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Chapter 3

Carbon Sources Attribute to Pathogenicity in *Candida albicans*

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Abstract

This topic was to examine the impact of galactose or fructose upon the assimilation of secondary carbon sources by *Candida albicans*. *C. albicans ICL1* gene is repressed upon addition of 2% galactose or fructose to lactate- and oleic acid-grown cells. Further studies on Ca*FOX2*, Ca*FBP1* and Ca*MLS1* transcripts in response to galactose or fructose on assimilation of lactate and oleic acid resulted in repression of these genes. The Ca*ICL1* gene, which encode the glyoxylate cycles enzymes isocitrate lyase are required for growth on non-fermentable carbon sources. However, the enzyme CaIcl1 was not destabilized by galactose, but was degraded in response to fructose. In contrast, *S. cerevisiae* Icl1 has retained the molecular apparatus of protein degradation 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 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 speed of protein degradation.

Keywords: *Candida albicans*, galactose, fructose, glyoxylate cycles, isocitrate lyase, gluconeogenic, metabolic adaptation, protein degradation, ubiquitination

1. Introduction

Candida albicans is eukaryotic diploid (2N) sexual yeasts of the kingdom fungi [1]. *C. albicans* can divide asexually or can undergo parasexual reproduction, heterothallic or homothallic mating [2]. *C. albicans* genome sequencing project revealed the presence of sequences homologous to

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the *Saccharomyces cerevisiae MAT* (mating type) loci, *C. albicans MTL* (mating-type-like) loci [3]. Members of the genus *Candida* are very heterogeneous, can grow in at least three different morphologies; yeast, pseudo-hyphae and hyphae, such as *C. albicans* and *C. dubliniensis* [4, 5]. Further morphological forms exist during colony switching, such as white-opaque switching system involves switch between white domed colonies and opaque flat colonies [6], mating type-like locus a (*MTLa*) and *MTLα* cells must switch from white to opaque to become mating-competent [7]. Opaque cells secreted pheromones resulting in the formation of conjugation tubes, and subsequently, cell and nuclear fusion occur to form tetraploid (4N) cells. Mating products can be induced to undergo concerted chromosome loss to return to the diploid state [8, 9].

In order to proliferate in a wide range of environmental niches, pathogens must not only depend on certain virulence factors, it is also important to have a flexible metabolism; therefore, they can assimilate a variety of carbon sources that are scarce or the only available carbon source at a specific environmental niche. The primary and preferred sources of metabolic carbon for most organisms are carbohydrates; it is used for generating energy and producing biomolecules. Before entering the glycolytic pathway, most sugars are converted to fructose-6-phosphate or glucose-6-phosphate. ATP and NADH are produced from the conversion of hexose phosphates into the key metabolite pyruvate in glycolysis pathway. From there, two major strategies of energy production (fermentation and respiration) are carried out by cells. Although NAD+ is regenerated by both processes, respiration is more efficient than fermentation as it produces additional ATP through the oxidative phosphorylation and tricarboxylic acid (TCA) cycle. Glycolysis is the central, common pathway for both processes and it is critical for carbon assimilation; the pathway has been shown to be up-regulated during infections and important to the virulence in pathogenic bacteria, parasites, and fungi [10–12]. Glycolysis, gluconeogenesis, and the glyoxylate cycle are part of *C. albicans* central metabolism.

Central metabolic pathway such as glycolysis is strictly regulated, transcript of glycolytic enzymes is regulated in response to environmental conditions such as carbon source and availability, oxygen levels, and to cellular demands such as energy needs and metabolite concentrations. However, the regulators of glycolytic gene expression in most species have not been identified; for eukaryotes understanding of transcriptional control of glycolysis is mainly based on the non-pathogenic yeast *Saccharomyces cerevisiae* [13]. The transcription regulators (Gcr1 and Gcr2) are responsible for inducing the expression of the glycolytic genes in *S. cerevisiae* [14, 15], Gcr1p binds to CT boxes (*5′-CTTCC-3′*) upstream of the glycolytic genes and Gcr2p acts as a co-activator by forming a complex with Gcr1p [16]. Inactivation of either gene (*GCR1* and *GCR2*) result in growth defects during culture on glucose, due to the decreasing expression levels of the glycolytic genes [15]. However, on non-fermentable carbon sources, the mutant strains display wild type growth rates [17]. Another glycolysis-specific regulator in *S. cerevisiae* is Tye7 (also known as Sgc1); it is involved in the activation of several glycolytic genes, although not to the extent of Gcr1 and Gcr2, this activation is independent of *GCR1.* No growth defects under any carbon source regime are detected in *tye7* mutant strain [18].

C. albicans is an opportunistic fungus that is capable of metabolizing carbon sources through respiration and fermentation pathway similar to that of a typical eukaryotic cell. The culprit for more than half of all *Candida* infections is *C. albicans* [19]; this shows the importance of studying the metabolism of this pathogen for the development of effective antifungal treatments. *C. albicans*

is a Crabtree-negative organism that lacks *GCR1/2* homologs and must control transcription of glycolytic genes differently as compared to *S. cerevisiae*. Tye7 and Gal4 are identified as two fungal-specific activators of the glycolytic pathway in *C. albicans*, severe growth defects were observed in the mutant strains (*tye7* and *gal4*) cultured on fermentable carbon sources when respiration was inhibited or oxygen was limited. Furthermore, chromatin immunoprecipitation coupled with microarray analysis (ChIP-CHIP) and transcription profiling showed these factors bind to and regulate expression of the glycolytic pathway genes. *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* [20].

Gluconeogenesis is required for yeast cells to generate sugar phosphates for the synthesis of essential cellular components, during the growth on non-fermentable carbon sources. Under physiological conditions with two exceptions, most of the glycolytic reactions are reversible. In many yeasts including *S. cerevisiae* and *C. albicans*, due to unfavorable thermodynamic balances, pyruvate kinase and phosphofructokinase have to be bypassed, this is achieved by phosphoenolpyruvate carboxykinase (Pck1, building phosphoenolpyruvate), and fructose-1,6-bisphosphatase (Fbp1, building fructose-6-phosphate). The enzymes of the glyoxylate cycle are necessary for gluconeogenesis, such as malate synthase (*MLS*) and isocitrate lyase (*ICL*). Moreover, in *S. cerevisiae,* in other to replenish TCA cycle intermediate (anaplerotic reactions), the succinate/fumarate transporter, Acr1p, has been shown to be a link between the glyoxylate cycle and the TCA cycle [21]. Not much is known about gluconeogenesis in *C. albicans*, whereas carbon sources/sugar phosphate may become available during lymphatic and blood infection, on skin after incorporation in macrophage, gluconeogenesis and the glyoxylate cycle may be depended for providing carbohydrates for cell wall biosynthesis [22].

The glyoxylate cycle is a "modified tricarboxylic acid (TCA) cycle," instead of the two decarboxylation steps of the TCA cycle the key enzymes of the glyoxylate cycle (isocitrate lyase, *ICL1* and malate synthase, *MLS1*) convert isocitrate and Acetyl-CoA into succinate and malate. *ICL1* splits the isocitrate, C_6-unit into succinate and glyoxylate, which in turn is condensed by malate synthase, *MLS1* with Acetyl-CoA generating free CoA-SH and malate. Malate is then processed malate dehydrogenase to continue the cycle and succinate is released as net product, which can be used to replenish the TCA cycle or to function as precursors for carbohydrate biosynthesis or amino acid biosynthesis. C_4-units (succinate) are generated by converting two Acetyl-CoA units generated by various catabolic processes. Therefore, the glyoxylate cycle enable cells to utilize fatty acids or C_2-units such as ethanol or acetate as sole carbon source [23].

Fatty acids are broken down in the mitochondria by catabolic process (beta oxidation) to generate two-carbon units (Acetyl-CoA), which can be oxidized to CO_2 and H_2O via the TCA cycle or used to generate hexose via the glyoxylate and gluconeogenesis, and NADH and $FADH_2$, which are co-enzymes used in electron transport chain. In yeast *S. cerevisiae*, β-oxidation and peroxisome biogenesis are regulated by transcription factors, *OAF1* and *PIP2*. However, such homolog is not present in *C. albicans.* Instead, *C. albicans* share a single homolog of transcription factors FarA/FarB with *A. nidulans*, *CTF1* which regulate genes for β-oxidation enzymes, glyoxylate cycle, and the gluconeogenesis in the presence of fatty acids. *CTF1* is important for growth on fatty acids and regulation of several genes encoding enzymes associated with β-oxidation, including *FOX2*. Deletion of *FOX2* (*fox2*Δ/*fox2*Δ (*fox2*Δ/Δ) strains) confers a mild

attenuation of virulence [24, 25]. Therefore, *C. albicans* appears to share more similarity to that of filamentous fungi than to that of budding yeast at phenotypic and genotypic level in the regulation of alternative carbon assimilation pathways, but there are clearly *Candida*-specific adaptations in these regulatory networks [26].

Glyoxylate cycle is present in fungi but not in mammals, and β-oxidation of fatty acids develop in different directions between mammals and fungi. Therefore, in order to have a better understand on the importance of these pathways, physiological and virulence of mutant strains lacking of these genes encoding key enzymes the β-oxidation multifunctional protein (*FOX2*), glyoxylate cycle (isocitrate lyase, *ICL1*), and gluconeogenesis (fructose-1,6-bisphosphatase, *FBP1*) was studied. Deletion of *ICL1* or *FOX2* or *FBP1* confers attenuation to some degree in a mouse model of disseminated candidiasis. Therefore, confirming that alternative carbon sources are relevant nutrients in vivo, and *C. albicans* probably consume multiple carbon sources during infection. Finally, in vitro genomic and phenotypes analysis indicate that the regulatory networks that control alternative carbon metabolism in *S. cerevisiae* differ significantly from the paradigms developed in *C. albicans* [25, 27].

In the present of glucose, *C. albicans* undergoes yeast-to-hyphal transition which is crucial for virulence and invasion of host cells [28]. The hyphal form can diffuse through the tissues or form mycelial biofilms, and the yeast form is thought to disseminate easily via body fluids [29]. Glucose is an important carbon sources in central metabolism pathway, thus glucose sensing and response is closely regulated and highly evolved in most organisms. For example, *C. albicans* appears to have over 20 different hexose transporters that are expressed under different conditions and *S. cerevisiae* has at least 17 [30], it is presumed that this large number of hexose transporters is required because *S. cerevisiae* prefers to ferment glucose. *S. cerevisiae* demands a high influx of glucose because for each molecule of glucose metabolized, it yields only two molecules of ATP. In contrast, *C. albicans* also has many hexose transporters although it is presumed to prefer respiration and thus can yield up to 38 ATPs per glucose molecule metabolized [31]. This might be the reason why *C. albicans* can thrives in various niches, which likely release many different sugars as carbon sources.

Not much is known about how *C. albicans* senses and responds to sugars, therefore *S.* cerevisiae a paradigm for non-conventional yeasts is used as a model to elucidate sugar-sensing and signaling pathway that primarily regulates glucose transport. In yeast *S. cerevisiae*, glucose is detected by two glucose sensors in the cell membrane, which are Snf3 and Rgt2. The glucose sensors are orthologs of transporters with long cytoplasmic tails for intracellular signaling, which are missing from glucose transporters. The sensors cannot transport sugar but they control sugar acquisition by regulating the expression of genes encoding hexose transporters (*HXTs*) [32]. A single point mutation in the *S. cerevisiae* glucose sensors Snf3 (R229K) or Rgt2 (R231K) resulted in constitutive expression of genes encoding hexose transporters, because these mutations may convert the sensors into their glucose-bound (signaling) conformations [33]. *C. albicans* possesses glucose sensors, Hgt4 (orf19.5962) which is an ortholog of the Snf3 and Rgt2, Hgt4 generates an intracellular signal to induce expression of certain *HGT* genes encoding hexose transporters which is required for growth on glucose, for optimal virulence, and for filamentation [34].

Similar observation is observed between *S. cerevisiae* and *C. albicans*, *ICL1* and *PCK1*, whereby upon exposure to 2% glucose the transcription from DNA to mRNA are repressed. However, in contrast to *S. cerevisiae*, Icl1 and Pck1, which are degraded rapidly upon exposure to glucose. *C. albicans*, Icl1 and Pck1 remain stable [35]. This suggests that *C. albicans* undergoes catabolite inactivation on transcriptional level but not on the protein degradation level, which further implicate that the proteins (Icl1 and Pck1) are subjected to post-translational modifications. Post-translational modifications of proteins, such as acetylation, phosphorylation and ubiquitination, play important roles in generating and relaying signal in almost every cellular pathway as well as cellular adaptation of all organisms, which includes their growth, differentiation, division and development. These protein modifications are important for the regulation of the pre-existing proteins' functions, activities and stabilities, thereby controlling dynamic cellular processes [36].

Similar to that in *S. cerevisiae*, *C. albicans* polyubiquitin is encoded by the *UBI4* gene, which has three rather than five tandem repeats in a consecutive head-to-tail arrangement that is found in *S. cerevisiae* [37]. Decrease in the polyubiquitin encoded by the *UBI4* gene in *C. albicans* induces the growth of hyphae and pseudohyphae [38]. This was compatible with previous studies indicating that the ubiquitination via the E2 enzyme, Rad6 inhibits hyphal development in *C. albicans* [39]. Furthermore, polyubiquitin is presumed to contribute to stress responses in *C. albicans*, because downregulation of *UBI4* resulted in mild temperature sensitivity in stationary cells grown in glycerol, but not during growth on glucose [38]. Therefore, it has been implied that the *UBI4* gene is necessary for viability in *C. albicans* [38]. For example, recent studies show that ubiquitination sites are present in glyoxylate cycle and gluconeogenesis enzymes from *S. cerevisiae* but absent from their *C. albicans* homologs [35]. This indicates that evolutionary rewiring of ubiquitination targets upon glucose exposure; this implies *C. albicans* proteins remain functional, allowing it to continue metabolize alternative carbon sources [35]. This metabolic flexibility is presumed to be important during infection and colonization of dynamic host niches with variable carbon sources.

Lorenz and Fink [27] have proved the importance of key enzyme, *ICL1* of glyoxylate cycle to the virulence and pathogenicity of *C. albicans*. Deletion of the gene cause attenuated virulence in mice model. This highlights the importance of *ICL1* as a factor that contributes to the virulency and viability of *C. albicans* and provides a new target site for antifungal drugs test. For example, caffeic acid, rosmarinic acid, and apigenin were found to have antifungal activity against *C. albicans ICL1* when tested under glucose-depleted conditions [40].

Thus, it is important to have a better understanding and studying the mechanism involved, and the fitness attribute of the key enzymes in central metabolism of *C. albicans*. Recent studies by Sandai et al. [35] indicate that glucose trigger degradation of transcript in *C. albicans*, *ICL1* and *PCK1* but not the protein. This suggests that *C. albicans* have undergone evolutionary rewiring and lacks ubiquitination site as compared to *S. cerevisiae*. As such, this study was mainly focused on the effects of galactose and fructose on the central metabolism of *C. albicans*. Galactose is metabolized to the more metabolically useful glucose-6-phosphate by the enzymes of the Leloir pathway. This pathway is necessary as the initial enzymes of glycolysis are unable to recognize galactose. Of all the sugars found in nature, only D-fructose feeds directly into glycolysis, the central pathway of carbohydrate metabolism.

2. Effect of galactose or fructose on *C. albicans FOX2, FBP1, MLS1* and *ICL1* mRNAs

There are reports that state repression on transcriptome by glucose in *S. cerevisiae* [41]. For example, study by Yin et al. [42] using northern blotting and transcriptomic analyses showed that transcripts encoding the gluconeogenic enzymes (FBP1 and PCK1) are repressed by glucose in *S. cerevisiae*. Furthermore, previous work by Sandai et al. [35] reported that *ICL1* and *PCK1* are repressed by glucose in *C. albicans* and *S. cerevisiae*. To reconfirm this report and to compare it with the galactose and fructose responses of *C. albicans* more directly in this study, we first examined the responses of *S. cerevisiae* glyoxylate cycle (Sc*ICL* and Sc*MLS1*), β-oxidation (Sc*FOX2*) and gluconeogenesis (Sc*FBP1*) mRNAs using the following experimental approach.

S. cerevisiae ICL1 mRNA levels showed a dramatic decrease within 1 hour of galactose or fructose addition to the cells growing on lactate or oleic acid. Similarly, Sc*FBP1*, Sc*FOX2* and Sc*MLS1* mRNAs levels declined after galactose or fructose addition to the cells growing on lactate or oleic acid media. This strong repression occurred 1 hour after galactose or fructose addition. These confirmed that in *S. cerevisiae*, the *ICL1, FBP1, FOX2* and *MLS1* transcripts are strongly repressed by galactose and fructose [43].

Global transcriptional responses of *C. albicans* to low (0.01%), medium (0.1%) and high (1%) glucose concentrations by microarray analysis indicated that a total of 347 *C. albicans* gene were up-regulated and 344 genes were down-regulated in responses to at least one of the glucose concentration examined. There are 170 of these genes were up-regulated and 180 genes were down-regulated by 0.01% glucose, indicating about half of glucose-regulated genes are responsive to low glucose levels [42]. Therefore, it is concluded that *C. albicans* and *S. cerevisiae* is acquisitively sensitive to glucose. Hence, at the start of this study, an aim was to confirm the impact of galactose and fructose upon specific mRNAs that encode enzymes required for the assimilation of alternative carbon sources. The transcripts encoding the glyoxylate cycle enzyme isocitrate lyase (Ca*ICL1*), fructose-1,6-biphosphatase (Ca*FBP1*), malate synthase (Ca*MLS1*) and multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway (Ca*FOX2*) were the main focus here.

C. albicans cells were grown to mid-exponential phase in media containing lactate or oleic acid as the sole carbon source using the same procedures [35]. Galactose or fructose was then added to a final concentration of 2%, samples taken for RNA analysis at various times thereafter, and the levels of the Ca*ICL1*, Ca*FOX2*, Ca*MLS1*, Ca*FBP1* and Ca*ACT1* mRNAs were measured. The relative expression of Ca*ICL1*, Ca*FBP1*, Ca*FOX2* and Ca*MLS1* (compared to the internal Ca*ACT1* control) was high in lactate- and oleic acid-grown cells compared to cells that were exposed to either galactose or fructose.

3. Role of ubiquitination in sugar phosphate-accelerated protein degradation in *C. albicans*

Research done by Sandai et al. [35] suggested that *S. cerevisiae* has retained the ability to degrade target proteins in response to glucose, but that *C. albicans* isocitrate lyase (*ICL1*) has

(A) *C. albicans* and *S. cerevisae* Fbp1

```
CaFbp1      MSGPVNSVSK--QMNVDTDIITLTRFILQEQQTVAPTATGELSLLLNALQFAFKFIAHNI 58
ScFbp1      MPTLVNGPRRDSTEGFDTDIITLPRFIIEHQKQFK-NATGDFTLVLNALQFAFKFVSHTI 59

CaFbp1      RRAELVNLIGVSGSANSTGDVQKKLDVIGDEIFINAMRSSNNVKVLVSEEQEDLIVFPG- 117
ScFbp1      RRAELVNLVGLAGASNFTGDQQKKLDVLGDEIFINAMRASGIIKVLVSEEQEDLIVFPTN 119

CaFbp1      GGTYAVCTDPIDGSSNIDAGVSVGTIFGVYKLQEGSTGGISDVLRPGKEMVAAGYTMYGA 177
ScFbp1      TGSYAVCCDPIDGSSNLDAGVSVGTIASIFRLLPDSSGTINDVLRCGKEMVAACYAMYGS 179

CaFbp1      SAHLALTTGHGVNLFTLDTQLGEFILTHPNLKLPDTKNIYSLNEGYSNKFPEYVQDYLKD 237
ScFbp1      STHLVLTLGDGVDGFTLDTNLGEFILTHPNLRIPPQKAIYSINEGNTLYWNETIRTFIEK 239

CaFbp1      IKK-------EGYSLRYIGSMVADVHRTLLYGGIFAY------PTLKLRVLYECFPMALL 284
ScFbp1      VKQPQADNNNKPFSARYVGSMVADVHRTFLYGGLFAYPCDKKSPNGKLRLLYEAFPMAFL 299

CaFbp1      MEQAGGSAVTIKGERILDILPKGIHDKSSIVLGSKGEVEKYLKHVPK-- 331
ScFbp1      MEQAGGKAVNDRGERILDLVPSHIHDKSSIWLGSSGEIDKFLDHIGKSQ 348
```

(B) *C. albicans* and *S. cerevisae* Fox2

```
CaFox2      MS-PIDFKDKVVIITGAGGGLGKYYSLEFAKLGAKVVVNDLGGALDGQGGNSKAADIVVD 59
ScFox2      MPGNLSFKDRVVVITGAGGGLGKVYALAYASRGAKVVVNDLGGTLGGSGHNSKAADLVVD 60

CaFox2      EITKNGGVAVADYNNVLD-GAKIVETAVKSFGTVHIIINNAGILRDSSIKKMTEKDFKLV 118
ScFox2      EIKKAGGIAVANYDSVNENGEKIIETAIKEFGRVDVLINNAGILRDVSFAKMTEREFASV 120

CaFox2      IDVHLNGAYAVTKAAWPYFQKQKFGRVVNTSSPAGLYGNFGQTNYSAAKSALLGFAETLA 178
ScFox2      VDVHLTGGYKLSRAAWPYMRSQKFGRIINTASPAGLFGNFGQANYSAAKMGLVGLAETLA 180

CaFox2      KEGDRYNIKANAIAPLARSRMTESILPPPILEKLGPEKVAPLVLYLSSAENEVTGQFFEV 238
ScFox2      KEGAKYNINVNSIAPLARSRMTENVLPPHILKQLGPEKIVPLVLYLTHESTKVSNSIFEL 240

CaFox2      AAGFYAQIRWERSGGVLFKPD-QSFTAEVVAKRFSEVLNFDDSGKPEYLKNQHPFMLNDY 297
ScFox2      AAGFFGQLRWERSSGQIFNPDPKTYTPEAILNKWKEITDYRD--KP-FNKTQHPYQLSDY 297

CaFox2      TTLTTEARKLPSNDASGAPKVTLKDKVVLITGAGAGLGKEYAKWFARYGAKVVVNDFKDA 357
ScFox2      NDLITKAKKLPPNEQGSVKIKSLCNKVVVVTGAGGGLGKSHAIWFARYGAKVVVNDIKDP 357

CaFox2      TKTVEEIKAAG--GEAWADQHDVASQAEEIIKNVIDKYGTIDVLVNNAGILRDKSFAKMS 415
ScFox2      FSVVEEINKLYGEGTAIPDSHDVVTEAPLIIQTAISKFQRVDILVNNAGILRDKSFLKMK 417

CaFox2      DQEWDQVQKVHLLGTFNLSRLAWPYFAEKKYGRIVNISSTSGIYGNFGQANYASAKAGIL 475
ScFox2      DEEWFAVLKVHLFSTFSLSKAVWPIFTKQKSGFIINTTSTSGIYGNFGQANYAAAKAAIL 477
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CaFox2    GLSKTLAVEGARNNIKVNVVAPHAETAMTLTIFREQDK-NLYHADQVAPLLVYLGSEEVE 534
ScFox2    GFSKTIALEGAKRGIIVNVIAPHAETAMTKTIFSEKELSNHFDASQVSPLVVLLASEELQ 537

CaFox2    ------VTGETFEAGGGWIGNTRWQRAKGAVSHDEHTTVEFIRDNLKDITNFDSDTENPK 588
ScFox2    KYSGRRVIGQLFEVGGGWCGQTRWQRSSGYVSIKETIEPEEIKENWNHITDFSRNTINPS 597

CaFox2    STTESSMAILSAVGGDDDDDDDEEEEDEGDEEEEEEDEEEDDPVWRFNDRDVILYNIALG 648
ScFox2    STEESSMATLQAV----------------QKAHSSKELDDGLFKYTTKDCILYNLGLG 639

CaFox2    ATTKQLHYVYENDSDFQVIPTFGHLITFNSGKSQNSFAKLLRNFNPMLLLHGEHYLKVHK 708
ScFox2    CTSKELKYTYENDPDFQVLPTF-AVIPFMQATATLAMDNLVDNFNYAMLLHGEQYFKLCT 698

CaFox2    WPPPTEGAIKTTFEPISTTPK-GSNVVIVHGSKSVDNDSGEVIYSNEATYFIR--NCQAD 765
ScFox2    PTMPSNGTLKTLAKPLQVLDKNGKAALVVGGFETYDIKTKKLIAYNEGSFFIRGAHVPPE 758

CaFox2    NKVYAERRS-FATNPFPAPK-RAPDYQVDVPISEDLAALYRLTGDRNPLHIDPNFAKGAK 823
ScFox2    KEVRDGKRAKFAVQNFEVPHGKVPDFEAEISTNKDQAALYRLSGDFNPLHIDPTLAKAVK 818

CaFox2    FPKPILHGMCTYGLSAKVLIDKFGMFDEIKARFTGIVFPGETLRVLAWKESDDTVVFQTH 883
ScFox2    FPTPILHGLCTLGISAKALFEHYGPYEELKVRFTNVVFPGDTLKVKAWKQGS-VVVFQTI 877

CaFox2    VVDRGTIAINNAAIKLVGDKAKI 906
ScFox2    DTTRNVIVLDNAAVKLSQAKSKL 900
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(C) *C. albicans* and *S. cerevisae* Icl1

```
CaIcl1    MPY----TPIDIQKEEADFQKEVAEIKKWWSEPRWRKTKRIYSAEDIAKKRGTLK-INHP 55
ScIcl1    MPIPVGNTKNDFAALQAKLDADAAEIEKWWSDSRWSKTKRNYSARDIAVRRGTFPPIEYP 60

CaIcl1    SSQQADKLFKLLEKHDADKTVSFTFGALDPIHVAQMAKYLDSIYVSGWQCSSTASTSNEP 115
ScIcl1    SSVMARKLFKVLEKHHNEGTVSKTFGALDPVQISQMAKYLDTIYISGWQCSSTASTSNEP 120

CaIcl1    SPDLADYPMDTVPNKVEHLWFAQLFHDRKQREER-LTLSKEERAKT-PYIDFLRPIIADA 173
ScIcl1    GPDLADYPMDTVPNKVEHLFKAQLFHDRKQLEARSKAKSQEELDEMGAPIDYLTPIVADA 180

CaIcl1    DTGHGGITAIIKLTKMFIERGAAGIHIEDQAPGTKKCGHMAGKVLVPVQEHINRLVAIRA 233
ScIcl1    DAGHGGLTAVFKLTKMFIERGAAGIHMEDQTSTNKKCGHMAGRCVIPVQEHVNRLVTIRM 240

CaIcl1    SADIFGSNLLAVARTDSEAATLITSTIDHRDHYFIIGATNPEAGDLAALMAEAESKGIYG 293
ScIcl1    CADIMHSDLIVVARTDSEAATLISSTIDTRDHYFIVGATNPNIEPFAEVLNDAIMSGASG 300

CaIcl1    NELAAIESEWTKKAGLKLFHEAVIDEIKNGNYSNKDALIKKFTDKVNPLSHTSHKEAKKL 353
ScIcl1    QELADIEQKWCRDAGLKLFHEAVIDEIERSALSNKQELIKKFTSKVGPLTETSHREAKKL 360

CaIcl1    AKELTGKDIYFNWDVARAREGYYRYQGGTQCAVMRGRAFAPYADLIWMESALPDYAQAKE 413
ScIcl1    AKEILGHEIFFDWELPRVREGLYRYRGGTQCSIMRARAFAPYADLVWMESNYPDFQQAKE 420
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CaIcl1      FADGVKAAVPDQWLAYNLSPSFNWNKAMPADEQETYIKRLGKLGYVWQFITLAGLHTTAL 473
ScIcl1      FAEGVKEKFPDQWLAYNLSPSFNWPKAMSVDEQHTFIQRLGDLGYIWQFITLAGLHTNAL 480
             **:***  .*************** ***..*** *.*:*.***.***:***********.**

CaIcl1      AVDDFSNQYSQIGMKAYGQTVQQPEIEKGVEVVKHQKWSGATYIDGLLKMVSGGVTSTAA 533
ScIcl1      AVHNFSRDFAKDGMKAYAQNVQQREMDDGVDVLKHQKWSGAEYIDGLLKLAQGGVSATAA 540
             **.:**.:::: *****.*.*** *:. **:*:******** *******:..***:.***

CaIcl1        MGQGVTEDQFKESKAKA 550
ScIcl1        MGTGVTEDQFKENGVKK 557
             ** *********. .*
```

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 ≤ s ≤ 0.69	0.464	0.903
Medium confidence	0.69 ≤ s ≤ 0.84	0.346	0.950
High confidence	0.84 ≤ s ≤ 1.00	0.197	0.989

(D)

Strain	Number of high confident ubiquitination site			
	Icl1	Mls1	Fox2	Fbp1
C. albicans	0	0	1	0
S. cerevisae	2	0	0	0

Figure 1. Comparison of an amino acid pile-up of (A) CaMls1 and ScMls1. (B) CaFbp1 and ScFbp1. (C) CaFox2 and ScFox2. (D) CaIcl1 and ScIcl1. In which the high confidence ubiquitination sites are highlighted. (E) Compilation of high ubiquitination site in *C. albicans* and *S. cerevisiae* Icl1, Mls1, Fox2 and Fbp1.

lost the specific signal(s) that trigger this glucose-accelerated protein degradation. However, studies have not been done on the effect of other sugar phosphate such as galactose or fructose, both are important carbon sources that are absorbed directly into the blood from intestine. This studies focus on whether CaIcl1 protein has lost the specific signal that trigger protein degradation with the formation of ubiquitin-proteasome complex.

Ubiquitination play an important role in the glucose-accelerated degradation of gluconeogenic enzymes in *S. cerevisiae* [20] by acting as a degradation signal in *S. cerevisiae* [44]. This fact is supported by Eschrich et al. [22], in which ubc8 functions in the catabolite degradation of fructose-1,6-biphosphatase in *S. cerevisiae*. Therefore, consensus ubiquitination target sites were examined in CaIcl1 and ScIcl1, CaMls1 and ScMls1, CaFox2 and ScFox2, and CaFbp1 and ScFbp1 using ubpred (predictor of protein ubiquitination site, from http://www.ubpred.org/index.html) [45, 46] (**Figure 1**).

Based on this bioinformatics comparison, the ScIcl sequence containing strong consensus ubiquitination sites at amino acid 158 and 551, but there is a lack of high confidence ubiquitin target in CaIcl1, CaMls1, ScMls1, CaFbp1 and ScFbp1 (**Figure 1**). Interestingly, CaFox2 contain one strong consensus ubiquitination site at amino acid 588, but there is none high confidence ubiquitination target in ScFox2. This prediction was based on high level of confidence which is described in UbPred system containing score range 0.84 ≤ s ≤ 1.00, 0.197 for sensitivity and 0.989 for specificity. However, focus of this study is on Icl1 because it is more important for *C. albicans* virulency. Deletion of *ICL1* gene in *C. albicans* attenuated virulence [27] while deletion of FOX2 confers mild attenuation of virulence of *C. albicans* [26].

The bioinformatics screening of ubiquitination site includes the hydrophobic nature of the ubiquitination target site for high confidence prediction (TEDQFKENGVKK), which is contrast to the low and medium confidence sites which contain acidic and basic residues in the putative ubiquitination site (NGVKK; FNWPKAMSVD) [45, 46]. Therefore, the presence of consensus ubiquitination sites in these proteins correlated with glucose-accelerated degradation [35].

4. Overview of carbon sources attribute to the pathogenicity to *C. albicans*

The effect of galactose or fructose on the expression levels of the CaIcl protein in *C. albicans* and to compare this response with the corresponding situation in *S. cerevisiae* were tested. The effects of glucose on *S. cerevisiae* fructose-1,6-bisphosphatase (FBPase) have been intensively studied and it was reported that the FBPase protein (ScFbp1) is rapidly degraded upon addition of glucose [47]. It is also reported by Hammerle et al. [48] that the levels of cytosolic malate dehydrogenase, fructose-1,6-biphosphatase, isocitrate lyase and phosphoenolpyruvate carboxykinase are all low in *S. cerevisiae* after glucose addition. However, the degradation of FBPase is not correlated with the prediction of ubiquitination target site presence in the protein as ScFbp1 has only one low confidence ubiquitination target site at amino acid 250 (**Figure 1B**). The reason is not known whether or not FBPase had undergone ubiquitin-proteasome machinery although Regelmann et al. [49] have found that there are two different degradation pathways: cytosolic ubiquitin-proteasome machinery, and the other dependent on vacuolar proteolysis. Genes that are essential for vacuolar degradation are unnecessary for proteasome-dependent degradation [49].

Therefore, as a starting point whether Icl1 decline in *S. cerevisiae* upon galactose or fructose addition. To achieve this, the *S. cerevisiae* which is tagged at its 3′-end with Myc_9 is obtained from Sandai et al. [35]. Cells were grown on lactate, and then subjected to 2% of galactose or fructose. Samples were harvested at various times point and the Icl1 proteins expression was determined by western blotting and normalized by beta Actin. Clearly galactose or fructose addition led to the degradation of ScIcl1. These results confirmed that in *S. cerevisiae*, Icl1 are degraded in response to galactose or fructose. Similar results are observed in study done by Sandai et al. [35], whereby ScIcl1 and ScPck1 are degraded in response to glucose.

Next was to determine the effect of galactose or fructose addition to *C. albicans* cell growing on lactate. *C. albicans* strains expressing Myc_3-tagged Icl1 was confirmed by western blotting. *C. albicans* cells were grown in media containing the non-fermentable carbon sources lactate as sole carbon sources and the 2% galactose or fructose was added while cells were in exponential phase. Cells were then harvested at various times, and their proteins extracted and were loaded equally onto the SDS-PAGE gels, and expression of the Myc_3-tagged CaIcl1 protein is detected with anti-Myc antibodies and normalized by beta-actin. The *C. albicans* Icl1 protein was expressed during growth on lactate and was not destabilized by the addition of 2% galactose. Indeed CaIcl1 proteins levels were not significantly different from the control (lactate) even after 4 hours. Likewise, similar result was obtained by Sandai et al. [35] in which

CaIcl1 proteins remained stable after addition of 2% glucose to lactate grown cells. However, interestingly CaIcl1 protein is destabilized after addition of 2% fructose to lactate grown cells. This result contradicts the finding that CaIcl1 has lost the specific signal(s) that trigger protein degradation. This might prove the points by Regelmann et al. [49] that except from ubiquitin proteasome pathways the protein can undergo degradation through vacuolar proteolysis. However, the exact mechanism of why fructose triggers such pathway is not known.

Testing was carried out to see whether *C. albicans* is able to degrade proteins in response to galactose or fructose, the *S. cerevisiae ICL1* gene is expressed in *C. albicans*. *C. albicans ICL1* allele was replaced with a tagged *S. cerevisiae ICL1*. Ca(ScIcl1-Myc_3) was then grown in lactate before subjected to 2% galactose or fructose. Western blot was performed to confirm the expression of the Myc_3-tagged ScIcl1 which will be normalized to housekeeping protein beta-actin. From the results, ScIcl1 protein expression remained stable in *C. albicans* cells grown on lactate. However, following addition of 2% galactose or fructose to *C. albicans* cells, ScIcl1 was degraded. This indicates that *C. albicans* has retained capacity to destabilize target proteins in response to galactose or fructose. In other words, ScIcl1 retained its molecular apparatus for protein degradation even if it is expressed in *C. albicans* cells.

Testing was also carried out to determine whether *C. albicans* Icl1 expression remains stable in response to galactose or fructose, the *C. albicans ICL1* gene was expressed in *S. cerevisiae*. *S. cerevisiae ICL1* allele was replaced with a tagged *C. albicans ICL1*. Sc(CaIcl1-Myc_3) was then grown in lactate before subjected to 2% galactose or fructose. Cells were harvested at different time points thereafter and western blot was performed to confirm the expression of the Myc_3-tagged CaIcl1 which is normalized to beta-actin. From the results, CaIcl1 protein expression remained stable in *S. cerevisiae* cells grown on lactate. However, following addition of 2% fructose to *S. cerevisiae* cells, CaIcl1 was degraded. This supports the previous result that *C. albicans* Icl1 although lacks ubiquitination site but is sensitive to fructose and might undergo a different proteolysis. *C. albicans* has lost capacity to destabilize target proteins in response to 2% galactose. In other words, CaIcl1 was not degraded and remained stable in *S. cerevisiae*.

S. cerevisiae Icl1 has two high confidence putative ubiquitination sites located at residues 551 and 158, whereas CaIcl1 has no such sites (**Figure 1**). Therefore, ubiquitination plays a role in glucose-accelerated protein decay in *C. albicans*; the addition of a ubiquitination site to CaIcl1 would confer glucose-accelerated degradation upon this protein Sandai et al. [35]. Therefore carboxy-terminal ubiquitin site from ScIcl (TEDQFKENGVKK) was added into CaIcl1, together with the Myc_3 tag into the wild type polyubiquitin containing *C. albicans* cells. The function of ubiquitination in regulation rewiring of *C. albicans* Icl1 in response to galactose or fructose was tested. Cells were grown in lactate and galactose or fructose was added to final concentration of 2%. Cells were harvested at different time points thereafter, protein extracted and quantified, and these subjected to western blotting. The CaIcl-Ubi-Myc_3 proteins are normalized to housekeeping protein beta-actin. The result obtained shows that CaIcl-Ubi-Myc_3 is degraded upon addition of galactose or fructose to non-fermentable carbon sources, lactate. The results confirm a finding in a research done by Sandai et al. [35] whereby additions of glucose to lactate grown DSCO4 cells degrade the protein. This has also proven that ubiquitination plays a significant role in regulation of central metabolism enzymes in both *C. albicans* and *S. cerevisiae*.

5. Conclusion and future perspective

The assimilation of carbon sources is fundamentally important for the growth of *C. albicans* and for the establishment of infections in the human host [10]. For most yeast, such as *S. cerevisiae*, glucose is generally a more favorable carbon sources and the chosen mode of metabolism is often fermentative via glycolytic pathway-Embden-Meyerhof fermentation. In the presence of excess glucose, *S. cerevisiae* utilize glycolytic pathways and produce ethanol. *S. cerevisiae* is Crab-negative yeast that has the ability to produce ethanol even in the absence of oxygen [50]. In contrast, *C. albicans* is Crab-positive yeast because it retains respiratory capacity in the presence of excess glucose [51].

C. albicans is glucose Crabtree positive yeast which retains respiratory activity even following exposure to glucose [51]. During growth on glucose, *ADH1* mRNA levels rise to maximum levels during late exponential growth phase and the decline to low levels in stationary phase [52]. The *ADH1* mRNA is relatively abundant during growth on galactose, glycerol, pyruvate, lactate or succinate, and less abundant during growth on glucose or ethanol. However, alcohol dehydrogenase levels do not correlate closely with *ADH1* mRNA levels. This locus may be controlled in both transcriptional and post-transcriptional levels, or other differentially regulated *ADH* loci may exist in *C. albicans* [51]. Interestingly, a significantly smaller proportion of glucose is fermented to ethanol by *C. albicans* than by *S. cerevisiae* [51].

S. cerevisiae is not able to assimilate both non-fermentable carbon sources and galactose/fructose at the same time because of repression. Hence, these yeasts have evolved different responses to galactose/fructose. Therefore in this analysis was done on regulation of carbon assimilation in *C. albicans* focusing on genes/enzymes involved in gluconeogenesis, glyoxylate cycle and β-oxidation. *ICL1*, *FOX2*, *MLS1*, and *FBP1* gene expression and the impact of galactose or fructose on the assimilation of non-fermentable carbon sources were analyzed. *C. albicans* responses were then compared to those of *S. cerevisiae* under equivalent conditions. The following conclusions can be drawn from these findings;

First, gluconeogenic, glyoxylate cycle and β-oxidation mRNAs are sensitive to galactose or fructose in both *C. albicans* and *S. cerevisiae*. This reconfirmed previous finding, whereby the transcriptome, such as *PCK1* and *FBP1* in *S. cerevisiae* [42], *ICL1* and *PCK1* in both *S. cerevisiae* and *C. albicans* [35], was degraded upon exposure to glucose. The dramatic decreases in *ICL, MLS1, FOX2* and *FBP1* mRNA levels in *C. albicans* after exposure to 2% galactose or fructose to lactate- and oleic acid-grown cells was observed. It is already known that *C. albicans* sensitive to glucose concentrations even as low as 0.01%, this value is within the physiological range of blood glucose (about 0.1%) [10, 12]. Therefore, *C. albicans* is able to respond to blood glucose levels during disseminated hematological infections. Interestingly, diabetic patients who often have elevated blood glucose levels have a higher risk of systemic *Candida* infections [53] and dietary glucose enhances *C. albicans* colonization and invasion [54]. Galactose and fructose are both important sugar phosphate to human and yeast. Galactose can be converted to glucose through Leloir pathway which utilized three enzymes in human liver [55] and five enzymes in yeast [56]. In mammary gland galactose covalently bound to glucose to formed disaccharide lactose [57]. Moreover individual with galactosemia, a rare inherited metabolic disorder that affects the body ability to metabolize galactose properly and have high content

of galactose in both urine and blood. This increases the risk of developing *E. coli* sepsis and sometimes or rarely fungal sepsis, such as *Candida* species [58].

Second main observation was that the Icl1 proteins are stable in *C. albicans* following galactose exposure but not fructose. The addition of 2% galactose to *C. albicans* cells growing on lactate did not trigger the degradation of the Icl1 proteins, but the protein was degraded upon addition of 2% fructose, at least 4 hours examined. This is in contrast to the situation in *S. cerevisiae*, where the addition of 2% galactose or fructose triggered the rapid degradation of the Icl1 proteins. The estimated half-lives for these proteins in *S. cerevisiae* are more than 20 hours [46] indicating that these proteins are very stable. This probably represents a significant difference in the physiological responses of these pathogenic and benign yeasts to glucose. *C. albicans* is able to establish infections in complex niches, many which contain a rich mixture of alternative carbon sources [10]. The stability of the Icl1 protein in *C. albicans*, even in the presence of galactose and glucose [35] might suggest that this pathogen is capable to assimilate alternative carbon sources at the same time as galactose or glucose in these carbon-rich niches. The observation from addition of galactose and glucose is similar in *C. albicans*, this may be because galactose and glucose is reversibly convertible in Leloir pathway, and galactose is first converted to glucose to enter the glycolysis pathway.

Interestingly there is a finding that fructose increases phosphorylation/activation of hypothalamic AMP kinase causing phosphorylation/inactivation of Acetyl-CoA carboxylase, whereas glucose has the inverse effects [59]. This finding is interesting because acetyl-coenzyme A (Acetyl-CoA) is an essential cofactor in central metabolism, this molecule is the entry point to the tricarboxylic acid (TCA) cycle that generates energy, biomass, and intermediates for macromolecules [59]. This means that addition of fructose might repressed Acetyl-CoA in *C. albicans* which will in turn affects the TCA and glyoxylate cycle. However, this might not be the only reason for Icl1 protein decay in *C. albicans* in response to fructose. Glucose enters the glycolytic pathway via glucokinase-catalyzed phosphorylation and its further metabolism is subjected to control in phosphofructokinase (PFK) in the glycolytic pathway, which is considered to be rate-limiting step. In contrast, fructose is metabolized faster than glucose because it bypasses this step entering the glycolytic pathway at the level of the triose phosphates. This showed that fructose is a much efficient carbon sources compared to glucose or alternative non fermentable carbon sources such as lactate.

The third observation whether *C. albicans* has lost signal that trigger destabilization in response to galactose and retained molecular capability of destabilizing target proteins in response to fructose. *C. albicans ICL1* ORF was expressed in *S. cerevisiae* and vice versa *S. cerevisiae ICL1* ORF was expressed in *C. albicans*. The CaIcl1 in *S. cerevisiae* remained stable even after addition of 2% galactose but was degraded after addition of 2% fructose was tested. The finding supported the previous report. This is interesting because it proved that *C. albicans* Icl1 is sensitive to fructose but not to galactose. In contrast, ScIcl1 in *C. albicans* was degraded in response of 2% galactose or fructose.

The analysis of ubiquitination in *C. albicans* was done by exposing CaIcl1-Ubi-Myc_3 to 2% galactose or fructose. *C. albicans* Icl1 is destabilized by galactose because of the addition of carboxyl-terminal ubiquitination site. In previous finding the fructose destabilize CaIcl1 and addition of ubiquitin site accelerated the destabilization of the protein. Cells

can no longer able to metabolize via the glyoxylate cycle following galactose and fructose addition due to the degradation of Icl1 protein. As a result, those cells would presumably be less able to cause infections and would be less able to compete for available nutrients against other microorganisms such as endogenous bacteria in the gastrointestinal (GI) tract compared to their wild type. It is likely to show less successful colonization, virulence and fitness due to this defect in its ability to assimilate both glucose and alternative carbon sources at the same time. Similarly, mutant *C. albicans* cell with deletion of *ICL1* attenuated virulence [27].

It is interesting to further study the mechanism involved in protein degradation in response to fructose. Why is only fructose and not glucose or galactose that triggers protein degradation of CaIcl1? And which pathways could CaIcl1 take for proteolysis? Is it vacuolar proteolysis or ubiquitin mediated proteolysis, such as ubiquitin conjugating enzyme Ubc8p [43] and E3 ubiquitin ligase, Gid complex [49, 60]? It is also interesting to explore other central metabolism enzymes that carry the same characteristic of CaIcl1 and also to determine the effect of exposure to other carbon sources such as malate and succinate.

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