Enhancement of Acetic Acid Tolerance in *Saccharomyces cerevisiae* by Overexpression of the HAA1 Gene, Encoding a Transcriptional Activator

Koichi Tanaka, Yukari Ishii, Jun Ogawa, and Jun Shima

Research Division of Microbial Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan; and Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto, Japan

Haal is a transcriptional activator required for *Saccharomyces cerevisiae* adaptation to weak acids. Here we show that the constitutive HAA1-overexpressing strain acquired a higher level of acetic acid tolerance. Under conditions of acetic acid stress, the intracellular level of acetic acid was significantly lower in HAA1-overexpressing cells than in the wild-type cells.

Bacterial contamination is the main culprit behind the reduction of ethanol yield during bioethanol production by yeast (15). Many bacteria, thriving in the ethanol fermentation environment, disturb the ethanol yield by consuming nutrition from the medium, but lactic acid bacteria (LAB) may be the most serious threat, because of their rapid growth (17). So far, various agents have been examined to prevent the LAB contamination. Although antibiotics such as penicillin and virginiamycin effectively inhibit the growth of LAB (1,6), their addition to the broth may not be desirable from an ecological viewpoint; i.e., exposure to antibiotics leads to the emergence of antibiotic-resistant mutants, and spreading them into the environment has a direct impact on food and animals. It is therefore particularly desirable to establish a method preventing bacterial contamination without using antibiotics. Weak organic acids, such as acetic acid and lactic acid, are effective and safe inhibitors used in the prevention of such contamination (14,18). However, the fermentation ability of yeast is also limited by the presence of high concentrations of weak organic acids. We therefore propose a novel strategy to employ acid-resistant yeast strains for bioethanol fermentation under acidic conditions (14,18). To date, we have reported several acid-tolerant yeasts, including an acetic acid-tolerant strain of *Saccharomyces cerevisiae* (4), an acetic acid-tolerant strain of *Schizosaccharomyces pombe* (14), and the lactic acid-tolerant yeast *Candida glabrata* (18).

At an external pH below the pKa value, the weak organic acids predominantly remain as the lipophilic undissociated form and can permeate the plasma membrane by simple diffusion. At natural cytosolic pH, however, the dissociation of the acids leads to the release of protons and the respective anions, which induce intracellular acidification (7). To overcome stress damage by organic acids, two mechanisms can be considered. During the first step, called the “acid shock response,” cells arrest their growth and adapt to the acidic conditions. The second step involves “acid adaptation,” in which the cells maintain the acid adaptation integrity and growth may resume (7). The *S. cerevisiae* genes involved in acid adaptation and “acetic adaptation” have been clarified by several research groups (3,13).

Recently, we identified a novel acetic acid-tolerant *S. cerevisiae* strain, ATCC 38555, which exhibits a shorter duration of the period of adaptation to acetic acid. Transcriptome analysis revealed that AFT1- and HAA1-regulated genes are clearly upregulated in this strain (4). Aft1 is a transcription factor involved in iron utilization and is activated by treatment with weak organic acids (12). HAA1 is a transcriptional activator involved in the adaptation to weak acid stress (3,9,10) which binds to an acetic acid-responsive element (ACRE), activating the expression of several targets, including TPO2 and TPO3, the membrane transporter genes (3,8,10). However, it is still unclear whether the acetic acid resistance of ATCC 38555 strain is the outcome of the upregulation of AFT1- and/or HAA1-regulated genes. In the present study, to gain insight into the acid-tolerant mechanisms of Haa1 and Haa1-regulated genes, the effects of HAA1 overexpression on the acetic acid tolerance and intracellular levels of acetic acid were examined by using a haploid laboratory strain, S288C (MATa prototroph), as a host.

A HAA1-overexpressing strain was constructed on the basis of a method previously reported (5) using a primer set listed in Table S1 in the supplemental material. Briefly, the promoter region of the TDH3 gene, which allows constitutive expression at a high level, was fused with the URA3 marker gene and then inserted upstream of the start codon of the HAA1 gene. The resultant HAA1-overexpressing strain (MATa ura3Δ0 URA3-P_TDH3-HAA1) is designated HAA1-OP in this paper.

To determine the expression level of HAA1 and its target genes in the wild-type (S288C) and HAA1-OP strains, real-time PCR analysis was performed. Total RNAs were extracted from logarithmically growing cells using the hot phenol method. Synthesis of the cDNAs from the total RNAs was performed using a PrimeScript II High Fidelity reverse transcription-PCR (RT-PCR) kit (TaKaRa, Ohtu, Japan) with an oligo(dT) primer. Real-time PCR was carried out using a LightCycler FastStart DNA Master SYBR green I kit (Roche, Mannheim, Germany) and LightCycler II instrument (Roche). Data analysis was performed using LightCycler software version 3.5 (Roche). The primers used for the real-time PCR analysis are listed in Table S1 in the supplemental material. TAF10 was employed as a control housekeeping gene based on the report of Teste et al. (16).

Received 27 July 2012 Accepted 4 September 2012 Published ahead of print 7 September 2012
Address correspondence to Jun Shima, shima@kais.kyoto-u.ac.jp.
Supplemental material for this article may be found at http://aem.asm.org/
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.02356-12
The expression data of the HAA1-OP strain were compared with those of the wild-type strain and are indicated as relative expression levels (Fig. 1). The data show that the transcriptional level of HAA1 was approximately 2.5-fold higher in the HAA1-OP strain. Expression of TPO2 and TPO3, which encode the plasma membrane transporter (3), was also more prominent (~4-fold) in the HAA1-OP strain. In addition, YRO2, which encodes a putative membrane protein of unknown function (3), and YGP1, which encodes a cell wall-related secretory glycoprotein (2), were dramatically upregulated (30-40-fold). These data indicate that the overexpression of HAA1 induced the expression of Haa1-regulated genes, suggesting that the Haa1 regulon is activated in the HAA1-OP strain even in the absence of acetic acid stimulation. In contrast, no significant differences between HAA1-OP and the wild-type strain in the expression of FPS1, which encodes the major channel for the entry of undissociated acetic acid (13), were observed (Fig. 1). These results suggest that transcriptional regulation of FPS1 was independent of the presence of Haa1.

To examine the phenotype of HAA1 overexpression, weak organic acid tolerance of HAA1-OP was compared with that of the wild type (Fig. 2). Each strain was precultured in YPD medium (2% glucose, 1% yeast extract, 2% peptone) until the late log phase, and serially diluted cells were spotted onto YPD agar plates containing 0.7% (wt/vol) lactic acid (pH 2.9). The data clearly show that the HAA1-OP strain exhibited an extremely increased resistance to acetic acid; conversely, lactic acid tolerance was not detected under our experimental conditions. Similar results were obtained in a viability test (data not shown). These findings suggest that HAA1 overexpression exerts a specific protective effect upon acetic acid challenge.

To gain further insight into the acetic acid-tolerant mechanisms governed by HAA1 overexpression, the intracellular level of acetic acid was evaluated. Cells precultured in YPD medium until an optical density at 600 nm (OD$_{600}$) of 2 was reached were harvested and reincubated into YPD medium (pH 4.2) containing acetic acid at various concentrations (0% to 1.0%). After incubation at 30°C for 60 min, the cells were harvested, washed twice with ice-cold distilled water, and suspended with an equal weight of 0.5% (wt/vol) arabinose solution. Low-molecular-weight intracellular components were extracted by boiling for 10 min and determined via high-performance liquid chromatography (HPLC) under the conditions described previously (18). As arabinose gave an independent signal from other cellular components under the experimental conditions, it was used for standardization. As shown in Fig. 3A, the intracellular acetic acid level was significantly lower in HAA1-OP cells than in wild-type cells. In wild-type cells, the intracellular acetic acid level gradually increased in a manner that was dependent on the extracellular acetic acid concentration. On the other hand, intracellular accumula-

FIG 1 Expression analysis of HAA1 and Haa1-regulated genes in the HAA1-OP strain. The expression data of the HAA1-OP strain were compared with those of the wild-type strain and are shown as relative expression levels. The values are the means of results from three independent experiments.

FIG 2 Growth phenotypes of the wild-type and HAA1-OP strains under conditions of acetic acid stress. Approximately $10^5$ cells and serial dilutions of $10^{-1}$ to $10^{-4}$ (from left to right) of wild-type and HAA1-OP strains were spotted on YPD plates in the absence or presence of 0.7% (wt/vol) acetic acid (pH 4.2) or 3% (wt/vol) lactic acid (pH 2.9). Plates were incubated at 30°C for 1 day (YPD), 4 days (0.7% acetic acid), or 6 days (3% lactic acid).

FIG 3 Comparison of the levels of intracellular acetic acid and cell viability after exposure to acetic acid. (A) Intracellular acetic acid levels. Wild-type and HAA1-OP cells precultured in YPD medium (pH 6.8) were reincubated into YPD medium (pH 4.2) in the absence (0%) or presence (0.2% to 1.0%) of acetic acid and incubated at 30°C for 60 min. Intracellular acetic acid was extracted and quantified using HPLC. The acetic acid concentration of each sample was compared with that of the wild-type sample grown in normal YPD medium (0% extracellular acetic acid, pH 6.8), and the results are shown as relative intracellular acetic acid levels. The values are the means of results from three independent experiments. (B) Viability of cells after exposure to acetic acid. Wild-type and HAA1-OP cells precultured in YPD medium (pH 6.8) were reincubated into YPD medium containing 1.0% acetic acid (pH 4.2) and incubated for 60 min at 30°C. Cells were harvested and resuspended in YPD medium, and approximately $4 \times 10^7$ cells and serial dilutions of $10^{-1}$ to $10^{-4}$ (from left to right) were spotted onto YPD plates (pH 6.8).
tion of acetic acid was significantly diminished in HAA1-OP cells. Because both strains retained full viability even in the 1.0% acetic acid medium (Fig. 3B), the different levels of intracellular acetic acid would not have been a consequence of viability loss. These data suggest that the HAA1-OP strain demonstrates a greater ability to reduce the intracellular acetic acid concentration, though it could not be determined whether HAA1-OP cells efficiently inhibit diffusional entry of acetic acid or activate the acetic acid extrusion mechanisms.

In the present study, the constitutive HAA1-overexpressing strain was constructed and it was found that the overexpression of HAA1 induced the expression of Haa1-regulated genes, including TPO2 and TPO3, the products of which are considered major facilitator superfamily transporters of the S. cerevisiae plasma membrane (3) (Fig. 1). Similar results have been obtained by the plasmid-borne overexpression of the HAA1 gene under the control of the galactose-inducible GAL1 promoter (8). However, our system (i.e., constitutive overexpression from the chromosomal locus) is more convenient and robust, making it suitable for industrial application. In addition, most importantly, the HAA1-OP strain showed a markedly higher acetic acid tolerance than the wild-type strain (Fig. 2). The acetic acid tolerance of the HAA1-OP strain is possibly related to its ability to reduce the intracellular accumulation of acetic acid (Fig. 3A). Consistent with our results, the cells in which the HAA1 gene was deleted showed an acetic acid-sensitive growth phenotype, with the accumulation of intracellular acetic acid, when cultivated in the presence of acetic acid (3). It is presumed that some of the Haa1-regulated genes have activities restricting the influx of the acetic acid and/or promoting a more efficient efflux of the counterion. One such candidate is the HRK1 gene encoding a protein kinase involved in the posttranslational regulation of plasma membrane transporters (9). Other Haa1-regulated genes which may also confer resistance to acetic acid include TPO2 and TPO3, whose gene products are proposed to mediate acetate export (3). We also suggest that expression of FPS1, which encodes the major channel for the entry of undissociated acetic acid (13), was not regulated by HAA1. Our data may support the report of Mollapour and Piper which suggests that inactivation of FPS1 occurred under the control of Hog1, which was activated by acetic acid (11).

Further work, such as DNA microarray analysis and evaluation of the intracellular pH, is needed for the clarification of the molecular mechanisms of acetic acid tolerance in the HAA1-OP strain; however, it is noteworthy that overexpression of HAA1 may be an effective method for the molecular breeding of acetic acid-tolerant S. cerevisiae strains, which are important for bioindustries, including those involved in bioethanol production. A slight growth delay in the HAA1-OP strain (Fig. 2,YPD), which was constructed using a strong constitutive promoter, was observed and may have been due to the excess expression of Haa1-regulated gene products. Optimization of the promoter used for the expression of HAA1 is in progress in order to construct commercial acetic acid-tolerant strains of S. cerevisiae for use in industrial ethanol production.

ACKNOWLEDGMENTS

This work was supported by the Institute for Fermentation, Osaka (IFO), Japan, a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Rural Biomass Research Project Bec-BC051), a grant from the Elizabeth Arnold Fuji Foundation, and a Grant-in-Aid for Scientific Research (C).

We thank Satoshi Harashima (Osaka University, Japan) for enormous encouragement.

REFERENCES