Functional and expression studies of two novel STL1 genes of the osmotolerant and glycerol utilization yeast Candida glycerinogenes

(Received February 23, 2017; Accepted October 25, 2017; J-STAGE Advance publication date: March 31, 2018)

Hao Ji,1,2 Xinyao Lu,1,2 Hong Zong,1,2 and Bin Zhuge1,2,*

1 The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China
2 The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Candida glycerinogenes is an osmotolerant yeast used for commercial glycerol production, as well as a glycerol utilization yeast which produces high biomass on glycerol medium. In the present study, two STL1 homologues CgSTL1 and CgSTL2 encoding the putative glycerol transporters were identified, and their products were found to be localized to plasma membranes by tagging GFP protein. The functions of CgSTL1 and CgSTL2 on glycerol transport were confirmed by their expression in S. cerevisiae STL1 null mutant and simultaneous deletion in C. glycerinogenes. The expression of CgSTL1 were osmotic-induced, whereas that of CgSTL2 was constitutive. Over-expression of CgSTL1 and CgSTL2 in C. glycerinogenes resulted in improved glycerol consumption rate and cell growth. Our study provided more details on the glycerol transporter of C. glycerinogenes, the potential cell factory for using glycerol as a carbon source.

Key Words: Candida glycerinogenes; glycerol transporter; glycerol utilization; hyperosmotic stress; osmotic response

Introduction

With the dramatic development of biodiesel industries, large amounts of crude glycerol as a by-product are generated, which inevitably leads to a decrease in the price of glycerol. To produce value-added products from the cheap resource of glycerol, many microorganisms have been studied regarding their glycerol metabolism (Clomburg and Gonzalez, 2013; Liu et al., 2015; Neves et al., 2004). Candida glycerinogenes, an osmotolerant yeast originally used for glycerol production on a commercial scale (Zhuge et al., 2001), could also utilize glycerol as a carbon source to obtain high biomass, whereas the versatile biotechnology host S. cerevisiae shows poor growth in glycerol medium (Liu et al., 2013). In our previous study, glycerol has been considered as a better co-substrate for xylose fermentation in C. glycerinogenes (Zhang et al., 2015b), and thereby C. glycerinogenes has a great potential to use glycerol as feedstock for bioprocesses.

Some specific channel proteins are considered to contribute to the glycerol transport, because the cell membranes have a low permeability for glycerol (Saito and Posas, 2012). The transmembrane channel protein Fps1, a member of the aquaporin family, mediates the glycerol export (Oliveira et al., 2003), and Stl1, a sugar transporter like protein might contribute to glycerol import (Ferreira et al., 2005). Although the activity of glycerol uptake is an important physiological mechanism in glycerol utilization and osmoregulation, STL1 genes have only been fully characterized in S. cerevisiae and C. albicans (Ferreira et al., 2005; Kayingo et al., 2009). STL1 was firstly identified in S. cerevisiae to be involved in active glycerol uptake. Deletion of STL1 abolishes activity of glycerol transport and causes impaired growth on glycerol-based medium (Ferreira et al., 2005). Furthermore, STL1 is involved in osmotic response depending on HOG pathway and the transcription factor Hot1 (Bai et al., 2015). The transcription levels of STL1 and the glycerol symporter activity are strongly induced under hyperosmotic stress.
(Ferreira et al., 2005; Posas et al., 2000). Recently, Duskova et al. have characterized two Stl1 proteins in a yeast utilized-incompetent yeast Zygosaccharomyces rouxii and described their roles in osmoregulation (2015); however, there are few relevant studies on glycerol utilization in unconventional yeast.

In this study, we describe for the first time the involvement of the genes CgSTL1 and CgSTL2 in the transport of glycerol in the osmotolerant yeast C. glycerinogenes. Experiments were performed demonstrating the functionality of both genes and some aspects regarding their transcriptional regulation.

The over-expression of endogenous glycerol transporters were shown to be an effective way for improving glycerol consumption and cell growth in C. glycerinogenes.

**Material and Methods**

**Strains, media and growth conditions.** All the strains used in this study are listed in Table S1. C. glycerinogenes UA5, *S. cerevisiae* W303-1A and their derived strains were cultured in YPD medium (2% glucose, 1% peptone, and 1% yeast extract), YPG (2% glycerol, 2% peptone, and 1% yeast extract) or synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids, and 2% glucose) supplemented with required nutrients for the auxotrophic mutants. SD medium supplemented with 0.1% 5-fluoroorotic acid (5-FOA) was used for genetic manipulation. For the growth test, 2% glycerol was added in the SD medium instead of glucose. *Escherichia coli* JM109 was cultured in LB medium (1% peptone, 0.5% yeast extract, and 1% NaCl) containing ampicillin (100 μg/ml) for plasmid propagation.

**Deletion of CgStls.** Two genes encoding CgStls were amplified from genomic DNA of *C. glycerinogenes* UA5 using oligonucleotide primers STL1-F, STL1-R and STL2-F, STL2-R. The gene fragments were cloned into pMDTM19-T simple vector (TAKARA) to generate pSTL1F, STL2-R. The gene fragments were cloned into using oligonucleotide primers STL1-F, STL1-R and STL2-R.

Deletion of CgSTLs

**Expression of CgSTL1 and CgSTL2 in *C. cerevisiae* STL null mutant.** CgSTL1 and CgSTL2 genes containing their native promoters were amplified from *C. glycerinogenes* genomic DNA using PrimeSTAR HS DNA Polymerase (TAKARA) and primers CSTL1Q-F, CSTL1Q-R and CSTL2Q-F, CSTL2Q-R, respectively. STL1 from *S. cerevisiae* ScSTL1 with its native promoter was amplified using SSTL1-F and SSTL1-R. The PCR products were inserted into multicopy shuttle plasmid YEplac181 after being digested with some restriction enzymes, the sites of which were attached to the primers (Table S2). The recombinant plasmids were transformed in *E. coli* JM109 and designated YEp181-CgSTL1, YEp181-CgSTL2 and YEp181-CgSTL1 and then they were introduced into the *S. cerevisiae* STL1 null mutant by a standard lithium acetate method (Schiestl and Gietz, 1989). *S. cerevisiae* W303 and the STL1 null mutant transforming with empty plasmid YEp181 serves as controls.

**Overexpression of CgSTL1 and CgSTL2 in *C. glycerinogenes.* For overexpression of CgSTL1 or CgSTL2 in *C. glycerinogenes* UA5, the integration expression vector pURGAPU (Fig. S2) was used in this study (Ji et al., 2016). CgSTL1 and CgSTL2 were amplified using primers STL1-F, STL1-KPN and STL2-BGL, STL2-SAC, and then linearized using the restriction enzyme Sac I for transformation.

**RNA extractions and Real-Time PCR.** *C. glycerinogenes* UA5 was grown up to the exponential phase in YPD medium at 30°C for 16 h, and then 1 M NaCl was added to the culture. After incubation for the indicated times, the cells were collected by centrifugation. The total RNA extraction and the synthesis of cDNA were performed as

---

**Fig. 1.** Sequence alignment of Stl proteins from *S. cerevisiae* and *C. glycerinogenes.* The alignment was performed by using an online Clustal Omega server (http://www.ebi.ac.uk/Tools/msa/clustalo/). The predicted transmembrane domains are present in the shadowed boxes.
Studies on STL1 genes of Candida glycerinogenes

Fig. 2. Subcellular localization of CgStl1 and CgStl2 in S. cerevisiae.
(A) Recombination strains containing vectors pYX212 (1), pS1GFP (2) or pS2GFP (3) were cultured in SD medium, then re-inoculated into YPG medium and cultured for 6 h at 37°C. After being washed by PBS buffer, ten microliters of the suspension were visualized by fluorescence microscopy (Leica TCS SP8, excitation wavelength 488 nm, emission wavelength 520 nm). (B) Recombinants were cultured in YPG medium for 12 h at 37°C and then collected for protein extraction by using glass beads. Ten microliters of the protein samples was loaded on SDS-PAGE gels. GFP fusions were monitored by Western blot analysis with an anti-GFP antibody. The lane numbers are the same as in panel A.

Described previously (Ji et al., 2016). Real-Time PCR was performed with 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). ACT1 was used as an endogenous reference with the primer RT-ACTr and RT-ACTf. The relative transcription levels were calculated via the 2–ΔΔCt method (Livak and Schmittgen, 2001).

Subcellular localization of CgSTLs by fusion expression of GFP. CgSTL1 and CgSTL2 were amplified using primers STL1-NCO, STL1-SAL and STL1-NCO, STL2-SAL, and inserted into pYX212 to generate pYX-CgSTL1 and pYX-CgSTL2 (Ji et al., 2014). The GFP coding sequence was amplified from a commercial plasmid pCAMBIA1302 using primers GFP-SAL, GFP-XHO and GFP-SAL, GFP-SAC. The PCR products were inserted into pYX212-CgSTL1 and pYX-CgSTL2 at restriction enzymes Sal I, Xho I and Sal I, Sac I to generate pS1GFP and pS2GFP. The fusion proteins were expressed under the control of Tpi 1 promoter. The plasmids were introduced into the S. cerevisiae W303-1A by the lithium acetate method.

Western blot assay. Cell extracts were prepared as described previously (Ji et al., 2014). Proteins were separated on the SDS-PAGE, and then transferred to PVDF membranes. GFP was detected using a mouse monoclonal GFP antibody and the secondary antibody conjugated with horseradish peroxidase (Beyotime Institute of Biotechnology). The detection was performed using an LAS-4000 luminescence camera system (Fujifilm, Japan).

Analytical methods. Samples were taken from the flask at the indicated times and filtered through a 0.22 μM filter membrane. Concentrations of the glycerol in supernatants were determined using high-performance liquid chromatography (HPLC; DIONEX; USA) equipped with an Aminex HPX-87H column (Bio-Rad; USA) and an RI-101 refractive index detector (Shodex; Japan). The mobile phase was 5 mM H2SO4 with a flow rate of 0.6 ml/min at 60°C.

Results and Discussion

Identification of STL1 genes in C. glycerinogenes
To obtain homologous STL1 genes of the glycerol utili-
zation in the osmotolerant yeast *C. glycerinogenes*, we searched the whole genome sequence of *Pichia kudriavzevii* (synonym for *C. glycerinogenes*) present in GenBank (ALNQ00000000) by the BLAST online program using the nucleotide sequence of *S. cerevisiae STL1* as queries. Two open-reading frames with high similarities were found in tandem on the chromosome. The former one, consisting of 1839 nucleotides, encodes a deduced protein with 612 amino acids which shares a 52% identity to Stl1 from *S. cerevisiae*, and this gene was designated *CgStl1* (GenBank: KU207227); the latter one, consisting of 1836 nucleotides, encodes a deduced protein with 611 amino acids which share a 78% identity to CgStl1 and a 48% identity to ScStl1, and this gene was designated *CgStl2* (GenBank: KU207228). Sequence alignments of Stl1 proteins from *S. cerevisiae* and *C. glycerinogenes* were performed by Clustal Omega and revealed the conservative regions (Fig. 1). As with Stl1 in *S. cerevisiae* which belongs to the HXT family, both CgStl1 and CgStl2 were predicted to be transmembrane proteins by using the online TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). To perform subcellular localization, GFP was fused to the carboxy-terminus of CgStl1 and CgStl2 and the fusion genes were then expressed in *S. cerevisiae* W303-1A, respectively. As shown in Fig. 2, both CgStl1 and CgStl2 were properly localized to the plasma membrane of *S. cerevisiae*. In addition, the fused proteins were expressed in *C. glycerinogenes* UA5 and also localized to the plasma membrane (Fig. S4). Western blotting was performed to check the degradation of the fusion proteins. The results in Fig. 2B and Fig. S4B showed that the fusion proteins were correctly produced in both *S. cerevisiae* and *C. glycerinogenes*, and no degradation of fusions was observed.

**Functional analysis of CgSTL1 and CgSTL2**

It is generally accepted that the glycerol transport is the first challenge for glycerol utilization in yeast cells. Deletion of the glycerol transporter gene *STL1* in *S. cerevisiae* resulted in the almost complete abolishment of growth with glycerol (Ferreira et al., 2005). We also constructed single (*CgSTL1Δ* and *CgSTL2Δ*) and a double deletion mutant (*CgSTL1ΔCgSTL2Δ*) in the uracil auxotrophic *C. glycerinogenes* UA5. When cultured in SD medium with glycerol as the sole carbon source, the cell growth and glycerol consumption of the mutants were investigated. As shown in Fig. 3, only the deletion of both CgSTL1 and CgSTL2 resulted in a dramatic reduction in cell growth and glycerol consumption, suggesting the overlapping roles of CgSTL1 and CgSTL2 in glycerol transport. To obtain more direct details about the function of CgSTL1 and CgSTL2, functional complementation was performed in a *S. cerevisiae STL1* null mutant. As shown in Fig. 4, the *S. cerevisiae STL1* null mutant harbouring Yep181-CgSTL1 or Yep181-CgSTL2 restored the growth on the glycerol based medium, indicating their functional complementation of the poor growth of the STL1 null mutant. In addition, the GFP fusion proteins also restored
the cell growth of *S. cerevisiae STL1Δ* and *C. glycerinogenes CgSTL1ΔCgSTL2Δ*, confirming that the fusion proteins Gfp-CgStl1 and Gfp-CgStl2 are functional in cells (Fig. S5). In *C. albicans*, only one of the two *STL1* homologues exhibited active glycerol transport (Kayingo et al., 2009), and in *Pachysolen tannophilus*, both of *PtSTL1* and *PtSTL2* genes had no obvious effect on glycerol growth (Liu et al., 2013).

Expression patterns of *CgSTL* genes

Several stress responsive elements (STRE) characterized by feature sequences AGGGG or CCCCT can be found in the 5′-flanking non-coding regions of *CgSTL1* and *CgSTL2*, suggesting that the expression of *CgSTL* genes might be stress-induced (Rep, 2000; Rep et al., 1999). Transcription factor Hot1 was shown to be required for the expression of a subset of HOG-dependent genes, including *STL1*, in recent studies (Bai et al., 2015; Rep, 2000). Gomar-Alba et al. (2015) described that the DNA-binding domain of transcription factor Hot1 recognized sequence 5′-GGGACAAA-3′ locating in the promoter region of *STL1*. We scanned the promoter regions of *CgSTL1* and *CgSTL2*, and found a similar sequence 5′-GGGAGAAA-3′ with four repeats at the position -652 to -618 relative to the translation start codon of *CgSTL1*, but no characteristic sequence can be found on the promoter region of *CgSTL2* (Fig. S3), implying that there are different transcriptional patterns between *CgSTL1* and *CgSTL2*. Surprisingly, although the analysis of the promoter sequences provided information suggesting the involvement of Hot1 in the transcriptional control of the *CgSTL1*, we failed to find a Hot1 homologue in the genome of *C. glycerinogenes* based on sequence alignment.

To elucidate the transcription patterns, the relative expression levels of *CgSTLs* were followed by quantitative Real-Time PCR (qRT-PCR). As shown in Fig. 5A, the expression level of *CgSTL1* was much higher than *CgSTL2*.

**Fig. 5.** Expression patterns of *CgSTL1* and *CgSTL2*. (A) relative expression levels of *CgSTL1* and *CgSTL2* in hyperosmotic shock condition. At time 0, 1M NaCl was added to the culture. The expression levels were represented as the multiple of the expression at time 0. (B) relative expression of *CgSTL1* and *CgSTL2* cultured with glucose and glycerol. The relative expression levels were normalized to *ACT* expression. All the values were the means of three biological replicates ± standard deviation.

**Fig. 6.** Glycerol consumption (A) and cell growth (B) of the *C. glycerinogenes* UA5 overexpressing *CgSTL* genes. Cells were grown on SD medium with glycerol as the sole carbon source at 30°C. All values from three independent replicates were used to calculate the standard deviation. Circle, UA5; Triangle, UA5-*CgSTL1*; Square, UA5-*CgSTL2*. 

---

Studies on *STL1* genes of *Candida glycerinogenes*
under basal conditions (time 0), and the expression of 
CgSTL1 were osmotic-induced, whereas that of CgSTL2 was
constitutive. The difference in kinetics of this induc-
tion between CgSTL1 and CgSTL2 implies their diverse
functions in hyperosmotic response, which might be due
to their difference in promoter elements. Interestingly,
although the expression level of CgSTL2 was constitutive
under hyperosmotic condition, it was upregulated when
the strain was cultured on glycerol compared with that on
glucose (Fig. 5B).

Overexpression of CgSTLs enhanced the glycerol con-
sumption of C. glycerinogenes

Although C. glycerinogenes produces much more
biomass on glycerol than another yeast such as S.
cerevisiae, the velocity of carbon source consumption and
biomass formation are much slower in a glycerol medium
than in a glucose medium. Here, CgSTLs were
overexpressed under the control of a native glyceralde-
hyde-3-phosphate dehydrogenase promoter to enhance the
glycerol transport. As shown in Fig. 6, overexpression of
CgSTL1 or CgSTL2 resulted in faster glycerol consump-
tion and improved cell growth, indicating that the gly-
erol import might be rate-limiting step for glycerol con-
sumption in C. glycerinogenes, and the overexpression of
dogenous glycerol uptake genes were desirable for gly-
erol utilization.

Heterologous expression of glycerol transporters from
glycerol utilization yeast could be an effective way for
improving the glycerol assimilation in S. cerevisiae. How-
ever, we failed to obtain positive results by overexpressing
CgSTL genes in S. cerevisiae (Fig. 3). Liu et al. (2013)
showed that the expression of exogenous STL1 genes from
P. tannophilus in S. cerevisiae resulted in no growth en-
hancement, whereas they have not raised the possibility of
overexpressing any transporters in the glycerol-utiliza-
tion yeast itself to improve its growth rate, probably be-
cause of their lack of available genetic tools for P. tannophilus.
Our study proposed a possible way to improve
the cell growth in glycerol of the potential biotech-
nology host C. glycerinogenes.

Acknowledgments

This work was funded by the National Natural Science
Foundation of China (Nos. 31570052, 31601456), the Natural Science
Foundation of Jiangsu Province (Nos. BK20140134, BK20140138), and the Six
Talent Peaks Project in Jiangsu Province (No. 2014-XCL-017).

Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site
(http://www.jstage.jst.go.jp/browse/jgam).

References

tion factor is critical for activating a single target gene, STL1. Mol.

Clomburg, J. M. and Gonzalez, R. (2013) Anaerobic fermentation of
glycerol: a platform for renewable fuels and chemicals. Trends

erol uptake systems contribute to the high osmotolerance of

Ferreira, C. et al. (2005) A member of the sugar transporter family, Stl1p
is the glycerol/H+ symporter in Saccharomyces cerevisiae. Mol. Biol.
Cell, 16, 2068–2076.

et al. (2015) The C-terminal region of the Hog1 transcription factor
binds GGACAAA-related sequences in the promoter of its target

of a novel HOG1 homologue from an industrial glycerol producer

Ji, H., Zhuge, B., Zong, H., Lu, X., Fang, H. et al. (2016) Role of
CgHOG1 in stress responses and glycerol overproduction of Can-

A permissive nucleoid STL1 is required for active glycerol uptake
by Candida albicans. Microbiology, 155, 1547–1557.

D-xylene by a Xylitol dehydrogenase gene-disruption mutant of

Liu, H., Ji, X. J., and Huang, H. (2015) Biotechnological applica-
Adv., 33, 1522–1546.

functional studies of genes involved in transport and metabolism

expression data using real-time quantitative PCR and the 2 −ΔΔCT
method. Methods, 25, 402–408.

ated with glycerol transport and metabolism. FEMS Yeast Res., 5,
51–62.

channel is the mediator of the major part of glycerol passive diffu-
sion in Saccharomyces cerevisiae: artefacts and redefinitions.
(BBA)-Biomembranes, 1613, 57–71.


to osmotic shock. Hot1p and Msn2p/Msn4p are required for the
induction of subsets of high osmolality glycerol pathway-depen-

cerevisiae requires Msn1p and the novel nuclear factor Hot1p. Mol.

Saito, H. and Posas, F. (2012) Response to hyperosmotic stress. Gen-
etics, 192, 289–318.

of intact yeast cells using single stranded nucleic acids as a carrier.

tion of xylitol from D-xylene by overexpression of xylene reductase

Glycerol production by a novel osmotolerant yeast Candida