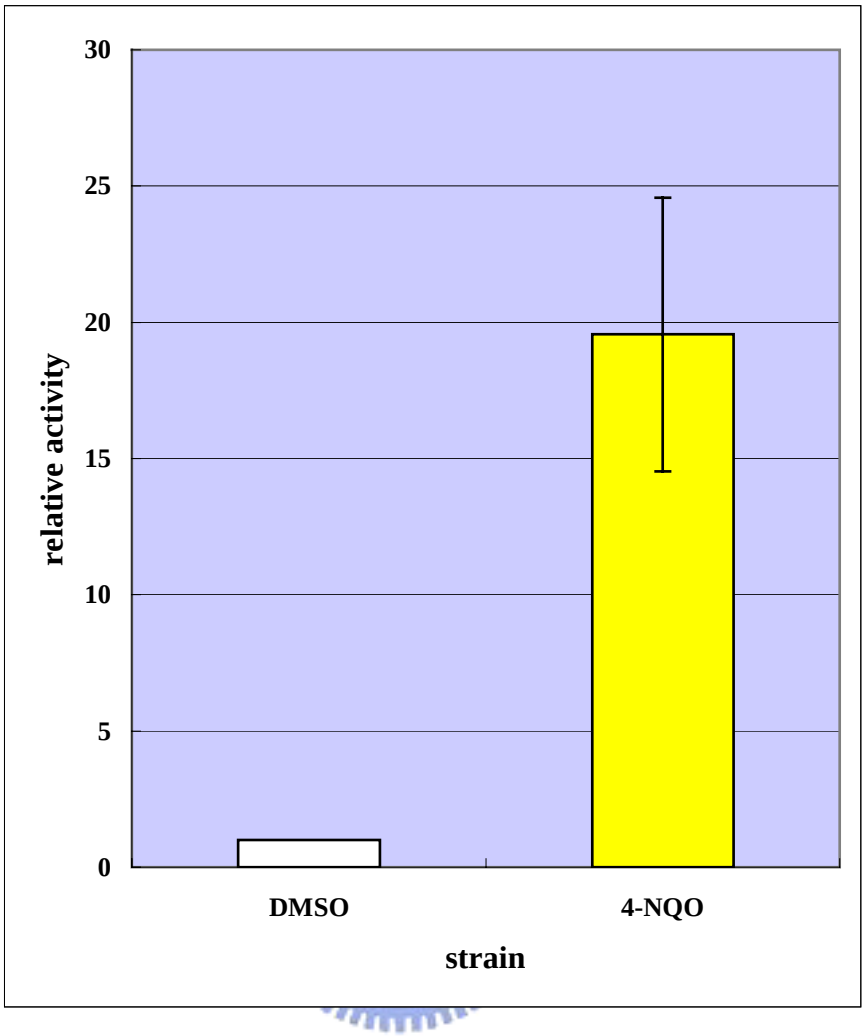


Figure 3-10. The *MDR1* promoter-luciferase activity can be induced by 4-NQO.

The wild type *MDR1p-RLUC* was integrated to *Candida* genome. The yellow bars represent activity from cells treated by 4-NQO dissolved in DMSO. The white bars represent activity from cells only treated by the DMSO. The activity of DMSO control is defined as 1 unit to standardize the other strains.

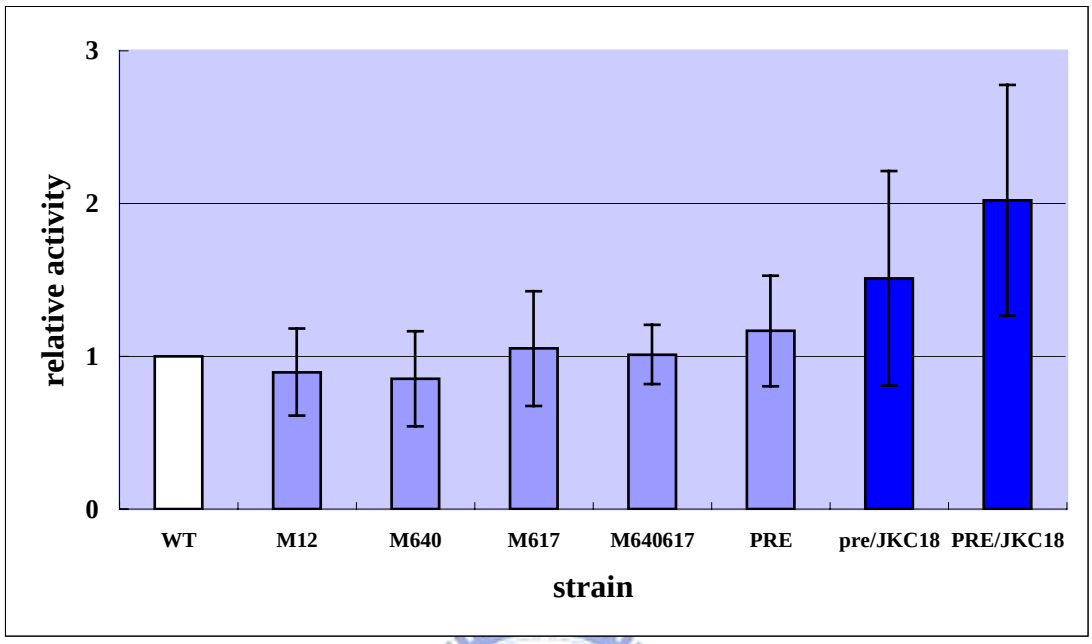


Figure 3-11. The basal relative activity of the integrated *MDR1* promoter-luciferase with different mutations.
WT represents the wild type *MDR1p-RLUC* which was integrated to *Candida* genome. WT is defined as 1 unit to standardize other strains. The numbers indicate the mutation sites on the *MDR1* promoter. M12 represents the *MDR1p-RLUC* with M12 (-736~-734) mutant; M640, the *MDR1p-RLUC* with -640 mutant. etc. PRE represents the *MDR1p-RLUC* with "perfect" PRE site. The dark blue bars are in JKC18 (*cph1/cph1* double mutant) strains and white and light blue bars are in CAI-4 strain.

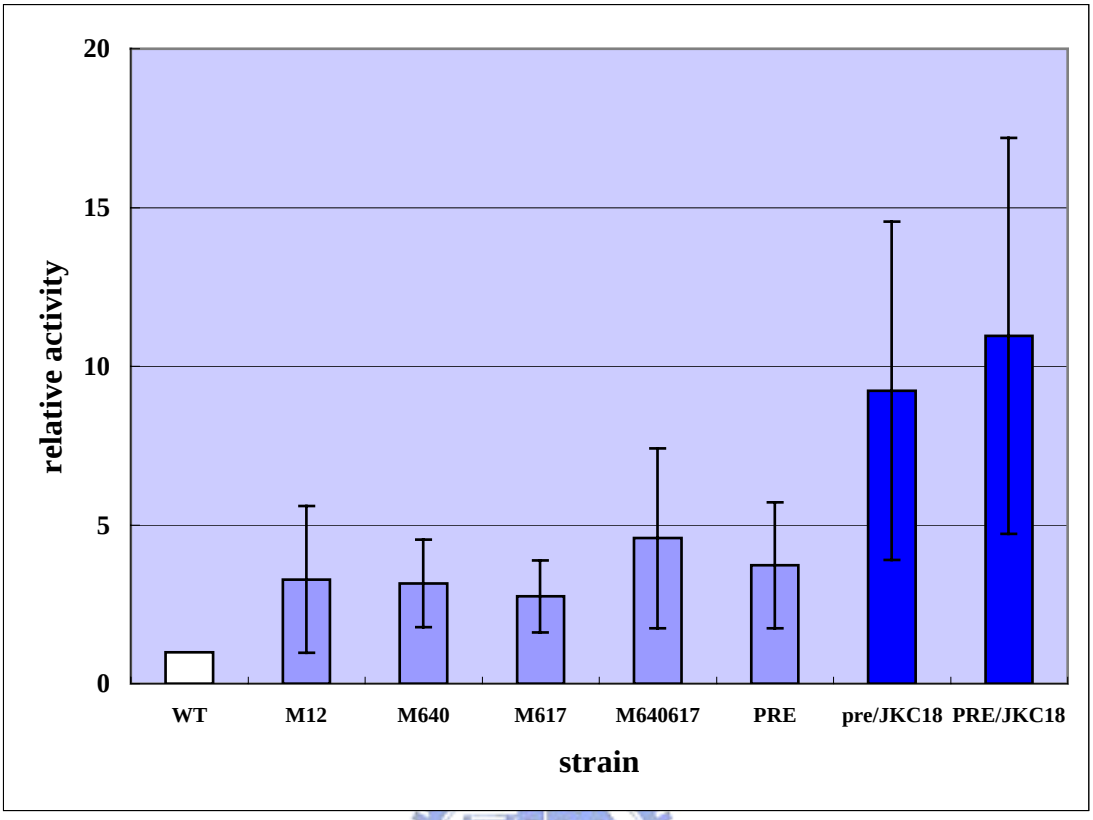


Figure 3-12. The relative luciferase activity of the integrated *MDR1* promoter with different mutations under induction of 4-NQO at 0.5 μg.

WT represents the wild type *MDR1p-RLUC* which was integrated to *Candida* genome. WT is defined as 1 unit to standardize other strains. The numbers indicate the mutation sites on *MDR1* promoter. The M12 represents the *MDR1p-RLUC* with M12 mutations. The M640 represents the *MDR1p-RLUC* with -640 mutation. etc. PRE represents the *MDR1p-RLUC* with "perfect" PRE site. The dark blue bars are in JKC18 (*cph1/cph1* double mutant) strain, others are in CAI-4 strain (white and light blue).

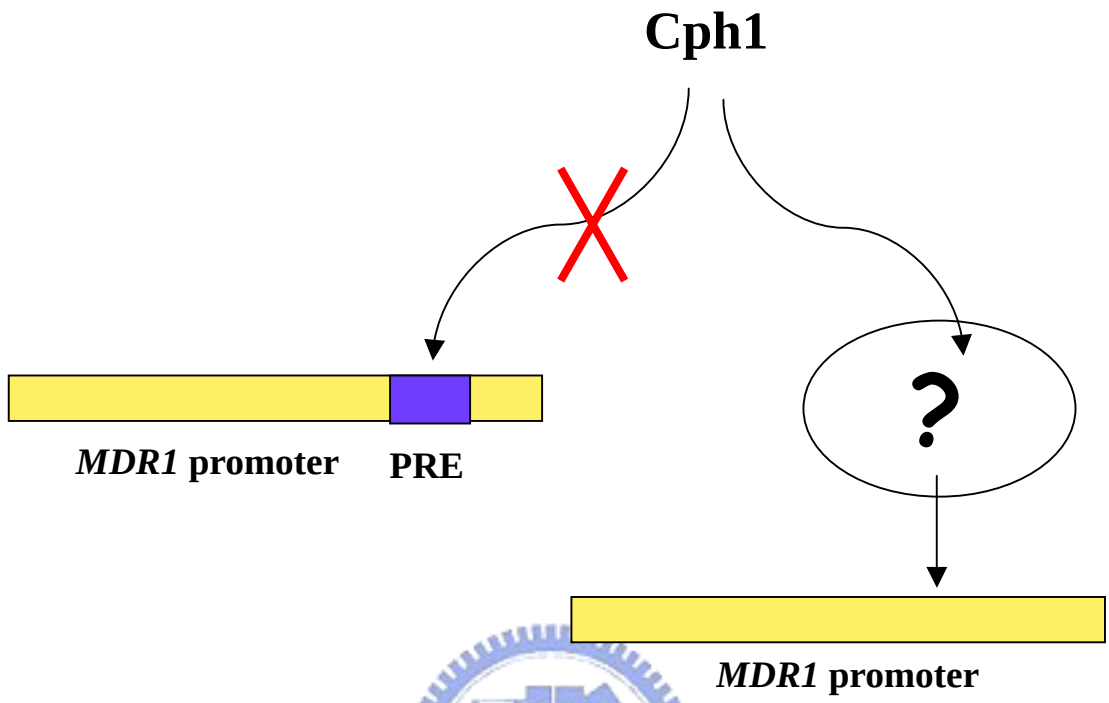
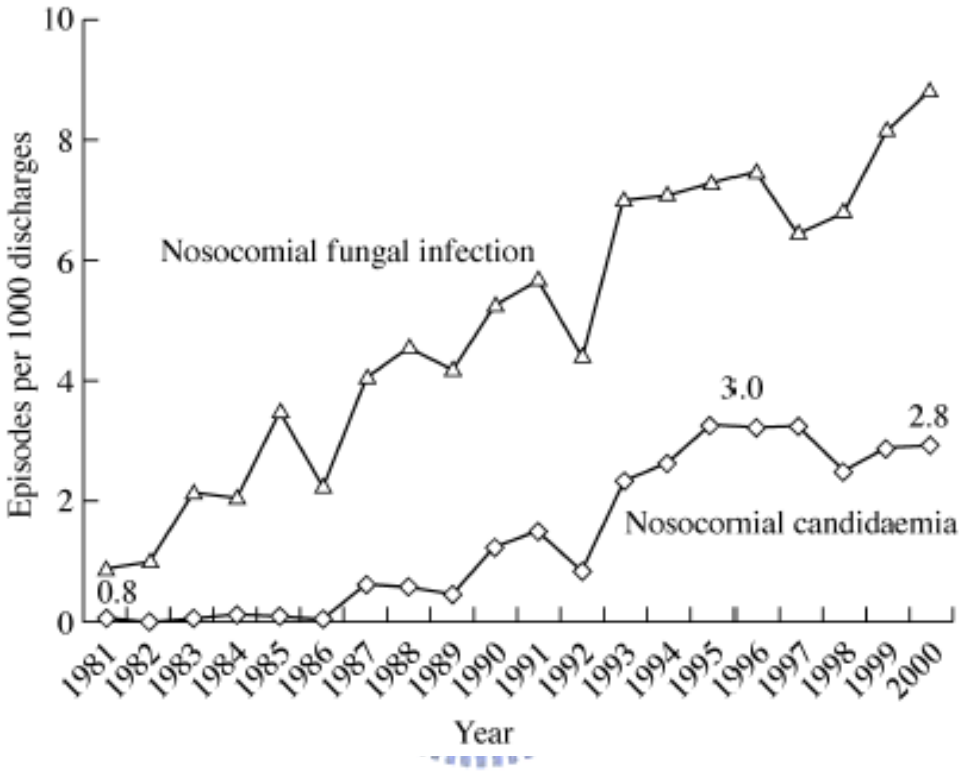


Figure 4-1. The regulation between the *CPH1* and *MDR1* is indirect. The yellow bars indicate the *MDR1* promoter. the blue bar indicates the *MDR1* promoter with a perfect PRE site. The Cph1 does not bind to PRE site in *MDR1* promoter directly (in the left). Instead, Cph1 may affect the *MDR1* indirectly.

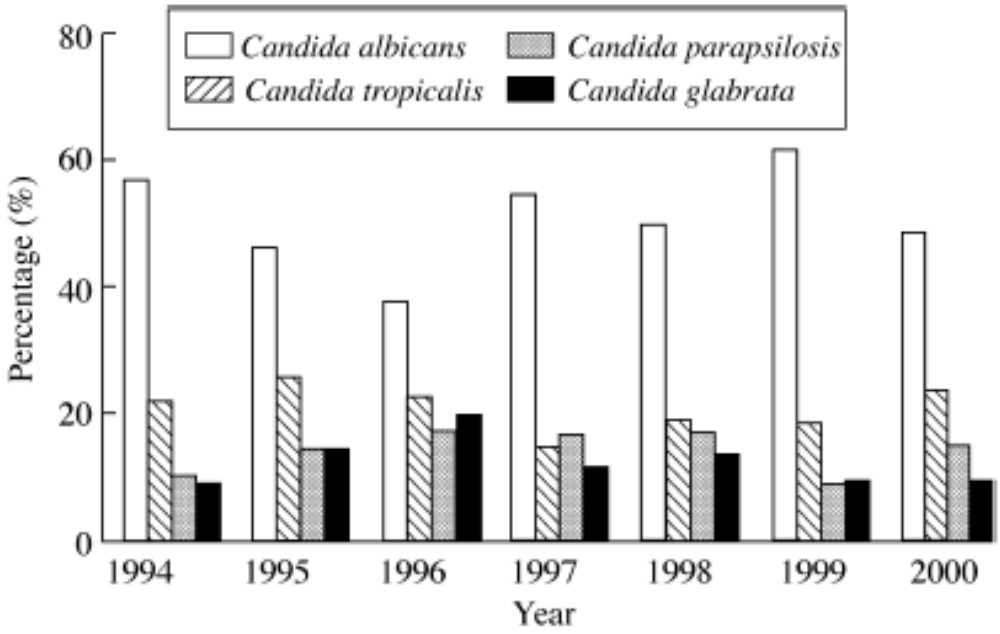
Appendix 1



Incidence of nosocomial candidaemia and overall nosocomial fungal infection during 1981–2000 at National Taiwan University Hospital. The incidence of nosocomial fungal infections increased gradually from 1981 to 2000. Nosocomial candidaemia increased rapidly during 1981–1996.

(Adapted from Chen et al., 2003)

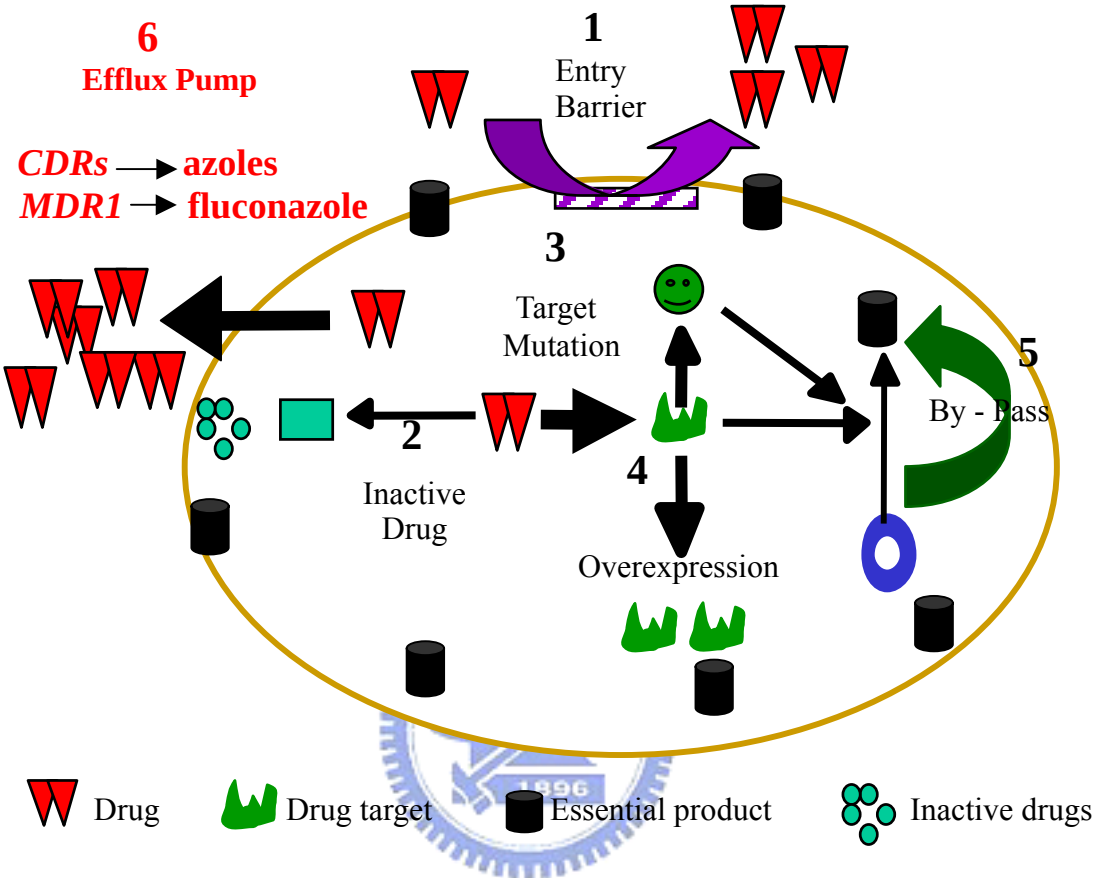
Appendix 2



Proportion of blood isolates of four *Candida* spp. during 1994–2000. During 1994–2000, a total of 1095 episodes of nosocomial candidaemia occurred. *Candida albicans* was the leading species (50.4%), followed by *Candida tropicalis* (20.5%), *Candida parapsilosis* (14.2%) and *Candida glabrata* (12.0%).

(Adapted from Chen et al., 2003)

Appendix 3

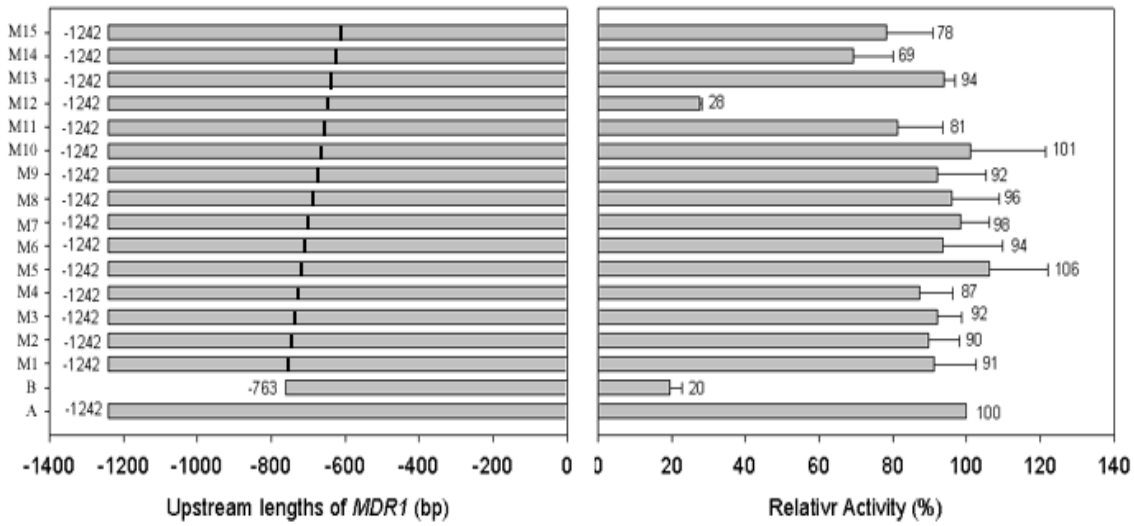


Potential molecular mechanisms of antifungal agent resistance.

1. Alteration of cell membrane and/or cell wall to prevent drug from entering.
2. Inactivation or degradation of the drug.
3. Mutations in the drug target to prevent the drug from binding to the target.
4. Overexpression of the drug target such that the drug fails to inhibit the biochemical reaction completely.
5. Mutations in the other gene in the same pathway such that the cell bypasses the requirement for the product of the drug target gene.
6. Overexpression of an efflux pump to reduce the accumulation of drug in the cell.

(Adapted from Yang et al., 2001)

Appendix 4



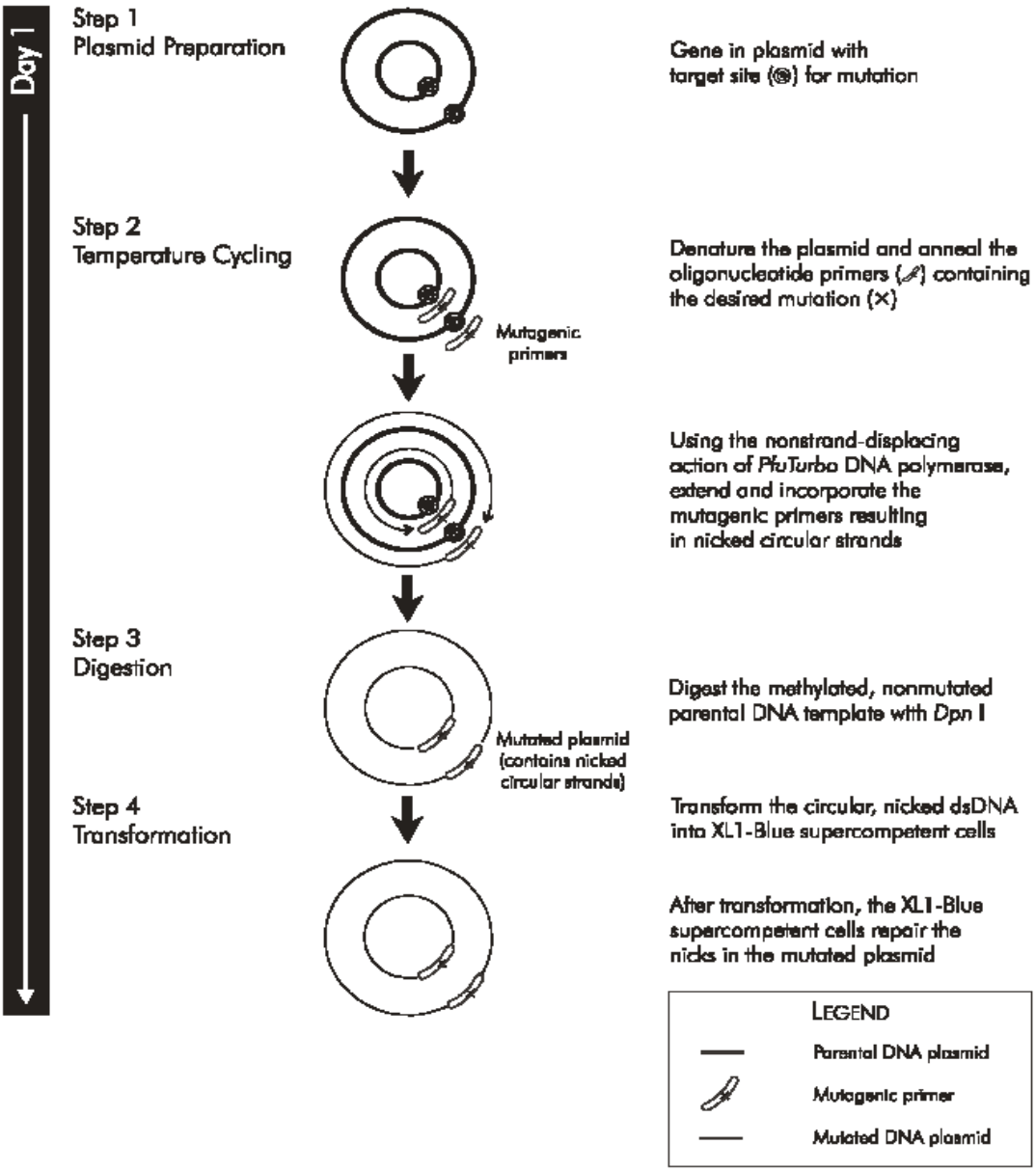
The site-directed mutagenesis of *MDR1* promoter activity.

The *lacZ* activity from each promoter was shown in relative percentage using *MDR1p* 1242 as 100%. The numbers in the names of the strains represent the different mutation *MDR1* promoter in the recombinant construct carried within the cells. The names are listed in the below.

M1	-769/-767	M6	-754/-752	M11	-739/-737
M2	-766/-764	M7	-751/-749	M12	-736/-734
M3	-763/-761	M8	-748/-746	M13	-733/-731
M4	-760/-758	M9	-745/-743	M14	-730/-728
M5	-757/-755	M10	-742/-740	M15	-727/-725

(Adapted from Lo, 2002)

Appendix 5



Overview of the QuikChange™ site-directed mutagenesis method.
(Adapted from Promega, 2002)

Appendix 6

Comparison site					sequence site
1	ctcggcttgc	taaacattat	caagatcaac	aaagttgaat	gaataacgct -1193
51	tttgtacaat	tgtattattg	ctatatatat	atttttgca	ttttttaat -1143
101	agaaacggaa	acatgattaa	catggtgtta	agataacgta	aatctgtaga -1093
151	aacggactcc	gtgaaaacta	gagctacaaa	tgacacactt	tcactttaag -1043
201	taactcattc	ttatctagtc	gtcgatgatt	acaaataaac	atggaaaaca -993
251	aaaaggaaat	aatacacaca	gccgtgaatc	ttagacttac	ttatatccgt -943
301	ataatccagc	tatatataag	aacactaatc	tagaattaaa	aagataagtt -893
351	gagtggcgat	caaatacttt	aaacattaga	ttagatacct	aaacgtgtaa -843
401	gaattgcgca	attctttagg	ttgcgccaaa	aaaaccatat	ttccaatttg -793
451	taagcaaagt	gaagtgttta	tatatccccg	tgtaatcttt	tatacggtga -743
501	taatgatgac	tcatcacacc	aacaaaaaca	acccaataaa	acttatatac -693
551	tgaagaaata	agttgtttaa	tagtgttgta	accacagttc	ttacaacctt -643
601	caagtaacct	tgcaatcctg	tcccactatg	ccaaataaaa	gaaaaaagca -593
651	tctagaaaaa	aaggcggatt	tactcctgat	acaactctac	tggtaactat -543
701	tggcgaaaga	ttagtaaata	aagttgctat	ttttgttaca	tcaattttca -493
751	ttttaggaaa	tttaccgagt	ttttagctcg	tttagttgtt	cccattcgca -443
801	tcaatcttat	aaatcaaaat	agtttactca	acagcccgtc	gccatcgccg -393
851	tttttccttg	ccgtggcatt	tttccgtggc	tacttttaa	ggttttgtta -343
901	tctgtttttg	tacaacaatt	atgggtgttg	ctaccagtta	atcacaacgg -293
951	taaaatccta	attgggaaaa	ataccgagaa	tgacacaacc	taagattttg -243
1001	cactcggaaa	ttatattatt	cttcatcgct	tattttctat	aaacttctat -193
1051	cgcgaaatga	ataatatcct	tatgattatt	aatagcaaaa	ttcaaacacc -143
1101	aagcaatgtt	ttggaaacat	atttaaggga	tgggatatcc	ttttcagttt -93
1151	ccaacaattc	tacttttttt	tattccttaa	caatcatatt	ataattttat -43
1201	attgccccaa	tagcaataca	tatacttaca	tagaacttca	caatg +3

(+3 was the third base of translation)

The sequence of *MDR1* promoter 1.2 kb of SC5315 wild type strain.
(Adapted from Lo, 2002)