Journal of Biomedical Science (2006) 13:313–321
DOI 10.1007/s11373-005-9054-6

Mutations on *CaENO1* in *Candida albicans* inhibit cell growth in the presence of glucose

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Key words: *Candida albicans*, cell growth, enolase, glucose, inhibition

Summary

Enolase (2-phospho-D-glycerate hydrolase) is an enzymatic component of the glycolytic pathway and is conserved through evolution. The *TR-CaENO1/Caeno1* stain, of which the expression of *CaENO1* is under control of the tetracycline-regulatable (TR) expression system, is utilized for elucidating the functions of *CaENO1* in *Candida albicans*. As expected, there was no detectable *CaENO1* mRNA when the *TR-CaENO1/Caeno1* cells grew on media containing doxycycline repressing the expression of *TR-CaENO1*.The *TR-CaENO1/Caeno1* cells were arrested in media containing doxycycline in the presence of glucose but not in non-fermentable carbon sources, such as glycerol. Furthermore, the *TR-CaENO1/Caeno1* cells were also arrested in media containing 4% serum. In this study, we have showed that *CaENO1* is required for the cell growth of *C. albicans* in the presence of glucose. Our findings may help us to design new and more effective antifungal agents for preventing and treating bloodstream fungal infections by blocking the function(s) of enolases.

Introduction

Enolase (2-phospho-D-glycerate hydrolase) is an enzymatic component of the glycolytic pathway, catalyzing the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate. It is a highly conserved protein throughout the kingdoms of life from plants to animals and from bacteria to humans [1].

Enolase and other glycolytic enzymes are the most abundant proteins in *Saccharomyces cerevisiae* [2, 3]. There are two non-tandemly repeated enolase structural genes, *ENO1* and *ENO2*, in the *S. cerevisiae* genome. These two genes share high degree of similarity within the coding sequences [4]. The amount of enzyme encoded by *ENO1* increases during stationary phase while the amount of enolase encoded by *ENO2* increases when glucose is present in the growth media [5, 6]. Unlike other genes involved in glycolytic pathway, single mutation on either *ENO1* or *ENO2* in *S. cerevisiae* are viable in the presence of glucose [6, 7].

In *Candida albicans*, the gene encoding enolase is named *CaENO1*, of which the *Ca* stands for *C. albicans* [8]. Enolase has been estimated to comprise 0.7% of the total proteins in the yeast-form cells and 2% in the hyphae [9]. Antagonizing *CaENO1* expression by antisense RNA causes reduction of the growth rate in *C. albicans* [10].

The tetracycline-regulatable (TR) expression system is a well-established system for gene expression among eukaryotic cells [11–13]. To investigate the functions of enolase in *C. albicans*, we have constructed a *TR-CaENO1/Caeno1* *C. albicans* strain, of which one copy of the

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CaENO1 gene is replaced by the *ARG4* selective marker and the other copy is under control of the TR expression system [14]. In the present study, we have showed that, unlike *ENO1* and *ENO2* of *S. cerevisiae*, *CaENO1* is essential for cell growth in glucose-contained media.

Materials and methods

Strains and media

Candida albicans strains used in this study are as following: BWP17, *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG* [15], YLO139 (*CaENO1/CaENO1*), *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CaENO1/tetR-HIS1-CaENO1* as the wild-type *CaENO1* strain; YLO165 (*CaENO1/Caeno1*) *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CaENO/tetR-HIS1-Caeno1::ARG4* as the heterozygous mutant; and YLO166 (*TR-CaENO1/Caeno1*), *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG TR-CaENO1/ tetR-HIS1-Caeno1::ARG4*. All media described below were prepared as deletion in Sherman [16]: Basal medium (BASAL, 1% Nutrient broth, 1% mannitol, 0.2% K_2HPO_4), Yeast Peptone Dextrose (YPD, 1% yeast extract, 2% peptone, and 2% glucose), Yeast Peptone Glycerol (YPG, 1% yeast extract, 2% peptone, and 2% glycerol), Synthetic Dextrose (SD, 0.67% yeast nitrogen base without amino acid and 2% glucose), Brain Heart Infusion (BHI, 20% infusion from calf brain, 15% infusion from beef heart, 1% proteose pepton, 0.2% glucose, 0.5% NaCl, 0.25% Na_2PO_4), and Mueller Hinton (MH, 0.03% beef extract, 1.75% Acid Hydrolysate of casein, and 0.015% starch). Equal volume of 2× media were mixed together to prepared for the mixed media. All agar plates were prepared with addition of 2% agar in media. Agar serum plates were prepared by mixing 1 ml of serum into 25 ml of 2% agar.

Construction of the CaENO1/Caeno1 heterozygous mutant

Candida albicans strain YLO139 (*CaENO1/CaENO1*) was used for the construction of *CaENO1/Caenol* heterozygous mutant (a gift from Dr. H.-J. Lo, NHRI, Taiwan). YLO139 is based on BWP17 [15] with the integration of the gene encoding the activator for the TR promoter, *tetR*, into the promoter of *CaENO1* alone with a $His1^+$ marker. Hence, it has the genotype of *ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CaENO1/tetR-HIS1-CaENO1* (Figure 1) and the expression of *tetR* was under the control of the *CaENO1* promoter. To construct the *CaENO1/Caeno1* heterozygous mutant, the plasmid pRS-ARG4 SpeI [15] was used as the template DNA. The primer ENO1KOF 5′d(ATGTCT TACG CCACTAAAAT CACGCCAGA TAC GTCTACG ACTCCAGAGG TTGGGTTGGCA ACTTCAAC GTTTTCCCAG CACGACGTT) and primer ENO1KOR 5′d(AGAGGCAAAC TTACAATTGA AAGCCTTTT GGAAATCT TT ACCAGCGTAG TAGCTTCAGAACCTAA TTC TGTGGAATTG TGAGCGGATA) were used to amplify a DNA fragment containing the *ARG4* gene sequence flanked by short homologous regions (70 bp) of the *CaENO1* gene at the two extremities. This DNA fragment was then transformed into the YLO139 strain, containing the TR transactivator gene, *tetR* [14] by the LioAC method. The open reading frame of *CaENO1* from position +1 to +1264 (The A of the translation start codon ATG as +1) in the *tetR-HIS1::CaENO1* allele was then replaced by the *ARG4* gene to generate the heterozygous mutant, YLO165 (*CaENO1/Caeno1*), *CaENO1/tetR-HIS1-Caeno1::ARG4* (Figure 1). We named this mutant allele as *Caeno1*.

Construction of the TR-CaENO1/Caeno1 strain

The primers ENO1AF 5′d(TTT*GGTACC*ATTA AGCCGTGGGTTCTCA) and ENO1AR, 5′d(T TT*CTCGAG*CAATAAAGGGAGTAAGGTGG) were used to amplify a *CaENO1A* region from the 175th to the 580th bp upstream of the translation initiation codon of the *CaENO1* gene. The primers ENO1BF, 5′d(GC*TCTAGA*TGT CTTACGC CACTAAAATCC) and ENO1BR, 5′d(TTT*CCG CGG*CAATGATGTCATTAACAT T) were used to amplify a *CaENO1*B region from the first to the 225th bp downstream of translation initiation codon of the *CaENO1* gene. These two regions were digested with restriction enzymes and ligated in the cloning regions of the p99CAU1 plasmid as described previously [14]. The strain YLO166 (*TR-CaENO1/Caeno1*) (Figure 1) was generated

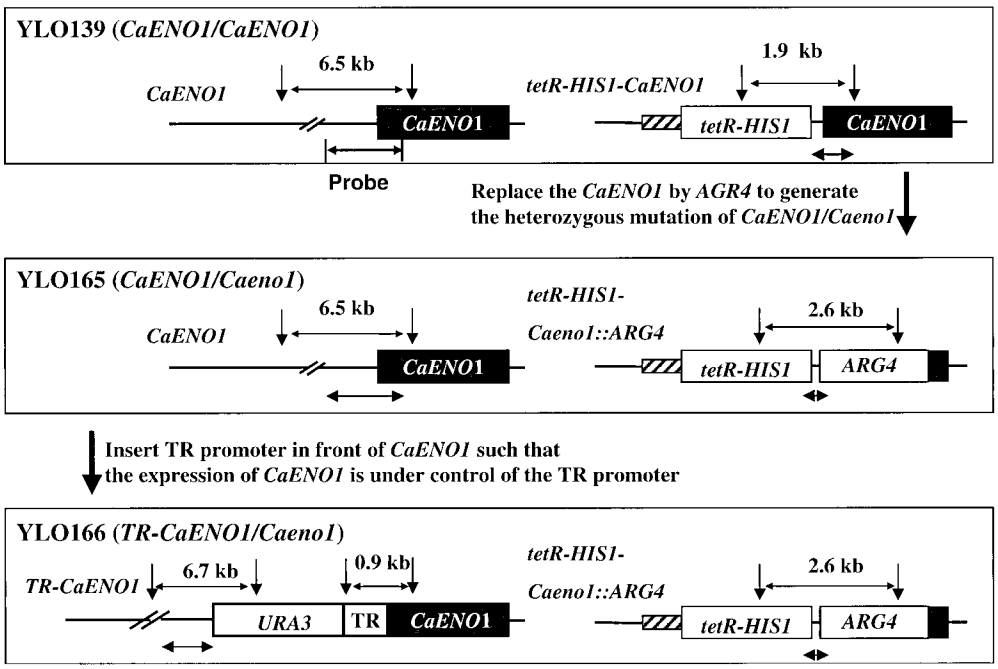


Figure 1. Scheme for the construction of the *TR-CaENO1/Caeno1* strain. The *C. albicans* YLO139 strain contains the activator gene of TR, *tetR* in its genome alone with a His+ marker (*tetR-HIS1*) at the promoter of *CaENO1*to create the *CaENO1/tetR-HIS1-CaENO1* genotype. The open reading frame (solid bar) of *CaENO1* in the *tetR-HIS1-CaENO1*allele was then replaced by the *ARG4* gene (open bar) to generate the heterozygous mutant *CaENO1/tetR-HIS1-Caeno1::ARG4*, YLO165. Then, the TR promoter was inserted in front of the wild-type copy of *CaENO1* to generate*TR-CaENO1/tetR-HIS1-Caeno1::ARG4*strain, YLO166. Thus, the expression of *CaENO1* in the YLO166 strain was regulated by the TR expression system. Vertical arrows represent the restriction enzyme sites of *Eco*RI. The predicated sizes (in kb) of DNA fragments hybridized with the probe were indicated between two vertical arrows. The detecting regions of the probe are showed as double-headed arrows beneath the constructs. Probe, the region used for synthesized labeled DNA probe for Southern analysis.

by transforming the entire promoter-replacing construct into the YLO165 strain, such that the expression of *CaENO1* is under control of the TR expression system.

Southern blot analysis

Total genomic DNA was isolated from each *C. albicans* strain and subjected to *Eco*RI digestion. The digested genomic DNA was then resolved in 0.7% agarose gel and transferred to a nylon membrane. The DNA fragment from 580th bp upstream to 205th bp downstream of the translation initiation codon of *CaENO1* was used as the template to synthesize the DIG-labeled (Cat. No. 1175033. Roche, US) probes for Southern analysis. The experiment was performed according to the instruction from the manufacturer.

Isolation of total RNA by hot acidic phenol

The *C. albicans* cells were grown in 30 ml of YPD liquid medium to the OD_{600} of 0.5. The cells were then harvested after being incubated at 30 °C for 1 h in the absence or presence of 20 $\mu g/ml$ of doxycycline (Sigma D9891). The cell pellet was resuspended in RNA isolation buffer (50 mM NaOAc, 10 mM EDTA, 1% SDS). Acid-washed glass beads (425–600 μm) and 500 μl of 65 °C acid phenol were added to the cell pellet. The cell suspension was vortexed vigorously for 10 s and then incubated at 65 °C for 10 min, followed by two extractions with acid phenol/chloroform and one extraction with chloroform. The RNA was then precipitated with ethanol and the quality determined by both the ratio of the absorption of OD_{260}–OD_{280} and by electrophoresis in agarose gels.

Northern blot analysis

Approximate 12 μg of total RNA was resolved in a 1% agarose gel under denaturing conditions and then transferred to a nylon membrane. The *CaENO1B* region, from the first to the 225th bp downstream of translation initiation codon of *CaENO1*, was used as the temple to generate a labeled probe for detection of *CaENO1* expression by northern blot analysis using the DIG system (Cat. No. 1175033, Roche, US). The experiment was conducted according to the instruction supplied by the manufacturer.

Growth assay on different media

Both the wild-type strain, YLO139, and the *TR-CaENO1/Caeno1* strain, YLO166, were patched onto YPD agar medium without the doxycycline and then incubated at 30 °C for 1 day. Then they were replica-plated onto different agar media either in the absence or presence of 20 μg/ml doxycycline. All cells were incubated at 30 °C for 1 day. For the agar serum medium, cells were streaked onto agar plates containing 4% serum and then incubated at 37 °C for 2 days.

Results and discussion

Construction of the TR-CaENO1/Caeno1 strain

To utilize the TR expression system [14] for the study, we have obtained the YLO139 strain (Figure 1), a strain based on BWP17 with the integration of the gene encoding the trans-activator of the TR promoter, *tetR*, immediately downstream to the promoter of *CaENO1*. Then, the *CaENO1* in the *tetR-HIS1::CaENO1* allele was replaced by the *ARG4* gene to generate the heterozygous mutant (Figure 1, YLO165). Finally, the promoter region of the other copy of *CaENO1* was replaced by the TR promoter and hence under the TR system (Figure 1, YLO166). Doxycycline binds the *tetR* trans-activator of the TR system and will prevent the interaction between the trans-activator and the *TR* promoter [11–13]. As a consequence, in the presence of doxycycline, the gene expression from the *TR* promoter is inhibited [14]. The genotypes of all constructed strains were verified by Southern analysis (Figure 2). In the YLO139 strain, the probe detected bands at the positions of 6.5 and 1.9 kb. In the YLO165 heterozygous mutant strain, bands were at 6.5 and 2.6 kb. And

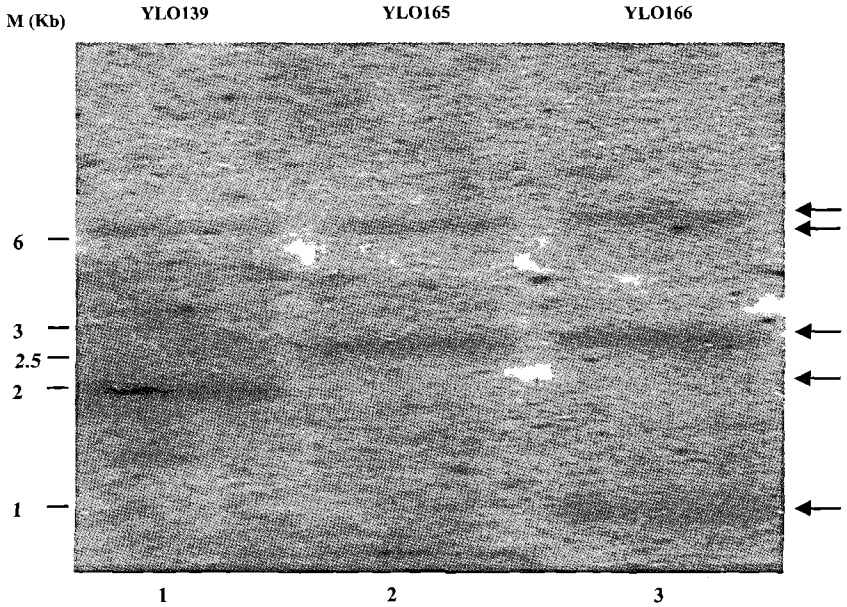


Figure 2. Result of Southern blot analysis of genomic *CaENO1* in various strains. Genomic DNA digested with *Eco*RI was from the following strains: lane1, wild-type strain, *CaENO1/CaENO1* (YLO139); lane2, heterozygous mutant, *CaENO1/Caeno1*(YLO165); lane 3, TR regulating *CaENO1* strain, *TR-CaENO1/Caeno1* (YLO166). The sizes (in kb) of the molecular weight standards are labeled on the left. The sizes of the bands (arrows on the right) were indicated in Figure 1 above each chromosomal construct between vertical arrows (the *Eco*RI restriction fragment).

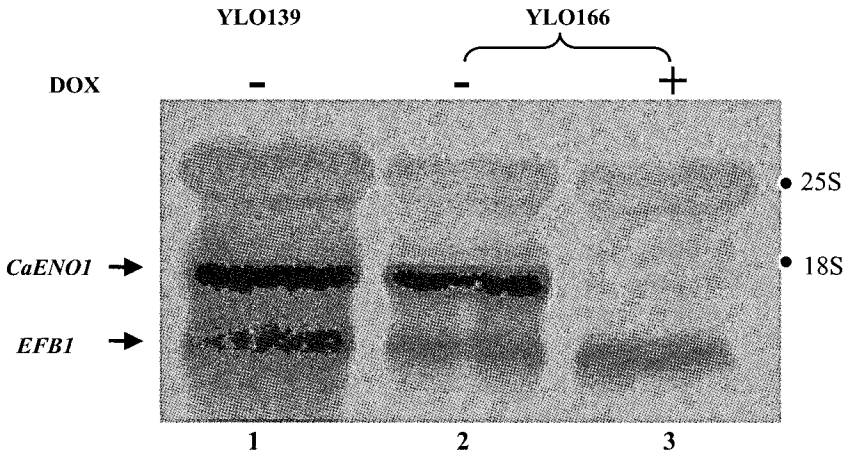


Figure 3. Northern blot analysis of the *CaENO1* mRNA. Total RNA was isolated from the *C. albicans* strains for the analysis to assess the mRNA levels. (1), wild type (YLO139); (2), *Caeno1/TR-CaENO1* (YLO166) without doxycycline; (3), *TR-CaENO1/Caeno1*(YLO166) with doxycycline. The *EFB1* gene was used as the loading control. The size of the mRNA for *CaENO1* is about 1.5 kb and the loading control *EFB1* 0.7 kb. DOX+, the medium containing 20 μg/*ml* of doxycycline; DOX −, in the absence of doxycycline.

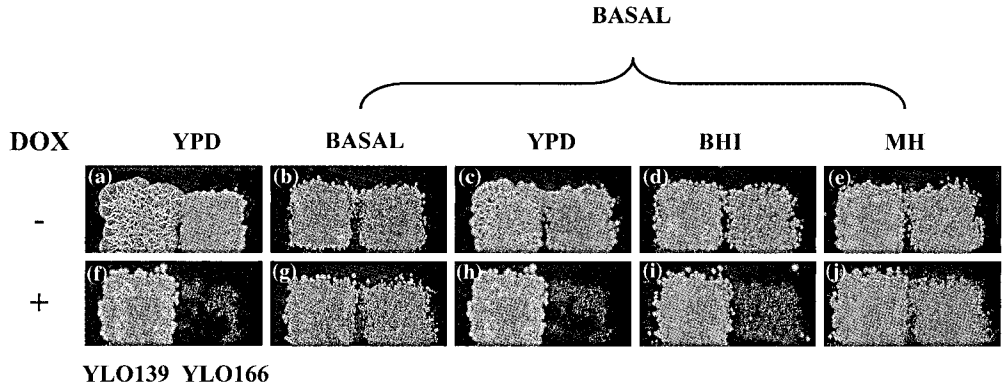


Figure 4. Cell growth on different media. The wild-type strain, YLO139 and the *TR-CaENO1/Caeno1* strain, YLO166 were patched onto the YPD media and then cells were replica-plated onto different media after incubation at 30 °C for 1 day. The photographs were taken after the replica were incubated at 30 °C for 1 day. The cells were grown on media in the absence of doxycycline (a–e) and in the presence of 20 μg/ml of doxycycline (f–j). Media included YPD (a and f), BASAL (b and g), BASAL plus YPD (c and h), BHI (d and i), and MH (e and j).

in the YLO166 strain, three bands positioned at 6.7, 2.6, and 0.9 kb. The results of Southern analysis were as expected.

To elucidate the functions of enolase, we have to be able to turn off the expression of the *CaENO1* in the YLO166 cells. Thus, to assess the expression of the gene in different strains, we applied Northern analysis to determine the mRNA level of the *CaENO1*. The level of *CaENO1* mRNA of *TR-CaENO1/Caeno1* YLO166 strain was determined after this strain was grown on media in the absence and presence of doxycycline. As expected, *CaENO1* was expressed in the YLO166 strain grown in the absence of doxycycline (Figure 3, lane 2); whereas, there was no detectable *CaENO1* mRNA when the strain was grown in the medium containing 20 μg/ml of doxycycline (Figure 3, lane 3). These results suggest that the expression of *CaENO1* in the YLO166 strain is indeed tightly regulated by the TR expression system.

Investigation of cell growth on different media

Both the wild-type, YLO139, and the *TR-CaENO1/Caeno1*, YLO166, were grown on different

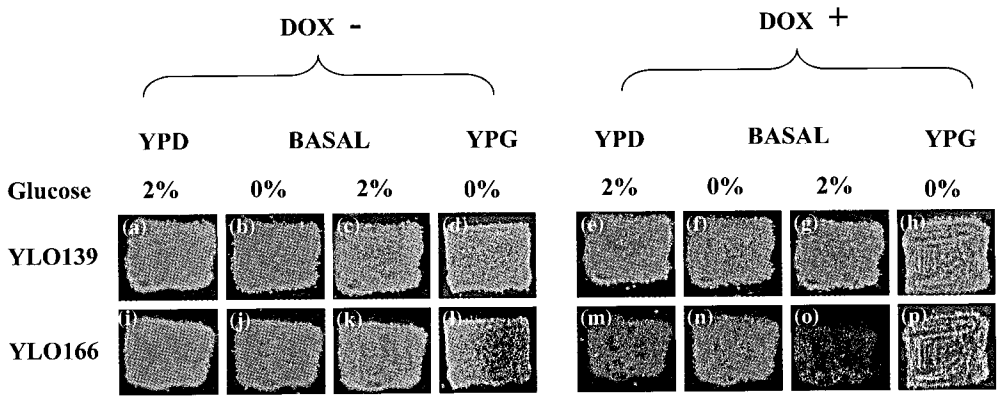


Figure 5. Enolase is required for cell growth in the presence of glucose. The wild-type strain, YLO139 (a–h) and the *TR-CaENO1/Caeno1* strain, YLO166 (i–p) were patched onto the YPD media and then cells were replica-plated onto different media after incubation at 3 °0C for 1 day. The photographs were taken after the replica were incubated at 30 °C for 1 day. The cells were grown on media in the absence of doxycycline (a–d and i–l) and in the presence of 20 μg/ml of doxycycline (e–h and m–p). Media included YPD plus 2% glucose (a, i, e, and m), BASAL (b, j, f, and n), BASAL plus 2% glucose (c, k, g, and o), and YPG (d, l, h, and p). DOX +, the medium containing 20 μg/ml of doxycycline; DOX–, in the absence of doxycycline.

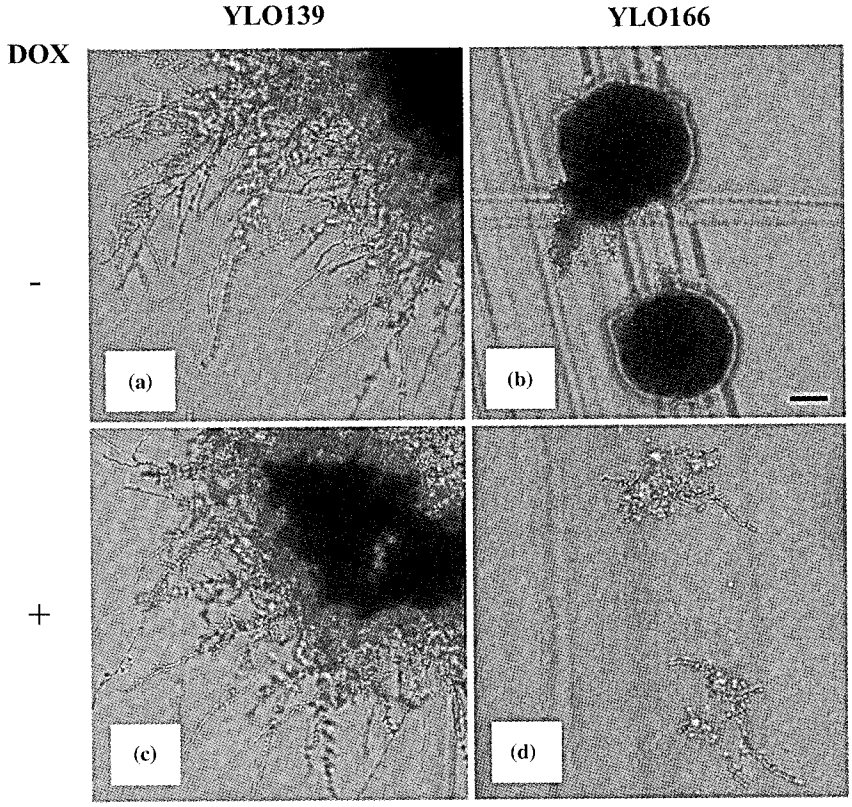


Figure 6. Cell growth on agar serum medium. The wild-type strain, YLO139 (a and c) and the *TR-CaENO1/Caeno1* strain, YLO166 (b and d) were streaked on agar plates containing 4% serum media. The photographs were taken after incubation at 37 °C for 2 days. The cells were grown on media in the absence of doxycycline (a and b) and in the presence of 20 μg/ml of doxycycline (c and d). Scale bar is 30 μm.

media to determine the functions of enolase in *C. albicans* (Figure 4). The wild-type strain grew on all media tested. And the YLO166 strain also grew well on all media in the absence of doxycycline. Interestingly, in the presence of doxycycline, the YLO166 strain grew on BASAL (Figure 4g)

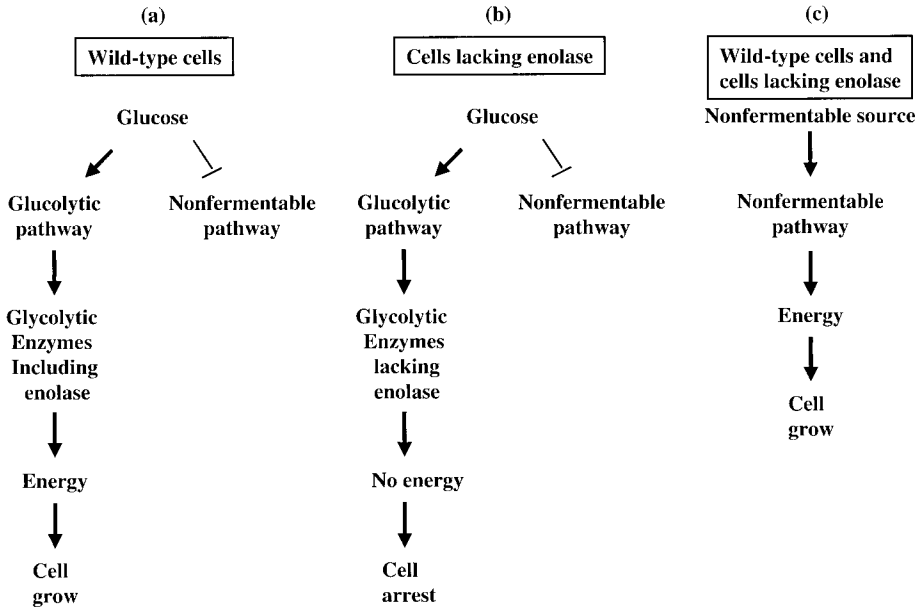


Figure 7. The schematic model for causing the arrest of cell growth by glucose repression. (a) The wild-type cells grow in media containing glucose. (b) Cells lacking enolase are arrested in media containing glucose. (c) Both the wild-type cells and cells lacking enolases grow in media containing only non-fermentable carbon sources.

but on YPD there was only a layer of arrested cells (Figure 4f). There are at least two explanations for this result. One is that the BASAL has positive factors required for the growth of cells lacking enolase. The other is that YPD has negative factors inhibiting the growth of cells not having enolase. To distinguish between these two possibilities, the YLO166 cells were grown on media with different combinations. The result showed that in the presence of doxycycline, cells grew on BASAL mixed with MH (Figure 4j) but not on BASAL with YPD (Figure 4h) or BHI (Figure 4i). One difference among these media is that both BASAL and MH do not contain glucose, whereas YPD and BHI do. Worthy mentioning is that BHI contains only 0.2% glucose. To determine if glucose is the factor affecting the growth of cells lacking enolase, we have patched the *TR-CaENO1/Caeno1* YLO166 cells on BASAL plus 2% glucose (Figure 5c, k, g, and o). Again, the wild-type cells grew on all media tested. In the presence of doxycycline, the YLO166 cells grew on BASAL medium in the absence of glucose (Figure 5n) but not on BASAL medium with 2% glucose (Figure 5o). As showed in the Figure 5o, there was only a layer of arrested cells. To further assess our hypothesis that glucose inhibits the growth of cells lacking enolase, we have grown the *TR-CaENO1/Caeno1* YLO166 cells on YPD and YPG media. Both the wild-type and the YLO166 cells grew on YPD and YPG in the absence of doxycycline (Figure 5a, i, d, and l). In the presence of doxycycline, the YLO166 cells could grow on the YPG medium (Figure 5p) but on the YPD medium there was only a layer of arrested cells (Figure 5m). The only difference between the YPD and the YPG is that the former has glucose and the later has glycerol as the carbon source. Therefore, we concluded that enolase is required for the cell growth of *C. albicans* in media containing glucose. This datum also suggests that *S. cerevisiae* cells containing single mutations on either *ENO1* or *ENO2* are viable in the presence of glucose [6, 7] may have resulted from the functional redundancy of these two isoforms.

Our findings may help us to design new and more effective antifungal agents for preventing and treating bloodstream fungal infection by blocking the functions of enolase since serum also contain glucose. To investigate if this is a possibility, we have streaked *C. albicans* cells on agar serum plates. Again, the wild-type cells (Figure 6c) but not cells lacking enolase (Figure 6d) grew on media containing serum.

Glucose is the most abundant monosaccharide in nature and it is also the primary fuel for yeasts. Although glucose and other sugars are the preferred carbon source by the yeast, non-fermentable substrates such as ethanol and glycerol can also be used for generating energy and cellular biomass [17–20]. Several regulatory networks of glucose repression are involved in coordinating the biosynthesis of enzymes required for the utilization of non-fermentable substrates [18, 21–24]. Thus, although yeasts can utilize various carbon sources, glucose-signaling pathways in yeasts ensure that they use up all the available glucose before turning toward alternative fuels (Figure 7a). When media contain only non-fermentable carbon sources, both the wild-type cells and the cells lacking enolase can utilize these sources to produce energy (Figure 7c). For the wild-type cells, glycolytic enzymes including enolase are important for generating energy while utilizing glucose. Therefore, cells lacking enolase cannot grow in media containing glucose because they are not able to utilize glucose. Neither can they utilize the non-fermentable carbon sources since genes involved in non-fermentable pathways are repressed by the presence of glucose (Figure 7b), which is consistent with the results in Figures 4 and 5.

In this study we have demonstrated that *CaENO1* is essential for the *C. albicans* cells to grow on glucose. In the presence of glucose, lacking the *CaENO1* gene became a limiting factor for cell growth due to glucose (catabolite) suppression. This also suggests that there is no other functional homolog of *CaENO1* in *C. albicans*, which is different from that of *S. cerevisiae*. Finding genes whose products affect the cell growth of *C. albicans* in the presence of glucose may help to design new and more effective antifungal agents for preventing and treating bloodstream fungal infection since serum also contains glucose. It may be a possibility to reduce or block infections of *C. albicans* by using compounds that suppress *CaENO1* activity.

Acknowledgements

We would like to thank Dr. H.-J. Lo and Dr. C.-G. Chen for their helps and the *C. albicans* strains YLO139. We would also like to thank Dr. H. Nakayama for the TR expression system. This work was supported in part by the Brain Research Center, National Chiao Tung University, University System of Taiwan, under grant 92B-711, and also in part by National Science Council under grants 94-2320-B-009-001.

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