รายงานการวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่อง

ผลกระทบของแหล่ง carbon sources ต่อการแสดงออกของยีนที่เกี่ยวข้องกับ metabolism และการติดเชื้อโรค ในยีสต์ *Saccaromyces cerevisiae*

(Effects of carbon sources on metabolic adaptation and virulence in the yeast *Saccharomyces cerevisiae*)

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บทคัดย่อ

การศึกษาผลกระทบของแหล่งคาร์บอนต่อลักษณะทางฟีโนไทป์ของยีสต์ *Saccharomyces cerevisiae* สายพันธุ์ที่ขาดยีนซึ่งอาจทำหน้าที่เป็นตัวควบคุมลักษณะพันธุกรรม ในกลุ่มของ Zinc cluster โปรตีน โดยพบว่ายีสต์สายพันธุ์ *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* และ *Δyfl052w* มีการเจริญเติบโตได้น้อยในแหล่งอาหารที่ใช้แหล่งคาร์บอนทางเลือกประเภท non fermentable คาร์บอน เช่น เอธานอล แลคเตท หรือกลีเซอรอล เมื่อเทียบกับการเจริญของสายพันธุ์ปกติ นอกจากนี้ยีสต์สายพันธุ์เหล่านี้ ยังทนต่อสภาวะเครียดได้ลดลง เช่น การทนต่ออุณหภูมิที่เพิ่มขึ้น การทนเกลือ และการเจริญในสภาวะที่เป็นกรดหรือด่าง โดยเฉพาะเมื่อเปลี่ยนจากแหล่งอาหารกลูโคสเป็น เอธานอล แลคเตท หรือกลีเซอรอล ซึ่งแสดงให้เห็นถึงบทบาทที่สำคัญของยีน Zinc cluster เหล่านี้ต่อการใช้แหล่งคาร์บอนทางเลือกของยีสต์ ทั้งในสภาวะปกติและสภาวะเครียด ดังนั้นคณะผู้วิจัยจึงได้ทำการติดฉลากตัวควบคุมลักษณะพันธุกรรมที่เกี่ยวข้องเหล่านี้ เพื่อศึกษาลักษณะการจับกับยีนเป้าหมาย ในสภาวะที่แหล่งคาร์บอนเป็นประเภท non fermentable คาร์บอน จากการศึกษาพบว่า ตัวควบคุมลักษณะพันธุกรรม Yfl133c หรือเรียกอีกชื่อว่า Rds2 มีความเกี่ยวข้องกับการแสดงออกของยีนในกลุ่มเมทาบอลิซึม เช่นยีน *TOR2* และยีนที่เกี่ยวข้องกับการสังเคราะห์ไขมัน ergosterol และการดื้อยา เช่นยีน *ERG11* เป็นต้น จากผลงานวิจัยทั้งหมด จึงสรุปว่า งานวิจัยนี้ทำให้ค้นพบ ตัวควบคุมลักษณะพันธุกรรมใหม่ที่เกี่ยวข้องกับการใช้แหล่งคาร์บอนทางเลือก รวมทั้งสิ้น 5 ตัวคือ Yil130w, Ypl133c, Yer184c, Yor380w และ Yfl052w ซึ่งยังมีหน้าที่ในการตอบสนองต่อสภาวะเครียดอีกด้วย เนื่องจากปัจจัยเหล่านี้ส่งผลต่อความสามารถในการก่อโรคของยีสต์ ดังนั้นงานวิจัยนี้จึงสามารถนำไปใช้ต่อยอดในการศึกษายีสต์เมทาบอลิซึมเพื่อการพัฒนายาต้านเชื้อราต่อไป

คำสำคัญ: แหล่งคาร์บอน/ เมทาบอลิซึม/ การควบคุมการแสดงออกของยีน/ *Saccharomyces cerevisiae*

Abstract

In this study, we reported the phenotypes of zinc cluster deletion strains under various physiological conditions. Our results indicated that the deletion strains namely *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* strains are sensitive to high temperature (37^{O}C or 39^{O}C), LiCl, NaCl or KCl salt stress and acidic or alkaline pHs when grown in glucose and more severe defective phenotypes are observed during growth in ethanol, glycerol or potassium acetate. Interestingly, these deletion strains were hypersensitive to acetic acid when non-fermentable carbon source was used as a sole source of carbon. Thus, to better understand about the roles of these unknown zinc cluster proteins, *YIL130W, YPL133C, YER184C* and *YFL052W* were selected for generation of the integrated Myc epitope tagging proteins via PCR and homologous recombination method. We obtained positive transformants for *YIL130W YFL052W* and *YPL133C* genes. These Myc-tagged *YIL130W, YPL133C* or *YFL052W* were confirmed epitope tagging at correct location by using specific primer-oligonucleotides via PCR and sent for sequencing. The results of alignment DNA sequences showed that DNA sequence of Myc-tagged *YPL133C* is completed with two positive clones and Myc-tagged *YFL052W* is completed with one positive clone. In parallel, we performed a standard genome-wide location analysis for the tagged *YPL133c* which also known as Rds2-tagged protein and examined the expression of its target genes during the shift from glucose to ethanol, lactate or glycerol. Our results showed that expression of some metabolic genes such as *TOR2* and *ERG* genes in the biosynthesis of ergosterol and drug resistance is altered upon removal of Rds2. In summary, our results indicated new zinc cluster proteins involved in the utilization of non-fermentable carbon metabolism, drug resistance and stresses response. Clearly, the characterization of these new regulatory proteins will be essential for better understanding of yeast metabolism and development of novel antifungal agents.

Keywords: Carbon source/ Metabolism/ Gene regulation/ *Saccharomyces cerevisiae*

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คำอธิบายสัญลักษณ์และคำย่อ (List of Acronyms)

4-NQO = 4-NitroQuinolone-*N*-oxide
ABC = ATP-Binding Cassette
ADR1 = Alcoholdehydrogenase Regulator gene
ALD6 = ALdehyde Dehydrogenase gene 6
ATP = Adenosine TriPhosphate
AQR1 = Acids Quinidine Resistance gene 1

BLAST = Basic Local Aligment Search Tool
Bp = base pair
bZip = basic leucine Zipper

CAT8 = CATabolite repression gene 8
CDR1, *2* = Candiad Drug Resistance gene 1,2
ChIP = Chromatin Immunoprecipitation
Chip = microarray chip
CIT1 = CITrate synthase gene 1
COX6 = Cytochrome c Oxidase gene 6
CSRE = Carbon Source Responsive Element
CWT1 = Cell Wall Transcription factor 1
CYB2 = CYtochrome B gene 2
CYC1 = CYtochrome C isoform 1 gene

DBD = DNA-binding domain
DHAP = DiHydroxyAcetone Phosphate
DLD1, *2* = D-Lactate Dehydrogenase genes 1,2
DNA = DeoxyriboNucleic Acid

EMSA = Electro Mobility Shift Assay
ERG1-25 = ERGosterol biosynthesis genes 1-25
EtOH = Ethanol

F-6-P = Fructose-6-Phosphate
F-1,6-bP = Fructose-1,6-bisPhosphate
F-2,6-bP = Fructose-2,6-bisPhosphate
FBP1 = Fructose-1,6-BisPhosphatase gene 1
FOA = Fluoro-Orotic Acid

G-6-P = Glucose-6-Phosphate
GAL4, *80* = GALactose metabolism genes 4, 80
GID8 = Glucose Induced Degradation deficient gene 8
GUT1,2 = Glycerol Utilization gene 1,2

h = hour
HA = Hemmaglutinin
HAP1, *4* = Heme Activator Protein genes 1, 4
HIS3 = HIStidine requiring gene 1
hisG = Salmonella enteric serovar typhirium hisG gene

HXT9, 11 = HeXose Transporter genes 9, 11

ICL1 = IsoCitrate Lyase gene 1
IDP2 = Isocitrate Dehydrogenase, NADP-specific gene 2
INO1, 2, 3 = INOsitol requiring genes 1, 2, 3

kD = kilo Dalton
KGD2 = alpha-KetoGlutarate Dehydrogenase gene 2

LEU3 = LEUcine biosynthesis gene 3
LSC2 = Ligase of Succinyl-CoA gene 2

MAE1 = MAlic Enzyme gene 1
MAPK = Mitogen-Activated Protein Kinase
MAT = MAting Type
MCS= Multiple Cloning Site
MDH2 = Malate DeHydrogenase gene 2
MDR = Multi-Drug Resistance
MDR1 = Multi-Drug Resistance gene 1
MET3 = METhionine requiring gene 3
MFS = Major Facilitator Superfamily
MHR = Middle Homology Region
MLS1 = MaLate Synthase gene 1
MPK = mitogen-activated protein kinase
mRNA = messenger RNA

OAF1 = Oleate-Activated transcription Factor gene 1
ORE = Oleate Response Element
ORF = Open Reading Frame

PCK1 = Phosphoenolpyruvate CarboxyKinase gene 1
PCR = Polymerase Chain Reaction
PDC1 = Pyruvate DeCarboxylase gene 1
PDR = Pleiotropic Drug Resistance
PDR1, 3, 5, 11, 12 = Pleiotropic Drug Resistance genes 1,3, 5, 11, 12
PDRE = Pleiotropic Drug Response Element (or Pdr1/3 Drug Response Element)
PEP = PhosPhoenolPyruvate
PFK1, 2 = PhosphoFructoKinase genes 1,2
PFK26, 27 = 6-PhosphoFructo-2-Kinase genes 26, 27
PIP2 = Peroxisome Induction Pathway gene 2
PKA = Protein Kinase A
PUT 3 = Protein Utilization gene 3
PYC2 = PYruvate Carboxylase gene 2

qPCR = Quantitative Polymerase Chain Reaction
OPI1 = OverProducer of Inositol gene 1

RGT1 = Repressor of Glucose Transport gene 1

S. cerevisiae = *Saccharomyces cerevisiae*
SDH4 = Succinate DeHydrogenase gene 4
SFC1 = Succinate-Fumarate Carrier gene 1
SIP4 = Snf1-Interacting Protein gene 4
SNF1 = Sucrose NonFermenting gene 1
STRE = Stress Response Element (or Sterol Response Element)

TCA = TriCarboxylic Acid
TKL1 = TransKetoLase gene 1
TOR2 = Target of Rapamycin

UAS = Upstream Activating Sequence
URA3 = URAcil requiring gene 3

WAR1= Weak Acid Resistance gene 1
WCE = Whole Cell Extract

YAP1 = Yeast AP-1 gene 1
YEP = Yeast Extract Peptone
YEPD = Yeast Extract Pepteon Dextrose
YFG = Your Favorite Gene
YPD = see YEPD

บทที่ 1

บทนำ (Introduction)

1.1 การทบทวนวรรณกรรม/สารสนเทศ (information) ที่เกี่ยวข้อง

Zinc cluster proteins form one of the largest sub-class of sequence-specific transcriptional factors in the yeast *S. cerevisiae*, consisting of over fifty members. They are characterized by the presence of a well conserved zinc cluster motif, $CysX_2CysX_6CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$, located in the DNA binding domain [1] The proper folding of this domain is coordinated through the binding of the conserved cysteine residues to two zinc atoms. Mutation or deletion of these cysteines, or the absence of zinc results in loss of DNA-binding [2].

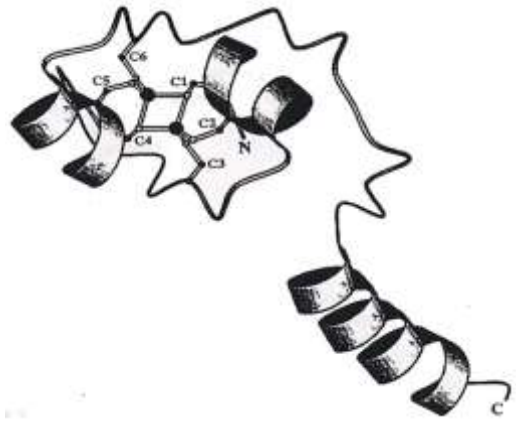


Figure 1. DNA binding domain (DBD) of zinc cluster proteins. This ribbon model illustrates the binding of highly conserved six cysteine residues to two zinc atoms. Their coordination is important for a proper folding of the DBD, located at the N-terminus [3].

Generally, zinc cluster proteins contain three major domains: the DNA-binding, the regulatory (MHR), and the acidic activation domains. The DNA binding domain (DBD) is composed of the zinc finger motif, the linker region and the dimerization sub-domain. Within the zinc cluster motif, each alpha helix strand contains three of the six conserved cysteine residues bound to two zinc atoms [4-6]. The linker region shows no sequence similarity among members of this class, giving rise to diversified forms of

linkers. Lastly, the dimerization region contains a coiled-coil structure, consisting of heptad repeats (reminiscent to the leucine zipper motif), may also be found in some zinc cluster proteins. This region is involved in mediating the protein-protein interactions through homo- or heterodimerization, such as in the case of zinc cluster Gal4, Ppr1 or Oaf1 and Pip2 [6-9]. The middle homology region (MHR) or the regulatory region is situated in between the DBD and the activation domain although it is not always present in all members. Several deletion studies suggest a transcriptional regulatory role for this region [1, 10]. For instance, removal of the MHR from the zinc cluster protein Hap1 (heme-activating protein) deregulates its transcriptional activation, resulting in a constitutively active activator even in the absence of its inducer, heme [11]. Typically, the C-terminal region is where the acidic activation domain is located.

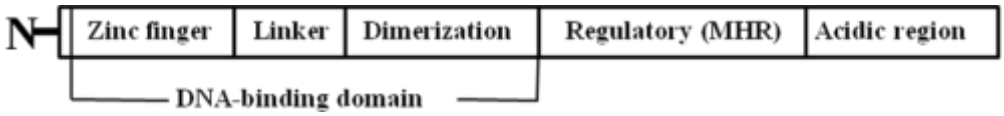


Figure 2. Structural and functional domains of zinc cluster proteins. Zinc cluster proteins are subdivided into three main sections: the DNA-binding domain, the regulatory domain and the acidic region [10].

Roles in nonfermentable carbon metabolism and connection to virulence

The yeast *S. cerevisiae* prefers a fermentable sugar like glucose over other carbon sources as glucose can directly enter the glycolysis pathway. However, when glucose becomes unavailable, other alternative carbon sources can be used for the efficient production of metabolic energy and cellular biomass. To metabolize these poorer carbon sources, the expression of genes encoded for specialized metabolic enzymes is activated. Unlike glucose, a nonfermentable carbon compound, such as ethanol, acetate, lactate or glycerol, can be converted directly to acetyl-CoA or pyruvate and therefore bypassing glycolysis.

A shift from a fermentative to a nonfermentative mode of growth is referred to as a diauxic shift. During this transition, a dramatic change in expression of genes in various pathways such as in carbon metabolism, protein synthesis and carbohydrate storage, occurs [12]. The up-regulation of gluconeogenic gene expression is indispensable for the production of glucose-6-phosphate which is critical for cell growth. For instance,

glucose-6-phosphate is required for nucleotide metabolism, glycosylation, cell wall biosynthesis and the storage of carbohydrates.

The expression of gluconeogenic genes is co-regulated with the expression of many respiratory genes, as respiration is necessary in order to obtain energy by oxidative phosphorylation during gluconeogenic processes [13]. In fact, one study showed that respiratory-deficiency mutants are unable to utilize or grow on the nonfermentable carbon sources [14]. Another study showed that the biosynthesis of these mitochondrial proteins also depends on the availability of carbon source in addition to the presence of oxygen and heme [15]. However, the expression of genes involved in metabolism of nonfermentable involves different sets of transcriptional factors than those implicated in respiration, and requires distinct regulatory DNA elements. Interestingly, our previous work identified many deletion strains of zinc cluster proteins showing growth defects on the nonfermentable carbon sources such as glycerol and lactate, suggesting putative roles for these proteins in the metabolism of nonfermentable carbons [16].

Glycerol uptake is mediated by two specialized glycerol symporters called Gup1 and Gup2. Following its uptake, glycerol is converted to triose phosphate (dihydroxyacetone phosphate; DHAP). As glycerol enters the glycolytic pathway, it is transformed to DHAP which is further converted to pyruvate and, to a certain extent, to glucose-6-phosphate (Fig. 3). Similarly, lactate is taken up in the cells through a specific permease called Jen1 which also transports pyruvate [17, 18]. In addition, *JEN1* expression is repressed in the presence of glucose and is induced by lactate. Its transcription is under the regulation of the gluconeogenic activator Adr1 and the zinc cluster protein, Cat8 [19]. Unlike glycerol or lactate, ethanol and acetate are thought to enter the cells by passive diffusion, although an acetate carrier has been identified [20]. Moreover, ethanol is also produced routinely in the cells as a consequence of excess glucose by alcoholic fermentation. Following its uptake, ethanol is sequentially oxidized to yield acetate and eventually acetyl-CoA; this enters the metabolic pool of acetyl-CoA, which can be used to generate sugar phosphates and energy. Generation of acetyl-CoA also occurs via the peroxisomal β-oxidation of fatty acids. The expression of the peroxisomal genes is regulated by a pair of zinc cluster proteins called Oaf1 and Pip2 through the consensus oleate response element (ORE) $CGGN_3TNAN_{9\text{-}12}CCG$ found in the promoters of β-oxidation genes [9].

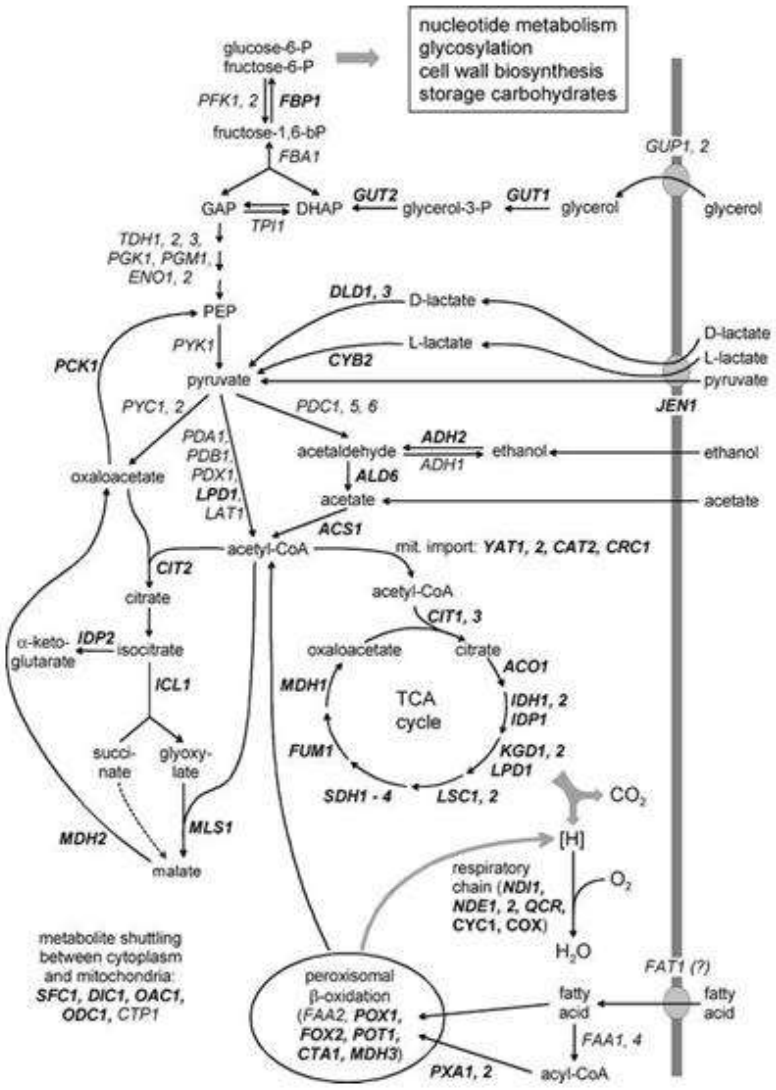


Figure 3. Examples of genes require for the utilization of nonfermentable carbons (such as glycerol, lactate, ethanol and fatty acids) for growth during gluconeogenic and oxidative metabolism in *S. cerevisiae.* The expression of genes indicated in bold is subjected to glucose-mediated repression (adapted from [15]).

Interestingly, recent expression profiling studies of yeast cells upon internalization by mammalian macrophages showed that the expression of genes required for utilization of nonfermentable compounds are up-regulated, suggesting for glucose-deficient environment [21]. Examples of these genes are gluconeogenic and glyoxylate cycle genes. Importantly, these genes have been shown to contribute to virulence in mice

model as *C. albicans* cells lacking these genes are avirulent [22]. Since the role of these two pathways is to assimilate the two carbon-compound which is thought to be the relevant carbon source used by fungal during phagocytosis or during nutrient starvation. Similarly, the role of glyoxylate cycle genes in virulence has been shown for other fungal and bacterial pathogens, for examples *Magnaporthe grisea*, *Leptosphaeria maulans*, *Stagonospora nodorum*, *Rhodococcus equi* and *Mycobacterium tuberculosis* [23]. Since deletion of zinc cluster genes results in impaired growth on nonfermentable carbon sources, their functions are likely linked to virulence. This may be due to defects in the gluconeogenic, glyoxylate cycle or other related pathway.

1.2 ความสำคัญ และที่มาของปัญหาที่ทำการวิจัย

Disseminated hematogenous candidiasis (bloodstream fungal infection) is a severe form of infection found in individuals with compromised immunity such as AIDS and cancer patients. Prostitutes and drug users who share infected needles are major contributors to growing incidence of HIV and, along the line, fungal infections. In fact, bloodstream fungal infection currently ranks the forth most common acquired infection in the hospitals and has the mortality rate of approximately 40%. *Candida* species such as *C. albicans* are responsible for most infections. Numbers of studies show that the disease progression depends on both the status of host immunity and the pathogen. In fact, in healthy people, *C. albicans* is benign commensal microbe present in the human flora such as in the gut, mouth, vagina and the skin. The interaction between *C. albicans* and host's innate immune system determines the outcome of the infection.

The genomic expression profiling of macrophage- and neutrophil-induced phagocytosis of *C. albicans* cells indicate a major reprogramming of gene expression for various metabolic pathways, including the glyoxylate cycle, β-oxidation and gluconeogenesis. These three interconnected pathways are required for the conversion of non-sugar compounds such as non-fermentable carbons or lipids to glucose. Interestingly, the expression profiling seen here is similar to the one observed after nutrient starvation. Importantly, the data indicates the necessity of cells to utilize alternative carbon sources from nonsugar compounds for growth which suggests for the unavailability of sugar *in vivo*. Indeed, deletion of genes encoding for the enzymes in these metabolic pathways (eg.

Isocitrate lyase, Icl1; fructose-1,6-bisphosphatase, Fbp1 or β-oxidation protein) attenuate virulence in the mouse model of disseminated candidiasis.

Since the yeast *Saccharomyces cerevisiae* is closely related to *C. albicans* and the pathways involved are very well characterized, it is best to first conduct the studies in this model organism and further characterize in detail in *C. albicans* whose genome is much more complex. Interestingly, in *S. cerevisiae*, the glyoxylate cycle, the gluconeogenic and the β-oxidation genes are under regulation of a group of transcription factors known as the zinc cluster proteins. Zinc cluster regulators activate their expression in response to glucose depletion or when the nonfermentable carbon source is the sole carbon source. A previous phenotypic analysis showed that deletion of open reading frames (ORFs) *YER184C*, *YFL052W*, *YIL130W*, *YLL054C*, *YLR266C*, *YOR162C*, *YOR380W* and *YPL133C* impairs growth on nonfermentable carbon sources such as lactate and glycerol. These respiratory-deficient mutants also show poor growth on medium containing cell wall perturbing agent calcofluor white, indicating alteration in the components of cell wall. Such pleiotropic effects imply the essentiality of these genes under certain growth conditions. As a result, a study on gene regulation is required to gain a better understanding of the mechanism behind metabolic adaptation. Importantly, these genes also link to virulence in the human pathogen. Since both the zinc cluster regulators and the enzymes in the glyoxylate cycle are unique in fungi, they represent promising new targets for antifungal drug development.

1.3 วัตถุประสงค์ของโครงการวิจัย

1. To identify zinc cluster genes involved in nonfermentable carbon metabolism and characterize their functions
2. To examine zinc cluster proteins' target genes and implicate pathways and regulatory network for the regulators
3. To investigate on the potential ability of zinc cluster protein as a new antifungal drug target

1.4 ขอบเขตของโครงการวิจัย

1. Perform a phenotypic analysis of yeast zinc cluster deletion strains on various nonfermentable carbon sources
2. Select zinc cluster regulators that show impaired growth on nonfermentable carbon sources and further characterize their roles in this process by ChIP
3. Examine the involvement of zinc cluster regulators in virulence through the identification of their target genes in comparison with known virulence determinants

1.5 ทฤษฎี สมมุติฐาน หรือกรอบแนวความคิดของโครงการวิจัย

Several classes of antifungal drugs are currently available for treatment of fungal infections. The most widely used antifungal drug azoles target a key enzyme the C-14α demethylase in the ERG pathway. In addition to azoles, six other classes of antifungal agents have been developed to target other fungal proteins or cellular components (i) pyrimidine (ii) polyene antibiotics (iii) allylamines, (iv) morpholines, (v) echinochandins and (vi) hydroxypyridone. The fungus-specific pyrimidine analogue 5-fluorocytosine targets enzymes required for pyrimidine metabolism and DNA and RNA synthesis. For instance, terbinafine inhibits the activity of squalene epoxidase that acts in the first step of this pathway. The mechanism of action involves is unknown although it is assumed that terbinafine binds to a lipid-binding site on the enzymes. Amorpholine is used to treat superficial infections. It inhibits the sterol Δ^{14}-reductase and $\Delta^{7,8}$-isomerase, encoded by *ERG24* and *ERG2*, respectively, in the ergosterol biosynthetic pathway . One of the latest classes of antifungal drugs used in the treatment of invasive fungal infection is echinocandin such as caspofungin, micofungin and anidulafungin. They target β-1,3 glucan synthase, involved in the biosynthesis of fungal cell wall. The last class of antimycotic agents is the hydroxypyridone such as ciclopirox olamide. Its mechanism of action is poorly understood and hypothesized to act as iron chelator.

Due to repetitive uses of antifungal drugs, the pathogen has developed many mechanisms to resist to the drugs. One of the most well characterized mechanism is the up-regulation of genes encoding for the efflux transporters which prevents the accumulation of drug inside the cells. Importantly, the expression of these transporters is under the control of a group of transcriptional regulators called the zinc cluster proteins

(see below). Azoles target C-14α demethylase, leading to the accumulation of lanosterol. This metabolite could then be converted to a toxic metabolite that interferes with membrane packing. However, nonfunctional mutation in the Erg3 enzyme of the ERG pathway prevents the formation of this toxic metabolite, resulting in resistance to the drugs. In addition, the Erg3 mutation also confers resistance to antifungal drug polyene due to the deletion of ergosterol. Furthermore, the mutation in the Erg11 (C-14α demethylase) also prevent the binding of the azole drug. Amplification of *ERG11* transcript has also been discovered in azole resistant isolates,

Currently, only three classes of antifungals are available for treatment of systematic fungal infections: the azoles, the echinocandins and the polyenes. To date, azole drug with a broad spectrum activity have been used successfully in immunocompromised patients. Example of new azole drugs are voriconazole, revuconazole and posaconazole. Caspofungin is also found to be effective for treatment of aspergillosis and in patients that do not respond to other drugs. Combinatorial therapy such as the use of azoles and polyenes is also used to control the infections. As a result of limited options of antifungal agents and recurrent infection, the development of new classes of antifungal agents is much needed. Several new targets of antifungal have been proposed. These include zinc cluster transcriptional regulators which are well-conserved across the yeast species but do not present in human. They are also control expression of genes, involved in many important pathways. Therefore, the characterization of these regulators may provide important insight and new ideas for antifugal drug development.

1.6 วิธีการดำเนินการวิจัยโดยสรุป

Experiments were performed in the gene/enzyme laboratory at School of Bioresources and Technology, King Mongkut's University of Technology Thonburi. Bang Khun Tean, Bangkok 10150. Tel: 0-2470-7765 Fax: 0-2452-3479

13.1 Generation of epitope tagging putative transcription factors

The open reading frame of gene of interest will be tagged at its natural chromosomal locations with either hemagglutinin (HA) or MYC epitope as described in (1). This is done by PCR, using gene specific primers and p3XHA or p3XMyc plasmids as template and subsequently by homologous

recombination. Yeast cells will be transformed with the resulted PCR products. We will test the resulting strains for their ability to grow on selective media lacking amino acid uracil (PCR product also contains the *URA3* marker) and check by gene-specific PCR for correct integration. Then, we will allow cells to undergo internal recombination and select for those with ability to grow on media containing 5-Fluoroorotic Acid (5-FOA) which selects against *URA3* gene. Correct integration of the epitope will be confirmed by gene specific PCR and, the expression of tagged protein will be checked by western blot with either HA or MYC antibodies.

13.2 Preparation of growth media and culture

Yeast strains isogenic to BY4741 will be grown routinely in rich YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) or in synthetic minimal (SD) media (0.67% bacto-yeast nitrogen base (without amino acids), 2% dextrose) at 30C. Media will be prepared according to Adams, et. al (2).

13.3 Chromatin immunoprecipitation (ChIP)

This technique is a powerful approach to identify gene promoters bound by given transcription factors. The detail method can be found in (1, 3). Briefly, cells from wild-type (non-tagged strain) and tagged strain will first be grown on rich YPD media or other selected media. Cells will be treated with formaldehyde to crosslink the transcriptional regulators with the DNA. Then, cells will be lysed by vortexing in the presence of glass beads. Chromatin will be shared to smaller fragments by sonication. The regulator of interest will be enriched with either anti-HA or anti-myc antibody coupled with magnetic beads. Following immunoprecipitation and cross-linking reversal, the DNA will be purified and can be use for gene-specific ChIP analysis by PCR and genome-wide location analysis (ChIP-chip). The ChIP array will be purchased from a commercial company. The data analysis will be performed using softwares as described in (1).

13.4 Gene induction conditions

Yeast cells will be grown overnight in YPD or SD media for 15 hours. Then, cells will be diluted 30 times and grow until mid-log phase (OD_{600} of 0.8-1.2). Growth conditions are varied, depending on the types of carbon sources. For instance, cells may be grown in media containing glucose and then shifted to different non-fermentable carbon sources such as ethanol, glycerol or lactate (3).

13.5 Analysis of gene expression by primer extension or alternative methods

Cells will be grown as described previously. RNA will be isolated by using hot-acid phenol/chloroform and purified (1). The total RNA will be used in the hybridization reaction which contains the radioactively-labeled oligonucleotides (1,3). These oligos are gene-specific synthetic oligos of approximately 20-30 bp in length. They are complementary to the most 5'end of the transcript of gene of interest. The enzyme reverse transcriptase will be used to extend the primers to produce cDNA from the RNA template. The amount of uncovered cDNA which is proportional to the amount of produced transcript (mRNA) will be analyzed on a denaturing polyacrylamide/urea gel.

13.6 Phenotypic analysis of zinc cluster deletion strains

Cells from wild-type and deletion strains will be grown overnight in YPD media. They will be diluted in fresh YPD and regrown until reaching log phase of growth (the OD_{600} is approximately 1.0). Then, they will be serially diluted and spotted onto plates, containing various carbon sources. Growth of yeast strains will be monitored over a period of 3-5 days.

References:

1. Larochelle, M., S. Drouin, F. Robert, and B. Turcotte. 2006. Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor

functions required for pentose phosphate pathway regulation and NADPH production. *Molecular & Cellular Biology* 26: 6690-701.

2. Adams, A., Gottschling, D. E., and Stearns, T., Methods in yeast genetics. Vol. 1. 1997, Cold Spring Harbor: Cold Spring Harbor Laboratory Press. 145-157.

3. Soontorngun, N., Larochelle, M., S. Drouin, F. Robert, and B. Turcotte. 2007. Regulation of gluconeogenesis in Saccharomyces cerevisiae is mediated by activator and repressor function of Rds2. Molecular & Cellular Biology **27**(22): 7895-905.

1.7 ประโยชน์ที่คาดว่าจะได้รับ เช่น การเผยแพร่ในวารสาร จดสิทธิบัตร ฯลฯ และหน่วยงานที่นำผลการวิจัยไปใช้ประโยชน์

At present, increased incidences of HIV infection and growing numbers of AIDS patients have put the studies of fungal infection at the forefront of today research. Due to the ability of fungi to resist to currently available antifungal drugs, the development of new better drugs are unavoidably required. The proposed study will provide important insight into the genes and mechanism involved in pathogenesis. Results obtained from this study will be published in international journals.

1.8 แผนการถ่ายทอดเทคโนโลยีหรือผลการวิจัยสู่กลุ่มเป้าหมาย

The new knowledge and techniques used in this study will be given to students at School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, in company with the lecture. After completion, this study will provide important insight into fungal physiology/ genes, enzymes and the pathways involved in virulence which will benefit researchers who work in the area of infections, drug design and other related topics.

บทที่ 2

วิธีการดำเนินงานวิจัย (Materials and Methods)

Table 1. Zinc cluster genes of *Saccharomyces cerevisiae* used in this study

Systematic name	Gene name	Strain name	Deletion*
YBR150C	*TBS1*	FA	aa 24-167
YBR240C	*THI2*	FB	aa 25-166
YCR106W	*RDS1*	FC	aa 23-206
YBR033W	*EDS1*	FI	aa 27-200
YIL130W	*ASG1*	FZG	aa 24-246
YPL133C	*RDS2*	FZH	aa 25-266
YLL054C	-	FZI	aa 25-220
YOL089C	*HAL9*	FZJ	aa 115-319
YNR063W	-	FZO	aa 25-166
YOR380W	*RDR1*	FZP	aa 25-196
YJL206C	-	FZQ	aa 28-149
YER184C	-	FZT	aa 26-225
YFL052W	*ROP1*	YZS	aa 24-173

The names of the targeted Open reading frame (ORFs) are given as systematic name. The wild-type (FY73) strain is used to perform the deletion analysis. *Deletion that means deleted ORFs in the cysteine-rich region (putative DNA binding domain) of zinc cluster gene, encoding for putative zinc cluster transcriptional regulator.

2.1 Yeast strains and media

Saccharomyces cerevisiae strains used in this study are listed in Table 1. Wild type strain used to generate zinc cluster deletion strains was FY73 (MATα; *ura3-52 his3-*Δ*200*) or W303 (MATa; *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15).* Media for routine growth were prepared according to Adam *et al*., 1997. Rich media (YPD) contained 1% yeast extract, 2% peptone and 2% dextrose. Selective media (SD) for spot assay contained 0.67% yeast nitrogen base without amino acid, 2% glucose, 2% bacto

agar. Amino acids histidine, leucine, lysine, methionine, adenine and tryptophan were added to the media at final concentration of 0.004% and uracil was also supplemented at a final concentration of 0.04%.

2.2 Phenotypic analysis

2.2.1 Utilization of carbon sources

Wild type strain and zinc cluster deletion strains were grown in liquid YPD overnight (approximately 16 hours). Cells were spun, resuspended in water and diluted to optical density (OD_{600}) of 0.1. Then, they were serially diluted and spotted on appropriated plates. Growth was assayed on different fermentable carbon sources, including 2% glucose, galactose, mannose, xylose, arabinose, sucrose, maltose or cellobiose or on non-fermentable carbon sources glycerol, ethanol and potassium acetate, following incubation at 30^{O}C for a period of 3 days. Growth on non-fermentable carbon sources was compared with growth on glucose. Assays were done in triplicate.

2.2.2 Heat sensitivity

To test heat sensitivity, wild-type and deletion strains were grown on different carbon sources (glucose, glycerol, ethanol and potassium acetate) at temperatures of 30˚C, 37˚C, 39^{O}C, 41^{O}C and 42˚C as previously described. Assays were done in triplicate.

2.2.3 Anaerobic condition

To assay growth under anaerobic condition, wild type and deletion strains were grown on different non fermentable carbon sources in the absence oxygen (an anaerobic chamber, incubated at 30^{O}C for 3-7 days). Assays were done in triplicate and in parallel with growth in the presence of oxygen.

2.2.4 Osmotic stress

Sodium chloride (NaCl) was added to media containing different carbon sources (glucose, glycerol, ethanol and potassium acetate) at a final concentration 0, 1.0 or 1.2M. Lithium chloride (LiCl) was added to media containing different carbon sources at a final concentration 0, 0.1, 0.3, 0.5 or 1.0M. or potassium chloride (KCl) was added to

media containing different carbon sources at a final concentration 0, 0.5, 1.0 or 1.5M. Assays were done in triplicate.

2.2.5 Acidic or alkaline pH condition

To prepare media at acidic or alkaline pH, 0.4 M HEPES (free acid) was adjusted to the desired pH with 10M HCl or 5M NaOH, respectively, to obtain the final pH of 4.0 or 8.0, respectively. Then, the YPD media was mixed with agar pre-cooled to 50°C. Cells were grown on YPD media at pH of 4.0, 8.0 or 6.2 (regular YPD media). Assays were done in triplicate.

2.2.6 Acetic acid stress

2M Acetic acid was added to media containing glucose at a final concentration 0, 70, 90, 100, 110 and 130 mM. or added to media containing different carbon sources (glucose, glycerol, ethanol and potassium acetate) at a final concentration 0, 20 or 40mM. Assays were done in triplicate.

2.3 Construction of epitope tagging strains for Genome-Wide Location Analysis (ChIP-chip) and confirmation via PCR method

Primers of gene of interesting were designed to tag the *Saccharomyces cerevisiae* zinc cluster protein: YFL052W, YPL133C and YIL130W. The tagging cassette was obtained by PCR using the oligonucleotides listed in Table 2. Each pair of PCR epitope tagging (PET) primers were designed to complementary to the tag-URA3-tag cassettes on the plasmid p3XMYC as template and homologous to the target gene of interest. The target gene ORF was N-terminally tagged at its natural chromosomal location with a triple MYC epitope. The correct integration and sequence of the 3XMYC epitope tagging were confirmed by gene specific PCR of the PCR product. (Table 3 and Fig. 4)

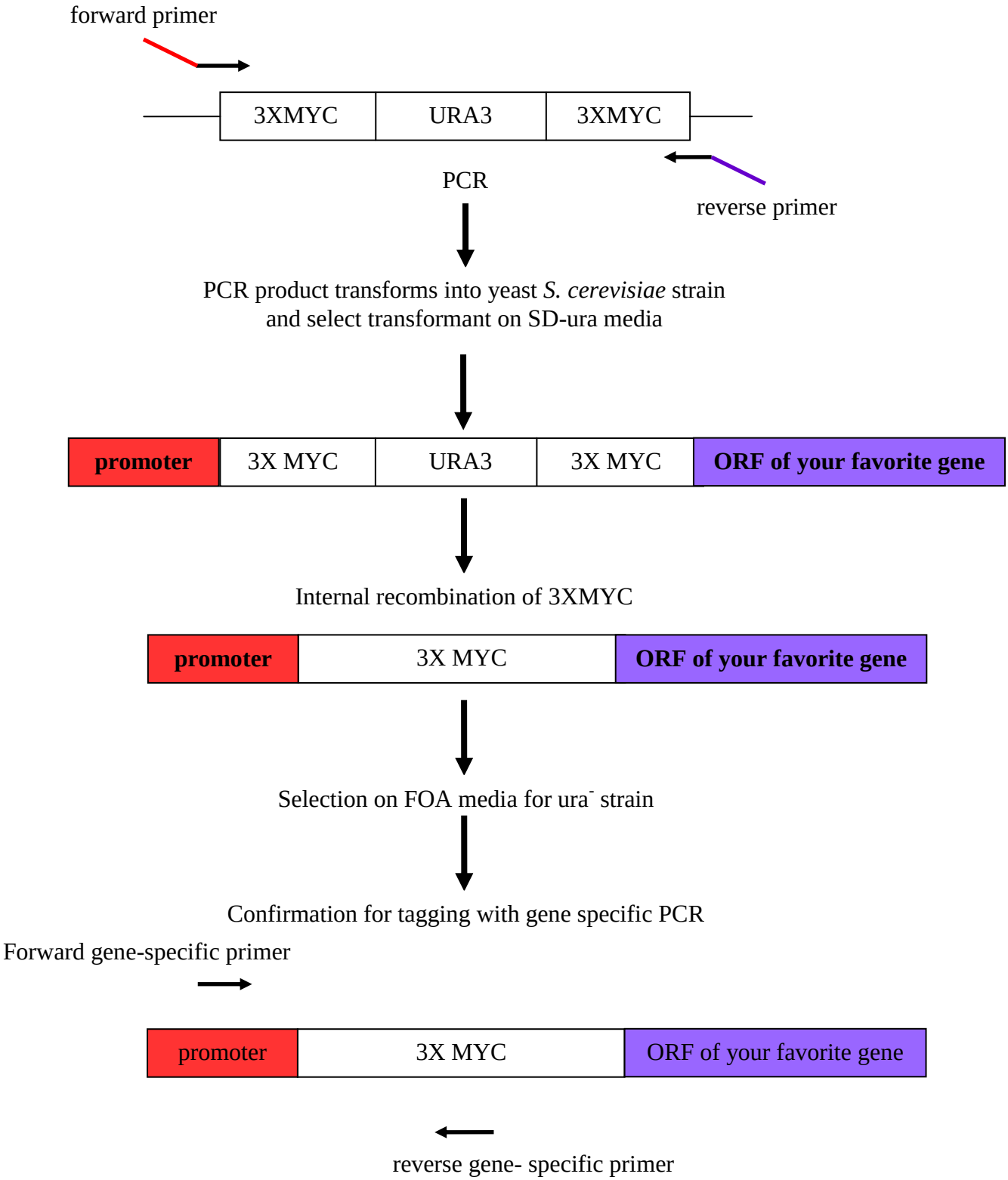


Figure 4 Illustration of epitope tagging of your favorite gene and confirmation with gene-specific PCR. The primers were used for tagging the N-terminus of YFG (Your Favorite Gene) with the 3XMYC epitope by PCR amplification of the tag-*URA3*-tag cassette.

Table 2. Oligonucleotides used for epitope tagging of your favorite gene via PCR amplification of the tag-*URA3*-tag cassette, containing the 3XMYC epitope

Primer name	Oligonucleotide sequence 5' to 3'
PET1-*YFL052W*	5' TTGTCAAAAGAGCTAAAGTAAAAGAGCTCTAGTTCG AAG**ATG**AGGGAACAAAAGCTGGAG 3'
PET2-*YFL052W*	5' TCGACGAATGCAACAACAGTCGCACGCTTGTGTATT GCGGGCTAGGGCGAATTGGGTACC 3'
PET1-*YFL052W*add60	5' GAAGAAAACCTTCAAGAAATGCTTCATTGTCTTGTC AAAAGAGCTAAAGT 3'
PET2-*YFL052W*add60	5' TACATGGTTTTTTACGATCACACTTTACTCGACGAA TGCAACAACAGTCG 3'
PET1-*YIL130W*	5' AAAGAAAAAGGAGCGCATATTATAATTGATAAGGGC GTT**ATG**AGGGAACAAAAGCTGGAG 3'
PET2-*YIL130W*	5' TCTTCTCTTGACAGACTGTTCCCCTTGTTGCGCTTGTTC TGGTAGGGCGAATTGGGTACC 3'
PET1-*YIL130W*add60	5' CTTTTTATTTGAGTTCCATAAGGTAAGAGACAAAGA AAAAGGAGCGCATA 3'
PET2-*YIL130W*add60	5' TGCATTCATCACAGGCTCTAGTGACCCTTCTTCTCT TGACAGACTGTTCC 3'
PET1-*YPL133C*	5' CAACACAAAATACACATATTTATATAAACTGACGAA ATA**ATG**AGGGAACAAAAGCTGGAG 3'
PET2-*YPL133C*	5' TTTAAAAGCCTTACTGGCTCGTTTTACACCACTGTT TGCTGATAGGGCGAATTGGGTACC 3'
PET1-*YPL133C*add60	5' CCAGTGAGATAAAGTTTTTTTTAATTTTAATCAACA CAAAATACACATAT 3'
PET2-*YPL133C*add60	5' CATACAACATGTGACCGTTTACAAAATAAACATGTT TTAAAAGCCTTACT 3'

Underlined sequences indicate sequence complementary to the sequence on the p3XMyc plasmid

Table 3. Oligonucleotides for confirmation of Myc epitope tagging within gene of interest for PCR amplification

Primer name	Oligonucleotide sequence 5' to 3'
PROM550*YFL052W*	5' GCTCGAGAGTTACTTAGTAG 3'
PROM550*YIL130W*	5' TTACTGTGTCTCTCCAAGCC 3'
PROM550*YPL133C*	5' TTGTGCGCTGAAGTGCTATT 3'
REVTAGKOKO	5' TCCAGCTTTTGTTCCCTCAT 3'
REV120code*YFL052W*	5' CAAAGGTCGAAGATATGTAC 3'
REV120code*YIL130W*	5' ACAATGGATACATGGTTGCT 3'
REV120code*YPL133C*	5' GCAATATCCCTCTTGACGCA 3'

PCR conditions were as following:

template p3XMYC	1 µl
5X forward primer (10 pmol)	5 µl
5X reverse primer (10 pmol)	5 µl
5X phusion buffer	10 µl
10 mM dNTPs	2 µl
50 mM $MgCl_2$	2 µl
1 unit phusion DNA polymerase	0.5 µl
To total volume	50 µl

Conditions for PCR reactions were:

Denature at 98^{O}C	0.5 min.	1 cycle
Denature at 98^{O}C	10 sec.	34 cycles
Annealing at 55^{O}C	0.5 min.	
Extension at 72 ^{O}C	1 min. 40 sec.	
Extension at 72 ^{O}C	1 min. 40 sec.	1 cycle

2.3.1 Lithium Acetate transformation (modified method from Grietz and Wood, 2002)

The yeast cells were grown overnight in 5 ml YPD liquid medium. Next step, the yeast cells were measured optical density (OD) 600 nm and calculated concentration at 0.1 for transfer to fresh YPD liquid 50 ml, shaking at 200 rpm, incubation at 30^{O}C for 4 hours. The final optical density of yeast cells approximately 0.6-0.7. The yeast cells were collected by centrifugation at 3000 rpm, for 5 minutes at room temperature, washed cells pellets in 10 ml of sterile water, resuspended in 5 ml of TE/LiOAc solution (made fresh from 10X TE [1M Tris-HCl, 0.5M EDTA, pH 7.5] with 10X LiOAc [1M LiOAc pH 7.5, adjusted with acetic acid]), resuspended in 500 µl of TE/LiAc solution. Each of sample was added 50 µl of yeast suspension, 5 µl denatured salmon sperm carrier DNA (boiled for 5 minutes and quick on ice) and 10 µl of PCR product. Yeast suspensions were mixed tap in a 1.5 ml micro-centrifuge tube. Then, 300 µl of filter sterile 40% PEG 4000 solution (40% PEG 4000 in TE/LiAc solution) was added and the tubes were incubated for 30 minutes at 30^{O}C with occasional gentle shaking. The cells were heat shocked for 30 minutes at 42 ^{O}C in water bath, quick centrifuged, remove supernatant, washed twice with 1 ml of sterile water, resuspended in 100 µl of 1X TE and plated onto SD selective medium lacking uracil, incubated at 30^{O}C for 3-5 days. Transformants contained Myc tag-*URA3*-tag were confirmed by specific oligonucleotides listed in Table 2 via PCR of the PCR product.

2.3.2 Yeast's genomic DNA extraction (Tiny Prep. mixed) for sequencing

The yeast cells were grown overnight in 5 ml YPD liquid medium. The samples were collected by centrifugation at 5000 rpm, for 5 minutes, remove YPD medium in 1.5 ml micro-centrifuge tube. The cell pellets and remain medium were mixed with vortex. Each of samples was added 200 µl of sterile Tiny Prep. mixed solution (2% triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0 adjust volume by sterile water), added glass beads three-fourth of suspension, added 200 µl of cooled phenol: chloroform (1:1), continuous vortex for 2 minutes. The suspension was separated by centrifugation at 12,000 rpm, for 5 minutes. Upper supernatant was collected in new micro-centrifuge tube 1.5 ml, gently added absolute ethanol 2 fold of upper supernatant volume and keep on -20^{O}C overnight. The DNA pellets were collected by centrifugation at 12,000 rpm, for 5 minutes, controlling temperature at 4^{O}C continue

method, remove supernatant. The DNA pellets washed in 500 µl 70% cooled ethanol, spin down at 12,000 rpm, for 5 minutes, remove suspension. The dried DNA pellets were added 20-40 µl of sterile water and keep on -20^{O}C.

2.3.3 Selection on FOA media and confirmation via PCR condition

Each of transformants of Myc tagged YIL130W, YPL133C and YFL052W were cultured overnight on YPD liquid, shaking at 200 rpm. Cell pellets were washed twice of sterile water, serial dilutions 10X, 100X and 1000X, respectively and selected minus URA3 colony from the tag-*URA3*-tag cassette on SD plates containing 5-fluoroorotic acid (FOA) (Fig. 5). Confirmation of minus URA3 strains on SD media lacking uracil and SD media containing uracil and checking Myc tagged YIL130W, YPL133C and YFL052W by specific oligonucleotides listed in Table 3 via PCR of the PCR product.

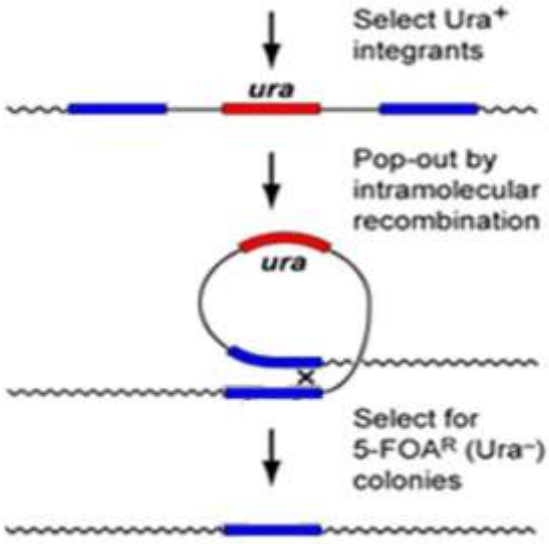


Figure 5 Selection of Myc epitope tagging of interesting gene without URA3 via internal recombination of sequences Myc epitopes and selected Myc tagged proteins on FOA media.

2.3.4 Western blotting analysis

The western blot is a technique used to detect specific proteins in the given sample extraction. It uses gel electrophoresis to separate native proteins. The proteins are transferred to a PVDF membrane, where they are probed using antibodies specific to the target protein.

The wild-type, Myc-*YPL133C*, Myc-*YIL130W* or Myc-*YFL052W* strains were grown in YP-media containing glucose, shaking 200 rpm for 6 hours.(approximately OD_{600} = 0.8) at 30^{O}C. After that cell pellets were washed twice with distilled water, shift to grown in YP-media containing either glucose or ethanol for 3 hours. The cell pellets were washed with ice-cold distilled water in 15 ml falcon. Proteins were extracted and immunoprecipitated. The IP buffer (15mM Tris-HCl pH 7.6, 150mM NaCl, 1% Triton-X and 10mM tetra-sodium pyrophosphate) added into cell pellets in falcon and added 2 ml of glass beads, vortex for 1 minute alternately on ice for 1 minute, fourth times. Then suspension was centrifuged at 5000 rpm for 5 minutes at 4^{O}C. The supernatant was transferred to new 1.5 ml eppendrof, spin down at 5000 rpm, for 20 minutes at 4^{O}C and collect supernatant. Protein extracts were incubated overnight with Myc antibody at 4^{O}C. The immunoprecipitated proteins were incubated with bead-slurry protein G-sepharose 4 fast flow for 2 hours at 4^{O}C. The samples were then washed five times with the IP buffer without protease inhibitors. Prior to being loaded, the proteins were dissociated from the beads by boiling them at 100^{O}C for 3 minutes in 50 µl of Laemmli buffer (40% glycerol, 400mM DTT, 240mM Tris pH 6.8, 0.004% Bromophenol blue). The samples were run on an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After that the protein was transferred to PVDF membrane. Blots were blocked in 12.5 ml of 1% blocking solution and shaking for 60 minutes at room temperature. Blots were then incubated overnight with primary Myc antibody at 4^{O}C. Next day, blots were washed with 30 ml of 1X TBST 20 for 10 minutes, 4 times and then washed with 12.5 ml of 0.5% blocking solution for 10 minutes, 2 times. Blots were incubated with 1 µl of POD-labeled secondary Myc antibody and 12.5 ml of 0.5% blocking solution. Blots were washed with 30 ml of 1X TBST 20 for 10 minutes, 4 times. Blots were detected with 2 ml of detection solution for 5 minutes and exposed on Kodak film.

2.4 Gene expression studies

2.4.1 Gene induction

The medium (5 ml) was added into a culture test tube, then, inoculated the yeast cell down and incubated at 30 ^{o}C overnight. This culture medium was transferred to flask that contained 100 ml YPD medium for 0.1 OD_{600} units and incubated at 30 ^{o}C for 5 h or to mid log phase. Then, the culture were divided in half to collect yeast cells (spin down at 3,000 rpm, washed twice with DEPC water) and transferred to YP medium containing

2% glycerol or 2% lactate. All of them were grown for additional 3 h. and collected yeast cells by spin down at 3,000 rpm, washed once with DEPC water, and keep at -80 ^{o}C.

2.4.2 RNA extraction

The yeast cells was taken out of -80 ^{o}C freezer and thaw on ice. Then, add 3 ml of Acid Phenol-Chloroform (5:1) (pre-warmed 10 min at 65 ^{o}C) and 3 ml of TES (RNA) per 200 OD_{600} units. Then, add 1 PCR tube glass beads and vortex tube at highest setting at angle (≈ 20^{o} angle), to suspend pellet. Incubate the tube at 65 ^{o}C in water bath for 1 h, incubate in beaker filled with hot water, vortex 20 sec (10 sec upright, 10 sec at angle) every 10 min. After that, vortex 20 sec, then aliquot in 1.5 ml eppendorf tube. Spin 20 min at 14,000 rpm at 4 ^{o}C. Transfer the top supernatant to new eppendorf, extract with 750 µl of Phenol-Chloroform (5:1) per tube, vortex 20 sec (10 sec upright, 10 sec at angle), and Spin 10 min at 4 ^{o}C. Transfer the aqueous phase to new eppendorf, extract with 750 µl of Chloroform:Isoamyl (25:1) per tube, vortex 20 sec (10 sec upright, 10 sec at angle), and Spin 10 min at 4 ^{o}C. Transfer aqueous phase to tube with 50 µl of 3M Sodium Acetate (NaAc) pH 5.2. Then, add 1 ml of 100% Ethanol (pre-cooled to -20 ^{o}C) (fill to top of tube) and store at -20 ^{o}C for longer than ½ h. Spin down RNA 20 min in microcentrifuge at 4 ^{o}C. Aspirate (leave a little left) and wash with 500 µl of 70% ethanol (temperature ≈ -20 ^{o}C) (no shaking, just add). Spin down 1 min, aspirate, remove last bit with pipette, let air dry 30 min, and add DEPC treated water to samples.

2.4.3 RNA purification

RNA product was transferred to 1.5 microcentrifuge tube, added 5 volumes of RNA Pure Buffer to 1 volume of the sample, and shake vigorously. Then, the each sample mixtures were added an equal volume of 70% ethanol and shake vigorously for break up any precipitate with pipetting. PR Column was placed in a 2 ml Collection Tube. Then, 500 µl of the ethanol-added mixtures were transferred to PR Column and centrifuge at full speed for 1 min. Then, discard the flow-through and transfer the remaining mixture to the same PR Column, centrifuge at full speed for 1 min, discard the flow-through, and place the PR Column back in the 2 ml Collection tube. 600 µl of Wash Buffer (ethanol added) into the center of the PR Column. Then, centrifuge at full speed for 30 second, discard the flow-through, place the PR Column back in the 2 ml Collection tube, centrifuge again for 3 min at full speed to dry the column matrix. The dried PR Column was transferred to a new 1.5 ml microcentrifuge tube and 50 µl of RNase-free water or TE (RNase-free) was added into the center of the column matrix. Let stand the tube for 10 min or until the

RNase-free water or TE (RNase-free) was completely absorbed by the matrix and centrifuge for 2 min at full speed to elute the purified RNA.

2.4.4 RNA gel and electrophoresis

2.4.4.1 Gel preparation: This method make for a Large-sized gel (20-40 samples), final volume is 300 ml. Weight 3 g of agarose. Add 30 ml of 10X MOPS and Complete to 207.5 ml with DEPC water. Then, microwave and cool down, add 62.5 ml of formaldehyde, add 5 µl of 10 mg/ml ethidium bromide and pour gel into gel box.

2.4.4.2 Sample preparation: In the sample tube compose 5.5 µl RNA, 1.0 µl 10X MOPS, 3.5 µl formaldehyde, 10 µl formamide, and 2 µl 5X loading dye. The final volume is 22 µl. Heat the sample at 65 ^{o}C for 15 min. Do a quick spin and keep the sample on ice until loading. Migrate the gel under the chemical hood. Wash the overnight with distilled water. Take the picture of the gel.

2.4.5 qRT-PCR analysis

2.4.5.1 First-Strand cDNA Synthesis: The synthesis start by combine the following in a 0.2- or 0.5-ml tube: 5 µg of total RNA, Primer 1 µl (50 µM of oligo(dT)$_{20}$, or 2 M gene-specific primer (GSP), or 50 ng/µl random hexamers), 10 mM dNTP mix, and up to 10 µl DEPC-treated water. All tubes were incubated at 65 ^{o}C for 5 min, then place the tube on ice for at least 1 min. Preparing the following cDNA Synthesis Mix by add each component in the indicated order (for 1 reaction): 10X RT buffer 2 µl, 25 mM $MgCl_2$ 4 µl, 0.1 M DTT 2 µl, RNaseOUT^{TM} (40 U/ µl) 1 µl, and Superscript^{TM} III RT (200 U/ µl) 1 µl (for control reaction, add DEPC-treated water instead Superscript^{TM} III RT (200 U/ µl)). 10 µl of cDNA Synthesis Mix was added to each RNA/primer mixture, mixed gently, and collected by brief centrifugation. All solutions were incubated as follow: 50 min at 50 ^{o}C for oligo(dT)$_{20}$, GSP primed, and for Random hexamer primed, incubate 10 min at 25 ^{o}C and follow by 50 min at 50 ^{o}C. After incubated, terminate the reactions at 85 ^{o}C for 5 min, chill on ice, collect the reactions by brief centrifugation, add 1 µl of RNase H to each tube and incubate for 20 min at 37 ^{o}C. cDNA synthesis reaction were made 10-fold dilution. Then, they were stored at -20 ^{o}C or were used for PCR immediately.

2.4.5.2 PCR: The transcripts of interested metabolic genes and *ACT1* gene were PCR amplified, using MxPro QPCR machine and MxPro QPCR software for analysis. The reaction mixtures were placed in preheated (95 ^{o}C) thermal cycler. Then, they are performed an initial denaturation step: 95 ^{o}C for 10 min. They perform 40 cycle PCR: Denature 95 ^{o}C for 30 sec, Anneal 55 ^{o}C for 1 min, and Extend 72 ^{o}C for 30 sec. Upon completion, reactions were maintained at 4 ^{o}C. For analysis, 10 µl of each sample were used for agarose gel electrophoresis and ethidium bromide staining for confirmation. The DNA sequences of the oligonucleotides used for RT-PCR analysis are given in Table 4.

Table 4. Oligonucleotides used for analysis by RT-PCR

Oligonucleotides	DNA Sequence
Ethanol	
MAE1……..	AAGAGAGGGCGACTCGAGAC and CGGTGTTCATCTACAATTGG
TKL1 ……..	CAATTGTCATTTAGAAAGCT and CAGTCTTAC CAGACA ACGTT
TOR2……	CAGCAATTAAATGAATAAAT and ATCTAGTTCTGAATCTAC AA
HXT9……	CACCACTTGGGTTCTGTTTC and ATACGCCCATTTATTTTCGA
HXT11……..	ACGTATTCTTAGGCTGTCTG and TTGCCGGAAGAGAACTTCAT
Lactate	
ERG1……	GTCGCTTTAAATGTCTGCTG and ACTAACTTGTTCGCCGTTGA
ERG3……	CATTCTTTAAAT GGATTTGG and AACAATAACCCATAGTGAGA
DLD1……	ATGGTAACTTCCATGCAT TC and CGACGACAAATGGTGCCGTA
ERG11 SOD…….	CAAGATCTGAATGTCTGCTA and CAGTCATGACTCTTCCTAAC
ERG11 YHR…….	AAGGTCGTAAATGTCTGCTA and CAGTCATGACTCTTCCTAAC
SUT1……	TTCACAATAAATGTCCACAA and CAAACAATGGTGGAGTGTAA
Glycerol	
OPI1…….	TGACAAATAAATGTCTGAAA and TAGTCGTAATCATCATGCTC
GUT1……	CGACTCCGCTTAAATG and GTTGTGAGCTCATTTA
GUT2……	CTGGATGACTAGCCAAGGTG and TCAACAAGGGCCACATTGAG
GLN1 ……..	AACAAACTAGATGGCTGAAG and GCTTGGTTGGTAGAAGAACC
FAS1 ……	GACAATGAATGAATGGACGC and AGAAGATACGTAGCCAAGGA

บทที่ 3

ผลการวิจัย (Results)

Part 1: Identification of zinc cluster genes involved in nonfermentable carbon metabolism and characterization of their functions through phenotypic analysis

Part 2: Examination of zinc cluster proteins' target genes and implicated pathways in the regulatory network

Part 1: Identification of zinc cluster genes involved in nonfermentable carbon metabolism and characterize their functions through phenotypic analysis

Growth on fermentable carbon sources

S. cerevisiae wild type and zinc cluster deletion strains were grown in SD medium containing 2% fermentable carbon source either glucose, galactose, mannose, xylose, arabinose, sucrose, maltose or cellobiose. The results showed that the deletion strains Δ*ybr150c,* Δ*ybr240c,* Δ*ycr106w,* Δ*ybr033w,* Δ*yil130w,* Δ*ypl133c,* Δ*yll054c,* Δ*yol089c,* Δ*ynr063w,* Δ*yor380w,* Δ*yjl206c,* Δ*yer184c* and Δ*yfl052w* c grew well on all fermentable carbon sources tested as the wild-type strain (data not shown).

Growth on non-fermentable carbon sources

Growth assays were performed with different non-fermentable carbon sources, using strains listed in Table 1. The results showed that the wild-type and the deletion strains grew normally on SD- media containing glucose as carbon source and showed moderate growth on media containing glycerol or potassium acetate as non-fermentable carbon sources. Some deletion strains namely *Δyil130w, Δypl133c, Δyer184c, Δyor380w* and *Δyfl052w* strains grew slightly on ethanol as non-fermentable carbon source, compared with growth of the wild-type strain (Table 5). Previous study showed that deletion of open reading frame (ORFs) *YER184C, YFL052W, YIL130W, YLL054C, YLR266C, YOR162C, YOR380W* and *YPL133C* which encode for putative zinc cluster regulators impair growth on glycerol or lactate as non-fermentable carbon sources (Akache *et al*., 2001). Our phenotypic analyses of these strains showed that deletion of five different zinc cluster genes sensitive to growth on ethanol as non-fermentable carbon source. These putative zinc cluster proteins or regulators may be involved in regulation of genes encoding for enzyme in the alternative carbon metabolism such as gluconeogenesis, glyoxylate cycle or the tricarboxylic acid cycle (TCA cycle). Zinc cluster Cat8 has an essential role during the adaptation of yeast on ethanol by controlling the induction of many genes in gluconeogenesis, glyoxylate cycle and ethanol utilization following the investigation on expression of genes affected by the deletion of *CAT8* [24]. The importance of Cat8 and another transcription factor Adr1 cooperatively control expression

of genes involved in ethanol utilization during the diauxic shift. Activation of these two transcriptional regulators requires the active Snf1 protein kinase complex at the diauxic transition [25]. Both Cat8 and Sip4 regulators involve in the derepression of *FBP1* gene in gluconeogenesis in response to glucose starvation [26]. To learn more about the function of these zinc cluster proteins, Chromatin immunoprecipitation on chip (ChIP-chip assay) of zinc cluster transcriptional regulator Rds2 (*YPL133C*) and showed that the Rds2 regulates genes in the gluconeogenesis, glyoxylate cycle or TCA cycle pathway. Moreover, Rds2 is also bound to the promoters of respiration genes whose expression is increased during a shift from glucose to ethanol [27]. Thus, at least three C_6 zinc cluster regulators, Cat8, Sip4 and Rds2, are required for expression of genes in alternative carbon metabolism and are under controlled by Snf1 kinase [26, 27, 28]. While the zinc cluster transcriptional regulators are dispensable for growth on rich carbon source such as glucose, they are important for growth, survival or gene regulation during growth on non-fermentable carbon sources.

Heat sensitivity

Sensitivity of the zinc cluster deletion strains to heat was tested at temperatures of 30˚C, 37˚C or 42˚C. Our results indicated that some deletion strains such as *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* show impaired growth on glycerol or potassium acetate containing media at 37˚C. Strikingly, slight growth of *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* strains was observed at this temperature during growth on ethanol. All deletion strains including the wild-type strain showed no growth on all carbon sources tested at 42˚C (Table 5). Heat shock transcription factor (HSF) is an evolutionally conserved protein that mediates eukaryotic transcriptional response to a variety of stress conditions, including heat shock, oxidative stress, glucose starvation, pH, pharmacological reagents and other conditions [29]. Yeast *S. cerevisiae* encodes a single HSF protein that responds to heat stress and glucose starvation and provides a simple model system to investigate how a single HSF is activated by multiple stresses and similar to mammalian *HSF1* [30]. The effect of heat sensitivity on growth of zinc cluster deletion strains was investigated at high temperatures (37^{O}C and 42^{O}C). Our results showed that the deletion strains showed impaired growth on media containing ethanol or acetate as a sole carbon source at 37^{O}C in comparison with growth on glucose (Table 5). We hypothesized that increased temperature may trigger cellular signals that

affect the binding of zinc cluster protein factor to DNA during the transcription process or interfere with the regulation of genes involved in the alternative carbon metabolism and thereby prevent normal growth. Moreover, all strains could not growth on all carbon sources tested at $42^{O}C$ indicating that yeast cells could not tolerate high temperature (data not shown). For instance, the HSF induced *CUP1* gene is expressed during glucose starvation and is dependent on the Snf1 kinase [31]. The heat shock-induced proteins are also controlled by other transcription factors Msn2 and Msn4 that activate transcription of target genes in response to various stresses. The roles of Hsf1 or Msn2/4 were observed by comparing the heat shock response between wild-type and deletion strains of these genes following a shift to high temperature. They found that these transcription factors regulate two different physiological roles in the heat shock cellular response [32]. The Hsf1p regulon is mostly composed of chaperons and associated proteins in the care of damage proteins [33], whereas the Msn2p/4p regulon includes antioxidants and enzymes involved in carbon metabolism. The Msn2/4p-dependent gene induction through the stress response elements (STREs) is sensitive to the cAMP-dependent protein kinase (PKA), which is under the control of the Ras/cAMP signaling pathway [34]. It surely will be interesting to further investigate the role of these zinc cluster proteins in heat shock response.

Anaerobic conditions

Yeast is a facultative anaerobe. Here, the effect of oxygen deprivation is studied using strains listed in Table 1. We tested the effect of growing zinc cluster deletion strains in absence oxygen. In the presence of oxygen, the wild-type and zinc cluster deletion strains grew normally on media containing glucose, and moderately on media containing glycerol or potassium acetate and slightly on ethanol as a sole carbon source. Under anaerobic condition, the wild type and zinc cluster deletion strains grew decreased compare with in presence oxygen (Table 5). Moreover, deletion strains were able to grow less well as wild type in absence oxygen after incubation in anaerobic chamber at $30^{O}C$ for 7 days. The previous reported that the effect of growing deletion strains under anaerobic conditions was tested to identify zinc cluster genes necessary for growth in phenotype. All deletion strains were found to be able to grow in well in anaerobic condition in both YPD rich or SD minimal media while deletion of *HAP1* which was reported the only zinc cluster protein is essential. In fact, Hap1 was later characterized and

implicated in respiration [35]. Rox1 and Mot3 transcriptional regulators are repressors of anaerobic genes in aerobic cells. Repressor synthesis is stimulated by heme, which de-repress a mechanism controlling expression of both *ROX1* and *MOT3* in anaerobic cells. Co-repressors of the anaerobic genes Tup1-Ssn6 which Rox1 and Mot3 dependent for repression of *ANB1* are also involved [35]. However, two transcription factors do not show synergistic repression under anaerobic conditions.

pH conditions

In pH conditions, the wild type and zinc cluster deletion strains grew normally on YP-media containing glucose, some deletion strains namely *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* grew moderately on media containing ethanol or glycerol and no grew on media containing potassium acetate as a sole carbon source in various pH 4.0 acidic conditions on YP-media (Table 5). Normally, in various pHs 6.2, the results showed that the wild-type and the deletion strains grew normally on YP-media containing glucose as carbon source and showed moderate growth on media containing glycerol or potassium acetate as non-fermentable carbon sources. Five deletion strains grew slightly on ethanol as non-fermentable carbon source (Table 5). In pH 8.0 alkaline condition, the result showed that zinc cluster deletion strains grew moderately on media containing glucose comparison to growth of wild type. Five deletion strains also grew slightly on YP-glycerol and no growth on YP-ethanol or YP-potassium acetate while wild type grew moderately on each media (Table 5).

Sensitivity to sodium chloride or lithium chloride salt

Growth of zinc cluster deletion strains was observed during growth on different non-fermentable carbon sources, containing sodium chloride (NaCl) at final concentration of 0, 1.0M or 1.2M, lithium chloride (LiCl) at final concentration of 0, 0.3M or 0.5M in comparison to growth of the wild-type strain. The results showed that some deletion strains showed moderate growth on glucose media containing 1.0M or 1.2M NaCl salt (Table 5). These deletion strains showed moderate growth on glucose media containing 0.5M LiCl while these deletion strains grow normally SD-media containing LiCl salt at concentration 0.3M (Table 5). In addition, the deletion strains such as *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* grew slightly on ethanol media increased salt concentrations as compared to the wild-type strain (Table 5). Similarly, growth of

these deletion strains on glycerol was also impaired. Interestingly, wild-type and all deletion strains could not grow on potassium acetate containing NaCl at concentration 1.0 or 1.2M. Whereas, some deletion strains such as *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* grew slightly on ethanol media or potassium acetate media and grew moderate on glycerol media at salt concentration 0.3M LiCl. Growth of these deletion strains on glycerol was also impaired at salt concentration 0.5M LiCl but five deletion strains cannot grew on ethanol or potassium acetate (Table 5). The abundance of salt such as sodium ions in natural environments allows yeast and many other eukaryotic cells to develop mechanisms to maintain a relatively low intracellular concentration of this cation or to sequester it into organelles. The yeast *S. cerevisiae* is a model organism used often for cation tolerance studies (Table 5). Our experiment on salt stress showed that deletion of some putative zinc cluster genes which encode for zinc cluster proteins exhibited less growth on glucose, ethanol, glycerol or potassium acetate media containing high salt concentrations (Table 5). The results were more pronounced when acetate was used as sole carbon source. Mendizabal *et al*. has identified genes encoding transcription factors relevant to halotolerance with similar amino acid sequences for four putative yeast transcription factors (Hal6-9) [36]. This study showed that Hal9 has a putative zinc C_2-Cys_6 binuclear cluster motif and disruptions of *HAL9* gene decreased both salt tolerance and *ENA1* expression. Ruiz and Arino reported that *ENA1* gene is most expressed and encodes for a putative ATPase necessary for Na+ and Li+ efflux. Moreover, *ENA1* expression increases in response to saline or alkaline pH stress [37]. Norbeck and Blomberg *et.al.* [38] have identified responsive proteins during growth in media containing NaCl and showed that many enzymes involved in central carbon metabolism in *S. cerevisiae* display salt dependence of expression. Thus, there appears to be a connection between carbon source utilization and salt stress, further examination on the role these putative zinc cluster regulators and their involvement of cell integrity or cell stress will be warrant.

Table 5. Summary of phenotypes resulting from deletion a gene encoding for zinc cluster protein in *S. cerevisiae* under various different conditions

		Carbon source				Increased sensitivity		Anaerobic	Salt tolerance			pH response		Acetic acid sensitivity
	Gene Name	Glucose	Ethanol	Glycerol	Potassium acetate	37^{O}C	39^{O}C		LiCl	NaCl	KCl	4.0	8.0	
Wild-type		+	+	+	+	+	+	x	xxx	+	+	+	+	+
YBR150C	*TBS1*	+	+	+	+	+	+	x	+	+	+	+	+	+
YBR240C	*THI2*	+	+	+	+	+	+	x	+	+	+	+	+	+
YCR106W	*RDS1*	+	+	+	+	+	+	x	+	+	+	+	+	+
YBR033W	*EDS1*	+	+	+	+	+	+	x	+	+	+	+	+	+
YIL130W	*ASG1*	+	xx	x	x	+	X	x	+++	x	x	+	x	xxx
YPL133C	*RDS2*	+	xx	x	x	x	Xx	x	+++	x	x	+	x	xxx
							x							
YLL054C	-	+	+	+	+	+	+	x	+	+	+	+	+	+
YOL089C	*HAL9*	+	+	+	+	+	+	x	+	+	+	+	+	+
YNR063W	-	+	+	+	+	+	+	x	+	+	+	+	+	+
YOR380W	*RDR1*	+	xx	x	x	+	Xx	x	x	x	x	+	x	xxx
YJL206C	-	+	+	+	+	+	+	x	+	+	+	+	+	+
YER184C	-	+	xx	x	x	+	X	x	+	x	x	+	x	+
YFL052W	*ROP1*	+	xx	x	x	+	Xx	x	+	x	x	+	x	xx

+ normal growth +++ better growth x moderate growth xx impaired growth xxx low growth

Part 2: Examination of zinc cluster proteins' target genes and implicated pathways in the regulatory network of non fermentable carbon metabolism

Genome-Wide Location Analysis (ChIP-chip) and confirmation via PCR method

Based on our results of phenotypic analysis, genes were selected for studies. *YIL130W, YPL133C* and *YFL052W* will be characterized. The design primers were used in PCR to generate Myc tag of *S. cerevisiae* zinc cluster proteins, Yil130w, Ypl133c and Yfl052w. Each pair of PCR epitope tagging (PET) primers (Table 2) were designed to complementary to the tag-URA3-tag cassettes on the p3XMYC and homologous to the target gene of interest. PCR products of *YIL130W, YPL133C* or *YFL052W* contained Myc tag-URA3-tag cassettes run on 0.8% agarose gel with 1X TAE buffer using electrophoresis machine, the result show *YPL133C* contained Myc tag-URA3-tag cassettes in Fig. 6. The PCR product compose of promoter of interesting gene segment, Myc tag-URA3-tag cassettes and Open reading frame (ORF) of interesting gene segment following illustration of Fig. 4.

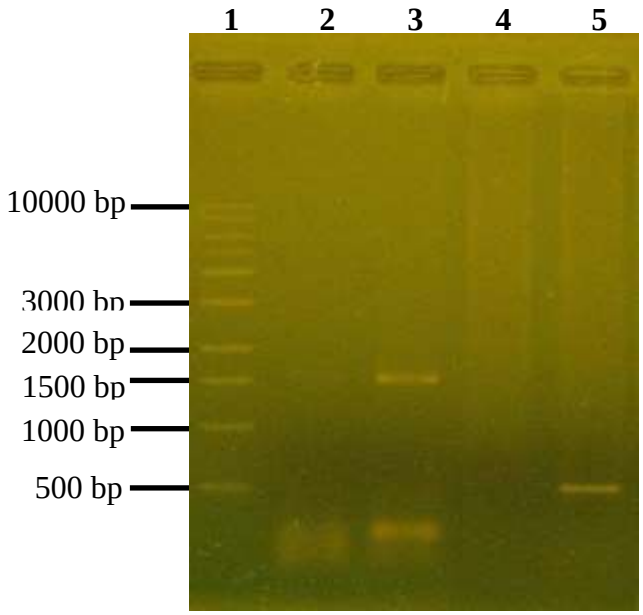


Figure 6 Each of cleaned PCR products of the zinc cluster proteins; YPL133C contained tag-URA3-tag cassettes. The size of PCR products estimates 1500 bp. From left to right; lane 1: 1 kb ladder marker, 2: negative sample, 3 cleaned PCR product of YPL133C, 4: negative control HA-RDS2, 5: positive control HA-RDS2, respectively.

Insertion each of cleaned PCR products of the YPL133C contained Myc tag-URA3-tag cassettes into yeast *S. cerevisiae* FY73 strain using chemical Lithium acetate transformation and selected transformant on SD selective media lacking uracil. The result showed transformants of Myc tagged YPL133C (fig. 7) on SD media plates lacking uracil.

Figure 7 Transformants of *YPL133C* contained Myc tag-URA3-tag cassettes on SD plates lacking uracil

The target gene ORF was N-terminally tagged at its natural chromosomal location with a triple MYC epitope. The correct integration and sequence of the 3XMYC epitope tagging were confirmed by gene specific PCR of the PCR product following illustration of Fig. 4. The specific oligonucleotides primer of Myc tagged YIL130W, YPL133C and YFL052W listed in Table 3. PCR products confirmed transformants contained Myc tag-URA3-tag cassettes into YPL133C show in fig. 8.

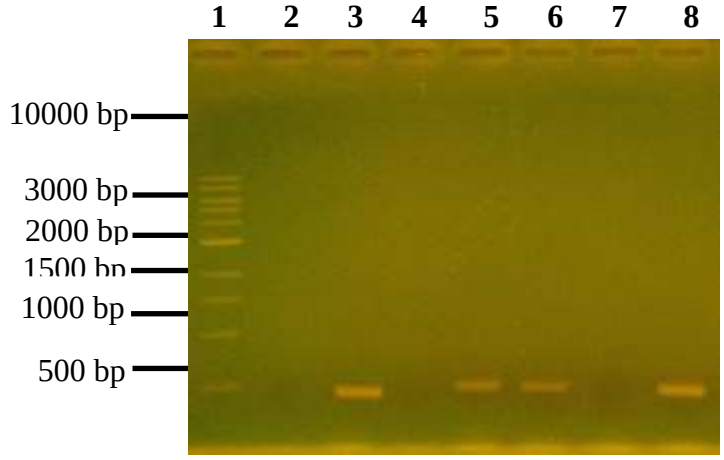


Figure 8 PCR products of the transformants; YPL133C contained Myc tag-URA3-tag cassettes. The size of PCR products estimates 550 bp. From left to right; lane 1: 1 kb marker, 2: negative control HA-RDS2, 3: positive control HA-RDS2, 4 : negative sample YPL133C, 5,6,7,8 : positive sample YPL133C transformants number 1, 2, 3 or 4, respectively.

Selection on FOA media and confirmation via PCR condition

Transformants of Myc tagged YPL133C were cultured on YPD liquid to allow internal recombination between sequences encoding Myc epitopes and *ura3*$^{-}$ strains, serial dilutions 10X, 100X and 1000X, respectively and selected *ura3*$^{-}$ strains on SD plates containing 5-fluoroorotic acid (FOA). Internal recombination of 3XMyc show that the *ura3*$^{-}$ strains grew on FOA media (Fig. 9). Only *ura3*$^{-}$ strains can grew on FOA media. The *ura3*$^{-}$ strains were confirmed Myc epitope tagging by gene specific PCR of the PCR product and oligonucleotides listed in Table 3, result show in Fig. 10. The Myc-tagged YPL133C were confirmed compared with non-tagged YPL133C (Fig.11)

Figure 9 The *ura3*$^{-}$ strains of Myc tagged YPL133C selected on SD media containing 5-floroorotic acid (FOA).

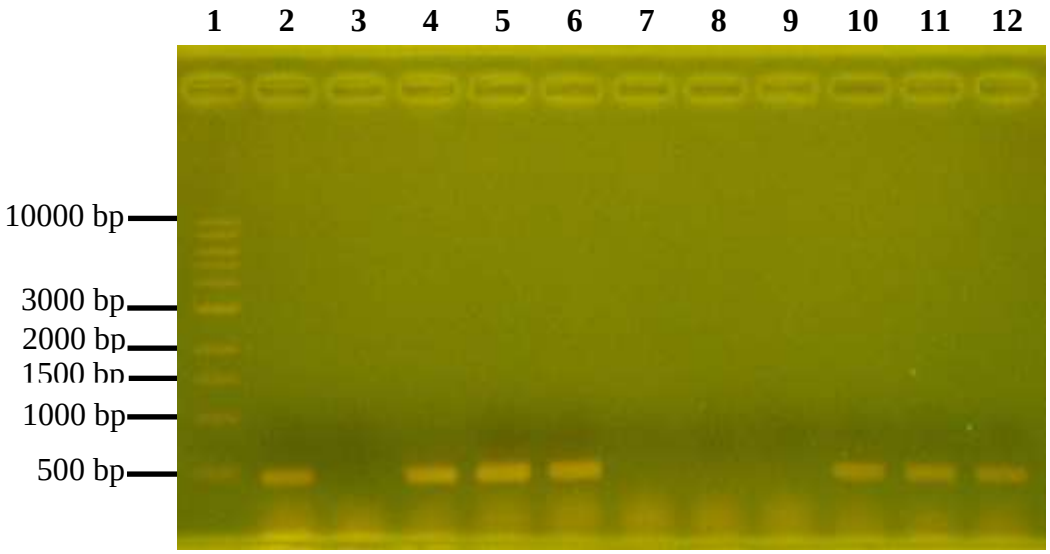


Figure 10 PCR products of the transformants; YPL133C contained Myc epitope tag without URA3. The size of PCR products estimates 550 bp. From left to right; lane 1: 1 kb marker, 2: positive control HA-RDS2, 3: negative control HA-RDS2, 4,5,6,: *ura3*$^{-}$ colonies of YPL133C clone 1, 7,8,9: *ura3*$^{-}$ colonies of YPL133C clone 2, 10,11,12: *ura3*$^{-}$ colonies of YPL133C clone 4, respectively.

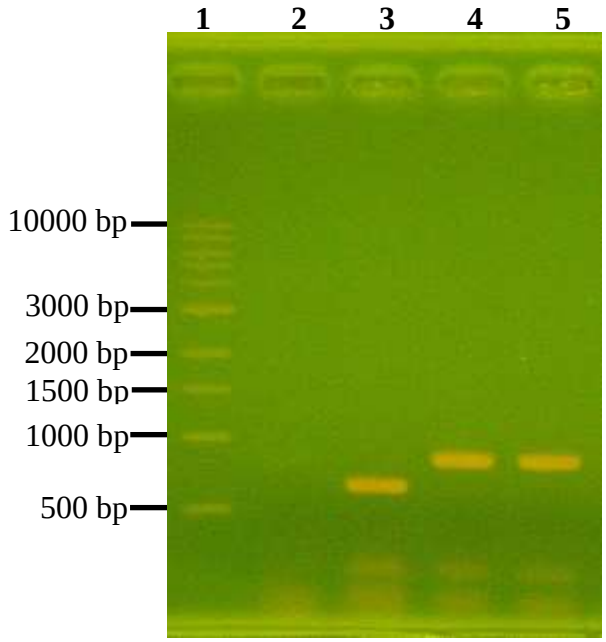


Figure 11 Confirmation of PCR products; YPL133C contained Myc epitope tag without compared with non-tag YPL133C. The PCR products size of Myc-tagged YPL133C or non-tagged YPL133C estimates 850bp or 550 bp, respectively. From left to right; lane 1: 1 kb marker, 2: non-tagged YPL133C, 3 and 4: Myc-tagged YPL133C, respectively.

Table 6. PCR epitope tagging primers sequence

Primer	Sequence
PET1-YLL054Cadd60	5’-AAC AAA GAT GAT GTG AAG TAA TTT ACC CAT CAG AAA TCT ATG ACA AG-3’
PET2-YLL054Cadd60	5’-TTA TCA CAT TTG ATT TTC CTT TGC TTA CACCGC AAGCAA ACA AAC GAT GG -3’
PET1-YER184Cadd60	5’-AAG CGC ATT AGG TTA ACG ACA TTA TTG TTG TTT AAA TTT TAA GCT TTT TA-3’
PET2-YER184Cadd60	5’-TTA ATC TTT TTC CTA TGG CAT CTA TCA CAG GCC TTG GTT ACC CTA GAT TT -3’
PET1-YOR380Wadd60	5'-TTC AAG TAG AAA CCT GAA AGA ACT TCA GTA AGA TAA CTT AGC GCA CAC TT-3'
PET2-YOR380Wadd60	5'-TTA CGT TCC CTA CAG GGC ACG CA A GCC TTT CGG ACT CTT TGT CGC TTG TG-3'

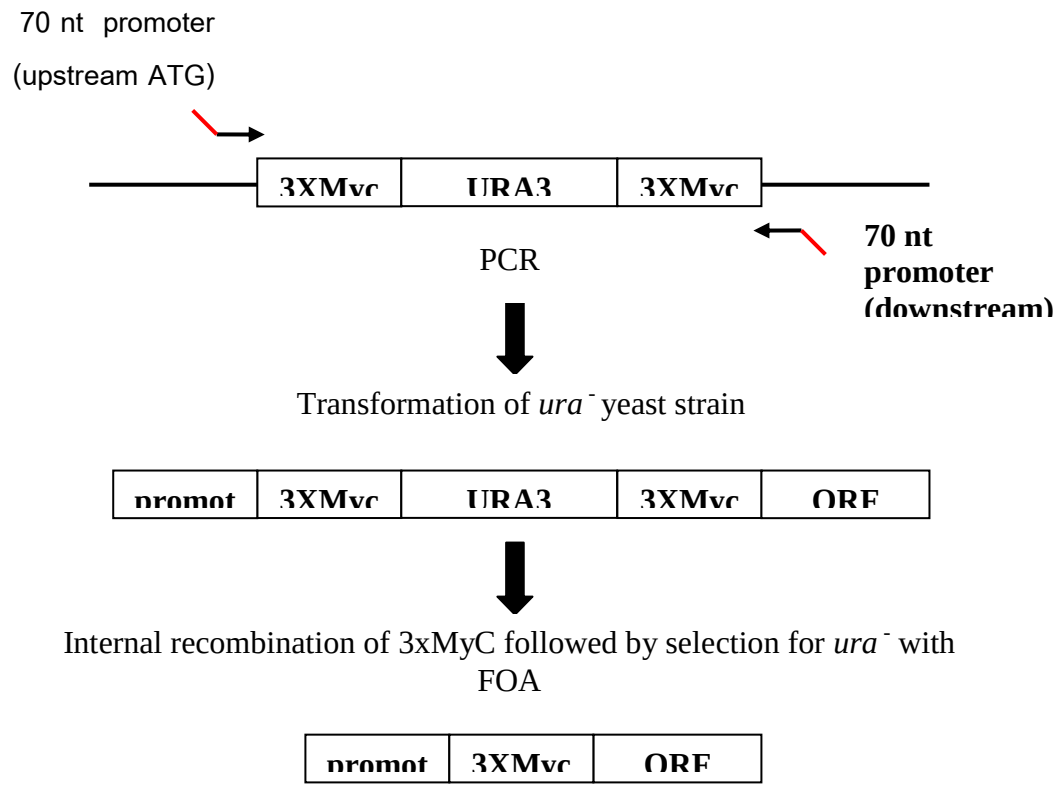


Figure 12. PCR epitope tagging (PET) strategy for your favorite gene (YFG).

Yeast transformation

Methods to transform DNA in to yeast cells is lithium acetate method. Yeast cells for transformation were grown in a fresh overnight culture 5 ml of YPD broth at 30°C with shaking at 250 rpm. Overnight culture was used to inoculate 50 ml YPD broth and to regrow the cells to OD_{600nm} of 0.6-0.7 at 30°C at 250 rpm. This lead to required two cell doublings and will take 4 hr. Cells were pelleted by centrifugation at 3,000 rpm for 5 min. at room temperature. The supernatant were poured off and the pellet was gently resuspended in 15 ml autoclaved water. Spinning was performed at 3,000 rpm 5 min. at room temperature.

Yeast cell were gently resuspended in 500 µl TE/LiAC. After that, 50 µl of suspension of yeast cells, 5 µl of boiled ssDNA and 10 µl of PCR product were mixed by several gentle inversions. Followed by addition of 30% PEG mixing the contents by several gentle inversions again. The mixture was then incubate at 30°C with shaking at 200 rpm for 1 hr. Heat shock at 42°C in a water bath for 15 min. Pellet were kept by quick centrifuge. Discard the supernatant and gentle suspend the pellet 100 µl of sterile water, using the end of a micropipet tip to agitate the pellet, followed by a few gentle pumps of the pipet. The cells can then be plated on to URA^- dropout plates (SD-URA media) and incubated at 30°C for 3-5 days to obtain transformants.

Selection of transformants and PCR confirmation

After transformation for 3-5 days, colonies usually appear. Ura $^+$colonies were tested individually by gene specific PCR. Extracted genomic DNA from

transformant colonies by the method as follow: Grow a fresh overnight culture of yeast in 5 ml of YPD broth at 30°C with shaking at 250 rpm incubator shaker. Transfer 1.5 ml of the culture into an eppendorf tube. Spin for 5 sec. in a microcentrifuge. Discard the supernatant and briefly vortex the tube to suspend the pellet in the residue. Add 200 µl Tiny prep mix (2% Tritron-X, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.1, 1 mM EDTA). Add glass beads until just below the level of the liquid. Add 200 µl phenol-chloroform. Vortex the content for 2 min. and spin for 5 min. Transfer the aqueous phase to new tube. Add 2 volumes of 100 % ethanol and precipitate for 20 min. at -20°C. Then, centrifuge for 15 min. at 4°C. Wash the pellet with 500 µl 70 % ethanol and spin for 5 min. Remove the supernatant and dry the pellet for 20 min. Complete use the pellet by suspend in 40 µl sterile water. Dilute the suspension of extract DNA to 100X-200X and then add the dilute to a typical PCR reaction describe above. Primers were directed against the target gene and the epitope tag such that only correctly integrated epitope tags would yield amplified bands. Genomic DNA obtained from the non-tagged strains was included in PCR reaction as negative control. While genomic DNA from the strain expressing tagged- zinc cluster protein was used as positive control.

Colonies shown to have integrated the epitope tags in the correct position were grown overnight in YPD liquid culture to allow *in vivo* recombination between epitope copies flanking the *URA3* gene. Cultures were washed with water and spread onto 5-fluoro-orotic acid (5-FOA) plates in three ten-fold dilution. PCR was used to ensure that 5-FOA-resistance colonies were result of the complete loss of the *URA3* gene. The gene specific PCR was used to check for correct integration (Design primers as shown in Figure 12 and Table 6). Expression of tagged target gene will be verified by Western blotting.

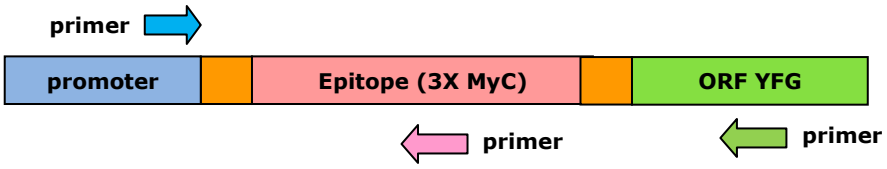


Figure 13. Design primer to check for correct integration shown in a pair of arrows.

Table 7. Design primers sequence to check for correct integration.

Primer	Sequence
PROM550RDS2	5' GTG CGC TGA AGT GCT A 3'
REVTAGKOKO	5' TCC AGC TTT TGT TCC CTC AT 3'
PROM550YER184C	5' GGC TTA GTC ATA ATA GAT CG 3'
REV107YER184C	5' TTG ATT GAG ACC CAA TAC AC 3'
PROM550YLL054C	5' CCA GAT ATT TCC CTC A 3'
REV110YLL054C	5’ AGC TAC AAA TCG AGG ATG AG 3’
PROM550YOR380W	5' GTG GTA AAC TAT TCG ATA TC3'
REV110YOR380W	5’ ATC CAT AGG CAA CGC ACA TT 3’
F730YER184C	5' CTG CAT GAA AGA CCT GAT TA 3'
F731YLL054C	5’ TTG GCA TAA CGC TCC CAT CG 3’
F738YOR380W	5’ GGA TCT CCG AAA AGC AGC A 3’
REVURA3	5’ GAA GAA GGC GCC GGA CGA 3’

Amplification of tag -*URA3* - tag cassette

Figure 13. Illustrates the basic strategy used for epitope tagging a interested gene (*YLL054C, YER184C, YOR380W*). The primers are designed such that 50 bases are homologous to target gene of interest. The remaining 18 bases of each PCR amplification primer complementary to vector sequences flanking the epitope tags. This sequence is identical in vector p3XMyc. Depending upon which tagging vector is used in the PCR reaction, PCR product approximately of 1.5 kbp for PCR epitope tagged product is amplified (as shown in Figure 14 and 15).

Transformation and Selection of tagged yeast strains

Yeast cells were grown overnight at 30°C and directly transformed with PCR amplification products by lithium acetated method. After transformation, strains were plate on $^{-}$URA dropout plates and incubated at 30°C for 3-5 days. The URA^{+} transformants were shown in Figure 16. The $^{+}$URA colonies of transformants were confirmed by streak on SD $^{-}$URA plate (as shown in Figure 17.) and were checked for the correct integration of epitope tags by PCR (as shown in Figure 18, 19 and 20). Colonies shown to have integrated the epitope tag correct position were grown overnight in YPD liquid culture to allowed *in vivo* recombination between epitope copies flaking the URA gene. The URA 'pop-outs' were selected on 5-FOA plate as shown in Figure 21. and confirmed again by streak on SD^{-}URA and SD^{+}URA plate (as shown in Figure 22). PCR was used to ensure that 5-FOA resistant colonies were the result of the complete loss of URA gene as shown in Figure 23, 24 and 25.

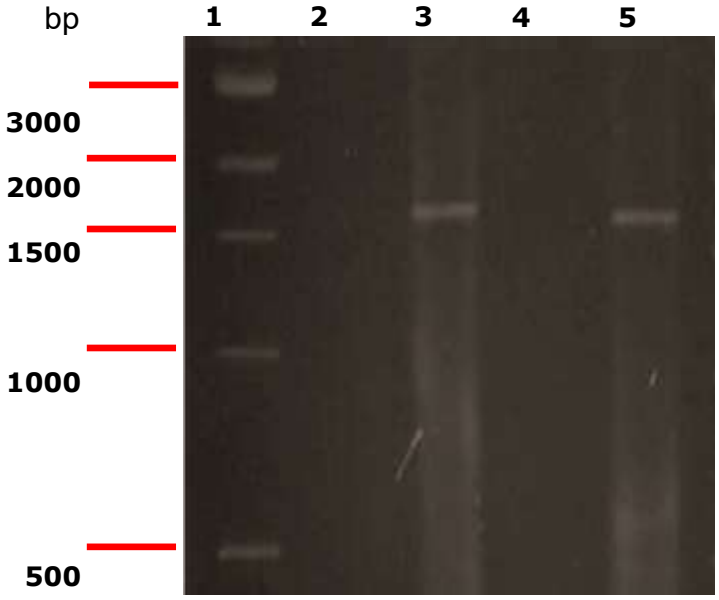


Figure 14. PCR amplification of PCR epitope tagged product, **lane 1** : molecular weight standard, **lane 2** : PCR epitope tagged product of *YLL054C* gene with no template (negative control), **lanne 3** : PCR epitope tagged product of *YLL054C* gene, **lane 4** : PCR epitope tagged product of *YER184C* gene with no template (negative control), **lane 5** : PCR epitope tagged product of *YER184C* gene.

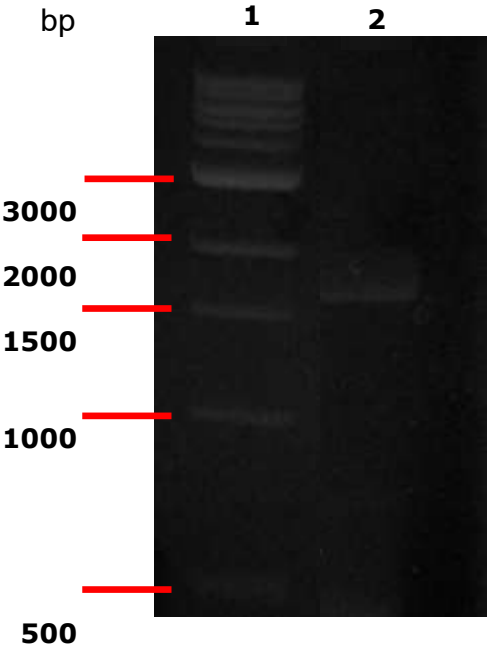


Figure 15. PCR amplification of PCR epitope tagged product, **lane 1** : molecular weight standard, **lane 2** : PCR epitope tagged product of *YOR380W* gene.

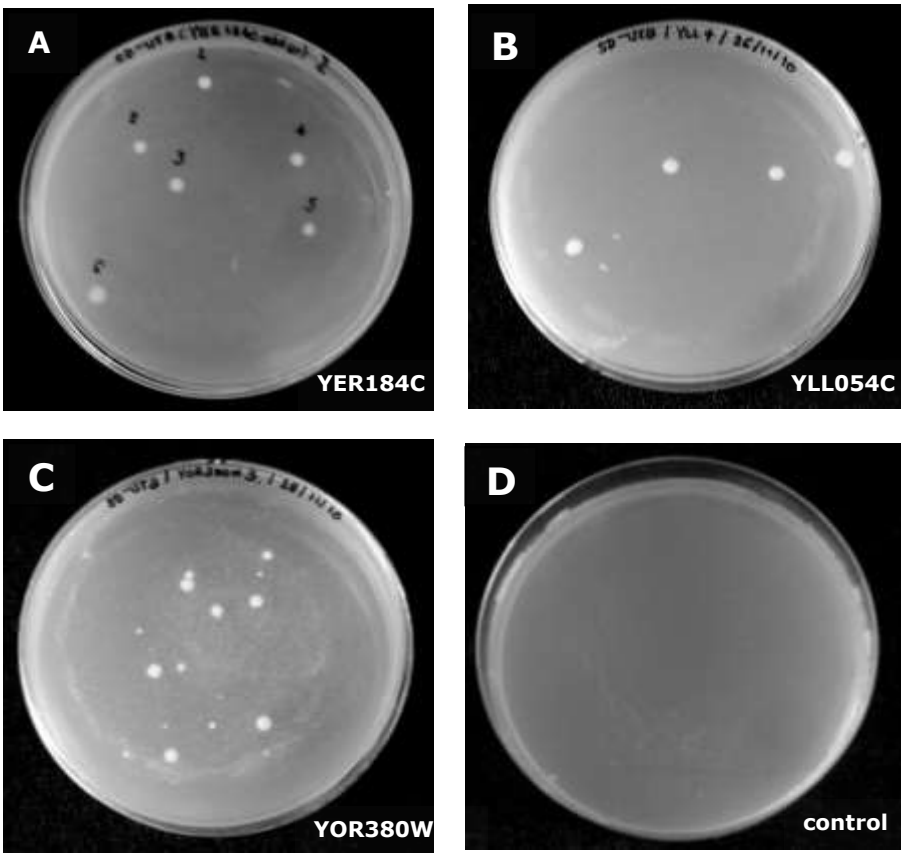


Figure 16. The colonies of $^{+}$URA transformants with *YER184C* (A),*YLL054C* (B), *YOR380W* (C) tagged with the 3x Myc PET construct on SD^{-}URA plate (A) and control plate (D).

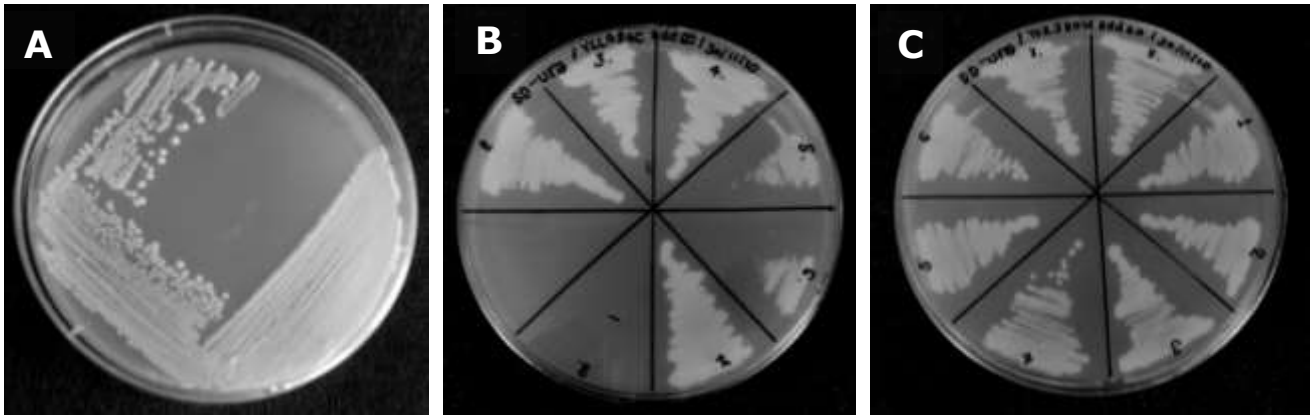


Figure 17. The colonies of $^{+}$URA tranformants with *YER184C* (A), *YLL054C* (B) and *YOR380W* (C) tagged with the 3xMyc PET construct on SD^{-}URA plate.

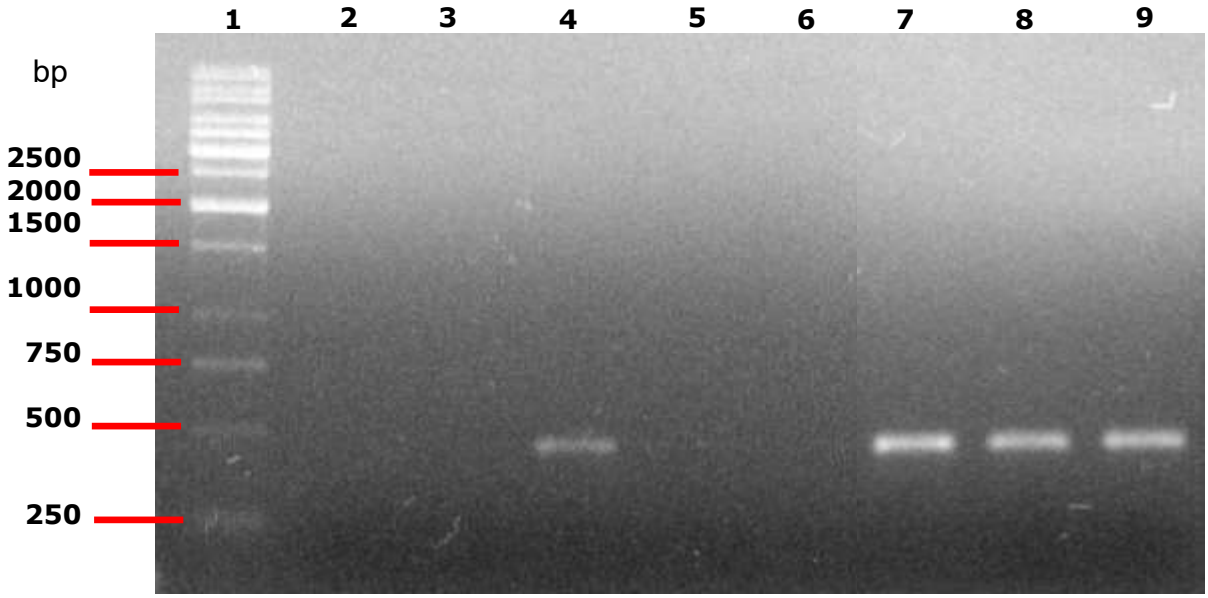


Figure 18. PCR products of transformants with *YER184C* tagged. **lane 1** : molecular weight standard marker, **lane 2** : negative control of tagged strain HA-RDS2, **lane 3** : negative control of tagged strain HA-RDS2 (with no template), **lane4** : tagged strain HA-RDS2 (positive control), **lane 5** : negative control of transformant with *YER184C* tagged, **lane 6** : negative control of transformant with *YER184C* tagged (with no template), **lane 7** : PCR product of transformant no.1 with *YER184C* tagged, **lane 8** : PCR product of transformant no.2 with *YER184C* tagged, **lane 9** : PCR product of transformant no. 3 with *YER184C* tagged.

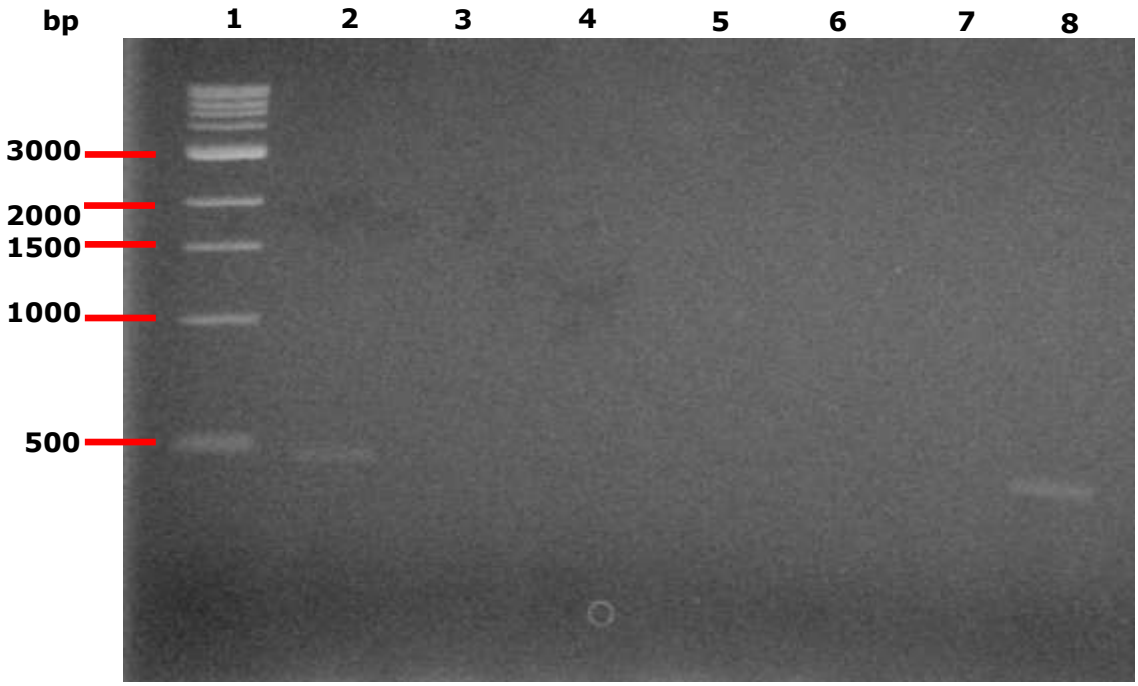


Figure 19. PCR products of transformants with *YLL054C* tagged. **lane 1** : molecular weight standard marker, **lane 2** : tagged strain HA-RDS2 (positive control), **lane 3** : negative control of tagged strain HA-RDS2, **lane 4** : negative control of transformant with *YLL054C* tagged (with no template), **lane 5-8** : PCR product of transformant no.1-4 with *YLL054C* tagged.

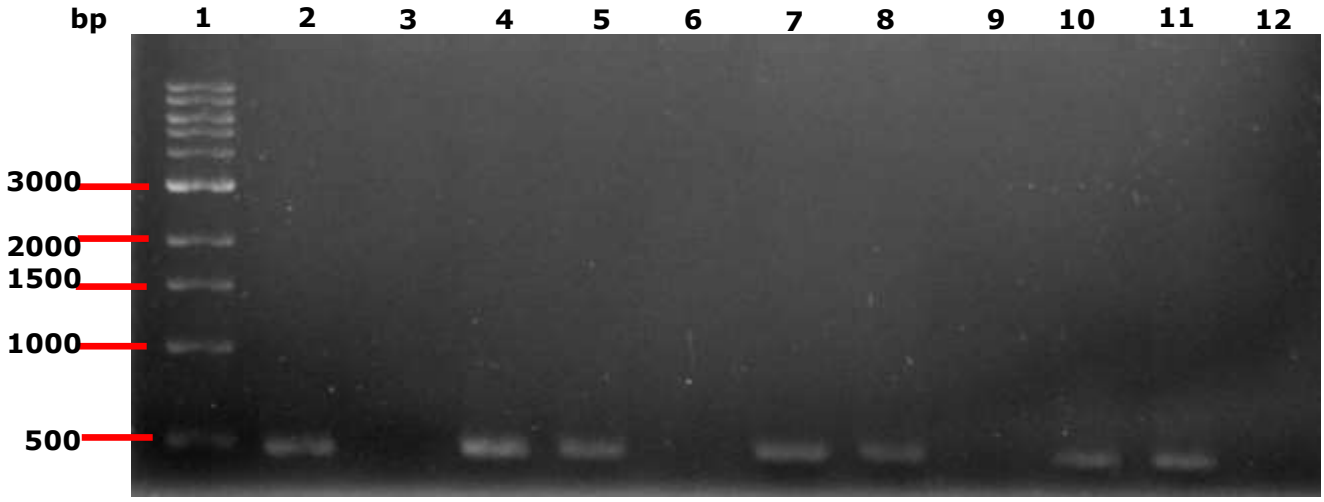


Figure 20. PCR products of transformants with *YOR380W* tagged. **lane 1** : molecular weight standard marker, **lane 2** : tagged strain HA-RDS2 (positive control) , **lane 3 -12 :** PCR product of transformant no.1-10 with *YOR380W* tagged.

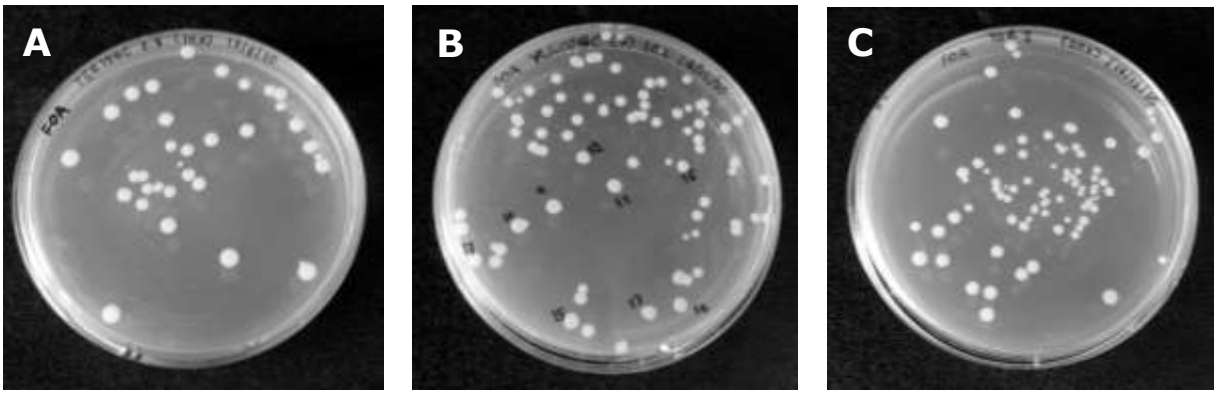


Figure 21. The colonies of transformants with *YER184C* (A), *YLL054C* (B) and *YOR380W* (C), tagged after selection on 5-FOA plate for URA pop-out.

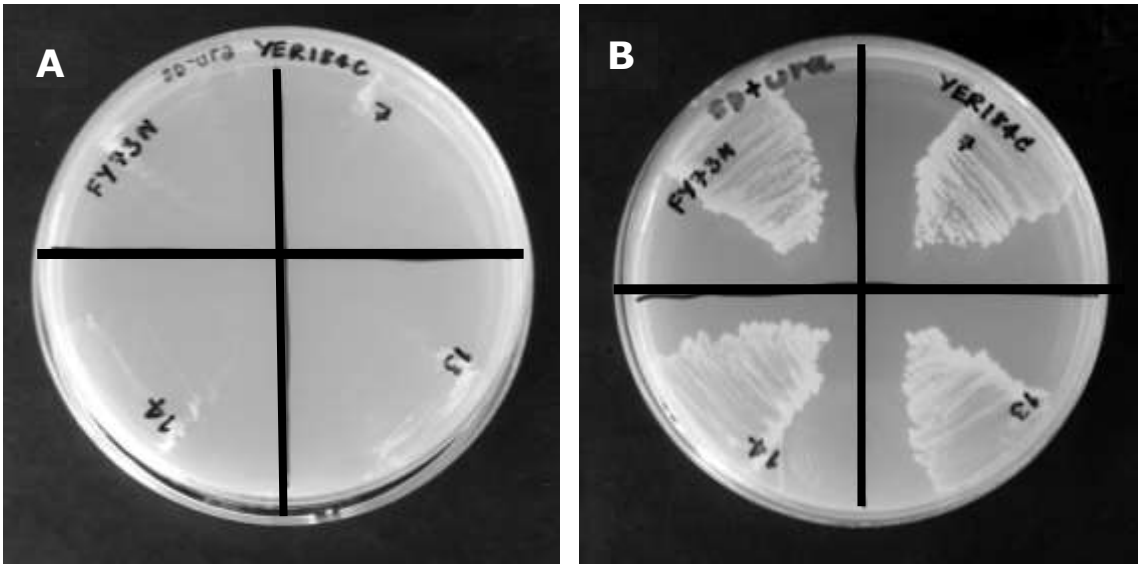


Figure 22. The colonies of transformants with *YER184C* tagged after selection on 5-FOA plate for URA pop-out streak on $SD^{-}URA$ (A) and $SD^{+}URA$ (B)

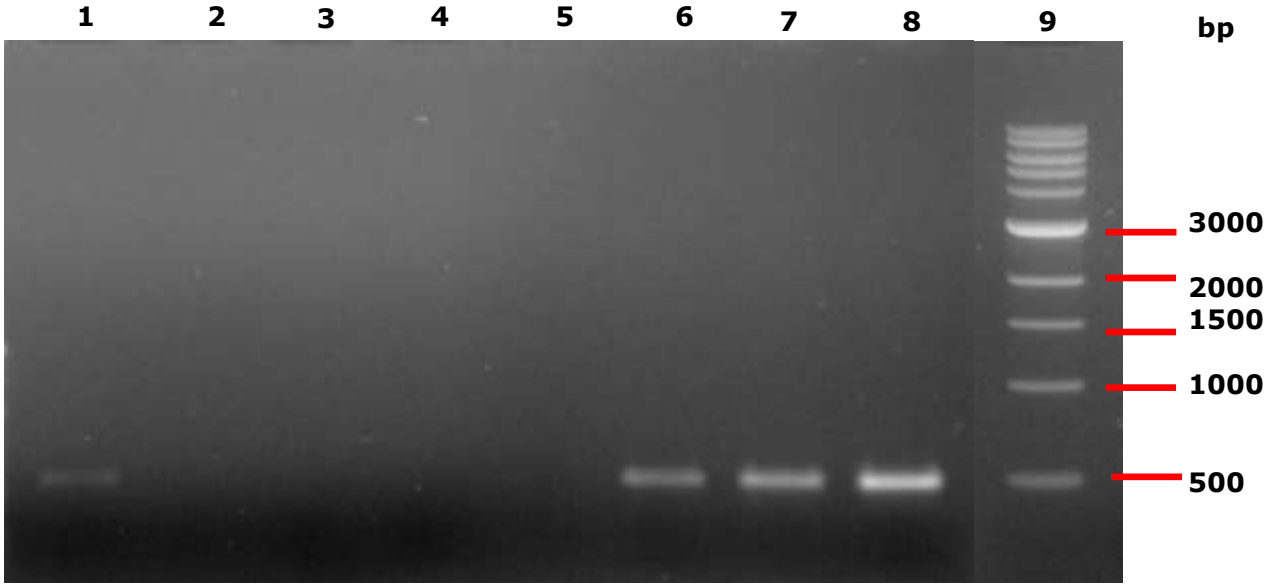


Figure 23. PCR products of transformants with *YER184C* tagged after selection on 5-FOA plate for URA pop-out. **lane 1** : tagged strain HA-RDS2 (positive control), **lane 2** : negative control of tagged strain HA-RDS2, **lane 3** : negative control of tagged strain HA-RDS2 (with no template), **lane 4** : negative control of transformant with *YER184C* tagged, **lane 5** : negative control of transformant with *YER184C* tagged (with no template), **lane 6-8** : PCR product of transformant no.1-3 with *YER184C* tagged, **lane 9** : molecular weight standard marker.

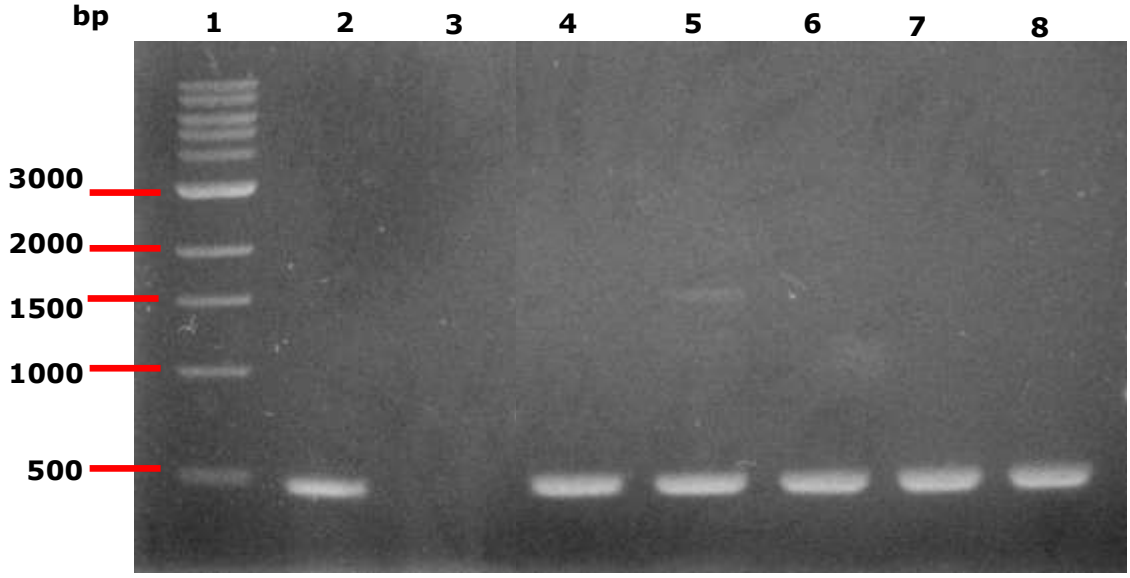


Figure 24. PCR products of transformants with *YLL054C* tagged after selection on 5-FOA plate for URA pop-out. **lane 1** : molecular weight standard marker, **lane 2** : tagged strain HA-RDS2 (positive control), **lane 3** : negative control of tagged strain HA-RDS2, , **lane 4-8** : PCR product of transformant no.1-5 with *YLL054C* tagged.

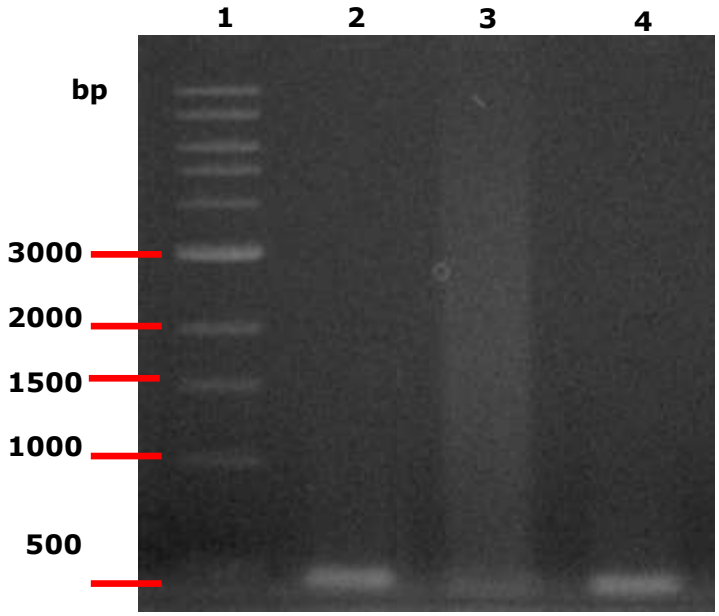
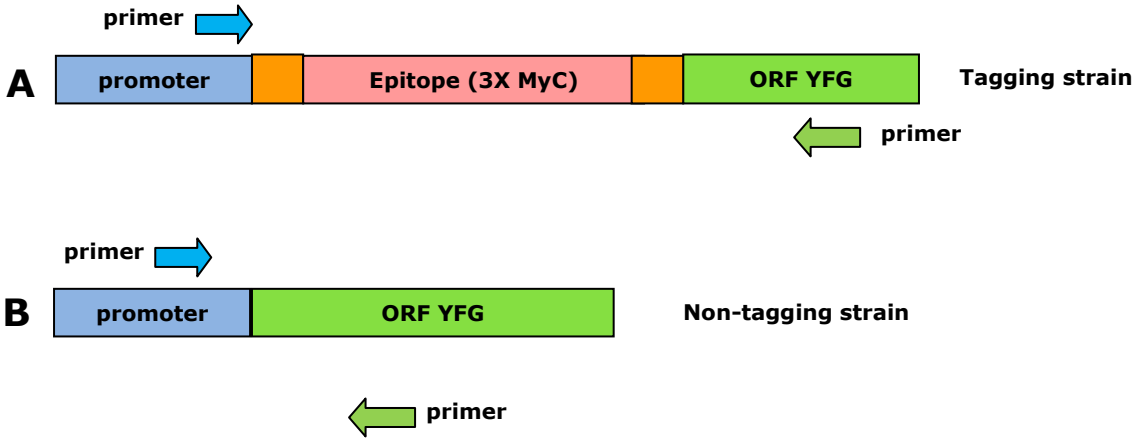


Figure 25. PCR products of transformants with *YOR380W* tagged after selection on 5-FOA plate for URA pop-out. **lane 1** : molecular weight standard marker, **lane 2** : tagged strain HA-RDS2 (positive control), **lane 3** : negative control of tagged strain HA-RDS2, , **lane 4** : PCR product of transformant with *YOR380W* tagged.

PCR confirmation

The gene specific PCR was used to check for correct integration. Design primers were shown in Figure13 and Table 7. The amplification primer (Table 7) was used in concert with primer homologous to sequence in the target gene. The PCR confirmation with design primer was represented in Figure 26 and 27.



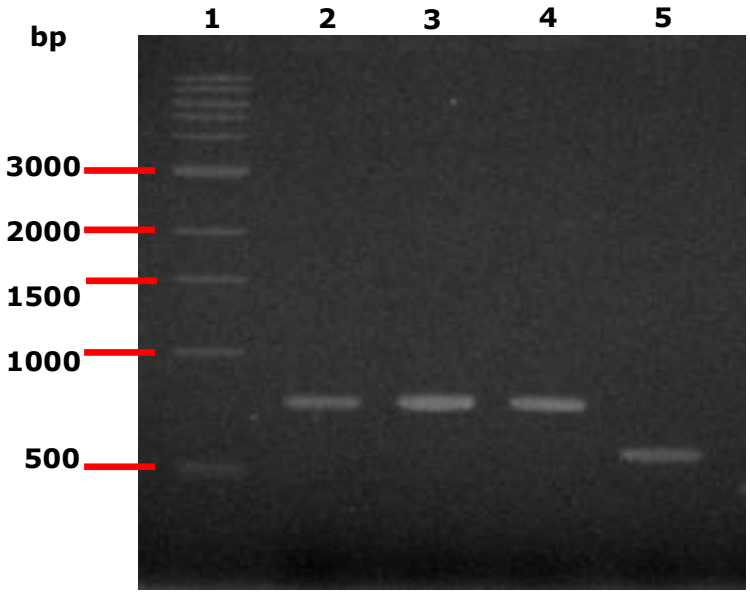


Figure 26. PCR products of transformants with *YER184C* tagged. **lane 1** : molecular weight standard marker, **lane 2-4** : PCR product of transformant no.1-3 with *YER184C* tagged, **lane 5** PCR product of strain with non-tagged.

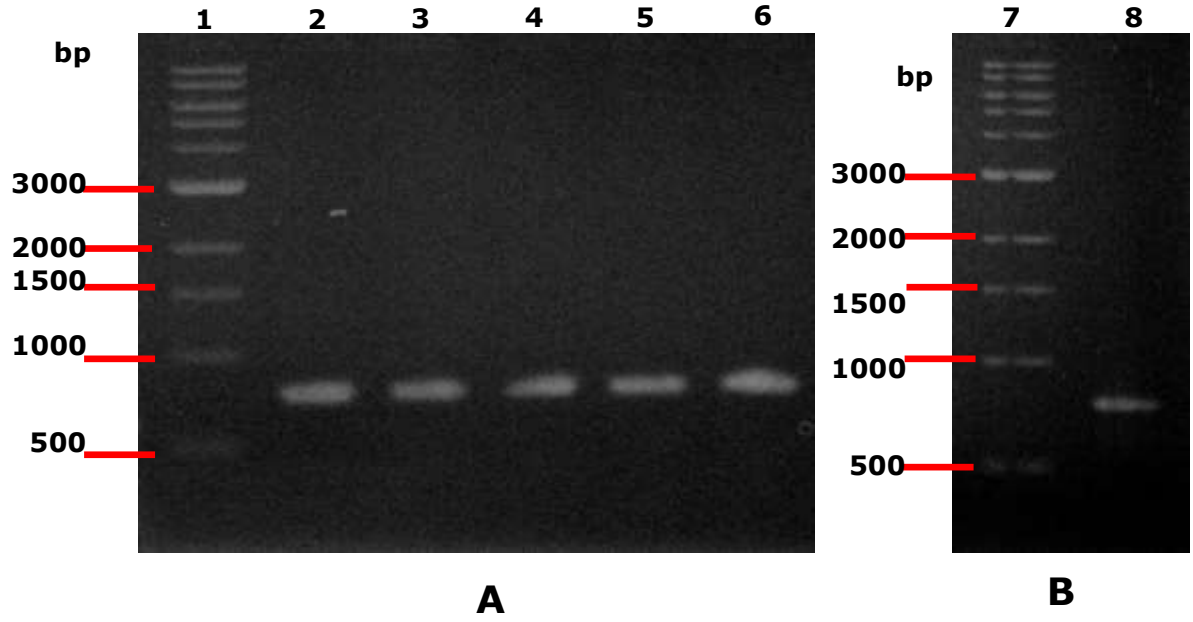


Figure 27. PCR products of transformants with *YLL054C* tagged (A) and *YOR380W* (B). **lane 1, 7** : molecular weight standard marker, **lane 2-6** : PCR product of transformant no.1-5 with *YLL054C* tagged, **lane 8** : PCR product of transformant with *YOR380W* tagged.

Sequence analysis of transformants with *YER184C* tagged

Analysis of the DNA sequence of PCR product of 3 transformants with *YER184C* tagged (YER184C-1MyC, YER184C-2MyC and YER184C-3MyC) revealed in Figure 25, 26 and 27. Comparison the DNA sequence with the design sequences (STD) indicated the similarity to *YER184C* tagging transformants. Schematic drawing of transformants with gene tagging containing region as shown in Figure A. The promoter region is blue box. The polylinker region is orange box. The pink box represented the epitope tag region. Whereas the ORF of *YER184C* region is in the green box. The same color between design sequence (STD) and *YER184C* tagging transformants indicated that the best fit of arrangement. The alignment was performed using EMbooss alignment program.

After the RNA extraction and purification, we run the RNAs on agarose gel via electrophoresis to check for the existence and purity of RNA samples. Fig.28 shows the example result after RNA extraction and purification. In each sample on the gel, we can observe two RNA bands which corresponding to the 18s and 28s ribosomal RNAs. The high purity RNA sample should result in clear and sharp RNA bands as shown on Fig. 28.

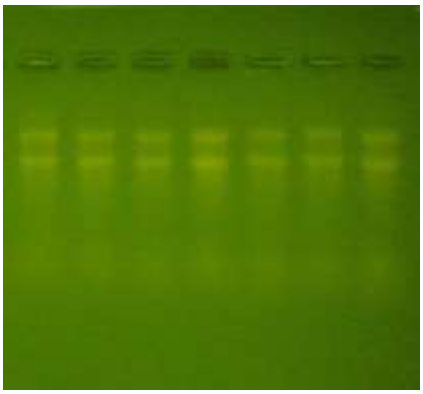


Fig. 28 Example results of our RNAs sample after extraction and purification on the RNA gel electrophoresis

Then, we used real-time PCR to compare the interested gene's transcription level between the wide-type *Saccharomyces cerevisiae BY4741* strain and the deletion strain, *Δrds2* during growth on alternative NF sources. In this research, we examined many genes which involve in the carbon source utilization, including glycerol, lactate or ethanol as a sole carbon source. The list of interested genes and their functions are shown in Table 8. These genes are binding target of *Rds2* regulator during the diauxic shift. [27] Moreover, some genes may also have multiple functions. For example, *OPI1* is encoded for the negative regulator of *GUT1* gene which encodes for the enzyme glycerol kinase. [39] and *TOR2* is also involved the nutrient response.[40]

Table 8. List of the genes used in qRT-PCR studied

Carbon source	Target genes for Rds2	Function
Ethanol	*MAE1*	MAlic Enzyme
	TKL1	TransKetoLase
	TOR2	Target Of Rapamycin
	HXT9	HeXose Transporter
	HXT11	HeXose Transporter
Lactate	*ERG1*	ERGosterol biosynthesis
	ERG3	ERGosterol biosynthesis
	DLD1	D-Lactate Dehydrogenase
	ERG11 (SOD)	ERGosterol biosynthesis
	ERG11 (YHR)	ERGosterol biosynthesis
	SUT1	Sterol UpTake
Glycerol	*OPI1*	OverProducer of Inositol
	GUT1	Glycerol Utilization
	GUT2	Glycerol Utilization
	GLN1	GLutamiNe metabolism
	FAS1	Fatty Acid Synthetase

Many scientific reports showed that the regulation of alternative carbon source utilization is highly complex, involving various relationship among the involved genes. As mentioned, *TOR2* regulates gene expression in response to nutrient starvation. The Tor proteins is itself activated by nutrients, and accordingly regulates the expression of its target genes involved in a wide variety of cellular processes such as in the utilization of nitrogen sources (Ure2 and Gln3), ribosome biogenesis by RNA polymerases, and amino

acid permease stability. [40] For glucose metabolism, The glucose (hexose) transporter such as *HXT* genes (*HXT1* to *HXT17*) and *GAL2*, *SNF3*, and *RGT2* are also involved. [41] We focused our study on the two HXT genes, namely the *HXT9* and *HXT11* because they are target genes of Rds2 regulator. Glucose-6-Phosphate is converted to Inositol-1-Phosphate by *INO1* gene product, encoding for inositol-1-phosphate synthase. This gene is constitutive derepressed by the regulator Opi1, in the presence of inositol. [42]

For glycerol metabolism, glycerol is transported into the cells by the symporter Stl1 (sugar transporter-like protein) and is changed to glycerol-3-phosphate and dihydroacetone phosphate by the glycerol kinase Gut1 and glycerol-3-phosphate dehydrogenase Gut2, respectively. The negative regulator Opi1 mediates repression of the *GUT1*, whereas the effects of the glucoses Mig1 and Mig2 are minor. [39]

Lactate is taken up in the cells through a specific permease called Jen1. *DLD1* and *CYB2* gene products, encoding for two distinct mitochondrial lactate cytochrome c oxidoreductases that can be metabolize D-Lactate and L-lactate to pyruvate, respectively. [43] Acetyl-CoA is a central intermediate in carbon metabolism. It is converted to malonyl CoA and is synthesized the fatty acid by enzyme fatty acid synthase *FAS1* encoded protein. Acetyl CoA is then coupled with oxaloacetate prior to the entrance of the TCA cycle. This TCA cycle pathway releases importance intermediate such as α-ketoglutarate and malate that can be converted to glutamine and pyruvate by *GLN1* and *MAE1* gene products, respectively. Moreover, acetyl CoA is also used in the ergosterol biosynthesis. This pathway involves several *ERG* genes, including the target of Rds2 such as *ERG1*, *ERG3*, and *ERG11*. [44] Yeast cells can also uptake sterol by utilize the *SUT1* gene product. The last interested gene is *TKL1* which encoding for transketolase enzyme in pentose phosphate pathway. It provides glycerol-3-P and fructose-6-P for glycolysis pathway.

Table 9 Results obtained from the qRT-PCR analysis showed the comparative Expression levels of Rds2 target genes the in term of average fold change (RNAs level of the wild-type versus the *Δrds2* strain) during the shift from glucose to other non fermentable carbon source. Detail procedures of gene induction and qRT-PCR condition is described under material and methods)

Carbon source	Target genes for Rds2	Total average fold changes
Ethanol	*MAE1*	-1.5
	TKL1	1.6
	TOR2	2.2
	HXT9	1.5
	HXT11	1.5
Lactate	*ERG1*	-1.4
	ERG3	-2.4
	DLD1	-2.9
	ERG11 (SOD)	2.0
	ERG11 (YHR)	1.8
	SUT1	2.0
Glycerol	*OPI1*	1.4
	GUT1	1.6
	GUT2	1.4
	GLN1	2.2
	FAS1	2.1

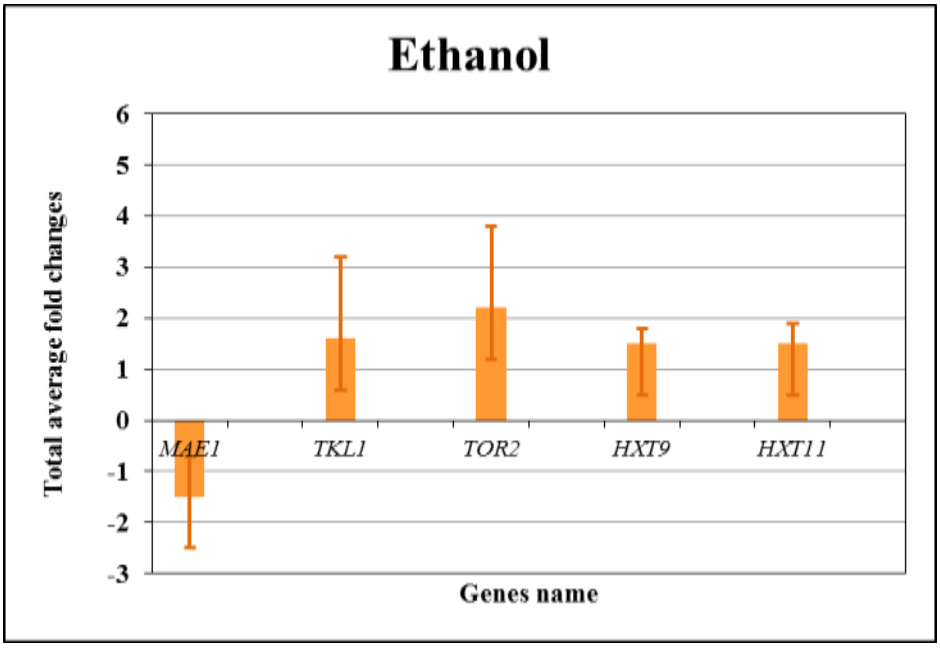


Figure 29. Summary graph of qRT-PCR results in ethanol

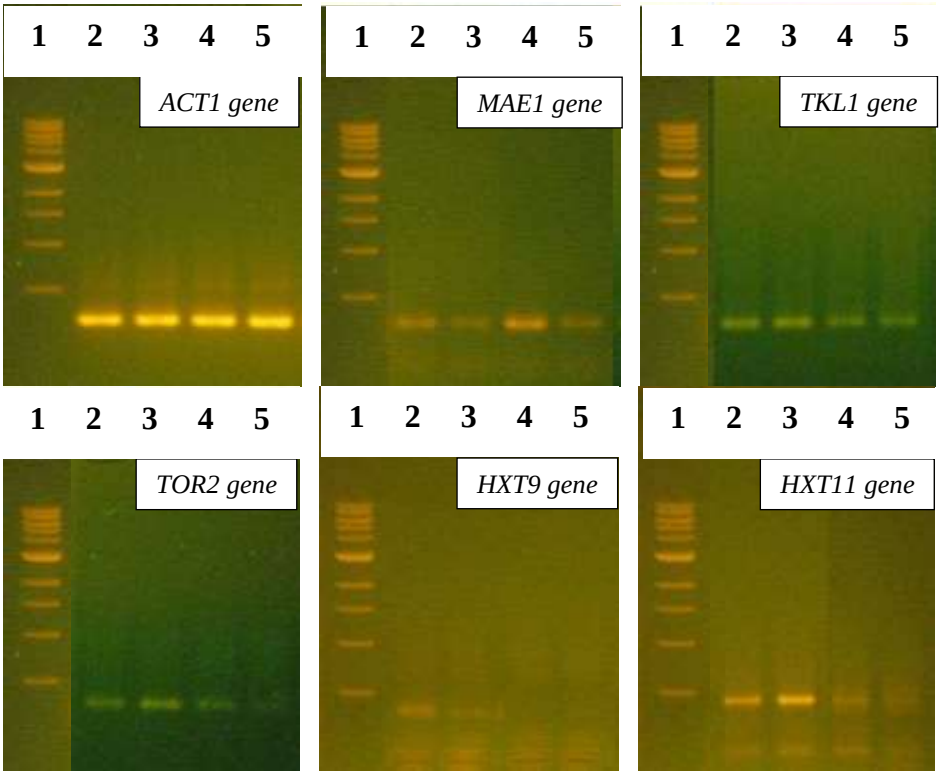


Figure 30. The mRNA levels for *S. cerevisiae* in the absence of Rds2 regulator upon the glucose-ethanol shift (experiment performed in triplicate). lane 1: 1 kb Laddar marker, lane 2-3: *BY4741*, lane 4-5: *Δrds2*

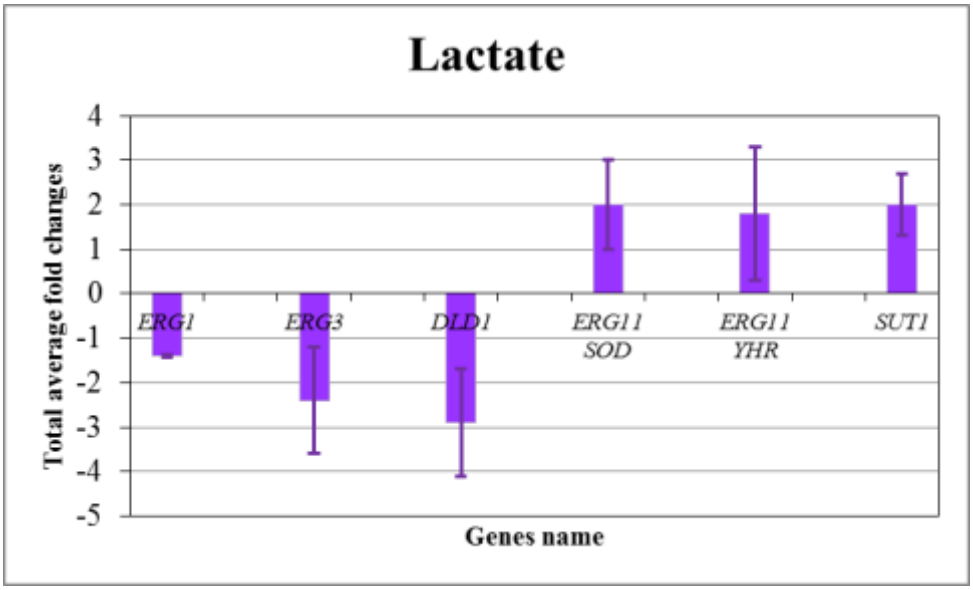


Figure 31. Summary graph of RT-PCR results in lactate

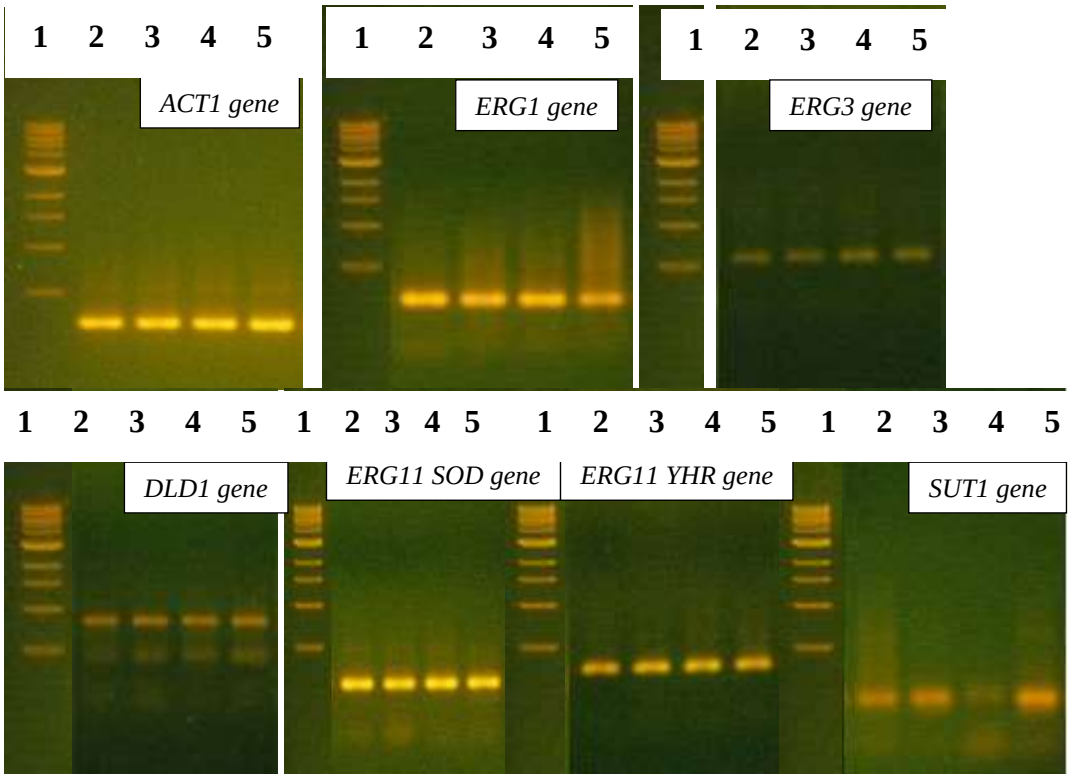


Figure 32. The mRNA levels for *S. cerevisiae* in the absence of Rds2 regulator upon the glucose-lactate shift (experiment performed in triplicate). lane 1: 1 kb Laddar marker, lane 2-3: *BY4741,* lane 4-5: *Δrds2*

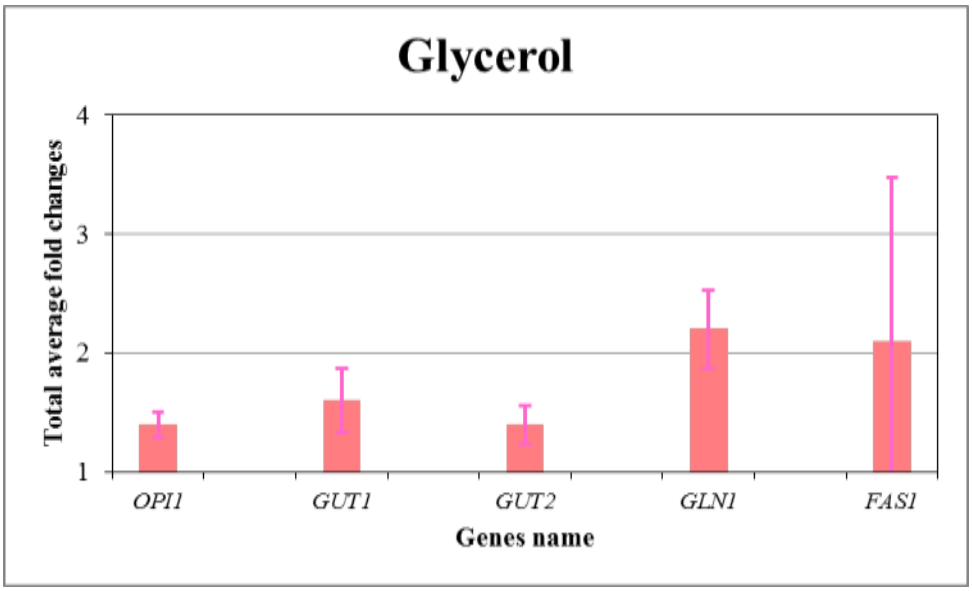


Figure 33. Summary graph of RT-PCR results in glycerol

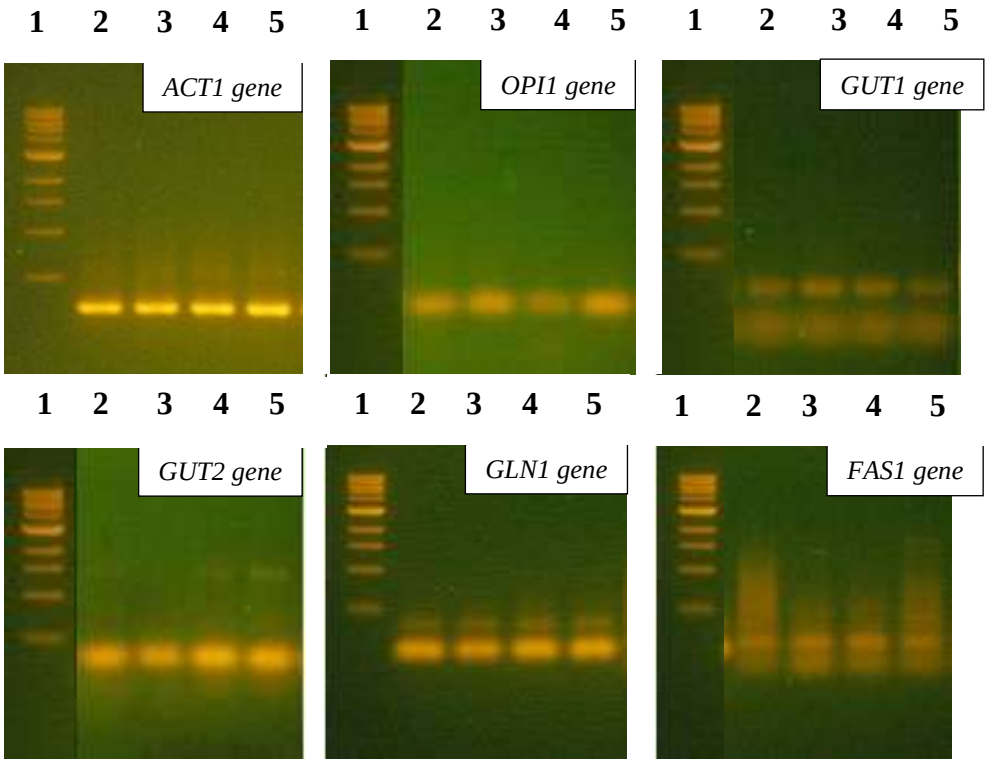


Figure 34. The mRNA levels for *S. cerevisiae* in the absence of Rds2 regulator upon the glucose-glycerol shift (experiment performed in triplicate). lane 1: 1 kb Laddar marker, lane 2-3: *BY4741*, lane 4-5: *Δrds2*

บทที่4

สรุปและข้อเสนอแนะ (Conclusion and Suggestions)

Our results from phenotypic analysis of zinc cluster deletion strains growing on different fermentable carbon sources, non-fermentable carbon sources under various conditions such as, heat sensitivity, anaerobic condition, osmotic stress, pH response and in the presence of acetic acid indicated the involvement of zinc cluster proteins in mediating carbon source utilization. Interestingly, deletion strains namely *Δyil130w*, *Δypl133c*, *Δyer184c*, *Δyor380w* and *Δyfl052w* strains are sensitive to increased temperatures (37^{O}C or 39^{O}C), LiCl, NaCl or KCl salt stress and acidic or alkaline pHs (summary of the phenotypes is given in Table 4). Especially, the same strains are also hypersensitive to combination of ethanol and other stresses. Interestingly, the *Δyil130w* strain is hypersensitive to acetic acid in glucose. In addition, *Δypl133c* and *Δyor380w* are sensitive to acetic acid in glucose. Moreover, both the wild-type strain and the zinc cluster deletion strains are sensitive to carbon sources in anaerobic condition (Table 4). Thus, these putative zinc cluster proteins or regulators may be involved in regulation of genes encoding for enzyme in the alternative carbon metabolism such as gluconeogenesis, glyoxylate cycle or the tricarboxylic acid cycle (TCA cycle). Increasing temperature may trigger cellular signals that affect the binding of zinc cluster protein factor to DNA during the transcription process or interfere with the regulation of genes involved in the alternative carbon metabolism and thereby preventing normal growth. In addition, these zinc cluster proteins may regulate genes involved in osmotic stress, acidic or alkaline pH response and weak acid stress.

To better understand about the function of these zinc cluster proteins, we performed chromatin immunoprecipitation on chip (ChIP-chip assay) to identify direct target genes of these zinc cluster transcriptional regulator. *YIL130W*, *YPL133C* and *YFL052W* were selected for integrated Myc epitope tagging via PCR and homologous recombination method. We obtain two transformants for *YIL130W* gene, two transformants of *YFL052W* gene and four transformants of *YPL133C* gene. These Myc-tagged *YIL130W*, *YPL133C* or *YFL052W* were confirmed epitope tagging at correct location by using specific primer-oligonucleotides via PCR and sent for sequencing. The results of alignment DNA sequences show that DNA sequence of Myc-tagged *YPL133C*

is completed with two positive clones and Myc-tagged *YFL052W* is completed with one positive clone. We assume that the zinc cluster proteins may be phosphorylated by protein kinases and activated in stress responses, including carbon limitation, salt stress or under acetic acid response. Thus, in our future study, we will disrupt function of *SNF1* or *KSS1* kinase gene with kanamycin resistance gene using electroporation then selection.

Secondly, we used qRT-PCR to examine the expression of some Rds2 (Ypl133c) target genes under the shift from glucose to other alternative non fermentable carbon sources such as ethanol, lactate and glycerol. We found that the expression of *TOR2*, *TKL1*, *MAE1*, *HXT9* and *HXT11* is altered in the absence of Rds2 regulator in ethanol. Also, the expression of *ERG* genes such as *ERG11* and *ERG3* as well as *DLD1* and *SUT1* is affected by the removal of Rds2 in lactate while the expression of *GLN1* and *FAS1* is affected in glycerol. Thus, the results indicated that Rds2 plays a role in mediating the utilization of these alternative carbon sources through the regulation of these metabolic genes.

Lastly, we examined some of the target genes of Rds2 (Ypl133c) and their involvement in virulence. Although these genes have not been shown to virulence genes, their implication is linked to virulence. It appears that the expression of these target genes is altered upon deletion of *RDS2* gene during growth on non fermentable compounds such as ethanol, lactate and glycerol. This suggests that they are involved in the utilization of alternative carbon sources which has a major contribution to virulence. Further investigation of the role of these new regulators of non fermentable carbon source shall be performed under virulence conditions such as in the presence of blood or immune cells. Such response will surely will lead to dramatic reprogramming of gene expression and enable us to gain further insight into the roles of these regulators in virulence.

Importantly, we also evaluated the possibility of Rds2 to function as new antifungal drug target. Since, Rds2 is involved in mediating sensitivity to antifungal drug particularly to azole which is widely used in the current clinical treatment and that our study shows that Rds2 regulates targets of azole drug such as *ERG11*. A drug that inhibits the activity of Rds2 will prevent the expression of *ERG11*. Thus, the combination of azole drug and the new antifungal drug which inhibits Rds2 function would lead to better treatment of antifungal infection.

Thus, we suggest further characterizing the roles of these regulators in the presence of azole drug and further identifying additional target genes, not only *ERG11*. In

addition, the crystal structure based study or other means to obtain the structure of these zinc cluster proteins shall be done to identify new compounds that will inhibit their functions/activities.

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