EFFECTS OF GALACTOSE AND FRUCTOSE ON THE EXPRESSION OF ISOCITRATE LYASE (*ICL1*) ENZYME IN *Candida albicans*

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2016

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by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

FEBRUARY 2016

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors, Dr. Doblin Anak Sandai and Dr. Hasni Arsad for their undivided support and advice. For without their encouragement, guidance and advice, this dissertation would not be completed. I would also like to thank my laboratory members: Puan Nur Hikmah, Laina Zarisa, Ishola Oluwaseun, Siti Fadhilah, Cheah Hong Leong, Khirun, Nalini and Muwafaq Adam for their support and company. Their company throughout this project keep me cheerful and motivated. I would also like to thank Dr. Kumitaa Theva Das, Dr. Amir Yunus and Dr. Citartan Marimuthu for their generous support and advice.

A special thank to MyBrain15 (Ministry of Higher Education, Malaysia) for the funding of MyMaster scholarship scheme. Thank also extended to the Advanced Medical and Dental Institute (AMDI, USM) for the funding of my project of study.

Last but not least, I would like to express my sincere gratitude to my family for their unconditional love and understanding. Their encouragement and continuous support is the main pillar that keeps me motivated.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADP	Adenosine 5’-diphosphate
ALS	Agglutinin like sequence
AMP	Adenosine monophosphate
ASM	American Society of Microbiology
ATP	Adenosine 5’-triphosphate
Bp	Base pair
cAMP	Adenosine 3’,5’- cyclic monophosphate
cDNA	Complementary DNA
CGD	*Candida* genome database
CLSI	Clinical and Laboratory Standards Institute
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytodine 5’ triphospahte
dGTP	2’-deoxycytosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleic triphosphate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix material
EDTA	Ethyenediaminetetraacetic acid
ESR	Environmental stress response
EtBr	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing

GFP	Green fluorescence protein
GPI	glycosylphosphatidylinositol
HBEC	human buccal epithelial cells
HIV	Human immunodeficiency virus
HOG	High osmolarity glycerol response
HRP	Horseradish peroxidase
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
IDSA	Infectious Diseases Society of America
kDa	kiloDaltons
mRNA	messenger RNA
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
MTL	mating-type-like
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OD	optical density
Oligo	oligonucleotide
ORF	open reading frame
PBS	phosphate buffer saline

PCR	polymerase chain reaction
PL	Phospholipase
PVDF	polyvinykidene difluoride
REC	Reconstitute human epithelium
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
Rpm	revolutions per minute
RT	reverse transcription
SAP	secreted aspartyl proteinase
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulphate
SGD	*Saccharomyces* Genome database
TAE	Tris-acetate/EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
Tris	Tris (hydromethyl)aminomethane
U	Unit (enzyme activity)
%	Percentage
^{o}C	Degree Celsius
w/v	Weight per volume
v/v	Volume per volume
mL	Milliliter
µL	Microliter
mm	Millimeter

Nm	Nanometer
cfu/mL	Colony forming unit per milliliter
mg/L	Milligram per liter
ng/µL	Nanogram per microliter
rpm	Rotations per minute
x *g*	Relative centrifugal force
mM	Millimolar
nM	Nanomolar
EDTA	Ethylenediaminetetraacetate
≥	Greater or equal to
MIC	Minimum inhibitory concentration
C_t	Cycle threshold
α	Alpha
β	Beta

KESAN GALAKTOSA DAN FRUKTOSA KE ATAS EKSPRESI ENZIM ISOSITRAT LIASE (*ICL1*) DALAM *Candida albicans*

ABSTRAK

Sifat kepatogenan dalam *C. albicans* bergantung kepada atribut kecergasan dan juga faktor-faktor virulen yang lain. Ini termasuk sifat kental terhadap tindak balas tekanan dan penyesuaian metabolik. *C. albicans* boleh menduduki kebanyakkan persekitaran dalam ruang badan manusia yang mengandungi kepelbagaian sumber karbon yang berbeza. Asimilasi sumber karbon adalah penting untuk pertumbuhan dan juga untuk meneruskan jangkitan. Tesis ini mengkaji kesan galaktosa atau fruktosa terhadap asimilasi sumber karbon sekunder seperti laktat oleh *C. albicans*. Gen *C. albicans* Isositrat liase (Ca*ICL1*) ditindas oleh penambahan 2% galaktosa atau fruktosa kepada sel-sel yang sedang membiak pada laktat. Gen Ca*ICL1* yang mengekod kitaran glioksilat enzim Icl1 diperlukan untuk pertumbuhan dalam mengunakan sumber karbon sekunder seperti laktat. Enzim CaIcl1 didapati stabil dalam galaktosa tetapi tidak stabil dalam fruktosa. Sebaliknya, kedua-dua galaktosa dan fruktosa mendegradasikan protein *S. cerevisiae* Icl1 (ScIcl1). Saringan laman pengubikuitinan oleh http://www.ubpred.org/ menunjukkan *C. albicans* tidak mempunyai tapak pengubikuitinan dalam enzim glukoneogenik dan kitaran glioksilat berbanding dengan *S. cerevisiae*. Penambahan tapak pengubikuitinan daripada ScIcl1 kepada CaIcl1 memberi kesan kepada degradasi protein Icl1 sebagai tindakbalas kepada galaktosa dalam sel *C. albicans* melalui satu proses yang bergantung pada ubikuitin. CaIcl selepas

penambahan tapak pengubikuitinan menunjukan peningkatan kepada kelajuan degradasi protein apabila terdedah kepada fruktosa. Dengan itu *C. albicans* telah kehilangan radas molekul yang mencetuskan ketidakstabilan protein sasaran sebagai tindak balas kepada galaktosa dan kesannya boleh mengasimilasikan sumber karbon alternatif dan galaktosa dalam masa yang serentak. Ini merupakan faktor dominan yang menyumbang kepada fleksibiliti metabolik seterusnya kevirulenan dalam *C. albicans*.

EFFECTS OF GALACTOSE AND FRUCTOSE ON THE EXPRESSION OF ISOCITRATE LYASE (*ICL1*) ENZYME IN *Candida albicans*

ABSTRACT

The virulence of *C. albicans* is dependent upon fitness attributes as well as virulence factors. These attributes include robust stress responses and metabolic flexibility. *C. albicans* can occupy a variety niches in human, many of which contain a range of different carbon sources. The assimilation of these carbon sources is important for growth and essential for establishment of infections by *C. albicans*. This thesis examines the impact of galactose or fructose upon the assimilation of secondary carbon sources such as lactate by *C. albicans* Isocitrate lyase gene (Ca*ICL1*) is repressed upon addition of 2% galactose or fructose to lactate- grown cells. The Ca*ICL1* gene, which encode the glyoxylate cycles enzymes isocitrate lyase are required for growth on non-fermentable carbon sources such as lactate. However the enzyme CaIcl1 was not destabilized by galactose, but was degraded in response to fructose. In contrast, *S. cerevisiae* Icl1 (ScIcl1) was rapidly degraded in response to either galactose or fructose. Screening of ubiquitination site by http://www.ubpred.org/ showed that *C. albicans* lacks ubiquitination site in gluconeogenic and glyoxylate cycles enzymes as compare to *S. cerevisiae*, Addition of a putative *S. cerevisiae* ubiquitination site at carboxy terminus of CaIcl1 led to galactose- accelerated degradation of this protein in *C. albicans* cell via a ubiquitin-dependent process. In the other hand, CaIcl prior to addition of ubiquitination site was degraded upon exposure to fructose; addition of

S. cerevisiae ubiquitination site to CaIcl1 further increased the rate of protein degradation. Thereby in conclusion, *C. albicans* has lost the molecular apparatus that triggers the destabilization of target proteins in response to galactose and can simultaneously assimilate alternative carbon sources and galactose, a dominant factor that contribute to metabolic flexibility subsequently virulency of *C. albicans*.

CHAPTER 1: INTRODUCTION

1.1 *Candida* spp. and Candidiasis

Approximately 200 species of fungi were recognized as human/animal pathogen (Kwon-Chung & Bennett, 1992; Rippon, 1988). Fungal diseases (mycosis) are often caused by around 50 of these species. Yeast such as *Candida* species, *Cryptococcus (Cr.) neoformans*, and *Cr. gattii* are the well known for causing diseases (Kurtzman *et al.*, 2011). *Candida* spp. are normally harmless and exist in a symbiotic mutualistic relationship with humans and inhabited skin, mucocutaneous tissues, and gastrointestinal tract (Kurtzman *et al.*, 2011). However given opportunity, they can take advantage of local and systemic weakness in host immune system, such as patients undergoing cancer chemotherapy treatment, or infected with HIV or in neonates or when environmental niche become available (for example, after antibiotic treatment), to cause superficial infections, such as vaginitis and oral thrush, and blood stream infection (Pappas, Silveira, *et al.*, 2009; Berman & Sudbery, 2002; Kao *et al.*, 1999). Such infections, termed candidiasis, are the fourth most common hospital-acquired infection in USA (8 out of 100,000 per annum), with 40% mortality rate and approximately 10,000 death per year (Edmond *et al.*, 1999; Kao *et al.*, 1999). Out of all the disease causing *Candida* spp., *C. albicans* is accounted for 40-60% of the cases (Bassetti *et al.*, 2006).

1.1.1 Asexual and Parasexual Reproduction in *C. albicans*

Candida albicans are eukaryotic diploid (2N) sexual yeasts (Kurtzman & Fell, 1998) and can divide asexually or can undergo parasexual reproduction (Ene & Bennett, 2014). *C. albicans MTL* (mating-type-like) loci is homologous to the *Saccharomyces cerev*isiae *MAT* (mating type) loci (Hull & Johnson, 1999). Members of the genus *Candida* are very incongruous and can grow in at least 3 different structures; yeast, pseudohyphae and hyphae, such as *C. albicans* and *C. dubliniensis* (Sudbery *et al.*, 2004; R.A. Calderone, 2002). Other morphology occur during colony switching, white domed colonies switch reversibly to opaque flat colonies (white-opaque switching system) (Slutsky *et al.*, 1987). To enable *Candida* cells to mate, mating type-like locus a (*MTLa*) and *MTLα* cells must switch from white to opaque (Butler *et al.*, 2009). Opaque cells secreted pheromones to form conjugation tubes, and subsequently, tetraploid (4N) cells through nuclear fusion. In order to return to the diploid state, mating products can be induced to undergo concerted chromosome loss (Forche *et al.*, 2008; Bennett & Johnson, 2003).

1.1.2 Clinical Manifestation

Mycoses are generally categorized into three groups: systemic mycose, dermotophytose and superficial mycoses. Although these categories are useful, some mycoses overlap and fall into all three categories, e.g. candidiasis (Bulmer, 1995).

Systemic candidiasis or invasive candidiasis occurs when *Candida* invade and spread via the bloodstream to multiple organs (Parker *et al.*, 1976). *Candida* contamination of

indwelling intravascular catheters may result in Candidemia (Karlowsky *et al.*, 1997). Candidiasis can also occurs after surgical procedures, or injuries to the skin, respiratory tract, or gastrointestinal (Wenzel, 1995) and lengthy therapy with wide range of antibiotics or corticosteroids (Pfaller, 1996). Initially, the patients have irregular, lengthy, or continuous fever and are unresponsive to antimicrobial chemotherapy. Many organ ailment may rise from haematological dissemination, some of these ailment may resolve impromptu under antimycotic therapy; others may result in permanent defects such as loss of vision after endophthalmitis. Substantial *Candida* colonization of the gastrointestinal, respiratory, or urinary tract of a post-surgical or impaired immune system patients, may cause invasion of mucosa or serosa subsequently producing infections such as, cystitis, esophagitis, peritonitis or pyelonephritis via penetration into adjacent deeper tissue. In small children, blockage or unsuccessful discharge in the gastrointestinal or urinary tract be capable of leading to gathering of *Candida* biomass and subsequently forming fungus balls in the the stomach, renal pelvis, or other natural body cavities. There is normally no invasion of the deeper tissue or mucosa. Removal of the fungal mass through surgical drainage should resolves the problem (Jucker, 2003).

1.2 Antifungal Agents

Candida spp. produce a broad spectrum of infections, from non-lethal mucocutaneous disease to invasive process that involve wide range of organs. In order to counter such a wide range of infections, a variety of diagnostic and treatments were required. According to the latest guidelines of the Infectious Diseases Society of America (IDSA), the choice of anti-fungal treatment should be based on the clinical condition of the patient, information on the species and antifungal susceptibility of the fungus isolated

from the patient, drug toxicity level, signs of organ failure and secondary affected drug clearance, and the condition of patient before exposure to anti-fungal drugs (De Rosa *et al.*, 2009; Pappas, Kauffman, *et al.*, 2009). The major groups of antifungals are polyenes, azoles, echinocandins, allylamine and morpholine, and antimetabolite, such as 5-fluorocytosine. These groups are distinguished primarily by the chemical structure and mechanism of action.

Polyene antifungal agents, such as Amphotericin B, pimaricin and nystatin interact with cell membrane components, such as ergosterol in fungi and cholesterol in humans, to form channels, thereby perturbing membrane function causing small leakage of cellular contents from the inside of the fungal cell to the outside (F. C. Odds *et al.*, 2003). Amphotericin B has a relatively broad spectrum of action against dimorphic fungi, molds (e.g. *Aspergillus* spp.), and yeasts (e.g. *Candida* spp., *Cryptococcus neoformans*). Amphotericin B is the only fungal polyene that can be administered systemically to treat visceral infection. The only disadvantages from the use of Amphotericin B are its poor aqueous solubility and toxicity to mammalian cells (F. C. Odds *et al.*, 2003).

Azole antifungal agents inhibit cytochrome P-450-dependent enzyme lanosterol demethylase (also known as 14α-sterol demethylase), which involved in the biosynthesis of ergosterol. Ergosterol is required for construction of fungal cell membrane structure and function (Neely & Ghannoum, 2000; Georgopapadakou & Walsh, 1996). As a result, ergosterol in the cell membrane is depleted; membrane functions and structure are altered, thus inhibiting the fungal growth (Pfaller, 2012).

Echinocandins (anidulafungin, caspofungin and micafungin) are fungal secondary

metabolites, they have a cyclic hexapeptide core with a lipid side-chain responsible for anti-fungal activity by targeting and inhibits the synthesis of β-1,3-D glucan (F. C. Odds *et al.*, 2003). β-1,3-D glucan is essential to the function and structure of the fungal cell wall. Echinocandins causes the formation of a faulty cell wall and leads to instability subsequently cell rupture in yeasts, and abnormal growth of hyphae in molds. Echinocandins have no effect against, *Fusarium*, *Cryptococcus*, *Scedosporium* and *Trichosporon* species or zygomycetes but are are highly effective against *Candida* and *Aspergillus* species (Espinel-Ingroff, 2003).

Allylamine, such as naftifine and terbinafine inhibit squalene epoxidase in ergosterol biosynthesis pathway. Similarly amorolfine, the morpholine drug inhibits downstream of the same pathway (Ryder & Mieth, 1992). The allylamine antimycotic terbinafine is efficient *in vitro* against a variety of disease causing fungi. It can be administered orally and can be applied directly to body surface in the therapy of fungal infections of the hair, skin and nails (Balfour & Faulds, 1992). Clinical study have shown topical and oral terbinafine to be effective in *Candida* nail infections and skin candidiasis (Ryder *et al.*, 1998).

Antimetabolite fungal agents, 5-Fluorocytosine (5-FC) functions by preventing fungi's DNA and RNA synthesis, 5-FC can be used in combination with Amphotericin B to treat serious systemic infection, such as candidosis, cryptococcosis, aspergillosis and chromoblastomycosis. 5-FC is rarely used alone (monotherapy) because of frequent development of resistance. However usage of 5-FC has severe side effects such as hepatoxicity and bone-marrow depression (Vermes *et al.*, 2000).

1.3 Antifungal Resistance

Clinical and Laboratory Standards Institute (CLSI) in the United States has issued standardized methods to test *in vitro* susceptibility of antifungal resistance. Similar testing protocol was issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe (Cuenca-Estrella *et al.*, 2010). A fungus strain is classified as resistance when the fungus are no longer susceptible to an antifungal agent or when the Minimal Inhibitory Concentration (MIC) of the drug exceeds the susceptibility breakpoint for the organism (Kanafani & Perfect, 2008). Antifungal resistance can be categorized into microbial or clinical resistance, or mixed of both category (Turnidge & Paterson, 2007). Clinical resistance occurs when the administration of an antifungal drugs fails to treat a fungal infection (Kanafani & Perfect, 2008). Resistance can be intrinsic (primary) or acquired (secondary). Intrinsic resistance belongs naturally to the fungi. Fungi with intrinsic resistance are resistance to the antifungal agent even before coming in contact with the drug. Example includes resistance of *C. krusei* to fluconazole. Fluconazole resistance among *C. albicans* is an acquired resistance due to the exposure of susceptible strain to the antifungal agent (Marichal *et al.*, 1999). Resistance also occurs when environmental factors causes the replacement of the susceptible species with a resistant one (Pfaller, 2012). MIC values do not always correspond to antifungal therapy and MIC levels are not always reliable (Rex & Pfaller, 2002). The difference between *in vivo* and *in vitro* data followed a pattern of "90-60" rule, infection due to non-resistant isolate react to therapy ~90% of the time, whereas infections due to resistant isolate react ~60% of the time (Rex & Pfaller, 2002).

Better understanding for mechanisms of action of different antifungal agents is

necessary to understand the mechanism of resistance. Mechanisms of resistance in fungi, are caused by mutation in gene encoding target proteins, development of active efflux pumps to reduce concentration of the drug, development of detour pathways and up-regulation of target enzyme. For example, depletion of ergosterol from the fungal membrane is consequence of exposure to azole anti-fungal agent and leads to accumulation of toxic product 14α-methyl-3,6-diol, therefore resulted in redundant growth. *erg3/erg3* mutant inhibits formation of 14α-methyl-3,6-diol (Spampinato & Leonardi, 2013; Pfaller, 2012; Peman *et al.*, 2009; Kanafani & Perfect, 2008).

1.4 Virulence and Pathogenicity in *C. albicans*

Pathogen is microorganism that has gained ability to cause infection of a host and yield disease. It depends on the expression of virulence factors on both sides of host and pathogen. A fungus pathogen can acclimate to the tissue environment and confront the lytic activity of the host's defense. Virulence is the ability of a pathogen to multiply, cause harm to its host and produce disease in patient (Casadevall, 2007). Virulence is attributed to several factors including adherence, secreted degradative enzymes, morphology switching, biofilms and quorum sensing (Fig. 1.1) (Lim *et al.*, 2012).

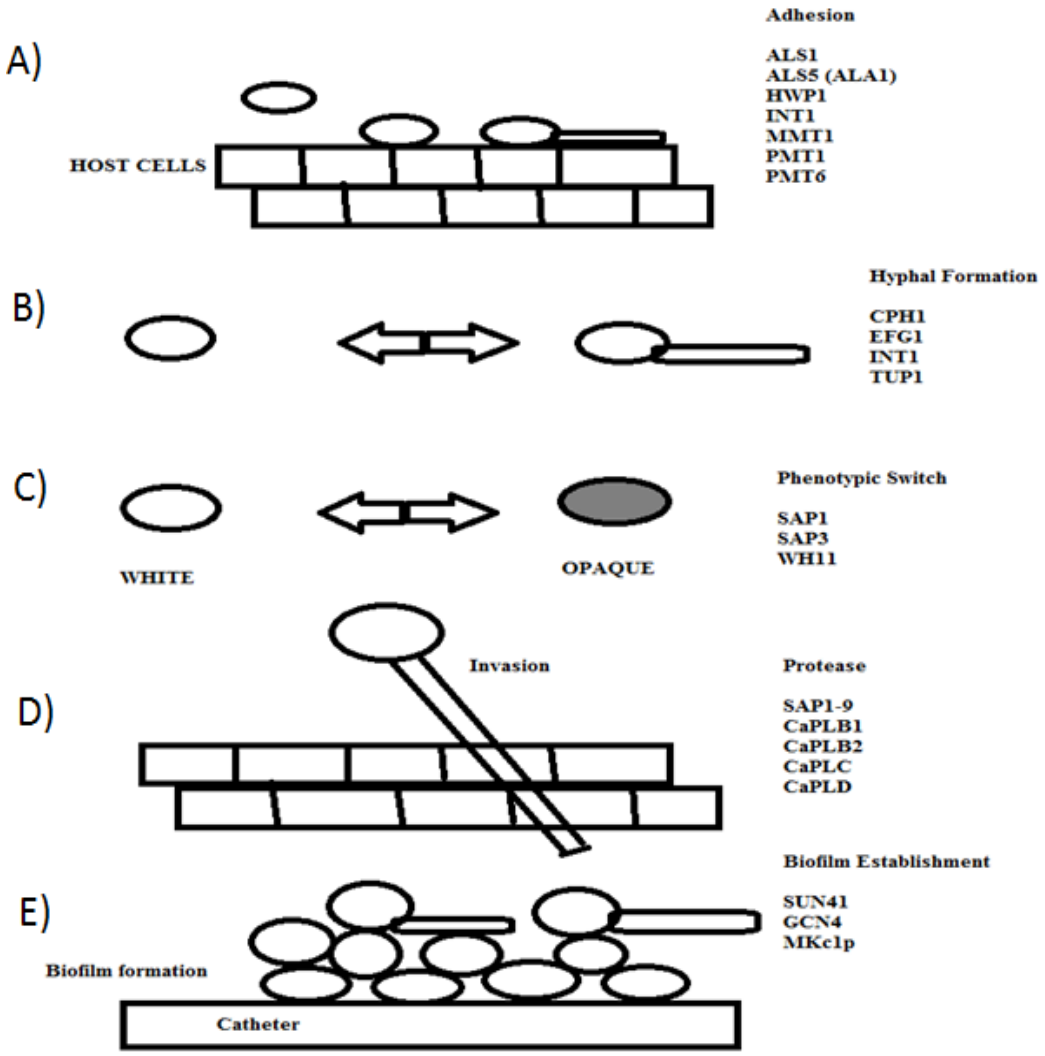

Figure 1.1 Virulence factors of *C. albicans***.** Factors and genes involved in virulence of *C. albicans*. Genes discussed are listed under each category of virulence factors:

A) Genes associated with adhesion of *C. albicans* to host cells
B) Genes associated with formation of hyphal formation of *C. albicans*
C) Genes associated morphological transition and phenotypic switching
D) Genes associated with secretion of protease by hyphae
E) Genes associated with biofilm formation

1.4.1 Adhesion

Adherence of *C. albicans* to host cells is important for commencing and maintaining a commensal relationship (Gaur *et al.,* 1999), as well as for establishment of disease through the settlement of host niches (Chaffin *et al.,* 1998). It is a complex mechanism utilizing several types of adhesins, including agglutinin- like sequence (*ALS*) and hyphal wall protein (Hwp1) (Mayer *et al.,* 2013; Yang, 2003). Glycosylphosphatidylinositol (GPI)- linked cell surface glycoproteins (Als1-7 and Als9) was encoded by *ALS* gene , these proteins are homologous to α-agglutinin, required for cell-cell recognition during mating in *S. cerevisiae* (R. A. Calderone & Fonzi, 2001). Als1, Als3 and Als5 (Ala1) have an attachment function to human buccal epithelial cells (HBEC) and fibronectin, collagen, laminin and endothelial cells (Hoyer, 2001; Hawser & Douglas, 1994). Als4p binds to endothelial cells, Als5 is for cell aggregation, Als6 bind to collagen and Als9 binds to laminin. Als1 is vital for the attachment of the microorganism to the oral mucosa during the initial phase of the infection (Kamai *et al.,* 2002). Hypha-associated adhesion Als3 is vital for adhesion (Murciano *et al.,* 2012; Phan *et al.,* 2007). *ALS3* gene was highly expressed during infection of *in vivo* vaginitis and during infection of oral epithelial cells *in vitro* (Naglik *et al.,* 2011; Wachtler *et al.,* 2011; Cheng *et al.,* 2005). *HWP1* encodes an outer surface mannoprotein on the hyphal wall (transglutaminase substrate, TGase) and form covalent binding with HBEC (Staab *et al.,* 1996). An *hwp1/hwp1* mutant strain was greatly defective in the ability to adhere to HBEC and display attenuated virulence in a murine model of systemic candidiasis (Staab *et al.,* 1999; Chaffin *et al.,* 1998).

1.4.2 Secreted Degradative Enzymes

Following attachment to host cell surfaces and hyphae growth, proteinases was secreted by pathogen's hyphae in order to degrade the tissue lining and acquire nutrition at the infection site (Wachtler *et al.*, 2012; Naglik *et al.*, 2003). Secreted aspartyl proteinases (*SAP*) from *Candida* dissolved many proteins at laceration sites, such as collagen, haemoglobin, keratin, albumin, Immunoglobulin A, fibronectin, cystatin A, salivary lactoferin, interleukin1β, mucin, and laminin (Hube *et al.*, 1998). *SAP* comprises of ten members, Sap1-10. Sap9 and Sap10 remain constrained to the cell-surface and Sap1-8 are released to the surrounding medium (Taylor *et al.*, 2005; Naglik *et al.*, 2003). Expression of Saps 1, 2 and 3 by the yeast phase are required for virulence in a murine model of systemic infection, and for invasion of reconstituted human epithelium (RHE) *in vitro* (Schaller *et al.*, 1999; Hube *et al.*, 1997). Saps 4, 5 and 6 are expressed in the passage from yeast to hyphae phase at neutral pH. Sap7 was never detected *in vitro* whereas, Saps 9 and 10 manifest in both forms (yeast and hyphae) (Albrecht *et al.*, 2006).

Enzymes such as phospholipases (PL) digest ester linkages of glycophospholipids and allow *Candida* cells to invade tissue. There are four different classes of phospholipases (*PLA*, *PLB*, *PLC* and *PLD*); out of these four classes only the five member of *PLB* (*PLB1-5*) are situated outside a cell and has both fatty acid release (hydrolase) and lysophospholipase- transacylase activities (Mavor *et al.*, 2005; Niewerth & Korting, 2001). There is higher level of phospholipases in *C. albicans* strains isolated from blood than commensal strains (Ibrahim *et al.*, 1995); cells producing less phospholipase are less likely to cause disease than strains producing high phospholipase in mouse model

(Theiss *et al.*, 2006; Ghannoum, 2000). Therefore high level of phospholipase activity is discovered at where the hyphae were in direct contact with the object (Pugh & Cawson, 1977).

1.4.3 Morphological Transition and Phenotypic Switching

Morphological transition in *C. albicans* is the ability to switch between the unicellular yeast form, such as blastospores, and the filamentous form, such as pseudohyphae or hyphae. Out of all the *Candida* spp., only *C. dubliniensis* and *C. albicans* can undergo morphogenesis. Morphological conversion from yeast cells (round/oxoid in shape and readily separate) to pseudohyphae (extended ellipsoidal cells with constraints at the septa) or parallel-walled true hyphae is facilitated by ~pH 7, nutrients, temperature of 37-40ºC, approximately 5.5% of CO_2 concentration and presence of biotin, amino acid, serum and N-acetyl-D-glucosamine. Reverse conversion from hyphae to yeast form occurs when cells are grown in higher concentration of glucose, absence of serum, acidic pH, and lower temperature (Eckert *et al.*, 2007; Corner & Magee, 1997). In *C. albicans*, this conversion is controlled by 2 regulatory proteins, Cph1 and Efg1, which are homolog to *S. cerevisiae*, Ste12 and Phd1 respectively (Lewis, Lo, *et al.*, 2002). *C. albicans* mutant strains (*cph1* and *efg1*) have morphogenesis defects, such as defective in filamentous growth and are unable to form filaments when it is exposed to many stimuli including macrophage and serum. *Candida* mutant strains, *cph1/cph1* and *efg1/efg1* are avirulent in mouse model (Lewis, Lo, *et al.*, 2002). This transition (yeast to psedohyphae or hyphae and vice versa) is termed dimorphism and it is required for pathogenesis, as yeast forms are suited for dissemination in tissue and hyphae forms are required for invasion and tissue damage. The yeast cell that develops into hyphae is able

to kill macrophages (upon phagocytozed by macrophages) by secreting hyphae associated proteinases; these factors also help hyphae cells in resist to neutrophils. Hyphae cells also induce transmigration through endothelial cells from bloodstream (Hube, 2004; Gow *et al.*, 2002; Molero *et al.*, 1998).

The colonies of *C. albicans* show distinct morphological form, including white round-oxoid cells, gray colonies with elongated shaped cells (opaque), hat, irregular wrinkle, strippled, fuzzy, star, and rough at high frequency (10^{-4} to 10^{-1}) (Sudbery *et al.*, 2004). It is not known what causes phenotypic switching and how switching affect virulence of *C. albicans*. Switching happen more frequently in cells isolated directly from vaginitis or systemically infected patients (Jones *et al.*, 1994; Soll, 1988). White-phase cells have lower frequency for mating and lower capability to colonize skin in a cutaneous model than opaque-phase cells. White-phase cells are more virulent in systemic infection while opaque-phase cells in cutaneous infection of murine model (Kvaal *et al.*, 1999). Expression of gene *SAP1* and *SAP3* only occur in opaque cells, while expression of *SAP2*, *WHI1*, and *EFG1* occur specifically in white-phase cells (Miller & Johnson, 2002). Morphology transition is important for *C. albicans* in its adaptation or infection of the fungus to specific organs, such as skin, kidney and endothelial lining in blood vessels.

1.4.4 Biofilms

A vital virulence factor of *C. albicans* is its ability to forms biofilms. Formation of biofilms starts with adherence of *C. albicans* cells to non biotic material, such as catheters and dentures, or to biotic surfaces, such as mucosal cell surfaces. Biofilms

results in an increase in candidemia and antifungal resistance (Chandra, Kuhn, *et al.*, 2001; Hawser & Douglas, 1994). Devices like catheters and dentures provide a platform for *Candida* cells to form biofilms. Formations of biofilms proceeds in three development phases: early (attachment of *Candida* cells to abiotic or biotic platform and expansion of these cells), intermediate (accumulation of extracellular matrix material (ECM) and development of hyphae cells on upper part of the biofilm), and mature (biofilm complex distribute *Candida* cells to the surrounding). Mature biofilms are more resistant to the exposure of antifungal agents and host immune factors in comparison to planktonic cells (Finkel & Mitchell, 2011). *C. albicans* biofilm is resistant to variety of azoles, including voriconazole (Lewis, Kontoyiannis, *et al.*, 2002), miconazole (Lamfon *et al.*, 2004), ketoconazole, itraconazole and fluconazole, (Hawser & Douglas, 1995), polyenes nystatin and Amphotericin B, (Chandra, Kuhn, *et al.*, 2001; Chandra, Mukherjee, *et al.*, 2001) and flucytosine (Hawser & Douglas, 1995). Azole resistant in *C. albicans* biofilms may be attributed to the high levels expression of the drug efflux determinants *MDR1*, major facilitator superfamily transporter, and the ATP-binding cassette transporters *CDR1* and *CDR2* (Lupetti *et al.*, 2002).

One important component of *C. albicans* biofilms is ECM, which is proposed to provide a structural scaffold and protection for biofilm cells (Taff *et al.*, 2012). ECM is mainly made from β-1,3 glucan (encoded by *SKN1* and *KRE1*). Higher Amphotericin B resistance corresponded to upregulation of *KRE1* transcript in yeast (Breinig *et al.*, 2004). β-1,3 glucan in ECM also protect *C. albicans* from neutrophils attacks and do not trigger production of reactive oxygen species (ROS) (Z. Xie *et al.*, 2012).

1.5 Transmigration

Adhesion of *C. albicans* to the endothelium of the blood vessels triggers the invasion or transmigration of *C. albicans* across the endothelium. Several mechanisms were proposed for *Candida* migrates across the endothelium, Two of the mechanisms are induced endocytosis and active penetration (Naglik *et al.*, 2011; Zhu & Filler, 2010). For induced endocytosis, adherent organism was endocytosed by endothelium allowing adherent cell to cross abluminal surface of the endothelium. On the cell surface of endothelium, the fungus synthesized specialized proteins that binds to ligand E-cadherin on host epithelium cells and N-cadherin on endothelium cells via the *Candida* protein Als3, thereby triggering endocytosis of the *C. albicans* across the endothelial barrier (Phan *et al.*, 2007; Phan *et al.*, 2005). So far two genes associated to invasion have been identified, *SSA1* and *ALS3* which functions as both adhesin and invasin (Sun *et al.*, 2010; Phan *et al.*, 2007). *SSA1* induced the expression of heat shock protein 70 (*HSP70*) family on the cell surface and deletion of both *SSA1* and *ALS3* genes resulted in reduced epithelial adherence and invasion. Similarly mutant strains show less frequency in causing oropharyngeal candidiasis in a mouse model (Naglik *et al.*, 2011; Sun *et al.*, 2010). Als1 and Ssa1 bind to host E-cadherin subsequently induces tyrosine phosphorylation of unknown intracellular endothelial cell proteins (Belanger *et al.*, 2002). These binding produce pseudopods through microfilament rearrangement subsequently trigger endocytosis of adherent *C. albicans* hyphae cells (Phan *et al.*, 2007; Filler *et al.*, 1995). Active penetration of *C. albicans* into endothelial cells requires the elongation of hyphae to penetrate through the endothelial cells and likely kill the cells along the process (Wachtler *et al.*, 2011; Dalle *et al.*, 2010). It is not well understood what exactly facilitates active penetration of host cells. However, it is presumed that

factors such as physical forces and fungal adhesion are crucial (Wachtler *et al.*, 2011). It is not only the hyphal form of *C. albicans* that can undergoes endocytosis; unchanged strains can also undergo endocytosis to a certain degree causing minimal damage to the endothelial cells (Klotz *et al.*, 1983). Induced endocytosis is a passive process and does not required living fungal cells as even dead hyphae cells are endocytosed (Dalle *et al.*, 2010; Park *et al.*, 2005).

1.6 Fitness Attribute

Adaptation to the environment is vital to pathogens such as *C. albicans* to allow them to grow and expand in wide range environments, such as within their mammalian host and cause infection. *C. albicans* can grows in variety niches in human and metabolize different carbon sources that are present in the host. Environment adaptation involves both metabolic and stress adaptation. Stress responses can be initiated in response to a wide-range of environmental stimulus including heat-shocks, osmotic and cationic, oxidation, nitrosative and cell wall stresses (A. J. Brown *et al.*, 2014). Regulation of *C. albicans* metabolism and activation of specific stress responses are initiated upon contact with host. Metabolic flexibility and stress response represent crucial fitness attributes that have evolves alongside with virulence attributes in *C. albicans* and is important for cells viability (Barelle *et al.*, 2006; Lorenz & Fink, 2001).

1.6.1 Heat-shocks

Numerous studies have shown that the pathogenicity of *C. albicans* is influenced by heat shock response. Stress response by heat shock proteins (Hsp70 and Hsp90) mark

damaged or aggregated proteins for degradation, or encourage the folding of target proteins (Feder & Hofmann, 1999; Parsell *et al.*, 1993). The response in *C. albicans* is regulated by the heat shock transcription factor Hsf1 (Nicholls *et al.*, 2009). Hsf1 is essential for cells viability in yeast. *C. albicans* exposure to acute-heat shock causes Hsf1 phosphorylation and through the canonical heat shock elements (*HSEs*) in their promoters promote the expression of target heat shock protein (*HSP)* genes (Nicholls *et al.*, 2009). Inductions of cellular adaptation to the heat shock are by refolding or degradation of damaged proteins through activation of *HSP* gene. Following thermal adaptation, heat shock protein 90 (*HSP90)* gene is activated and interacts with Hsf1 to repressed the stress response in *C. albicans* (Leach *et al.*, 2012). Hsp90 is required for *C. albicans* to establish systemic infection. Autoantibodies against Hsp90 gives mild protection against the infection (Matthews *et al.*, 1991).

Heat shock proteins are found on *C. albicans* cell surface and are immunogenic during infections (Lopez-Ribot *et al.*, 1996; Matthews *et al.*, 1987). Induction of heat shock proteins by mild thermal insult is associated with yeast-hyphae morphogenesis, which is considered to be virulence attribute (Swoboda *et al.*, 1996). A mutant strain with mutation that inhibits promotion of the heat shock response in *C. albicans* has attenuated virulence.

1.6.2 Osmotic and Cationic Stress

Prolonged exposure to salts, such as sodium chloride, NaCl and potassium chloride, KCl resulted in dehydration of cells, causing loss of turgor pressure and a reduction in cell size due to cationic and osmotic stress (Kuhn & Klipp, 2012). These events will

subsequently triggers the expression and phosphorylation of the stress-activated protein kinases (SAPK) Hog1 that results in reprogram of the gene expression pattern required for cell survival upon osmostress. Examples include regulation of gene encoding glycerol biosynthetic enzymes (Enjalbert *et al.*, 2006; Smith *et al.*, 2004). This will results in the build-up of glycerol in the cells which will then allow them to balance their osmotic pressure with that of surrounding and allowing the resumption of growth. Adaptation of *C. albicans* cells to osmotic/cationic stresses relies on *HOG1*. It is a major regulator of the osmostress-regulated transcription, including activation of glycerol biosynthetic gene which will then cause aggregation of glycerol (Smith *et al.*, 2004; San Jose *et al.*, 1996).

Common signaling modules found in both higher and lower eukaryotic cells, such as mitogen-activated protein kinase (MAPK) cascades are composed of three succesively activated tiers of kinases (MAP kinase kinase kinase, MAP kinase kinase and MAP kinase). Hog1 is a component of a highly conserved mitogen-activated protein (MAP) kinase pathway. In *C. albicans*, this MAPK is activated by the MAPKK, Pbs2, which in turn is activated by a single MAPKKK, Ssk2 (Arana *et al.*, 2005). The regulators network upstream of gene expressions that activate this MAPK module due to exposure to osmotic stress in *C. albicans* is open to interpretation.

Hog1 (SAPK) regulates both morphogenesis and stress response in *C. albicans* and is a dominant factor for virulence of *C. albicans (Cheetham et al., 2011)*. Adaptation to other stresses, such as regulation of cellular morphogenesis and metabolism, oxidative stress response, and influence cell wall performance required Hog1 (Alonso-Monge *et al.*, 2009; Eisman *et al.*, 2006; Alonso-Monge *et al.*, 2003). Based on several studies,

adaptation to osmotic and cationic stress are important in host niches, such as in the kidney, where the concentrations of NaCl in urine can approach 600 mmol l^{-1} (Z. Zhang *et al.*, 2004; Ohno *et al.*, 1997).

1.6.3 Cell Wall Stress

The fungal cell wall is important for survival and interaction with the surrounding environment; it is the point of contact between fungus and target surfaces, and processes such as adhesion, dimorphism, and biofilms formation take place. These factors are responsible for the pathogenicity of *C. albicans*.

Congo Red and Caspofungin are antifungal drug that inhibit and disturb synthesis and assembly of β-glucan, whereas Calcofluor White disrupt formation of chitin. These antifungal drugs are used to pressure the *C. albicans* cell wall *in vitro* (Eisman *et al.*, 2006; Wiederhold *et al.*, 2005). The Hog1 pathway regulates cell wall stress responses and enables the synthesis of chitin (Munro *et al.*, 2007; Eisman *et al.*, 2006).

Two additional highly conserved MAPK pathways are responsible for regulations of stress response in *C. albicans* cell wall. The first pathway, the Mkc1 facilitated MAPK or the cell integrity pathway, and a second pathway, involves in Cek1-mediated MAPK or yeast-hypha morphogenesis (Roman *et al.*, 2007).

Bck1-mediated MAPKKK, Mkk1-mediated MAPKK and Mkc1-mediated MAPK are MAPKK module that involves in cell integrity pathway (Navarro-Garcia *et al.*, 1998). Cascade of phosphorylation reactions, Mkc1 governed by protein kinase (Pkc1) plays an

essential role in the generation of a stable cell wall in yeast (Paravicini *et al.*, 1996). The inactivation of Mkc1 increases the cell wall sensitivity to thermal insult and other stresses (Navarro-Garcia *et al.*, 1998). Inactivation of Mkc1 does reduce and weaken the virulence of *C. albicans* (Diez-Orejas *et al.*, 1997). However inactivation of this protein does not contribute and increase the probability of cell death in response to neutrophils or macrophages (Arana *et al.*, 2007).

MAPKKK Ste11, MAPKK Hst7 and MAPK Cek1 are components of morphogenetic MAPK (Cek1) pathway. This pathway also regulate *C. albicans* mating response and are required for efficiency of mating (Chen *et al.*, 2002). Cell surface sensor Msb2 triggers Cek1 pathway upon exposure to antifungal agents that target cell wall integrity or mutation of gene required for integrity of cell wall (Cantero & Ernst, 2011; Roman *et al.*, 2009). Inactivation of Cek1 in *C. albicans* growing under certain conditions resulted in retarded growth of filament and is more prone to cell wall stresses (Eisman *et al.*, 2006; Csank *et al.*, 1998; Leberer *et al.*, 1996). *C. albicans cek1* mutants display attenuated virulence but does not affect sensitivity of the cells in response to macrophage or neutrophil (Arana *et al.*, 2007; Csank *et al.*, 1998).

1.6.4 Oxidative Stress

Reactive oxygen species (ROS) is an acting compound causing damage and inflammation to the tissue. Resistant to this compound by *C. albicans* require Cap1, an orthologue of AP-1-like transcription factor Yap1 in *S. cerevisiae* (Alarco & Raymond, 1999). Following oxidative stress, cysteine residues near the carboxyl terminus Cap1 which are redox-sensitive was oxidized and subsequently leads to the nuclear

accumulation of Cap1 and Yap1-responsive elements (YRE) in their promoters that will activates the target genes. These processes are Hog1 independent (Znaidi *et al.*, 2009; X. Zhang *et al.*, 2000). Upon accumulation of Cap1 in *C. albicans*, genes involved in the removal of oxidative stress, such as oxidative damage repair, superoxide dismutase and catalase, and redox homeostasis glutathione synthesis was expressed and functions by detoxifying ROS and regulates stress adaptation in cells. Deletion of *CAP1* will increase *C. albicans* sensitivity to oxidative stress due to decrease promotion of genes and enzymes mentioned above (Enjalbert *et al.*, 2006; Alarco & Raymond, 1999). Moreover *C. albicans* mutant strains (*cap1/cap1* and *hog1/hog/1*) display attenuated virulence and increased response to phagocytes (Arana *et al.*, 2007; Fradin *et al.*, 2005).

1.7 Central Metabolism Pathways

In order to proliferate in a broad range of environmental niches, metabolic flexibility and virulence factors are important so that they can metabolize different carbon sources that are scarce or the only available carbon source at a specific environmental niche. Carbohydrates are necessary to produce biomolecules and for generating energy for basic functions of cells. Before entering the glycolytic pathway, sugars undergo transition to fructose 6-phosphate or glucose 6-phosphate. ATP and NADH are produce from the conversion of sugar phosphates into pyruvate in glycolysis pathway. From there, fermentation and respiration are carried out by cells. Although NAD+ is regenerated by both processes, respiration produces more ATP than fermentation through the oxidative phosphorylation and tricarboxylic acid (TCA) cycle. Glycolysis pathway is important for carbon metabolism and it is frequently used for both fermentation and respiration. This pathway is important to the virulence in pathogenic

bacteria, parasites, and fungi, and was up-regulated during infections (Costa *et al.*, 2007; Barelle *et al.*, 2006; Rodaki *et al.*, 2006). Glycolysis, gluconeogenesis, and the glyoxylate cycle are part of *C. albicans* central metabolism (Fig. 1.2).

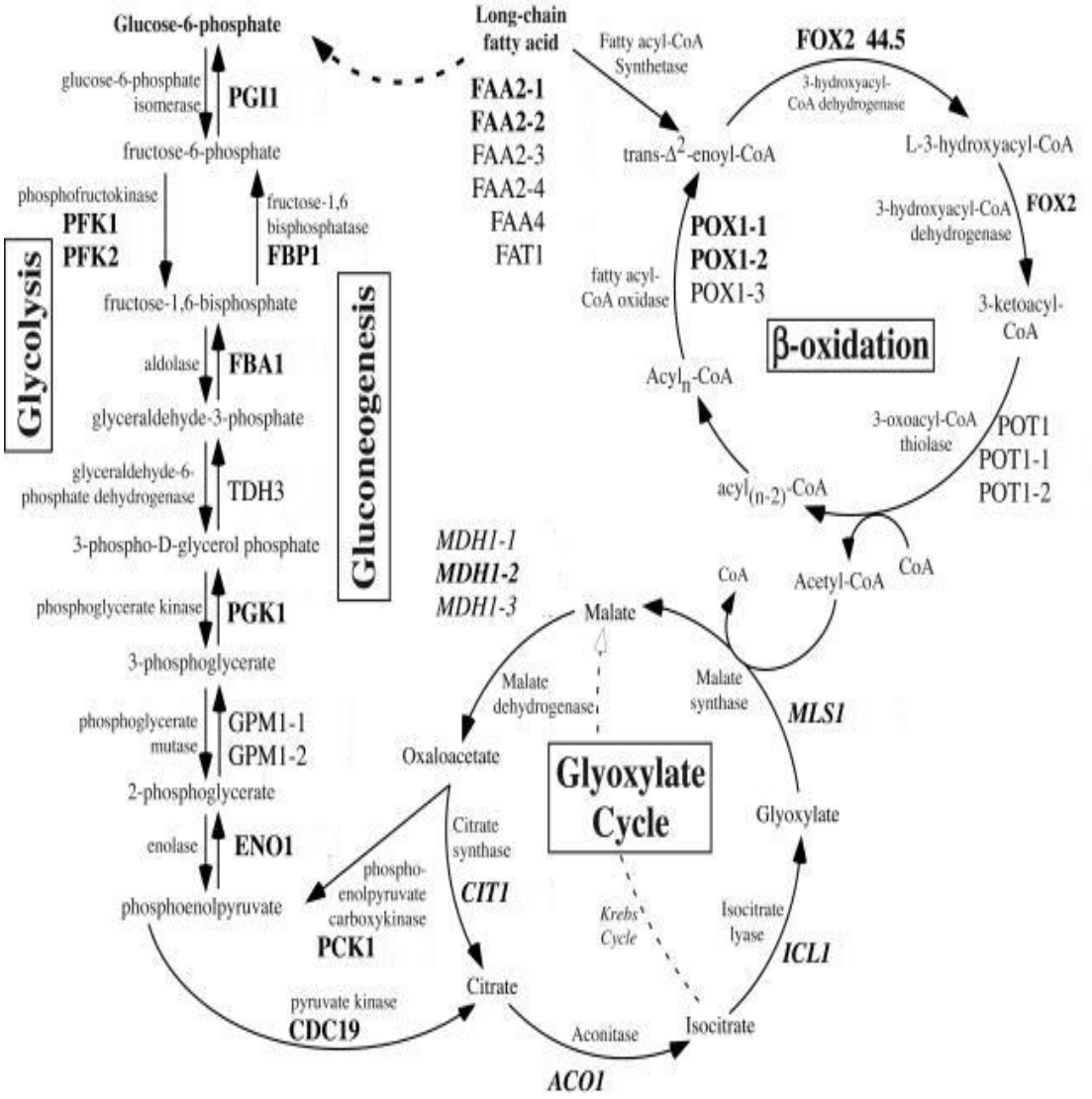

Figure 1.2: The pathways of ß-oxidation, the glyoxylate cycle, gluconeogenesis, and glycolysis are shown, with the *C. albicans* gene names (Taken from Lorenz et al., 2004).

1.7.1 Glycolysis and Gluconeogenesis

Transcript of central metabolic pathway such as glycolysis is strictly regulated in response to environmental conditions, such as availability of carbon source, oxygen levels, energy needs and metabolite concentrations. Most of the regulators of gene expression in glycolytic of other species have not been identified. Understanding of transcriptional control of glycolysis in eukaryotes is mainly based on the experimental paradigm *S. cerevisiae*; nonfermentable yeast (Chambers *et al.*, 1995). The transcription regulators (Gcr1 and Gcr2) are responsible for inducing the expression of the glycolytic genes in *S. cerevisiae* (Uemura & Fraenkel, 1990; Clifton *et al.*, 1978). Inactivation of either gene (*GCR1* and *GCR2*) result in growth defects in response to glucose due to unexpressed glycolytic genes (Uemura & Fraenkel, 1990).

The Crabtree-positive *Saccharomyces* yeasts is a facultative anaerobes and has glucose repression circuit. This is in contrast to that of most other obligate aerobes and facultative eukaryotes, which lack the glucose repression circuit. Pyruvate is oxidized to carbon dioxide through the TCA cycle in Crabtree-negative organism under aerobic conditions. Most aerotolerant organisms are able to metabolize energy anaerobically through fermentation pathways to a certain extent to subsequently regenerate NAD+, such as *Neurospora crassa, Aspergillus oryzae*, and *Trichoderma reesei.* These fungi metabolize energy aerobically and the TCA cycle is not affected during growth in glucose rich which is different in *S. cerevisiae* that rely heavily on fermentation pathway (Maeda *et al.*, 2004; X. Xie *et al.*, 2004; Chambergo *et al.*, 2002).

C. albicans is an opportunistic fungus that is capable of metabolizing carbon sources through respiration and fermentation pathway. *C. albicans* is a Crabtree-negative organism that does not have *GCR1/2* homologs and it regulates transcription of glycolytic genes in different way compared to *S. cerevisiae*. Tye7 and Gal4 activates the glycolytic pathway in *C. albicans* and severe growth defects was observed in the mutant strains (*tye7* and *gal4*) cultured on a certain condition. *C. albicans* mutant strains (*tye7* and *gal4*) showed attenuated virulence in *Galleria mellonella* infection model. Therefore *TYE7* and *GAL4* genes are required for pathogenicity and virulence of *C. albicans* (Askew *et al.*, 2009).

Gluconeogenesis is required for yeast cells to generate sugar phosphates for the synthesis of essential cellular components, during growth on non-fermentable carbon sources. During growth on unbalanced carbon sources, *S. cerevisiae* and *C. albicans* shunt glycolysis pathway, this is achieved by enzymes of gluconeogenesis, such as phosphoenolpyruvate carboxykinase, and fructose-1,6-bisphosphatase. The enzymes of the glyoxylate cycle are necessary for gluconeogenesis, such as malate synthase (*MLS*) and isocitrate lyase (*ICL*).

1.7.2 The Tricarboxylate Acid (TCA) and Glyoxylate Cycle

The glyoxylate cycle is also known as a "modified tricarboxylic acid (TCA) cycle". Isocitrate lyase, *ICL1* and malate synthase, *MLS1* are enzymes in glyoxylate cycle that functions by converting isocitrate and acetyl-CoA into succinate and malate respectively (Fig 1.2). In glyoxylate cycle, isocitrate was metabolized into succinate and glyoxylate which are subsequently condensed by malate synthase, *MLS1* and accompanied by

acetyl-CoA to produce free CoA-SH and malate. Consequently malate is further processed by malate dehydrogenase and produce succinate as a final product. Succinate can be reused in the TCA cycle or to operate as carbohydrate biosynthesis indicator or biosynthesis of amino acid. Fatty acids or C_2-units such as ethanol or acetate are utilized by glyoxylate cycle to produce succinate, a C_4-units carbon sources through various catabolic processes. Succinate is then metabolize by glyoxylate cycle to produce energy (Kornberg & Madsen, 1958). In contrast, carbon sources such as ethanol, acetate or oleic acid cannot be metabolized by *S. cerevisiae* because it has deficiency in Icl1 or Mls1. Moreover growth on these carbon sources greatly repress the expression of gene involved in metabolism of nitrogen, malate synthase (*DAL7*) (Kunze *et al.*, 2002; Fernandez *et al.*, 1992; Hartig *et al.*, 1992). In recent year, key enzyme such as isocitrate lyase in glyoxylate cycle is greatly studied. Mutant strain (*icl/icl*) has attenuated virulence in mouse model (Lorenz & Fink, 2001). This provide a platform for anti-fungal testing to reduce the virulency of *C. albicans*

1.8 Glucose Sensing

In the presence of glucose, *C. albicans* undergoes yeast-to-hyphal transition which is crucial for virulence and invasion of host cells (Hudson *et al.*, 2004), as explained previously (Section 1.4.3) morphological plasticity is important factor for *C. albicans* virulency. The hyphal form can diffused through the tissues or form mycelial biofilms, and the yeast form is presume to be able to propogate easily via body fluids (Bendel *et al.*, 2003).