Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2017

www.elsevier.com/locate/jbiosc

Evaluation of *Saccharomyces cerevisiae GAS1* with respect to its involvement in tolerance to low pH and salt stress

Akinori Matsushika,[1,2,*] Toshihiro Suzuki,[1] Tetsuya Goshima,[3] and Tamotsu Hoshino[1,2]

Research Institute for Sustainable Chemistry (ISC), National Institute of Advanced Industrial Science and Technology (AIST), 3-11-32 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan,[1] Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-8530, Japan,[2] and National Research Institute of Brewing (NRIB), 3-7-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan[3]

Received 22 November 2016; accepted 5 March 2017
Available online xxx

We previously showed that overexpression of *IoGAS1*, which was isolated from the multiple stress-tolerant yeast *Issatchenkia orientalis*, endows *Saccharomyces cerevisiae* cells with the ability to grow and ferment under acidic and high-salt conditions. The deduced amino acid sequence of the *IoGAS1* gene product exhibits 60% identity with the *S. cerevisiae* Gas1 protein, a glycosylphosphatidylinositol-anchored protein essential for maintaining cell wall integrity. However, the functional roles of *ScGAS1* in stress tolerance and pH regulation remain unclear. In the present study, we characterized *ScGAS1* regarding its roles in tolerance to low pH and high salt concentrations. Transcriptional analysis indicated that, as for the *IoGAS1* gene, *ScGAS1* expression was pH dependent, with maximum expression at pH 3.0; the presence of salt increased endogenous expression of both *GAS1* genes at almost all pH levels. These results suggested that *ScGAS1*, like *IoGAS1*, is involved in a novel acid- and salt-stress adaptation mechanism in *S. cerevisiae*. Overexpression of *ScGAS1* in *S. cerevisiae* improved growth and ethanol production from glucose under acid stress without added salt, although the stress tolerance of the *ScGAS1*-overexpressing strain was inferior to that of the *IoGAS1*-overexpressing strain. However, overexpression of *ScGAS1* did not result in increased tolerance of *S. cerevisiae* to combined acid and salt stress, even though *ScGAS1* appears to be a salt-responsive gene. Thus, *ScGAS1* is directly implicated in tolerance to low pH but does not confer salinity tolerance, supporting the view that *ScGAS1* and *IoGAS1* have overlapping yet distinct roles in stress tolerance in yeast.

[**Key words:** *Saccharomyces cerevisiae*; *Issatchenkia orientalis*; Acid tolerance; Salt tolerance; *GAS1*; GPI-anchored protein; Yeast stress response; Ethanol fermentation]

Biofuels and biochemicals produced from renewable lignocellulosic biomass have recently gained wide interest in industrial applications (1,2). In these bioproduction processes, industrial yeast strains, such as *Saccharomyces cerevisiae*, are subjected to various stresses, including heat, inhibitory compounds released by pretreatment procedures, osmotic pressure, and oxidative stress, all of which can negatively affect fermentation by yeast (e.g., reviewed by Caspeta et al. (3)). Acid stress is an especially serious problem in the industrial bioproduction of ethanol (4–6) and of lactic acid (7,8). Notably, during lactic acid production, decreased pH has been reported to cause a high level of stress, and an associated decrease in productivity, in *S. cerevisiae* cells (7). The most widely used method for the preparation of lignocellulose for ethanol production, dilute sulfuric acid-based chemical pretreatment (9), results in the presence of residual acid, which in turn decreases the rates of growth and fermentation and cell viability of yeast. Therefore, a neutralization step in which the residual acid is neutralized with alkalis to form salts, such as Na_2SO_4, is required prior to downstream enzymatic hydrolysis and fermentation. However, a number of salts are known to have significant inhibitory effects on the fermentation ability of *S. cerevisiae*, thereby increasing the levels of by-products, including glycerol (10,11). To avoid inhibition of fermentation and to decrease neutralization costs, the bioproduction industry has turned to salt- and acid- tolerant yeasts. In addition, fermentation by acid-tolerant yeast under acid stress conditions (i.e., below pH 4.0) decreases the risk of bacterial contamination and minimizes the cost of sterilization (12). However, a limited number of strategies are currently available for improvement of tolerance to acid and salt stress; further investigation of this process is needed.

It has been recently reported that *Issatchenkia orientalis*, also known as *Pichia kudriavzevii* or *Candida krusei*, exhibits tolerance to low pH, high salt concentrations, and high temperatures (13–16). For instance, *I. orientalis* strain MF-121, which was isolated from a river at pH 3.0, can ferment glucose to ethanol in medium containing 5.0% Na_2SO_4 at pH 2.0 (14). Based on these desirable characteristics, we subsequently isolated and characterized a novel gene related to low-pH and salt tolerance by screening a genomic DNA library of *I. orientalis* (17). This gene,

* Corresponding author at: Research Institute for Sustainable Chemistry (ISC), National Institute of Advanced Industrial Science and Technology (AIST), 3-11-32 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan. Tel.: +81 82 420 8289; fax: +81 82 420 8291.
E-mail address: a-matsushika@aist.go.jp (A. Matsushika).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.03.004

Please cite this article in press as: Matsushika, A., et al., Evaluation of *Saccharomyces cerevisiae GAS1* with respect to its involvement in tolerance to low pH and salt stress, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.03.004

named *I. orientalis GAS1* (*IoGAS1*), encodes a protein (IoGas1p) that is highly homologous (58–60% identity) to the Gas-family proteins of *S. cerevisiae* (18) and the pH-responsive Phr1/Phr2 proteins (CaPhr1p/CaPhr2p) of the pathogenic yeast *Candida albicans* (19); members of this family are known to be glycosylphosphatidylinositol (GPI)-anchored glycoproteins (20). *S. cerevisiae* Gas1p (product of the *ScGAS1* gene) is the best characterized member of the Gas family; together with CaPhr1p and CaPhr2p, these gene products belong to the GH72 family of β-1,3-glucanosyltransferases and play a crucial role in cell wall assembly (21,22). It has also been demonstrated that *CaPHR1*, a gene that is maximally expressed at pH values of 5.5–8.0 (23), and *CaPHR2*, whose transcription is greatest under acidic conditions (pH 4–5) (24), are part of a pH-dependent regulon required for virulence. We found that overexpression of the *IoGAS1* gene in *S. cerevisiae* significantly improved growth and fermentation by yeast under extreme acid stress (pH 2.0–2.5) and under combined acid and salt stress (pH 2.0–2.5, 5.0–7.5% Na_2SO_4) (17). We further observed that expression of *IoGAS1*, like that of *CaPHR1* and *CaPHR2*, was modulated in response to ambient pH conditions, and that heterologous expression of *IoGAS1* complemented the growth and morphological defects of an *S. cerevisiae gas1Δ* mutant (17). These results suggested that *IoGAS1* and the corresponding *S. cerevisiae* and *C. albicans* genes play similar roles in maintaining cell wall integrity during environmental stress. Nevertheless, to our knowledge, previous reports have not examined whether *ScGAS1* is involved in low-pH and salt tolerance in *S. cerevisiae*.

In the present work, we characterized the endogenous expression of the *ScGAS1* and *IoGAS1* genes in the respective organisms, and also investigated the potential role of *ScGAS1* in improving the acid- and salt-stress tolerance of *S. cerevisiae*. For this purpose, we first compared the gene expression profiles of *S. cerevisiae* and *I. orientalis* strains grown on medium with or without salt (7.5% Na_2SO_4) at different pH values (pH 2.0–6.0). Second, we evaluated the effect of overexpression (in *S. cerevisiae*) of *ScGAS1* on aerobic growth in acidic medium (pH 2.2–2.5) in the presence or absence of a high concentration of salt (2.5–5.0% Na_2SO_4). Third, we assessed the impact of overexpression (in *S. cerevisiae*) of *ScGAS1* on glucose fermentation under acid-stress (pH 2.1) or under combined acid- and salt-stress (pH 2.5, 7.5% Na_2SO_4) conditions. Based on these results, we consider the feasibility of using the GPI-anchored Gas1 proteins, including ScGas1p, as tools for improving the low-pH and salt tolerance of *S. cerevisiae* for bioprocess applications.

MATERIALS AND METHODS

Yeast strains and media For gene expression analysis using real-time PCR, the wild-type *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and *I. orientalis* NBRC1279 strains were used. *I. orientalis* NBRC1279 was obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan). *S. cerevisiae* BY4742 was also used as the recipient yeast strain for the overexpression of the *ScGAS1* or *IoGAS1* gene. The wild-type *S. cerevisiae* and *I. orientalis* strains were grown in yeast peptone dextrose (YPD) broth or on agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) unless otherwise noted. For real-time RT-PCR analysis, *S. cerevisiae* BY4742 and *I. orientalis* NBRC1279 were grown anaerobically in YP medium supplemented with 40 g/L glucose (YPD2 medium) and adjusted to different pH values (pH 6.0, 5.0, 4.0, 3.0, 2.5, and 2.0) with or without salt stress (7.5% Na_2SO_4). Recombinant *S. cerevisiae* strains (B4-CON, B4-ScGAS1, and B4-IoGAS1) were grown in synthetic complete (SC) minimal medium (6.7 g/L yeast nitrogen base without amino acids) supplemented with the appropriate amino acids and nucleic acids (25) and 20 g/L glucose (SCD medium). In anaerobic fermentation experiments using recombinant *S. cerevisiae* strains, glucose (40 g/L) was added to the SC medium (SCD2 medium). The pH values of the SC-based media (SCD and SCD2) were adjusted to 2.5, 2.4, 2.3, 2.2, and 2.1 by the addition of H_2SO_4. For salt stress experiments, appropriate amounts (5.0% or 7.5%) of sodium sulfate were added to the SC-based media prior to pH adjustment, and then the pH of the media was adjusted with H_2SO_4. Unless specified, the yeast strains were grown at 30°C either on agar plates or in liquid medium.

Plasmids *S. cerevisiae* (S288C) genomic DNA that was prepared using a MasterPure Yeast DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) was used for amplification of the *ScGAS1* gene. A DNA fragment that encodes ScGas1p was obtained by genomic PCR using the primers GAS1-F-Hind (5′-CAT*AAGCTT*ATGTTGTTTAAATCCCTTTCAAAGTTAGC, with the *Hind*III site indicated in italics), and GAS1-R-Bam (5′-A*AGGATCC*TTAAACCAAAGCAAAACCGACACC, with the *Bam*HI site indicated in italics). The pPGK-ScGAS1 plasmid for *ScGAS1* expression in yeast was constructed by inserting the 1.68-kbp *Hind*III-*Bam*HI amplified DNA fragment that comprises the *ScGAS1* coding region (26) into the *Hind*III-*Bam*HI sites of the pPGK shuttle vector (27), which is a multicopy (2-μm DNA-based) plasmid containing a *PGK* promoter and terminator and the *URA3* gene as a selective marker for yeast cells. The plasmid pPGK-IoGAS1 was used for expression of *IoGAS1* with the *PGK* promoter and terminator; construction of pPGK-IoGAS1 was previously described in detail (17).

Yeast transformation Yeast was transformed with pPGK-ScGAS1 or pPGK-IoGAS1 using the Yeastmaker Yeast Transformation System 2 (Clontech Laboratories, Mountain View, CA, USA). The wild-type *S. cerevisiae* strain BY4742 was transformed with pPGK-ScGAS1 and pPGK-IoGAS1 to generate the recombinant strains B4-ScGAS1 and B4-IoGAS1, respectively. Furthermore, pPGK (the empty vector) was transformed into BY4742 to create B4-CON, which was used as a control strain.

Quantitative real-time RT-PCR Expression of the two *GAS1* genes, i.e., the *ScGAS1* and *IoGAS1* genes, was analyzed by quantitative real-time RT-PCR using previously described procedures, with slight modifications (28). For analysis, yeast cells (BY4742 or NBRC1279 strains) were grown under the twelve different conditions described above. Total RNA was extracted using the FastRNA Pro Red kit (MP Biomedicals, Irvine, CA, USA) and then purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) as previously described (29). To generate first-strand cDNA, total RNA (1 μg) was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA samples (0.1 μg) were used as templates for real-time PCR analysis with appropriately designed gene-specific primer sets. These primer pairs were as follows: 5′-TGGTATGTGTAAAGCCGGTTTTG and 5′-CATGATACCTTGGTGTCTTGGTCTA (for *ACT1*), 5′-CCACTCTACAAACTGCCACCA and 5′-GAAGACCCCGAAGCGTTAGA (for *ScGAS1*), and 5′-CTGACGTTTGGTCTGGTGGT and 5′-TTGCATAGGTTGGGCTGATG (for *IoGAS1*). Real-time PCR was performed using a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) and in a final volume of 25 μL using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The PCR reactions were programmed as follows: initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 62°C for 30 s, and elongation at 72°C for 20 s. The amplification products were detected with SYBR Green, and the specificity of the amplification was confirmed by analysis of melting curves from 65°C to 95°C. Fluorescence did not increase in a control experiment without a template. The transcription level of each of the two genes was calculated using the $2^{-\Delta\Delta CT}$ method (30) as a ratio, relative to the expression level of the actin-encoding gene (*ACT1*). All data points were analyzed in triplicate.

Aerobic growth experiments B4-CON, B4-ScGAS1, and B4-IoGAS1 cells were pre-cultivated aerobically in SCD medium for 16 h at 30°C. The cells were then washed with sterile water and inoculated into SCD media without salt at each of the indicated differing low pH values (pH 2.2–2.5) and into SCD medium supplemented with 5.0% Na_2SO_4 at pH 2.5. The initial absorbance at 600 nm (A_{600}) in both cases was approximately 0.02. During cultivation, cell growth was monitored by A_{600} measurements using a bio-microplate reader (HiTS, Scinics Corporation, Tokyo, Japan) as described previously (17). All cultivations in 96-well microplates were performed at 30°C with mild agitation (150 rpm) using the HiTS microplate reader. Cultivation experiments were repeated three times and standard deviations were less than 10%.

Fermentation For anaerobic batch fermentation using B4-CON, B4-ScGAS1, and B4-IoGAS1, the recombinant yeast strains were first cultivated aerobically in 5 mL of SCD medium for 36 h at 30°C. Then, the culture was centrifuged at 6000 ×*g* for 5 min at 4°C, and the pelleted cells were washed and resuspended in distilled water. These cells were inoculated into 20 mL of fermentation medium (SCD2 medium without salt at pH 2.1 or with salt (7.5% Na_2SO_4) at pH 2.5). For all strains, the initial cell density in the fermentation medium was adjusted to an optical density at 600 nm (OD_{600}) of approximately 7.3. During fermentation, cell growth was monitored by measuring the OD_{600} with a WPA Biowave II (Biochrom Cambridge, UK). Anaerobic batch fermentations were performed at 30°C in sterile, closed 50-mL bottles with magnetic stirring, as described previously (31). Samples (0.3 mL) of fermentation broth (SCD2 medium without salt at pH 2.1 or with salt (7.5% Na_2SO_4) at pH 2.5) were removed at specified intervals and diluted four-fold with 8 mM H_2SO_4. These diluted samples were stored at −30°C pending high-performance liquid chromatographic (HPLC) analysis of the substrates and fermentation products. All experiments were performed in triplicate.

Analysis of substrates and fermentation products The concentrations of substrates (glucose) and fermentation products (ethanol, glycerol, and acetic acid) were determined using an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031Plus; Jasco), using an Aminex HPX-87H column

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