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ORIGINAL RESEARCH PAPER

A comparative proteomic analysis of *Bacillus coagulans* in response to lactate stress during the production of L-lactic acid

Xiuwen Wang · Jiayang Qin · Landong Wang · Ping Xu

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Abstract The growth rate and maximum biomass of *Bacillus coagulans* 2–6 were inhibited by lactate; inhibition by sodium lactate was stronger than by calcium lactate. The differences of protein expressions by *B. coagulans* 2–6 under the lactate stress were determined using two-dimensional electrophoresis coupled with mass spectrometric identification. Under the non-stress condition, calcium lactate stress and sodium lactate stress, the number of detected protein spots was 1,571 ± 117, 1,281 ± 231 and 904 ± 127, respectively. Four proteins with high expression under lactate stress were identified: lactate dehydrogenase, cysteine synthase A, aldo/keto reductase and ribosomal protein L7/L12. These proteins are thus potential targets for the reconstruction of *B. coagulans* to promote its resistance to lactate stress.

X. Wang · J. Qin (✉) · L. Wang
College of Pharmacy, Binzhou Medical University, Yantai 264003, People's Republic of China
e-mail: qinjysdu@gmail.com

X. Wang
e-mail: wangxiuwen0822@163.com

L. Wang
e-mail: 250493714@qq.com

P. Xu
State Key Laboratory of Microbial Metabolism, and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China
e-mail: pingxu@sjtu.edu.cn

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Introduction

As one of the three major organic acids, lactic acid can serve as an acid, preservative, plant-growth regulator, a biodegradable material, drug and pesticide (Okano et al. 2010). A major industrial application of lactic acid is to produce polylactic acid, a biodegradable polymer, that can replace non-degradable petrochemical materials (Corma et al. 2007; John et al. 2007).

Microbial fermentation is the main method for industrial production of lactic acid (Datta and Henry 2006). Due to the inhibitory effect of products, a neutralizer needs to be added in the producing process to maintain the neutral or mildly acidic condition for fermentation liquid. The commonly-used neutralizing agents include $CaCO_3$ and NaOH. Lactic acid in the fermentation liquid mainly exists as lactate. When the concentration of lactate reaches a limit lactic acid bacteria become stressed, resulting in a retardation of bacterial growth and the reduction of lactic acid yield. Maximum yields of lactic acid are higher when using calcium salts or alkali as neutralizer than that when using sodium salts or alkali as neutralizer (Bai et al. 2003; Ding and Tan 2006; Qin et al. 2010). Therefore, the response mechanisms of lactic acid bacteria to the

calcium lactate and sodium lactate stress may be different.

Bacillus coagulans is newly applied to the fermentation and production of L-lactic acid. Compared with conventional strains, it has a number of advantages: high fermentation temperature, no need for sterilization of culture media, and high optical purity of products (Budhavaram and Fan 2009; Michelson et al. 2006). All these features conform to the production requirements for low energy consumption and high quality.

To our knowledge, little is known of the response of *B. coagulans* to lactate stress. The objective of this article was to determine the differences of *B. coagulans* 2–6 in growth and protein expression under sodium lactate stress, calcium lactate stress and non-stress conditions.

Materials and methods

Bacterial strain and culture media

Bacillus coagulans 2–6 (Qin et al. 2009) was used. It was cultured in three types of medium at 50 °C. Non-stress culture media: 40 g glucose l^{-1}, 10 g yeast extract l^{-1}, 1 g $CaCl_2$ l^{-1}, pH 6.5; for sodium lactate stress: 40 g glucose l^{-1}, 10 g yeast extract l^{-1}, 1 g $CaCl_2$ l^{-1}, 0.5 M L-sodium lactate, pH 6.5; for calcium lactate stress: 40 g glucose l^{-1}, 10 g yeast extract l^{-1}, 1 g $CaCl_2$ l^{-1}, 0.25 M L-calcium lactate, pH 6.5.

Culture conditions

Bacillus coagulans 2–6 was activated in non-stress culture media and inoculated into 100 ml culture media for sodium lactate stress, calcium lactate stress or non-stress. The three cultures were shaken at 150 rpm and 50 °C. Three repeated experiments were performed and the cell dry weight (CDW) was measured every few hours. For two-dimensional electrophoresis (2DE), samples were taken when the CDW reached 0.4 g l^{-1}. The total protein was extracted from the samples to conduct 2DE.

Protein extraction and quantification

A bacterial sample, 200 µl, was added to 1 ml extraction buffer of 2DE (Sangon). The cells were oscillated at 4 °C for 2 h, and ultrasonically disrupted at 100 W for 3 min, then centrifugated at 12,000×*g* and 4 °C for 30 min. Then this procedure was repeated for the supernatant, and the final sediment was total protein.

Non-Interference Protein Assay Kit (Sangon) was employed to determine the protein concentration.

2DE

The loading amount of protein was 80 µg. IPG Ready Strip (pH 3–10 NL) was used to conduct first-dimension isoelectric focusing (IEF). The IPG strips were subjected to IEF with the following procedure: 30 V for 12 h, 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 8 h, and a final phase of 500 V for 4 h.

The second-dimension electrophoresis was performed on 12.5 % SDS-PAGE gels. The parameters of electrophoresis were set as follows: 15 mA for 30 min; 30 mA until the end. The temperature for cooling circulation was 10 °C.

After electrophoresis, silver staining was performed according to the instruction of Mass Spectrometry-Compatible Rapid Silver Staining Kit (Sangon). The decolorized polyacrylamide gel was scanned at the resolution of 300 dpi. Image Master 2D Platinum was used for the analysis of 2DE patterns.

Mass spectrometric identification of protein spots

The differential protein spots on the gel were collected and digested with enzyme. Maldi-TOF-TOF-MS was employed for mass spectrometric identification and comparison.

Results

Growth of strains under lactate stress

As shown in Fig. 1, under non-stress conditions, the CDW of *B. coagulans* 2–6 reached 0.83 g l^{-1} at 8 h, and a maximum of 1.22 g l^{-1} at 16 h. Under sodium lactate stress, the growth of *B. coagulans* 2–6 was the slowest; CDW was 0.24 g l^{-1} at 8 h and reached a maximum 0.5 g l^{-1} at 20 h. Under calcium lactate stress, CDW of *B. coagulans* 2–6 was 0.51 g l^{-1} at 8 h, and reached a maximum of 0.63 g l^{-1} at 16 h. Obviously, lactate stress had significant inhibitory

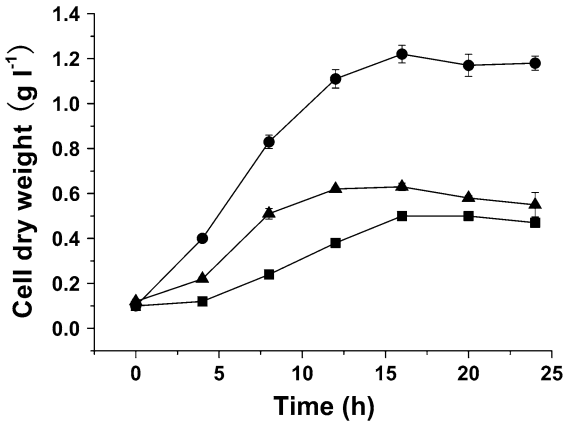

Fig. 1 Growth of *B. coagulans* 2–6 under different stress conditions. Three repeated experiments had been performed and the cell dry weight was measured every few hours. *Filled circle*, non–stress condition; *filled triangle*, calcium lactate stress; *filled square*, sodium lactate stress

effect on the growth of *B. coagulans* 2–6, and the inhibitory effect of sodium lactate was stronger than that of calcium lactate.

Influence of lactate stress on the protein expressions of *B. coagulans* 2–6

When the CDW of *B. coagulans* 2–6 cultured in sodium lactate stress, calcium lactate stress and non-stress conditions reached 0.4 g l^{-1}, the strains were called NA, CA and GY, respectively. By total protein extraction, the protein concentrations of NA, CA and GY were 1.21, 1.78 and 2.01 g l^{-1}, respectively. The two-dimensional electrophoresis (2DE) was conducted using 80 μg protein, with three replicates in each group. One of the electrophoregrams is shown in Fig. 2.

The analysis indicated that GY sample contained 1,571 ± 117 protein spots, NA sample 904 ± 127 protein spots, and CA sample 1,281 ± 231 protein spots. Compared with the non-stress condition, the number of proteins expressed by *B. coagulans* 2–6 may be fewer under the lactate stress. Moreover, the number of expressed proteins under the sodium lactate stress was less than that under the calcium lactate stress.

The comparison between the 2D electrophoregrams of samples indicated that there were 73 protein spots with significant differences between NA and GY. The expression amount of all the differential proteins in GY samples was higher than that in NA sample. The high expression of protein spots was not found under the sodium lactate stress.

There were 44 protein spots with significant differences between CA and GY samples; 35 differentially expressed proteins showed higher expression in GY samples than in CA samples, and nine showed higher expression under the calcium lactate stress. The nine differential proteins were identified by mass spectroscopy, and four of them were successfully recognized as lactate dehydrogenase (GenBank accession no. ACR02673), cysteine synthase A (GenBank accession no. AEH52120), aldo/keto reductase

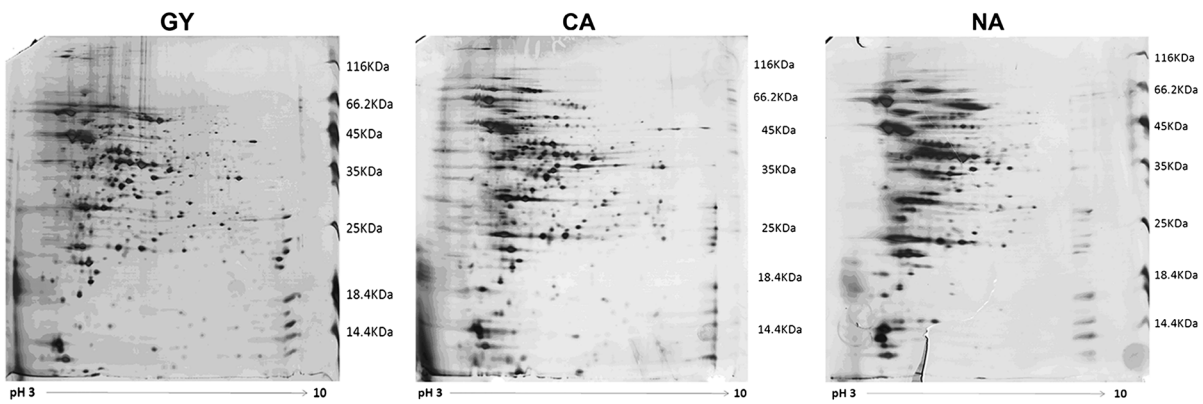

Fig. 2 Two-dimensional electrophoretogram of total soluble protein of *B. coagulans* 2–6 cultured under different stress conditions. The two-dimensional electrophoresis was conducted using 80 μg of protein, with three replicates in each group. *GY* non-stress condition, *CA* calcium lactate stress, *NA* sodium lactate stress

(GenBank accession no. AEH52329) and ribosomal protein L7/L12 (GenBank accession no. AEH52167).

There were 46 protein spots with significant difference between CA and NA samples; 45 differential proteins showed higher expression in CA samples than in NA sample, and one showed a higher expression in NA samples. Unfortunately, this differential protein could not be identified by mass spectroscopy.

Discussion

Lactate stress is inevitable for lactic acid bacteria in the production of lactic acid. However, the existing domestic and foreign researches pay little attention to the response mechanism of *B. coagulans* to the stress of lactate. In this article, *B. coagulans* 2–6 with high yield of L-lactic acid screened at early stage (Qin et al. 2009) was used to determine the influence of two lactate stress conditions on the cell growth and protein expressions.

The results indicated that under the same concentration of lactates, sodium lactate had stronger stress on the *B. coagulans* 2–6 compared with calcium lactate (Fig. 1). In early stages of fermentation with this strain, NaOH and $CaCO_3$ were used as neutralizer to produce L-lactic acid, respectively, and the yield was also lower in the former (Qin et al. 2009, 2010). It may be because the neutralization of 1 $C_3H_6O_3$ requires 1 NaOH, but only 0.5 $CaCO_3$. That is to say, if the concentrations of lactic acid are equal by using NaOH or $CaCO_3$ as neutralizer, Na^+ concentration should be twice that of Ca^{2+}. Therefore, the high ion concentration of sodium lactate may be one reason for stronger stress.

Fewer proteins were detected by two-dimensional electrophoresis (2DE) under lactate stress conditions compared with non-stress condition (Fig. 2). It is speculated that part of the biological processes and metabolic pathways of *B. coagulans* 2–6 were inhibited under the lactate stress. After the comparison of differential protein spots, four proteins with high expressions under the calcium lactate stress were identified. As a key enzyme in lactic acid metabolism, lactate dehydrogenase can mediate the mutual conversion between pyruvic acid and lactic acid. When the lactic acid concentration is relatively high in the reaction system, the high expression of lactate dehydrogenase will facilitate the conversion of lactic acid to pyruvic acid, therefore reducing the stress of high-concentration lactic acid on the strains. Cysteine synthase A is a crucial enzyme in the synthetic pathway of glutathione. Glutathione can help *Lactococcus lactis* resist the acid stress and oxidative stress (Li et al. 2003; Zhang et al. 2007). The excess expression of aldo/keto reductase in *Escherichia coli* leads to increased resistance to methylglyoxal (Grant et al. 2003), and the excess expression of aldo/keto reductase in *Clostridium beijerinckii* could improve its resistance to furfural (Zhang and Ezeji 2013). Ribosomal protein L7/L12 plays an important role in the binding of RNA and ribosome and the process of accurate protein translation (Rosen et al. 2001). The reason for the excess expression of this protein may be that under the stress condition, the cells have to rapidly synthesize some specific protective proteins to resist the stress.

In summary: the influence of sodium lactate and calcium lactate on the growth and protein expression of *B. coagulans* 2–6 was studied. Four proteins related to anti-lactate stress were found. These proteins are potential targets for the reconstruction of *B. coagulans* 2–6 by metabolism engineering, so as to improve its resistance to lactate stress and its lactic acid production ability.

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