J. Dairy Sci. 101:1–13
https://doi.org/10.3168/jds.2018-14594

Quantitative proteomics of *Lactococcus lactis* F44 under cross-stress of low pH and lactate

Hao Wu,*†[1] Yue Zhao,*†[1] Yuhui Du,* Sen Miao,*† Jingui Liu,*† Yanni Li,* Qinggele Caiyin,* and Jianjun Qiao*†‡[2]
*Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, and
†Key Laboratory of Systems Bioengineering (Ministry of Education), Tianjin University, Tianjin 300072, P. R. China
‡SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, P. R. China

ABSTRACT

Lactococcus lactis encounters 3 environmental stimuli, H^+, lactate, and undissociated lactic acid, because of the accumulation of lactic acid—the predominant fermentation product. Few studies have examined how these stimuli synergistically affect the bacteria. Herein, we analyzed the dissociation degree of lactic acid at different pH and investigated the cellular response to cross-stress in *L. lactis* ssp. *lactis* F44 through quantitative proteomic analysis using isobaric tags for relative and absolute quantitation of 3 groups: 0% lactic acid with pH 4.0 and 0% lactic acid with pH 5.0 for acid stress; 2% lactic acid with pH 7.0 and 3% lactic acid with pH 7.0 for lactate stress; and 2% lactic acid with pH 4.0, 2% lactic acid with pH 5.0, 3% lactic acid with pH 4.0, and 3% lactic acid with pH 5.0 for cross-stress. We observed that the metabolisms of carbohydrate and energy were inhibited, which might be due to the feedback inhibition of lactic acid. The arginine deiminase pathway was improved to maintain the stability of intracellular pH. Additionally, some differentially expressed genes associated with the general stress response, amino acid metabolism, cell wall synthesis, and regulatory systems played significant roles in stress response. Overall, we highlighted the response mechanisms to lactic acid stress and provided a new opportunity for constructing robust industrial strains.
Key words: *Lactococcus lactis*, stress response, quantitative proteomics

INTRODUCTION

Lactococcus lactis is a gram-positive bacterium that has great economic value because of its characteristics of simple metabolism, rapid growth, lack of toxic substances, and extensive usefulness in the food industry (Song et al., 2017). During fermentation, *L. lactis* is exposed to several environmental stimuli, especially lactic acid, that results in the inhibition of cell growth and the accumulation of important products, such as nisin (Shimizu et al., 1999; Guerra and Pastrana, 2003).

Lactic acid is a weak organic acid with a pK_a of 3.86 (Eyal and Canari, 1995). The lipophilic undissociated lactic acid can diffuse across the cell membrane freely; once inside the cell, this lactic acid dissociates and releases protons to acidify the cytoplasm (Rosengren et al., 2013). Therefore, H^+, lactate, and the undissociated lactic acid should be taken into account when assessing the detrimental effects of lactic acid. Lactic acid restrains cell growth by causing loss of membrane potential (Axe and Bailey, 1995), acidification of cytoplasm induced by dissociated protons (Shelef, 1994), and indirect suppression of NAD^+ regeneration via intracellular accumulation of anions (Russell, 1992; Russell and Diez-Gonzalez, 1998). In addition, a large number of acid-resistance mechanisms have been discussed, such as F_1F_0-ATPase, glutamate decarboxylase system, arginine deiminase pathway (Marquis et al., 1987; Gruening et al., 2006; Lucas et al., 2007; Vrancken et al., 2009), abundance of chaperones or DNA repair system (Hanna et al., 2001; Frees et al., 2003; Desmond et al., 2004), alterations in the composition of the cell envelope (Montanari et al., 2010), and transcriptional regulation (Zhai et al., 2014).

The effects of low-pH stress on proteome and transcriptome levels have been determined in multiple lactic acid bacteria (**LAB**), such as *L. lactis* (Frees et al., 2003; Budin-Verneuil et al., 2005), *Lactobacillus casei* (Broadbent et al., 2010), *Lactobacillus reuteri* (Wall et al., 2007; Lee et al., 2008), *Lactobacillus rhamnosus* (Koponen et al., 2012), *Streptococcus thermophiles* (Arena et al., 2006), and *Bifidobacterium longum* (Sánchez et al., 2007). High-throughput sequencing technology aids in systematic analysis of organisms under environmental stresses. Xie et al. performed the oligonucleotide microarray to profile gene expression levels under acid,

Received February 15, 2018.
Accepted April 13, 2018.
[1] These authors contributed equally to this work.
[2] Corresponding author: jianjunq@tju.edu.cn

osmotic, and heat stress in *L. lactis* ssp. *lactis* IL1403; simultaneously, the authors explained the differences of response mechanisms under acid stress between *L. lactis* strains MG1363 and IL1403 (Xie et al., 2004). Pieterse et al. studied the transcriptome of *Lactobacillus plantarum* in diverse concentrations of lactic acid, osmolality, and pH value, and many responsive mechanisms under lactic acid stress were identified (Pieterse et al., 2005). Desmond et al. revealed that the expression of GroEL in *Lactobacillus paracasei* NFBC338 was increased under heat adaptation conditions, and the *groESL*-overexpressing strains exhibited a higher tolerance to heat and butanol (Desmond et al., 2004). Sánchez et al. compared the protein maps of *B. longum* biotype *longum* NCIMB 8809 and its acid-resistant mutant, and results indicated that the AA deamination pathway could neutralize internal protons through the production of ammonium (Sánchez et al., 2007). Similarly, a comparative proteomic study of *L. casei* Zhang and its acid-resistant mutant showed that several key proteins associated with cellular metabolism, translation, DNA replication, and chaperones were induced to protect the cells of the mutant (Wu et al., 2012). Zhai et al. used the proteome complemented by the transcriptome to uncover the cellular response in *Lactobacillus delbrueckii* during acid adaptation and identified Ldb0677, a novel transcriptional regulator related to acid stress (Zhai et al., 2014). Moreover, they also found pyruvate kinase (Pyk) could increase acid resistance probably by enhancing the fatty acid biosynthesis, and they disclosed that it was regulated by the transcriptional regulator CcpA via bacterial one-hybrid technology (Zhai et al., 2015).

Although many studies have investigated the molecular basis of acid stress in LAB, response mechanisms under lactic acid stress are still poorly understood. In this study, we applied the isobaric tags for relative and absolute quantitation (**iTRAQ**) quantitative proteomics approach to determine the metabolic response of *L. lactis* ssp. *lactis* F44 under cross-stress of H^+ and lactate. This study will contribute to the construction of robust industrial strains.

MATERIALS AND METHODS

Strains and Growth Conditions

Lactococcus lactis ssp. *lactis* F44 was obtained by the genome shuffling of *L. lactis* ssp. *lactis* YF11 (accession number CGMCC7.52) in our previous study (Zhang et al., 2016). The inoculum was prepared at 30°C for 8 h (optical density at 600 nm ~1.5) in seed medium and was inoculated (10%) into the fresh fermentation medium with different 12 stress conditions: 1% lactic acid with pH 4.0, pH 5.0, pH 6.0, or pH 7.0; 2% lactic acid with pH 4.0, pH 5.0, pH 6.0, or pH 7.0; or 3% lactic acid with pH 4.0, pH 5.0, pH 6.0, or pH 7.0. Samples were taken at 0.5, 1, 1.5, and 2 h. After collection by centrifugation (8,228 × *g*, 5 min, 4°C), the cell suspension was serially diluted with 0.9% normal saline. A total of 100 μL of diluted samples was spread on seed medium agar plates in triplicate and incubated at 30°C for 48 h. Cell survival numbers were estimated by calculating the average number of colony-forming units per milliliter through the plates with colonies. The seed medium and fermentation medium followed the composition in our previous study (Liu et al., 2017). The mediums were adjusted to the particular pH values with 18% hydrochloric acid or 10 mol/L of sodium hydroxide. The growth characteristics were confirmed from 3 independent growth experiments. In the proteomic analysis, 8 conditions were carried out as follows: pH 5.0 without lactic acid (**L0P5**) and pH 4.0 without lactic acid (**L0P4**) for acid stress; 2% lactic acid with pH 7.0 (**L2P7**) and 3% lactic acid with pH 7.0 (**L3P7**) for lactate stress; and 2% lactic acid with pH 5.0 (**L2P5**), 2% lactic acid with pH 4.0 (**L2P4**), 3% lactic acid with pH 5.0 (**L3P5**), and 3% lactic acid with pH 4.0 (**L3P4**) for cross-stress. Cells for proteomic analysis were collected after 1 h of stress by centrifugation (8,228 × *g*, 5 min, 4°C).

iTRAQ Quantitative Proteomics

Protein preparation and digestion and iTRAQ labeling were conducted as in our previous study (Qiao et al., 2012). In brief, fermentation samples were frozen in liquid nitrogen after centrifugation (8,228 × *g*, 5 min, 4°C). Protein extraction, concentration measurement, SDS-PAGE, and protein digestion were then performed. Total proteins were digested by Trypsin Gold (Promega, Madison, WI) at 37°C for 16 h, and the iTRAQ labeling of peptide samples was carried out using an iTRAQ Reagent 8-plex Kit (Applied Biosystems, Foster City, CA). Eight samples were individually iTRAQ labeled as follows: sample L3P4 (113 tag), sample L2P4 (114 tag), sample L3P7 (115 tag), sample L2P7 (116 tag), sample L2P5 (117 tag), sample L3P5 (118 tag), sample L0P5 (119 tag), and sample L0P4 (121 tag). The labeled peptides were then incubated at room temperature for 2 h and vacuum dried.

An LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) was used to carry out strong cation exchange chromatography. The mixed peptides were resuspended in 4 mL of buffer A (25 m*M* NaH_2PO_4 in 25% acetonitrile, pH 2.7) and added to an Ultremex SCX column (4.6 × 250 mm) containing 5-μm particles (Phenomenex, Torrance, CA). The peptides were eluted