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New insights into the mechanisms of acetic acid resistance in *Acetobacter pasteurianus* using iTRAQ-dependent quantitative proteomic analysis

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ABSTRACT

Acetobacter pasteurianus is the main starter in rice vinegar manufacturing due to its remarkable abilities to resist and produce acetic acid. Although several mechanisms of acetic acid resistance have been proposed and only a few effector proteins have been identified, a comprehensive depiction of the biological processes involved in acetic acid resistance is needed. In this study, iTRAQ-based quantitative proteomic analysis was adopted to investigate the whole proteome of different acidic titers (3.6, 7.1 and 9.3%, w/v) of *Acetobacter pasteurianus* Ab3 during the vinegar fermentation process. Consequently, 1386 proteins, including 318 differentially expressed proteins ($p < 0.05$), were identified. Compared to that in the low titer circumstance, cells conducted distinct biological processes under high acetic acid stress, where >150 proteins were differentially expressed. Specifically, proteins involved in amino acid metabolic processes and fatty acid biosynthesis were differentially expressed, which may contribute to the acetic acid resistance of *Acetobacter*. Transcription factors, two component systems and toxin-antitoxin systems were implicated in the modulatory network at multiple levels. In addition, the identification of proteins involved in redox homeostasis, protein metabolism, and the cell envelope suggested that the whole cellular system is mobilized in response to acid stress. These findings provide a differential proteomic profile of acetic acid resistance in *Acetobacter pasteurianus* and have potential application to highly acidic rice vinegar manufacturing.

1. Introduction

Acetic acid bacteria (AAB) are gram-negative, strictly aerobic microorganisms belonging to *Acetobacteraceae* (Cleenwerck and De Vos, 2008; Yamada and Yukphan, 2008). The species belonging to genera *Acetobacter* and *Gluconacetobacter* (some species are now relocated into *Komagataeibacter*) are mainly used in industrial vinegar production because of their specific acetic acid and ethanol tolerance and remarkable ability to oxidize ethanol to acetic acid via alcohol dehydrogenase and aldehyde dehydrogenase (Matsushita et al., 1994; Thurner et al., 1997). In particular, *Acetobacter pasteurianus* is a predominant starter in traditional rice vinegar production in China and Japan, where the acetic acid titer does not often exceed 6% (v/v) (Matsutani et al., 2011). Because acetic acid is well known as a metabolite that produces cellular toxicity at concentrations as low as 0.5% (v/v), it is important to elucidate the molecular mechanisms of acetic acid resistance in terms of AAB exploration and industrial vinegar production (Steiner and Sauer, 2001).

During the past few decades, numerous studies have been performed to explore the mechanisms of acetic acid resistance in AAB, and *Acetobacter* and *Gluconacetobacter* are usually the predominant bacteria studied. Currently, proposals about acid resistance mechanisms have been focused on four main aspects: (i) proteins involved in alcohol oxidation into acetic acid (Nakano and Fukaya, 2008; Trcek et al., 2007; Trcek et al., 2006), (ii) pathways involved in the detoxification of intracellular acetate anion and H^+ by efflux pumps or assimilation of acetate through TCA cycle (Matsushita et al., 2005; Mullins et al., 2012; Nakano et al., 2006; Sakurai et al., 2013; Soemphol et al., 2015), (iii) acid shock responses, such as GroESL, DnaKJ (Ishikawa et al., 2010; Nakano and Fukaya, 2008), and (iv) cell envelope adaptions, such as outer membrane structures, pellicle polysaccharides, etc., which function as barriers to passive diffusion of undissociated acetic acid molecules into the cells (Deeraksa et al., 2005). Additionally, changes in the membrane lipids along with the titer stress accumulation, may suggest an underlying effect on the influx of undissociated acetic acid molecules, thus conferring acid resistance (Hanada et al., 2001; Trcek et al., 2007). By reviewing the bacterial acid tolerance mechanisms in *E. coli*, *Listeria monocytogenes*, and lactic acid bacteria, etc., the contribution of amino acid deamination to acid resistance of AAB was first proposed

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by Trcek et al. (2015). Although substantial progress has been achieved (Andres-Barrao et al., 2012; Wang et al., 2015a), the insights into the acetic acid resistance seem scattered, suggesting that multiple adaptions or modulatory pathways cooperate in the cellular acid resistance.

Actually, the available genomic, transcriptomic and proteomic data and methods are becoming feasible to support a more global and comprehensive study of the biological metabolism of AAB. So far, eighteen genomes of *Acetobacter* or *Gluconacetobacter* have been sequenced; and a transcriptome analysis of the ethanol and glucose switches further confirmed the role of energy metabolism and glyoxylate pathway in acid resistance (Sakurai et al., 2011; Zhong et al., 2014). Upon analysis of the functions of 52 identified proteins, it was concluded that *A. pasteurianus* LMG 1262^T differentially modulated the following processes: (1) protein folding, (2) stress response, (3) oxidation-reduction processes, (4) metabolic processes, (5) protein biosynthesis, and (6) membrane modifications. Recently, the comparative proteome studies of *A. pasteurianus* and *Komagataeibacter* spp., which face much higher acetic acid titers (>9%, w/v) during vinegar fermentation, were reported (Andrés-Barrao et al., 2015; Wang et al., 2015b). Although several novel protein targets were identified, the limited number greatly restricts any downstream analysis aiming at high resolution determination of the adaption mechanisms by Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Recently, the iTRAQ-based proteomic analysis has emerged to identify protein expression more qualitatively and quantitatively, and thus can provide more comprehensive proteome information (Evans et al., 2012; Miao et al., 2015). Compared to conventional proteomics techniques, iTRAQ possesses unique advantages in identifying and quantifying proteins by using labeled peptides identifiable by mass spectrometers (Evans et al., 2012; Zhang et al., 2014). In addition, iTRAQ analysis is further enhanced by adopting sturdy bioinformatics tools and statistical analysis to specifically annotate the results (Herbrich et al., 2013). In current study, the iTRAQ-based quantitative proteomic analysis technique was adopted to further examine the acetic acid resistance mechanism in *A. pasteurianus* Ab3.

2. Materials and methods

2.1. Bacterial strain and samples

A. pasteurianus Ab3 (deposited in the China Center for Type Culture Collection, NO M 2013116) was stored lyophilized in the laboratory. *A. pasteurianus* Ab3 was isolated from the traditional rice vinegar in Zhejiang Wuweihe Food Co. Ltd., Huzhou, China. Because of its high acetic acid resistance, strain Ab3 is applied to high titer rice vinegar manufacturing (>9%, w/w). To activate the lyophilized culture, it was first dissolved in 0.5 mL fresh sterilized YPD medium (10 g/L yeast extract, 5 g/L peptone, 10 g/L D-glucose, pH 6.5) without ethanol. The activated culture was then inoculated into fresh YPD medium containing 2% ethanol (v/v) and 0.5% acetic acid (v/v) with shaking (160 r/min) at 30 °C for 24 h. The seed culture are prepared in a 500 L self-priming fermenter (Nanjing Biological Engineering Equipment Co., LTD, Jiangsu, China). When the titer accumulates to ~3% (w/v), seeds are transferred into a 30 m^3 fermenter for vinegar fermentation. The ethanol concentration was automatically maintained at 3% (v/v) by addition of ethanol during cultivation. The acetic acid concentration was determined by titration. Three cell samples with acidic titers at 3.6%, 7.1%, and 9.3% (w/v) vinegar fermentation were collected and cognately prepared, with the pooled sample being designated as P3, P7 and P9 respectively. The P3 sample was used as the control and P7 and P9 were regarded as the experimental groups in the iTRAQ 8 plex quantitative analysis. All processes were repeated three times, and the sampled cells were mixed to prepare the pool for analysis (Fig. S1).

2.2. Protein extraction and quantification

The total cellular proteins from the pooled sample were extracted according to instructions provided with the bacterial protein extraction kit (Liu et al., 2015). Briefly, the cells were collected by centrifugation, and the precipitates were resuspended and washed 3 times with PBS buffer (phosphate buffer saline). The Lysis solution (1 mL of lysis buffer included 1 μL of protease inhibitor, 10 μL of DTT (1 M) and 10 μL of PMSF (100 mM)) was added to one-fifth of the original volume of the cell cultures, and the mixture was incubated at 4 °C for 10 min. The cells were further lysed by ultra-sonication and centrifuged at 14,000 r/min for 15 min at 4 °C. The supernatant was collected and the protein concentration was quantified using the Bradford method (Bradford, 1976).

2.3. Protein digestion and iTRAQ labeling

Protein digestion was conducted as previously described (Liu et al., 2015). Approximately 60 μg of protein were dissolved in a 5-fold volume of dissolution buffer (8 M urea and 100 mM Tris-HCl). Then, 4 μL of reducing reagent were added to the mixture and incubated at 37 °C for 2 h. Another 2 μL of cysteine-blocking reagent were added and incubated for 15 min at room temperature; the alkylated protein were collected by centrifugation at 12,000 r/min for 20 min. After three washes, the pellet was digested with Trypsin and incubated at 37 °C for 16 h. Following — trypsin digestion, the peptide samples were lyophilized and then labeled using the iTRAQ reagents 8-plex kit (AB Sciex) according to the manufacturer's instructions. iTRAQ reagents 113 and 114 were used to coordinately label the peptides from the control P3 samples, whereas iTRAQ reagents 115, 116 and 117 were used to coordinately label the peptides from the P7 sample and reagents 118, 119 and 121 were used to label the peptides from the P9 sample. The labeled peptides were incubated at room temperature for 2 h, and the reaction was stopped by adding 100 μL of ultrapure water. Then, 1 μL of sample was removed from each of the 8 groups and mixed to confirm that the samples had been successfully labeled, after which the labeled peptides were pooled and lyophilized.

2.4. SCX fractionation and LC-ESI-MS/MS analysis using a triple TOF 5600

The iTRAQ-labeled peptides were dissolved in 100 μL of buffer A (25 mM NaH_2PO_4 in 25% CAN, pH 2.7) and purified on a strong cation exchange chromatography (SCX) column (2.0 × 150 mm, 5 μm, michrom) using an Agilent 1200 HPLC (Agilent). The samples were eluted using a binary linear gradient of 5% buffer B (25 mM NaH_2PO_4 and 1 M KCl in 25% CAN, pH 2.7) for 5 min, 5–50% buffer B for 35 min, 50–80% buffer B for 5 min, 80% buffer B for 5 min, 80–5% buffer B for 0.01 min and 5% buffer B for 9.99 min. The flow rate was set at 0.3 mL/min, and the detection wavelengths were 215 nm and 280 nm. The first tube of eluted peptides was collected from 0 to 5 min, and the eluted peptides were collected every 4 min from 5 to 44 min. The peptides that eluted from 35 to 46 min were collected as one tube. Equal amounts of peptides from the first and second tubes were mixed. All of the eluted peptides were pooled in 10 fractions and dried using vacuum freeze drying. The dried peptides were re-dissolved in Nano-RPLC buffer A and desalted using a C18 column (100 μm × 3 cm, 3 μm) for 10 min. The analytical separation was performed using an Eksigent nanoLC-Ultra™ 2D system (AB SCIEX) coupled to a triple TOF 5600 system (AB SCIEX) fitted with a Nanospray III source (AB SCIEX, USA) and a pulled quartz tip as the emitter (New Objectives, USA). The system was equipped with a C18 reverse chromatography column (75 μm × 15 cm, 3 μm) for analysis. Separation was achieved using gradient elution and performed as previously reported (Miao et al., 2015), with some modifications. The data were acquired using an ion spray voltage of 2.4 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and a heater temperature of 150 °C. The information-dependent acquisition (IDA) method was used to acquire the data; the secondary scans were