Curr Microbiol (2016) 73:827–833
DOI 10.1007/s00284-016-1132-7

Role of *CgHOG1* in Stress Responses and Glycerol Overproduction of *Candida glycerinogenes*

Hao Ji[1] · Bin Zhuge[1] · Hong Zong[1] · Xinyao Lu[1] · Huiying Fang[1] · Jian Zhuge[1]

Received: 25 May 2016 / Accepted: 31 August 2016 / Published online: 12 September 2016

Abstract *Candida glycerinogenes*, the glycerol producer with excellent multi-stress tolerances, is considered to be a potential biotechnological host used in the production of glycerol and its derivatives under extreme fermentation conditions. In this study, to evaluate the multiple roles of mitogen-activated protein kinase *CgHOG1*, we constructed a gene disruption system in the diploid *C. glycerinogenes* to obtain *CgHOG1* null mutant. Pseudohyphae generation of the *CgHOG1* mutant under non-inducing condition indicated a repressor role in morphological transitions. Disruption of *CgHOG1* resulted in increased sensitivities to osmotic, acetic acid, and oxidative stress but not involved in thermotolerance. In the *CgHOG1* mutant, NaCl shock failed to stimulate the accumulation of intracellular glycerol and was fatal. In addition, the *CgHOG1* mutant displayed a significant prolonged growth lag phase in YPD medium with no decrease in glycerol production, whereas the mutant cannot grow under hyperosmotic condition with no detectable glycerol in broth. These results suggested that *CgHOG1* plays important roles in morphogenesis and multi-stress tolerance. The growth and glycerol overproduction under osmotic stress are heavily dependent on *CgHOG1* kinase.

Introduction

Yeast cells are challenged by the complex and dynamic factors, such as the weak acids, osmotic stress, and oxidative stress when exposed to the culture environment. Some efficient response mechanisms are employed to enable cells survival. The high-osmolarity glycerol (HOG) response pathway mediated by the key component of mitogen-activated protein kinase, HOG1, is demonstrated to involve in tolerance of the osmotic stress [20], even oxidative stress [11], acetic acid stress [17], and cold stress [23]. In *Saccharomyces cerevisiae*, ScHOG1 kinase is fully active on Thr174 and Tyr176 in the catalytic domain by MAPK signaling cascades, and then the phosphorylated ScHOG1 is transported and accumulated in nucleus to employ the downstream regulatory mechanism [1]. Under hyperosmotic condition, glycerol as the major compatible solute is overproduced and accumulated intracellularly by inducing the expression of glycerol biosynthesis genes and controlling the glycerol flux [9].

Candida glycerinogenes is a multi-stress-tolerant yeast that can survive in 55 % (W/V) glucose or 15 % (W/V) NaCl medium. When *C. glycerinogenes* is cultured in high-glucose medium, more than 120 g glycerol l^{-1} can be produced; therefore, it has been successfully used for commercial scale production of glycerol in the last decades [26]. However, there is no evidence that multi-stress tolerance is closely related to glycerol overproduction via the activated HOG pathway. In a previous study, we have cloned HOG1 homologous gene in *C. glycerinogenes* and characterized the kinase function via complementary expression in a *S. cerevisiae HOG1* △null mutant [12]. However, more information is needed to understand the key role of *CgHOG1* in stress tolerance and glycerol overproduction. Unlike *S. cerevisiae*, a genetic study on *C. glycerinogenes* is more difficult. In a previous study, we have developed the transformation method for *C. glycerinogenes* using phleomycin as a drug-resistance marker, and the parameters involved in transformation efficiency were optimized [4]. Integrative vectors were also

✉ Bin Zhuge
bzhuge@126.com

[1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Table 1 Primers used in this study

Primer	Sequence	Restriction site
Hog1-f	GACCAAGAGAGTTGCCACATAG	–
Hog1-r	CGGCATCAATGATAGAGTTCAAT	–
1Hog-f	TCTGGGCCCCGCACAAAATCTTGGTCGGTt	ApaI
1Hog-r	TCCCCGCGGCACGGCGGCGGAAGCATTGG	SacII
Ura5-F	GGGCCCATGAATTCCTCGAAAACGGCGACGGTAT	ApaI, EcoRI
Ura5-R	CCGCGGATCTCGAGTGAACACCATTGTACCAATG	SacII, XhoI
HisG-F1	TCTGGGCCCTTCCAGTGGTGCATGAACGC	ApaI
HisG-R1	CCGGAATTCGCTGTTCCAGTCAATCAGGGT	EcoRI
HisG-F2	CCGCTCGAGTTCCAGTGGTGCATGAACGC	XhoI
HisG-R2	TCCCCGCGGGCTGTTCCAGTCAATCAGGGT	SacII
RT-GPDf	GGCAAGGATATTGACCAT	–
RT-GPDr	CCATAGCAACGACATTCT	–
RT-ACTf	CATTGTTATGTCTGGTGGTA	–
RT-ACTr	TTGGAAGGTGGATAGAGAT	–

constructed for heterogenous gene expression in *C. glycerinogenes* [24, 25]. However, more available tools are required for facilitating the genetic engineering studies. Here, we constructed an effective gene disruption system to generate the *CgHOG1* mutant, and the characterization may provide more insight into the multi-roles of *CgHOG1* in stress tolerance of the industrial yeast *C. glycerinogenes.*

Materials and Methods

Strains and Media

A uracil auxotrophic mutant of *C glycerinogenes* WL2002-5 was isolated from 5-fluoroorotic acid (5-FOA) plate and used as a parental strain in this study. The strain was cultured in YPD medium (2 % glucose, 2 % peptone, 1 %yeast extract), or synthetic dextrose (SD) medium (2 % glucose and 0.67 % yeast nitrogen base without amino acid) and SD medium supplemented with 0.1 % 5-FOA and 0.005 % uracil during genetic manipulation. *E. coli* JM109 was cultured in LB medium (0.5 % yeast extract, 1 % tryptone, and 1 % glucose) to maintain plasmids at 37 °C.

Construction of *CgHOG1* Mutant

The *C. glycerinogenes HOG1* gene was disrupted by a URA-Blaster system with modifications [15]. A 1.6 kb URA5 gene was amplified with genomic DNA of *C. glycerinogenes* WL2002-5 using primer URA5-R and URA5-F. Two 1.1 kb his*G* fragments were amplified with plasmid pCUB6 using two group of primers (hisG-F1, hisG-R1 and hisG-F2, hisG-R2) and then inserted separately into the flank of URA5 gene under the assistance of a clone vector pMD-19T (Takara). Finally, a general vector pMD-HUH containing hisG-URA5-his*G* cassette was constructed. Two rounds of disruptions were performed as described previously to obtain the *CgHOG1* mutant [15]. DNA transformation of *C. glycerinogenes* was carried out by the method developed by Chen [4]. The integrative expression vector harboring phleomycin resistance gene as a selective marker and 5.8S ribosomal RNA (rRNA) gene region as a homologous arm was constructed to reintroduce *CgHOG1* under the control of P*CgGAP* promoter in *CgHOG1*△ mutant. All the primers can be found in Table 1.

Analysis of Cell Survival

The cells of wild-type *C. glycerinogenes* and *CgHOG1*△ mutant on exponential phase were collected in 1.5-ml tubes, washed twice with sterile water, and then incubated with 1 ml YPD or YPD supplied with 1 M NaCl for various time points. Tenfold serial dilutions were performed using saline solution after the supernatant was removed by centrifugation at 5000 rpm, and then 100 µl dilutions were spread on YPD plate to produce 20–200 colonies per plate. Colonies were then counted after 48 h of incubation at 30 °C. Five plates were used for each experiment and the average value was calculated. Relative survival (%) of the samples shocked with and without NaCl was expressed as the ratio of counting colonies.

Real-Time Quantitative PCR

Candida glycerinogenes was grown up to the exponential phase in YPD medium at 30 °C for 16 h, and then NaCl

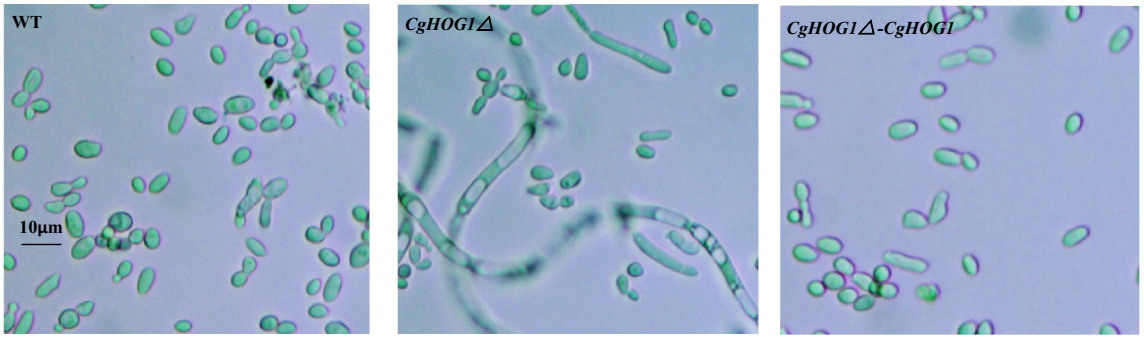

Fig. 1 The *CgHOG1*△ mutant generates pseudohyphae under non-inducing conditions. Micrographs illustrating the morphology of wild-type (WT) *C. glycerinogenes*, *CgHOG1*△ mutant, and *CgHOG1*△ rescued by wild-type *CgHOG1* gene with strains grown in YPD medium at 30 °C and pH 5.5. The figure shows the result of a representative experiment

was added to the culture. After incubation for the indicated time, the cells were collected by centrifugation. The total RNA extraction and the synthesis of cDNA were performed as described previously [12]. The expression levels of genes were determined on a Bio-Rad CFX96 Real-Time PCR system. Each reaction mixture contained cDNA (10 ng), 2× UltraSYBR Mixture with ROX (CWbiotech, 25 μl), 10 μM forward and reverse primers (1 μl), and RNase-free water (up to 50 μl). The primers RT-GPDr and RT-GPDf were used to study the transcription expression of *CgGPD*. *ACT1* was used as an internal reference with the primers RT-ACTr and RT-ACTf. All the primers are listed in Table 1. The relative transcription levels are analyzed via ΔΔCt method.

GPD Activity Assay

Treated cells were harvested by centrifugation, and cell extracts were prepared as described [6]. The cells were disrupted by agitation with glass beads (Sigama, 425 × 600 mm) in a vortex mixer, alternating 30 s on vortex and 1 min on ice 15 times. GPD activity assay was performed in the reaction solution containing 20 mM imidazole–HCl (pH 7.0), 1 mM $MgCl_2$, 1 mM DTT, 0.67 mM DHAP, and 0.09 mM NADH [5]. One unit (U) of enzyme activity is defined as the amount of enzyme for 1 μmol NADH extinction per minute at 25 °C. Protein content of extracts was determined using bicinchoninic acid method (CWBiotech, BAC protein assay kit).

Glycerol Quantification

Exponentially growing cells were collected, washed twice, and shifted to the fresh YPD medium with or without NaCl. The supernatant and the cells were separated and then used for intracellular glycerol quantification. For intracellular glycerol quantification, the cells were resuspended in 1 ml water and disrupted by glass beads. Glycerol content was determined by the method of Lambert and expressed as mg of glycerol per mg of dry cell weight [26].

Results and Discussion

Disruption of *CgHOG1* by Homologous Recombination

C. glycerinogenes was treated with ultraviolet light to generate uracil auxotrophic mutant for applying in further genetic manipulation. One uracil auxotrophic mutant was obtained on the positive selection using 5-FOA medium. We cloned genes in the UMP biosynthesis via de novo pathway and sequencing indicated that the unique URA5 (Orotate phosphoribosyltransferase 1) gene was broken on the orotate binding site in the mutant. Then the uracil auxotrophic mutant was employed as a host to construct the *CgHOG1* null mutant. Since *C. glycerinogenes* has been confirmed to be diploid with no sexual regeneration [22], the URA-Blaster disruption system widely applied in diploid yeast such as *Candida albicans* was used here with necessary modifications. Although the homologous recombination efficiency is relatively low, eight *CgHOG1*△ mutant clones were obtained on the selective medium after performing two rounds of homologous recombination. We have long been difficult on the genetic manipulation in the diploid *C. glycerinogenes*, this study provided a feasible procedure for gene disruption that might be applied in genetical engineering of the potential biotechnological host *C. glycerinogenes*.

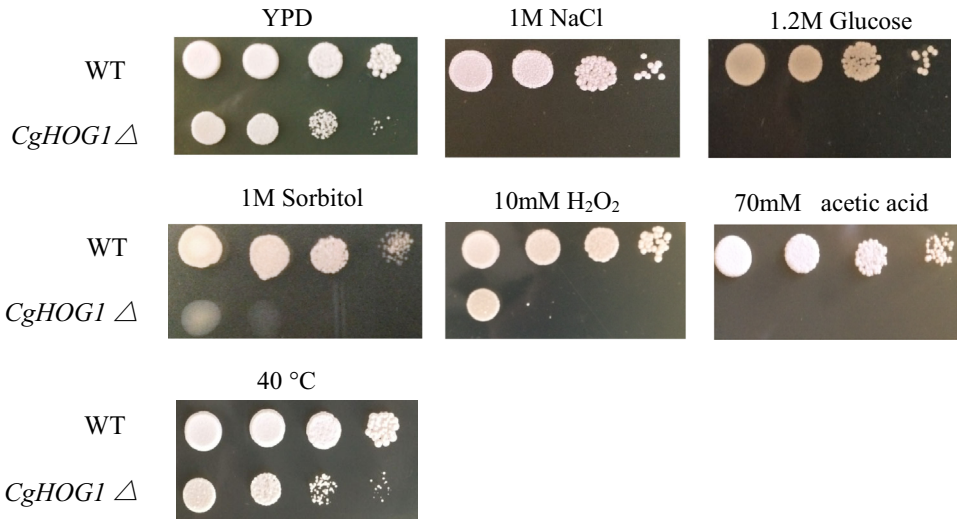

Fig. 2 Stress sensitivity phenotypes of *C. glycerinogenes CgHOG1*△ mutants. Serially diluted (tenfold) cell suspensions were spotted on YPD plate or YPD plate containing 1 M NaCl, 1.2 M glucose, 1 M sorbitol, 10 mM H_2O_2, or 70 mM acetic acid at 37 or 40 °C for 2 days

CgHOG1 Functions as a Repressor in Morphogenesis

When *CgHOG1* was disrupted, visible changes of cell morphogenesis can be observed under the microscope. Pseudohyphae generated under non hyphae-inducing conditions (Fig. 1). This morphological transition was also observed in the fungal pathogen *Candida albicans* in the absence of *HOG1* [3]. HOG1 kinase is considered to participate in the morphological transitions, and it prevents the yeast-to-hypha switch independent of any morphogenetic signal [3, 7, 8]. When the *CgHOG1* mutant was rescued by complementation of wild-type *CgHOG1*, the pseudohyphae generation was prevented. These results suggest that CgHOG1 might also function as a repressor of morphogenetic switching in *C. glycerinogenes*.

CgHOG1 Plays Important Roles in Stress Tolerance

Candida glycerinogenes can tolerate hyperosmotic stress, high temperature, and low pH. In addition, it has been reported to have excellent tolerance to 2-phenylethanol [16]. To investigate the potential roles of *CgHOG1* in stress signal transduction, the growth of mutant under various stress conditions was monitored. In agreement with the expected function in osmoadaptation, the *CgHOG1* mutant completely failed to grow on YPD plates containing 1 M NaCl or 1.2 M glucose, and showed impaired growth on 1 M sorbitol (Fig. 2). These indicate that the mutant displayed different tolerance to various osmolytes, which implies that the active *CgHOG1* differs depending on the osmolyte type, and this profile was also found in the halotolerant *Hortaea werneckii* [14]. *CgHOG1* mutant displayed increased sensitivity to 10 mM hydrogen peroxide and 70 mM acetic acid (Fig. 2). In contrast, the *CgHOG1* null mutation had no significant effect on growth at higher temperature, which was in agreement with the result observed in *C. albicans* HOG1 mutant [13] and the thermotolerant yeast *Kluyveromyces marxianus* [19]. The results suggest that *CgHOG1* is involved in multi-stress tolerance except thermotolerance.

Osmoadaptation Response Depending on *CgHOG1*

In *S. cerevisiae*, hyperosmotic shock causes a rapid loss of intracellular water and then the cells shrink lethally. To further confirm the role of *CgHOG1* in response to osmotic stress, the main mechanism of accumulation of intracellular glycerol was investigated in *C. glycerinogenes.* When incubated under hyperosmotic stress, the mutant lost the controlling of HOG pathway displayed poor survival to compare with the wild type (Fig. 3a). The production and intracellular accumulation of compatible solute is the main rapid response strategy to counteract the loss of turgor stress, this process is mediated by HOG pathway [9, 10]. In wild-type *C. glycerinogenes*, the amount of glycerol accumulated intracellularly rapidly and peaked soon after hyperosmotic shock for 15 min; meanwhile, in the *CgHOG1* mutant, intracellular glycerol remained at low levels and no obvious changes can be observed (Fig. 3b).

The transient increase in the intracellular glycerol can be caused by the effective modulation of enzymatic activity and gene expression of the key enzyme glycerol-3-phosphate dehydrogenase [2]. In *C. glycerinogenes*, a unique *CgGPD* gene encoding glycerol-3-phosphate dehydrogenase homologous to *GPD1/GPD2* from *S. cerevisiae* has been cloned and characterized in a previous study [17]. The specific activity of CgGPD was induced transiently by

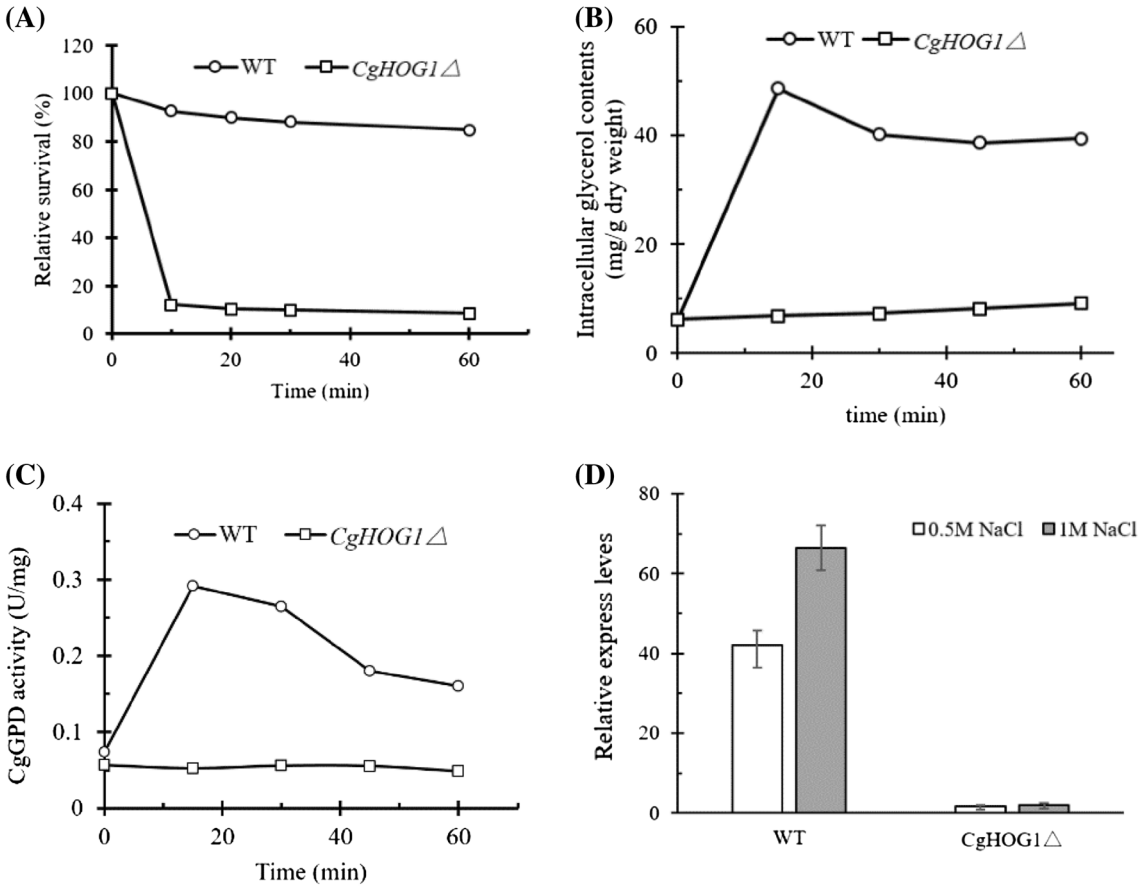

Fig. 3 Effect of *CgHOG1* mutation on the adaptive response to the hyperosmotic shock. Relative survival (**a**), intracellular glycerol accumulation (**b**), and activity of glycerol 3-phosphate dehydrogenase (**c**) of *C. glycerinogenes* or *CgHOG1*△ mutant were measured after shocked with 1 M NaCl for indicated time. **d** Relative expression levels of *CgGPD* in the presence of NaCl were reported. The expression levels of specific transcripts were normalized against their expression without osmotic shock. All data from three independent replicates were used to calculate the mean and standard deviation

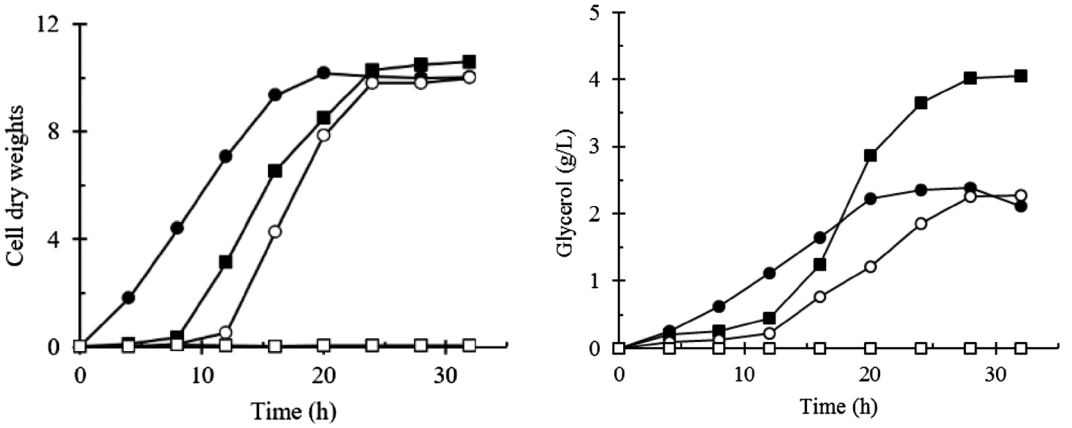

Fig. 4 Cell growth and glycerol production of *C. glycerinogenes CgHOG1*△ mutants. Wild-type (WT) or *CgHOG1*△ mutants were inoculated in YPD or YPD containing 1 M NaCl and incubated on a rotary shaker at 32 °C. The biomass and glycerol production was measured at the indicated time point; *closed circles* WT/YPD; *open circles* WT/1 M NaCl; *closed squares CgHOG1*△/YPD; *open squares CgHOG1*△/1 M NaCl. All data are the average values from three independent replicates

hyperosmotic shock with a significant increase in the first 15 min (Fig. 3c). The transcription level of *CgGPD* also increased greatly with NaCl incubation, as well as higher osmolarity made stronger induction (Fig. 3d). However, the enzyme modulation and gene transcription regulation mediated by HOG pathway were deactivated when C*gHOG1* is disrupted.

In traditional yeast *S. cerevisiae*, control of the glycerol flux is another important mechanism for the rapid accumulation of glycerol upon hyperosmotic stress. A member of the aquaporin family, transmembrane channel FPS1, closes to maintain intracellular glycerol in response to hyperosmotic stress [18]. It is considered to be the fastest mechanism to alter glycerol concentration [21]. We scanned the whole genome of *Pichia kudriavzevii* (synonyms for *C. glycerinogenes*); however, no *FPS1* homologous gene was found, which implies an untraditional mechanism of glycerol export in *C. glycerinogenes*. In conclusion, the results highlighted the conserved role of *CgHOG1* kinase in the osmotic stress response.

Effects of *CgHOG1* on the Cell Growth and Glycerol Overproduction in Broth

CgHOG1 mutant also showed a marked growth defect in the moderate YPD without any additional osmolytes. A further time course of growth assay indicated that the *CgHOG1* mutant displayed a significant prolonged growth lag phase than the wild type on YPD liquid medium (Fig. 4); however, finally the biomass is not influenced significantly. The data on transcription profiling in *C. albicans* revealed that the deletion of *HOG1* has significant effects on transcriptome in the absence of any stress [8]. Hence, a subset of growth-related genes in the absence of operation by HOG pathway in the mutant might contribute to the prolonged growth lag phase under basal conditions.

Although the *CgHOG1* mutant displayed a prolonged growth lag phase in moderate YPD medium, finally the glycerol production was not affected, indicating that glycerol production constitutively under non-osmotic stress is independent of HOG pathway. The glycerol production significantly increased when *C. glycerinogenes* was cultured in YPD with additional 1 M NaCl; meanwhile, no glycerol can be detected in the broth of mutant because the hyperosmotic stress is fatal in the absence of *CgHOG1*. *C. glycerinogenes* produces more than 120 g glycerol l^{-1} in high-glucose medium with 250 g glucose l^{-1} [26], in which the *CgHOG1* mutant cannot survive under this osmotic stress condition. In contrast, the deletion of HOG1 in *S. cerevisiae* resulted in a slight decrease in growth rate and only a 20 % decrease in glycerol production; hence, the results of this study demonstrate a different role of *CgHOG1* in the control of glycerol production [20]. In conclusion, the HOG pathway mediated by *CgHOG1* in *C. glycerinogenes* makes a significant contribution to cell growth and glycerol overproduction under hyperosmotic stress.

Acknowledgments This work was funded by China National "863" High-Tech Program (No. 2012AA021201) and supported by the National Natural Science Foundation of China (Nos. 31570052, 31601456), the Natural Science Foundation of Jiangsu Province (Nos. BK20140134, BK20140138), the Six Talent Peaks Project in Jiangsu Province (No. 2014-XCL-017), and the Fundamental Research Funds for the Central Universities (JUSRP11431). We thank Dr. Jiangye Chen (Chinese Academy of Sciences) for plasmid pCUB6.

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