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Investigation of protein expression profiles of erythritol-producing *Candida magnoliae* in response to glucose perturbation

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ABSTRACT

Protein expression patterns of an erythritol-producing yeast, *Candida magnoliae*, were analyzed to identify differentially expressed proteins in response to glucose perturbation. Specifically, wild type *C. magnoliae* was grown under high and low glucose conditions and the cells were harvested at both mid-exponential and erythritol production phases for proteomic studies. In order to analyze intracellular protein abundances from the harvested cells quantitatively, total intracellular proteins were extracted and applied to two-dimensional gel electrophoresis for separation and visualization of individual proteins. Among the proteins distributed in the range of pI 4–7 and molecular weight 29–97 kDa, five osmo-responsive proteins were drastically changed in response to glucose perturbation. Hsp60 (Heat-shock protein 60), transaldolase and NADH:quinone oxidoreductase were down-regulated under the high glucose condition and Bro1 (BCK1-like Resistance to Osmotic shock) and Eno1 (enolase1) were up-regulated. These proteins are directly or indirectly related with cellular stress response. Importantly, protein expression patterns of Hsp60, Bro1 and Eno1 were strongly correlated with previous studies identifying the proteins perturbed by osmotic stress for other organisms including *Saccharomyces cerevisiae*.

1. Introduction

A tremendous amount of genomic information from novel microbes has been obtained through the development of both advanced bioinformatics tools and next generation sequencing technologies [1]. Still, numerous uncharacterized microbes, which survive in extreme or specific environments, are waiting to be explored. Through analyzing these microorganisms exhibiting unique phenotypes at the systems level, we can identify proteins that play a pivotal role in cell survival in extreme environments. Furthermore, these newly identified proteins may also have unprecedented functions. Therefore, it is worthwhile to develop a large-scale proteomic approach combined with genomic information for cost effective ways to screen valuable genes from these novel microbes.

Candida magnoliae is an industrially important yeast that can produce erythritol with a high yield and productivity. Erythritol is a low calorie sugar alcohol naturally produced by algae, fruits and mushrooms [2,3]. Erythritol can be used as a sugar substitute to lower the risk of diabetes and obesity due to its low absorption in the human body [2–5]. *C. magnoliae* has several interesting physiological properties such as the ability to grow in a wide range of pH values (at least between 2.5 and 8.0) and an abnormal sugar preference for fructose rather than glucose as a primary carbon source [6,7]. It has the ability to grow in the presence of high sugar concentrations (≥300 g/L glucose and fructose), suggesting that this yeast obtained its ability to survive in a hyper-osmotic environment [8]. Importantly, high external osmotic stress promotes accumulation of erythritol in *C. magnoliae*, which may serve as an osmolyte to compensate for the high extracellular osmotic pressure [8]. Recently, our group has identified and characterized the enzyme erythrose reductase, which is responsible for the final step of erythritol production in *C. magnoliae* [9]. Erythrose reductase is specifically up-regulated under high osmotic and salt stress conditions [10]. In spite of intriguing industrial and biological features, the genome sequence of *C. magnoliae* has not been determined. Currently, 912 randomly sequenced EST clones are available [9].

Although cellular responses to osmotic stress have been investigated in various yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Zygosaccharomyces rouxii* and *Debrayomyes hansenii*, details of the molecular

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mechanism of a microbial response to high external osmotic stress is not understood clearly as it is highly complex and previous studies have only focused on specific aspects [11]. As compared to other yeasts and fungi, *C. magnoliae* also has interesting physiological properties that are quite unique to this microorganism such as erythritol-producing capability under high sugar concentrations [7,8]. Thus, we employed a proteomic approach to understand the osmotic stress mechanism of *C. magnoliae*. Fermentations with *C. magnoliae* strain were performed under low and high glucose conditions. To identify *C. magnoliae* proteins whose expression levels were changed under different glucose conditions, two-dimensional electrophoresis (2-DE) coupled with mass spectrometry was used. Expression levels of five osmo-responsive proteins showed significant variations in different glucose conditions. Heat-shock protein 60 (Hsp60), transaldolase and NADH:quinone oxidoreductase were down-regulated under the high glucose condition and Bro1 (BCK1-like Resistance to Osmotic shock) and Eno1 (enolase1) were up-regulated under the high glucose condition. Most of these proteins are directly or indirectly related to cellular stress response.

2. Material and methods

2.1. Materials and chemicals

All solutions were made with Milli-Q water (Millipore, Bedford, MA). All apparatuses for isoelectric focusing (dry strip holder, IPGphor) were from Amersham Pharmacia Biotech (Uppsala, Sweden). One-dimensional SDS-polyacrylamide gel electrophoresis was performed using standard methods with the Bio-Rad Protean 2 system (18 cm × 20 cm). Ethanol (HPLC-grade) was purchased from Merck and acetonitrile from J.T. Baker. Ammonium bicarbonate, ammonium persulfate (APS), bromophenol blue, agarose, glycine, DTT, glycerol, urea, CHAPS, Tris base, SDS, sodium thiosulfate and potassium ferricyanide were all ACS reagents and were purchased from Sigma (St. Louis, MO). Sequence-grade modified trypsin (porcine) was purchased from Promega (Madison, WI). Immobiline DryStrip, pH 3–10 L, 18 cm, was obtained from Pharmacia Biotech (Piscataway, NJ).

2.2. Fermentation conditions

A single yeast strain was isolated from honeycomb and identified as *C. magnoliae* (KFCC 11023) [12]. The wild type *C. magnoliae* strain was used for fermentation experiments. For preculture, the yeast was inoculated into a 500 mL flask containing 50 mL YPD (yeast extract 1%, bacto-peptone 2% and glucose 2%) and incubated at 30 °C with an agitation speed of 250 rpm for 18 h. The culture broth of 5% (v/v) was transferred to a 1 L jar fermenter (Braun, Melsungen, Germany) containing an initial volume of 500 mL fermentation medium including 300 g/L glucose, 10 g/L yeast extract and 20 g/L bacto peptone. For the low glucose stress condition the initial glucose concentration was adjusted to 20 g/L. Fermenter experiments were conducted at 30 °C, 700 rpm, 1 vvm (gas volume flow per medium volume per minute) and (initial) pH 7.

2.3. Analytical methods

Cell density was measured optically at 600 nm using a spectrophotometer (Shimadzu, Japan) or gravimetrically by measuring the cell dry weight (conversion factor from OD to cell density: 0.26) [8]. Extracellular metabolite samples were obtained by filtering the supernatant through a 0.45 μm syringe filter (MILLEX-GS, Millipore, France) and stored at −20 °C. Glucose, erythritol and glycerol were measured from filtered cultivation samples with a Waters HPLC system. Twenty microliters of the diluted sample was separated with an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA) heated at 60 °C and 5 mM of H_2SO_4 solution was used as a solvent at a flow rate of 0.6 mL/min. Detection was made using a reflective index detector.

2.4. Two-dimensional electrophoresis

We obtained samples from the fermenter under high and low osmotic conditions (17 and 27 h after inoculation). More than three samples were prepared independently for 2-DE as follows. Cells obtained from a fermenter were centrifuged at 12,000 rpm for 5 min at 4 °C. The cell pellet was washed using 2 mL ice-cold PBS buffer. Subsequently, the samples were resuspended with SDS sample buffer (1% SDS, 100 mM Tris–HCl, pH 7.0), while cell mass was adjusted to 5 mg Cells were boiled for 5 min and 200 μL cell suspension was disrupted five times at 20,000 psi using a French® Press (Thermo Spectronic, Rochester, USA), followed by boiling for 5 min and cooling on an ice bath. After the addition of 500 μL thiourea/urea lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 1% DTT and 2% carrier ampholytes; pH 3–10), the suspension was shaken moderately for 1 h and centrifuged at 16,000 rpm for 20 min. The supernatants were discarded and the pellets were stored at −70 °C until analysis. Protein amounts in the samples were assayed by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard. The first dimension of the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out on an IPGphor (Amersham Pharmacia Biotech AB, Sweden) isoelectric focusing apparatus. Non-linear, 18 cm long, pH 3–10 Immobiline gels (IPG-strips; Amersham Pharmacia Biotech AB) were rehydrated in a strip holder for 6 hr in 350 μL rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% pH 3–10 IPG buffer, a few grains of Bromophenol blue, HPLC water and 100 μg protein extract. Isoelectric focusing was carried out at 20 °C with the following settings: 30 V for 5 h; 500 V for 30 min; 1000 V for 1 h; 8000 V for 6.5 h until 80,000 Vh was reached. Prior to the second dimension, the IPG-strips were equilibrated for 15 min in 10 mL of buffer (1.5 M Tris–HCl, pH 8.8, 6 M urea, 22% glycerol, 2% SDS, bromophenol blue, double distilled H_2O, 1% (w/v) dithiothreitol (DTT)) and then a further 15 min in the same buffer in which DTT was replaced with 4.5% (w/v) iodoacetamide. The second dimension, 10% SDS-PAGE [13], was carried out in a Bio-Rad Protean II system (18 cm × 20 cm). After the electrophoresis, the gels were fixed for 1 h in 40% ethanol and 10% acetic acid and silver-stained using the method of O' Connell and Stults [14].

2.5. Image scanning and processing

Silver stained gel images were acquired by a GS-710 Imaging Densitometer (Bio-Rad, USA) and raw scans were processed using the 2D software PDQuest (Bio-Rad). The *M*r and p*I* of protein spots were calculated by comparing with a two dimensional gel electrophoresis standard marker (Sigma, USA) loaded on one edge of each gel and the theoretical pH gradient shape of IPG strips, using an ImageMaster 2D Elite V 3.10 (Amersham Biosciences). Using the ImageMaster 2D Elite program, we could detect approximately 1200–1400 spots on the 2-DE gel having less than 130 kDa *Mr* and a p*I* between 3 and 8 [15]. Synthetic gel images were built using a minimum of five different 2D PAGE migrations of the same sample. The matching images were then completed using the ImageMaster program. Matching was adjusted and corrected manually after each automated analysis. The comparison of each protein spot volume was calculated as a percentage of the total spot volume in the gel (%vol) [6]. Protein amounts corresponding to each spot were calculated and groups having at least a 2-fold consistent variation when comparing different samples were taken into consideration. Protein spots were identified based on our previous study using EIS-MS/MS

and microsequencing [15]. As *C. magnoliae* has not been sequenced, peptide sequences of the proteins rescued from the differentially expressed spot using ESI-MS/MS and microsequencing were compared to protein sequences of other sequenced yeasts and fungi. Peptide sequences from MS/MS spectra were searched against protein sequences of fungal origin from SWISS-PROT and the National Center for Biotechnology Information (NCBI). The latest versions of the NCBI non-redundant database were searched with the resulting peptide mass lists, manually using the search engine MASCOT (http://www.matrixscience.com) [15].

3. Results and discussion

Erythritol production by *C. magnoliae* is strongly associated with an osmotic stress response as erythritol may have properties of a compatible solute. A wild type *C. magnoliae* strain isolated from honeycombs showed a difference in growth and final erythritol concentration under low and high glucose conditions (Fig. 1 and Table 1) [12]. Initial osmotic pressure is 37.0 atm under the high glucose condition (corresponding to glucose 300 g/L, Table 1), whereas it is 2.46 atm under the low glucose condition (corresponding to glucose 20 g/L, Table 1). Because a bioreactor was employed for fermentations in this study, we speculated that osmotic pressure sensed by cells in the bioreactor was more considerable than the pressure sensed by cells on plates. Both final dry cell weight and productivity were higher under the high glucose condition than under the low glucose condition (Table 1). Interestingly, the final dry cell weight obtained under the high glucose condition was 1.9 times higher than that under the low glucose condition, while final erythritol concentration under the high glucose condition was 11 times higher as compared to the low glucose condition (Table 1). Under the high glucose condition, the metabolic flux appears to be directed toward erythritol production as erythritol may serve as a compatible solute. While only 0.81 g/L of erythritol was produced under the low glucose condition, 10.6 g/L of erythritol was produced at the high glucose condition. The erythritol yield per gram of glucose consumed (g erythritol/g glucose) was 0.04 and the biomas yield (g DCW/g glucose) was 0.79 under the low glucose condition. However, the yield of erythritol was 0.21 and the biomass yield was 0.63 under the high glucose condition, suggesting high glucose condition increased the carbon flux to erythritol production.

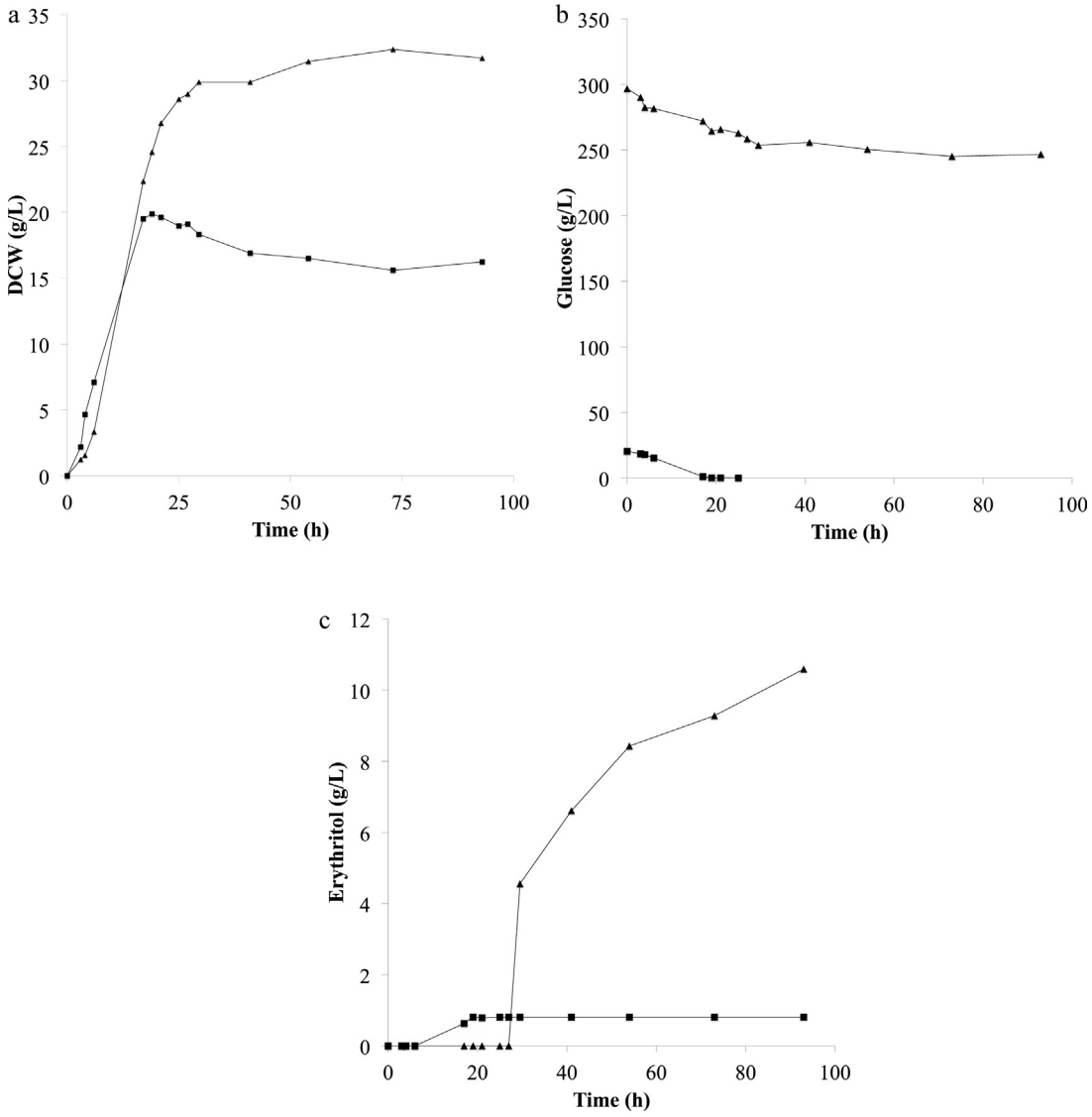

Fig. 1. Fermentation profiles of *C. magnoliae* under low (■) and high (▲) glucose conditions. (A): dry cell weight, (B): glucose and (C): erythritol. Fermentation patterns were reproducible in at least five independent experiments.

Table 1
Comparison of erythritolfermentation under different glucose conditions.

	Low glucose condition (20 g/L glucose)	High glucose condition (300 g/L glucose)
Initial osmotic pressure (atm)	2.46	37.0
Erythritol yield (g erythritol/g glucose)	0.04	0.21
Final erythritol concentration (g/L)	0.81	10.6
Dry cell mass (g/L)	16.2	31.7
Biomass yield (g DCW/g glucose)	0.79	0.63

The protein expression profiles of *C. magnoliae* grown under different glucose conditions were investigated using two-dimensional gel electrophoresis (Figs. 2 and 3). Two time points (17 and 27 h after inoculation) were chosen as the sampling times; 17 h after inoculation is at the mid-exponential phase and 27 h is at the beginning of erythritol production in *C. magnoliae* under the high glucose condition. When protein expression patterns in the 2D gels were compared between the low and high glucose conditions, intensities of five spots were significantly different. Three of the spots indicated up-regulation and two indicated repression under the low glucose condition compared to the high glucose condition at 17 h after inoculation (Fig. 3). Since glucose under the low glucose condition was depleted at 27 h after inoculation, protein response under the low glucose condition may be interfered by sugar starvation. However, the expression patterns of differentially expressed spots except for one spot (later identified as transaldolase) were consistent at 27 h after inoculation. Combination of 2-DE and mass spectrometry allowed identification of these spots using the methodology reported previously [15]. The bioinformatics search identified three up-regulated proteins under the low glucose condition as Hsp60 (LLQEVASKENESAGDGTTSATVLGR, AAVEEGILPGGGTALIK), transaldolase (LLEELYNSEEAVPK, SLNNGAFASFLDK) and NADH:quinone oxidoreductase (LSVFQAMLTEPAAGGALSK, AFSVDATGVAWAK, YDGFLLRPTR). Two down-regulated proteins were identified under the low glucose condition as Bro1 (VAKQTEDSTANSPAPPLPPLDSK, MQDVANK) and Eno1 (ALLGVAVAAAR, HSTGSWALELR, AFDDFLLK, TLLDLDNTR) (Table 2).

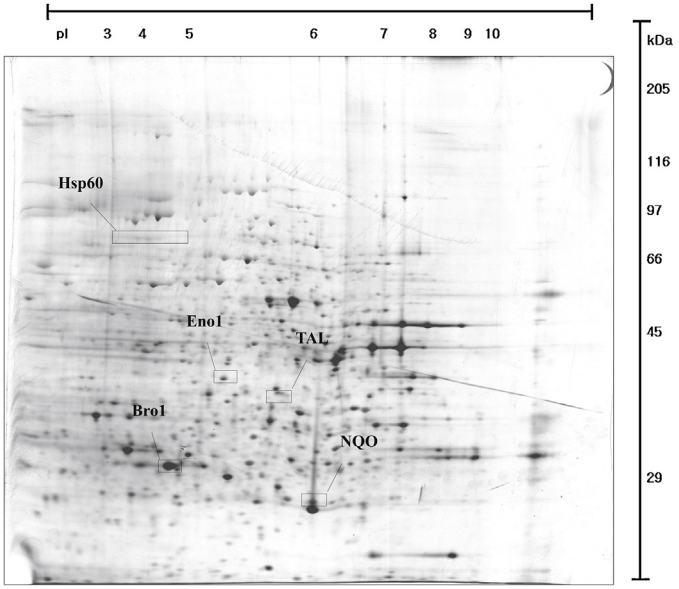

Fig. 2. Proteome maps of *C. magnoliae* in response to the low glucose conditions (300 g/L glucose) during the mid-exponential phase. Bro1, BCK1-like Resistance to Osmotic shock; Eno1: enolase1; Hsp60: heat shock protein 60; NQO: NADH:quinone oxidoreductase; TAL: transaldolase.

The three down-regulated proteins (Hsp60, transaldolase and NADH:quinone oxidoreductase) under the high glucose condition are directly or indirectly involved in stress response [16,17]. Hsp60 is a mitochondrial chaperone essential for the viability in *S. cerevisiae*, which is not limited to heat stress conditions [18]. Yeast Hsp60 exhibits highly conserved sequence similarity to 60 kDa heat-shock proteins from bacteria as well as fungi, animals and plants. Hsp60 appears to play an important role in the prevention of heat-induced aggregation of proteins in *S. cerevisiae* [18]. Hsp60 is also involved in the maintenance of DNA and protein stability in the mitochondria [19,20]. In addition, Hsp60 promotes resistance to oxidative stress. The amount of resistance displayed by cells in response to oxidative stress is correlated to the amount of Hsp60 in the cell [21]. Interestingly, expression levels of yeast Hsp60 decreased about 4-fold under hyperosmotic stress (growth in 1.4 M NaCl) compared to the hypoosmotic condition (growth in 0 M NaCl) when comparing the 2-dimensional electrophoresis [17].

Another important observation is the decreased amount of transaldolase under the high glucose condition during the mid-exponential phase (17 h). Transaldolase is an enzyme that is responsible for conversion of sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to fructose-6-phosphate and erythrose-4-phosphate in the pentose phosphate pathway (Fig. 4). Because erythrose-4-phosphate is considered as a starting material of erythritol-producing pathway, a decrease of transaldolase may inhibit erythritol production. This is consistent with our observation showing that erythritol production is detectable in the low glucose condition (0.63 g/L) but unnoticeable under the high glucose condition at the mid-exponential phase. Consequently, reduced amounts of transaldolase under the high glucose condition may detour the primary pathway through rearrangement of ribose-5-phosphate and xylulose-5-phosphate to form sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate by transketolase (Flux A, the red dashed arrows in Fig. 4), allowing for erythritol synthesis. The possible bypass for erythritol production under the high glucose condition is through the conversion of fructose-6-phosphate and glyceraldehyde-3-phosphate into erythrose-4-phosphate and xylulose-5-phosphate (Flux B, the blue bold arrows in Fig. 4). In accordance with this, erythritol production is dramatically increased in the late exponential phase of the high glucose condition (Fig. 1). Since fructose-6-phosphate and glyceraldehyde-3-phosphate in the altered pathway (Flux B) is provided from the glycolytic pathway, the procedure is shorter than the route through the Flux A.

Two protein spots were consistently induced under the high glucose conditions. One is Eno1 and the other is Bro1 (Fig. 3). Eno1 is an enzyme that converts 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). The increased abundance of Eno1 indicates that Eno1 is the main isoenzyme responsible for converting 2-PG to PEP under the high glucose condition. Eno1 was found to be up-regulated in *S. cerevisiae* under salt stress conditions [17]. The Eno1 in *S. cerevisiae* shares a consensus sequence with salt induced genes (Gpd1, Gpp2, Gcy1, and Dak1) in upstream repressor sequence. In addition, the amount of Eno1 increased 1.5-fold in respiratory phase cells and 2-fold in nitrogen-starved cells

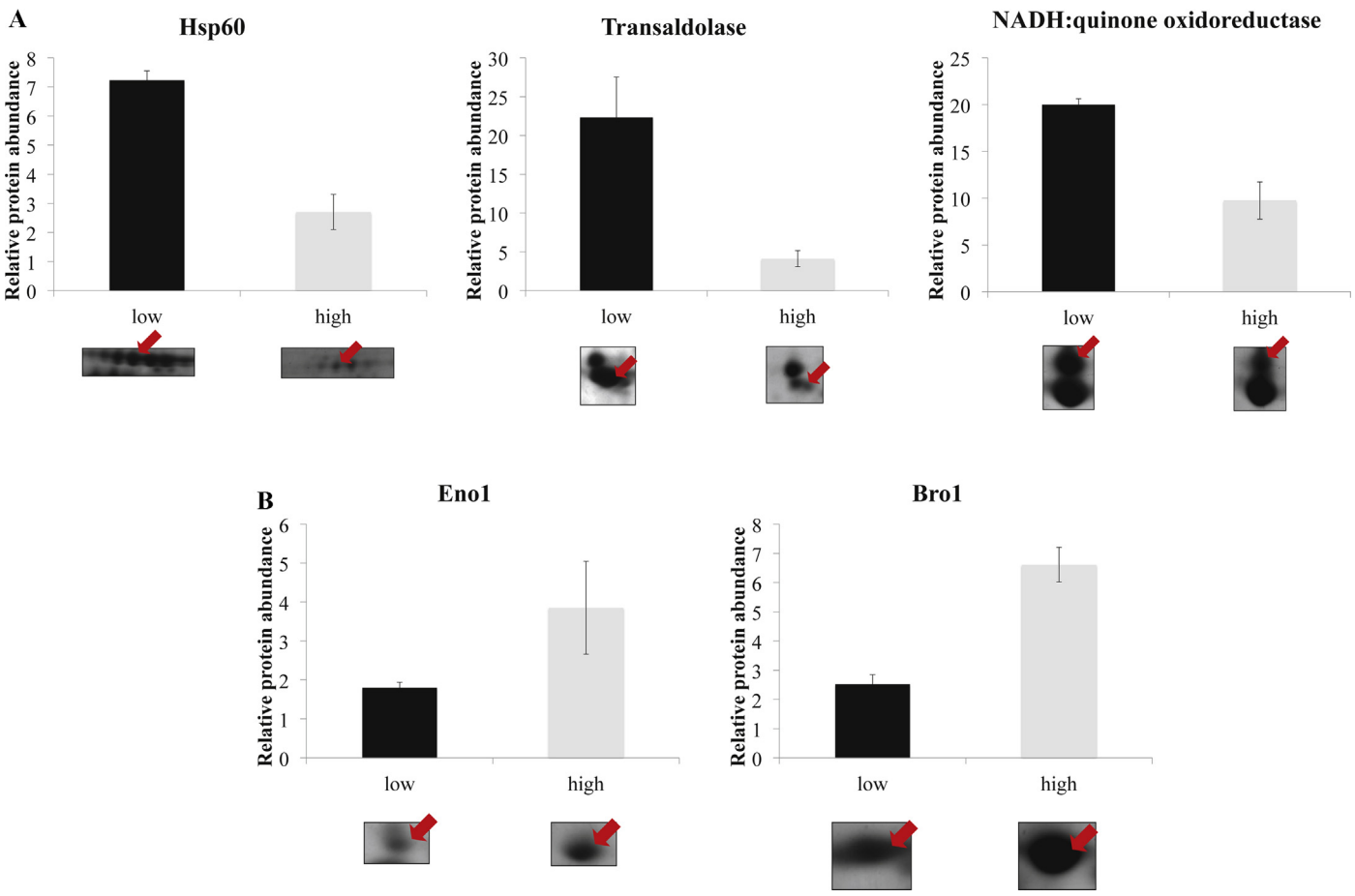

Fig. 3. Up-regulated proteins under the high glucose condition (A) and the low glucose condition (B). Relative expression levels of each spot denoted by arrows were calculated from image analysis program. Bar heights represent the averages of at least three independent experiments in designated conditions. The average value and the standard deviation are depicted. Bro1, BCK1-like Resistance to Osmotic shock; Eno1: enolase1; Hsp60: heat shock protein 60.

Table 2
Identification of protein spots significantly changed in response to glucose perturbation.

pI/M_r (kDa)	Protein	Fold Change[a]	Organ- Ism[b]	Cellular function
4.6/85	Hsp60	0.37	CA	Stress response
5.8/38	Transaldolase	0.18	PB	Carbohydrate metabolism & Stress response
5.3/38	Bro1	2.62	SP	Stress response
6.0/28	NADH:quinone oxidoreductase	0.49	GT	Electron transport
5.4/42	Eno1	2.14	NC	Carbohydrate metabolism & Stress response

Fold changes under the high glucose condition are represented, as compared to values under the low glucose condition, at 17 h after inoculation. Protein expression patterns are maintained at 27 h after inoculation except transaldolase. Expression levels of transaldolase proteins under the glucose depletion condition are decreased as similarly observed under the high condition at 27 h after inoculation. Abbreviated names of organisms: CA: *C.albicans*; GT: *Gloeophyllumtrabeum*; NC: *Neurosporacrassa*; PB, *Paracoccidioidesbrasiliensis*; SP: *S.pombe*. Due to the lack of the publicly accessible genome sequence for *C. magnoliae*, we identified proteins of *C. magnoliae* using cross-species protein identification based on publicly available genome database from the designated organisms [15].

compared to respiratory-fermentative cells [22]. The high energy phosphate of PEP is important for creating ATP by conversion to pyruvate. Therefore, up-regulation of enolase may be related to the increased primary metabolism to provide the ATP required for osmotolerance or other stress responses. The additional ATP might also be necessary for increased metabolic activity under the high osmotic condition. Taken together with our result, it suggests that Eno1 may be involved in cellular responses to various extracellular environmental perturbations such as osmotic stress, oxidative stress and nitrogen-starved stress. Under the high glucose conditions, the spot corresponding to Bro1 increased 2.6-fold. Generally, Bro1 is required for a normal response to nutrient limitation [23] and possible interactions with the protein kinase C-mitogen activated protein kinase pathway [24]. Interestingly, mutations in Bro1 result in a temperature-sensitive growth defect that can be rescued by addition of osmotic stabilizers or Ca^{2+} to the growth medium [23]. Therefore, our result and previous study reported for *S. cerevisiae* consistently indicate that Bro1 is involved in osmotic stress response [23].

This study was carried out to discover novel proteins that are involved in osmoregulation of *C. magnoliae*. Most of the proteins identified in this study are indeed related to stress responses. Moreover, the protein expression patterns of Hsp60, Eno1 and Bro1 in response to glucose perturbation are very consistent with previous studies reported for other organisms including *S. cerevisiae*. This suggests that those three proteins may play major roles in osmoregulation. It would be intriguing to introduce these proteins into other organisms that are not osmophilic to improve a fermentation performance under high glucose pressure. The strong correlation with other studies regarding sequenced

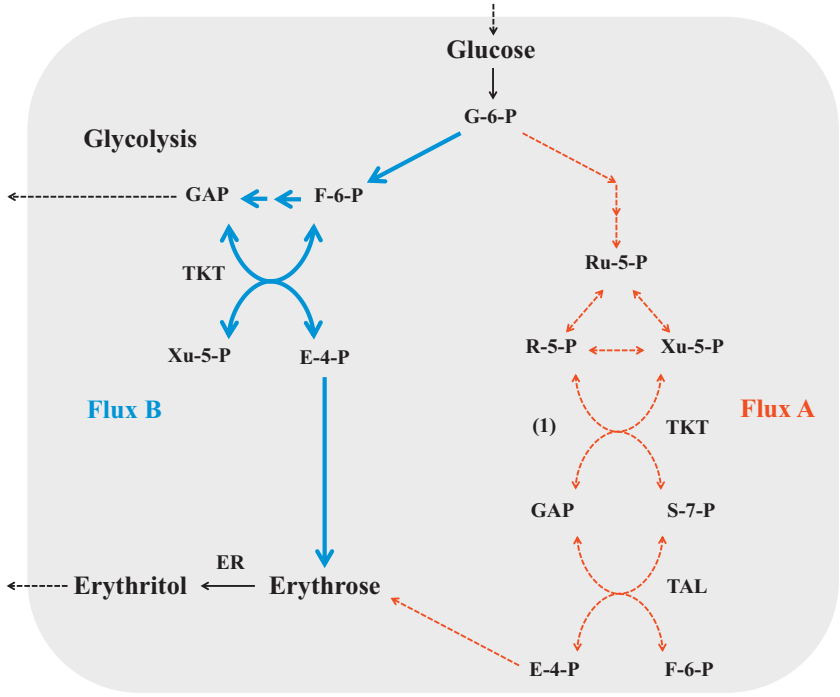

Fig. 4. Schematic representation of glycolysis and pentose phosphate pathway. Proposed reactions (Flux A and Flux B) leading to production of erythritol. In Flux A(the red dashed arrows), glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate generated from ribose-5-phosphate and xylulose-5-phosphate by the transketolase, and then converted into fructose 6-phosphate and erythrose 4-phosphate. Flux B(the blue bold arrows)represents a pathway through the synthesis of erythrose-4-phosphate and xylulose-5-phosphate from fructose-6-phosphate and glyceraldehyde-3-phosphate by transketolase. E-4-P: erythrose-4-phosphate; ER: erythrose reductase; F-6-P: fructose-6-phosphate; GAP: glyceraldehyde-3-phosphate; G-6-P: glucose-6-phosphate; S-7-P: sedoheptulose-7-phosphate; TAL: transaldolase; TKT: transketolase; R-5-P: ribose-5-phosphate; Ru-5-P: ribulose-5-phosphate; Xu-5-P: xylulose-5-phosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

organisms also verifies feasibility of our strategic approach to identify the proteome of *C. magnoliae* with the unsequenced genome [15].

4. Conclusion

Since *C. magnoliae* has not been sequenced yet, there is a limitation to understand osmophilic properties of the strain during fermentations. A proteomic approach was executed to circumvent this limitation. Five proteins were shown to be responsive to glucose perturbation during fermentations. These proteins are directly or indirectly involved in stress response; moreover, their variations show similar results to previous studies reported for other similar microorganisms. Therefore, proteins found in this study can possibly be employed for further applications to improve an osmo-tolerant property.

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