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Acid tolerance response of *Tetragenococcus halophilus*: A combined physiological and proteomic analysis

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ABSTRACT

Tetragenococcus halophilus is known to flourish in extreme salt environments, and frequently existed during the manufacture of fermented foods, where various environmental stresses are encountered. In this study, acid tolerance response of *T. halophilus* was investigated based on physiological and proteomic analysis. The optimal acid-adaptation condition was optimized to stimulate acid tolerance response effectively. After acid adaptation at pH 4.0 for 1 h, the highest survival rate at lethal pH 2.5 was observed. Analysis of the physiological data showed that higher intracellular pH and NH_4^+ pool were detected in cells adapted at pH 4.0 compared with cells without acid adaptation. In addition, the activities of hexokinase, pyruvate kinase, and lactate dehydrogenase were determined, and significantly higher activities were monitored in cells after acid adaptation. Comparison of the intracellular amino acid pools indicated that six amino acids (glutamate, aspartate, isoleucine, leucine, citrulline, ornithine) exhibited notable alternations during acid treatments, and acid pre-adaption led to higher pools of amino acid compared to that without acid pre-treatment. Proteomic analysis demonstrated that eight proteins (Glk, Pfk, Fba, Gap, Gpm, Eno, Pyk, and Ldh) involved in carbohydrate metabolism and three classic stress response proteins (Ssb, UspA, and GroL) were up-regulated after acid adaptation. Results presented in this study may help to further understand the acid tolerance response in *T. halophilus* and improve the industrial performance of this species.

1. Introduction

Tetragenococcus halophilus is one of the halophilic lactic acid bacteria found in extreme salt environments, and it is one of the most osmotolerant of all the prokaryotes [1]. This halophilic bacterium plays an important role in the production of soy sauce, soy paste, fish paste, and Chinese horsebean-chili-paste [2–4]. Previous research revealed that *T. halophilus* contributed to the formation of aroma in fermented foods as they produced numerous volatile compounds, including 2-methylpropanal, 3-methylbutanal, and 4-ethyl guaiacol [2,5]. Therefore, the addition of *T. halophilus* as starter culture for flavor improvement has been proposed in many fermented foods [3–5]. Such volatiles also act as antimicrobials that can confer a competitive advantage, via their chaotropic activities [6–8]. However, *T. halophilus*, similar to other lactic acid bacteria, encounters various environmental challenges including chaptropicity-induced, osmotic, acidic and oxidative stresses during the manufacture of fermented foods, which lead to the decrease of metabolic capacity and production efficiency. Thus, to engineer the robustness of lactic acid bacteria may contribute to enhance the industrial application and industrial functionality of this species.

To cope with the harsh conditions, lactic acid bacteria employed a variety of strategies, including the up-regulation of stress response proteins, maintaining the cell membrane functionality, and metabolic shifts [9–12]. Based on these results, many feasible strategies have been proposed to increase the stress resistance, and increased robustness of lactic acid bacteria was also acquired. For example, acid stress led to the up-regulation of DNA repair protein RecO in *Lactobacillus casei*, and the acid-resistant mutant exhibited higher expression level compared to the corresponding control strain [9]. To further validate the protective role of RecO, a RecO-overproducing mutant was constructed by using *Lactococcus lactis*, and the mutant exhibited higher tolerance to acid, salt and

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oxygen stresses [13]. Wu et al. [9] reported that acid stress induced the accumulation of aspartate in *L. casei*. Validation of the data was performed by the exogenous addition of aspartate to the medium, and at least 65.8% higher biomass was observed during growth at pH 4.3 [14].

Acid tolerance response is a widely existed phenomenon in LAB, which made these organisms less susceptible to lethal acid stress by prior exposure of the cells to acidic conditions [12,15–17]. Moreover, acid tolerance response appears to confer protection against other stresses in addition to severe acid stress [18]. However, information is still lacking on mechanisms used by lactic acid bacteria to modulate acid tolerance response.

In this study, we optimized the conditions for acid tolerance response induction in *T. halophilus*, and then analyzed its effects on physiological characters including intracellular pH, intracellular pools of amino acids and key enzymatic activities. In addition, a comparative proteomic analysis was performed between cells with or without acid adaptation. This result may gain insights into, and contribute knowledge to, the mechanisms underlying the acid tolerance response.

2. Materials and methods

2.1. Strains and growth conditions

The strain used in this study was *T. halophilus* CGMCC 3792, which was originally isolated from soy sauce moromi and then identified via physiological, biochemical, and 16 S rDNA sequence analysis [3]. Cultures were grown statically in GM17 (M17 broth (Oxide) supplemented with 0.5% (w/v) glucose) at 30 °C for 20 h.

2.2. Acid stress experiment

To investigate the acid tolerance response, cells grown to mid-exponential growth phase in GM17 were harvested, centrifugated at 10000 × *g* for 5 min, washed and resuspended in GM17 adjusted to different pHs with 1 mol/l hydrochloric acid for pre-adaptation. After pre-adaptation for 1 h, cells were harvested and washed as noted and suspended in GM17 adjusted to acid stress at pH 2.5. Cells challenged at pH 2.5 were serially diluted at spotted in triplicate onto GM17 agar plates to determine the viability.

2.3. Measurement of intracellular pH (pH_i)

pHi was measured by the fluorescence method developed by Breeuwer et al. [19] using 5 (and 6-)-carboxyfluorescein succimidyl ester as the fluorescent probe. Calibration curves establishing the relationship between extracellular pH and intracellular pH were established to exclude artifacts caused by environmental conditions. Loading of cells with 5 (and 6-)-carboxyfluorescein succimidyl ester, determination of pH_i, and calibration of pH_i all followed the procedure described previously [19].

2.4. Determination of intracellular ammonia

Cells subjected to different treatments were harvested by centrifugation at 12000 × *g* for 10 min, washed twice with 200 mM phosphate-buffer saline (PBS, pH 7.5), and then resuspended in the same buffer. The solution was sonicated on ice for 10 min and followed by centrifugation at 12000 × *g* for 10 min. The amount of ammonia in the supernatant was analyzed with the ammonia assay kit (Sigma, USA) according to manufacturer's instructions.

2.5. Determination of enzymes activities

Cells subjected to different treatments were harvested by centrifugation at 12000 × *g* for 10 min, washed twice with ice-cold saline (0.85% NaCl, w/v), and resuspended in an equal volume of phosphate-buffer saline (PBS, 0.2 M potassium phosphate, 2 mM EDTA, pH 7.0). The cell-free extract was prepared according to method described by Zhang et al. [20], and the cell-free extract was used for determination of enzyme activity. The activities of hexokinase, pyruvate kinase, and lactate dehydrogenase activities were analyzed with the assay kits (Nanjing Jiancheng, China) according to the manufacturer's instructions.

2.6. Determination of intracellular amino acid

For the extraction of amino acids, cells subjected to different treatments were harvested by centrifugation at 12000 × *g* for 10 min, washed twice, resuspended in 1 ml of 200 mM phosphate buffer solution (pH 7.0), and then boiled for 15 min. Cell debris was discarded by centrifugation (12000 × *g*, 10 min, 4 °C). The supernatants were treated at room temperature by addition of 1 ml of 10% tricarboxylic acid for 10 min. The mixture was then centrifugated (12000 × *g*, 4 °C) again for 10 min, and the supernatants were analyzed with HPLC according to the method of Fountoulakis and Lahm [21].

2.7. 2D gel electrophoresis, gel image analysis and protein identification

Cells treated with different pHs (7.0 and 4.0) for 1 h were centrifugated at 10000 × *g* for 5 min, washed for three times with distilled water to remove the residual acidified medium. The whole-cell extracts were prepared using the BioRad kit and the concentration of each protein sample was determined using the BioRad Protein Assay Kit (BioRad) with bovine serum albumin as a standard. Each sample was applied to immobilized pH gradient (IPG) strips (18 cm, pH 4–7, BioRad) with a final concentration of 600 μg protein in 350 μl rehydration buffer. Isoelectric focusing (IEF) and SDS-PAGE were performed according to the method described by Wu et al. [12]. After SDS-PAGE, the 2D gels were stained with 0.1% Coomassie blue R-250.

The stained gels were scanned using Imagescanner (GE Healthcare). Comparative analysis of the protein spots was performed using Image Master 6.0 2D platinum software (GE Healthcare). Proteins that displayed at least 2-fold variations were taken into account for further analysis. The proteins spots with significant change were excised using gel plugs, transferred to Eppendorf tubes, then digested with 20 μl of 10 ng/μl proteomics sequencing grade trypsin at 37 °C for 16 h and rehydrated in 500 μl of 50 mM NH_4HCO_3 (pH 8.0). Supernatants of 0.5 μl were spotted directly onto the MALDI plate for proteins identified by mass spectrometry as described previously [22].

2.8. Statistical analysis

Analysis of variance (one-way ANOVA) with Duncan's test was performed to evaluate statistical differences. Differences between samples with *p*-values $p \leq 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Acid tolerance response of T. halophilus CGMCC 3792

Acid tolerance response is widely reported in many microbes such as *L. casei* [12], *Lactobacillus sanfranciscensis*