Effect of the absence of the *CcpA* gene on growth, metabolic production, and stress tolerance in *Lactobacillus delbrueckii* ssp. *bulgaricus*

C. Li, J. W. Sun, G. F. Zhang, and L. B. Liu¹

Key Laboratory of Dairy Science, Ministry of Education, College of Food Science, Northeast Agricultural University, Harbin, China 150030

ABSTRACT

The catabolite control protein A (CcpA) is a kind of multi-effect regulatory protein. In the study, the effect of the inactivation of CcpA and aerobic conditions on the growth, metabolic production, and stress tolerance to heat, oxidative, and cold stresses in *Lactobacillus delbrueckii* ssp. *bulgaricus* was investigated. Results showed that inactivation of CcpA distinctly hindered growth. Total lactic acid concentration was significantly lower in aerobiosis for both strains and was lower for the mutant strain than *L. bulgaricus*. Acetic acid production from the mutant strain was higher than *L. bulgaricus* in aerobiosis compared with anaerobiosis. Enzyme activities, lactate dehydrogenase (LDH), phosphate fructose kinase (PFK), pyruvate kinase (PK), and pyruvic dehydrogenase (PDH), were significantly lower in the mutant strain than *L. bulgaricus*. The diameters of inhibition zone were 13.59 ± 0.02 mm and 9.76 ± 0.02 mm for *L. bulgaricus* in anaerobiosis and aerobiosis, respectively; and 8.12 ± 0.02 mm and 7.38 ± 0.02 mm for the mutant in anaerobiosis and aerobiosis, respectively. For both strains, cells grown under aerobic environment possess more stress tolerance. This is the first study in which the CcpA-negative mutant of *L. bulgaricus* is constructed and the effect of aerobic growth on stress tolerance of *L. bulgaricus* is evaluated. Although aerobic cultivation does not significantly improve growth, it does improve stress tolerance.

Key words: *Lactobacillus bulgaricus*, CcpA deletion, mutant, enzyme, stress resistance

INTRODUCTION

Carbon catabolite repression (CCR) is defined as a phenomenon that microorganisms preferentially use a rapidly metabolizable carbon source, along with inhibition of some gene expression and enzyme activities related to catabolism of nonpreferred carbon resources

(Magasanik, 1961). In low-GC gram-positive bacteria, the key regulator for exerting CCR is CcpA, which is a pleiotropic regulator involved in various physiological processes in addition to CCR, including central carbon and nitrogen metabolism, biofilm formation, and toxin gene expression.

The CcpA is a DNA-binding protein belonging to the LacI/GalR family of transcriptional regulators. The CcpA, linked to the serylphosphorylated form of phosphocarrier protein HPr (HPr-Ser-P), binds to a catabolite response element (*cre*) that is commonly located in the proximity of promoters, thereby either repressing or enhancing the transcription of downstream operons (Weickert and Adhya, 1992). The *cre*-like elements are identified in each of these DNA fragments. Catabolite control of *PepQ* is demonstrated in *Lactobacillus delbrueckii* ssp. *lactis* (Schick et al., 1999). *Lactobacillus bulgaricus* *PepR1* biosynthesis is not constitutive like those of several bacteria but is autoregulated and depends on the glucose concentration of the culture medium (Morel et al., 2001).

The CcpA protein has a key role in the regulation of the aerobic metabolism in *Lactococcus lactis* (Gaudu et al., 2003; Lopez de Felipe and Gaudu, 2009) and in *Lactobacillus plantarum* (Lorquet et al., 2004; Goffin et al., 2006). Moreover, CcpA is also involved in stress response mechanisms in *L. plantarum* by exerting positive control on the *DnaK* and *GroESL* operons (Muscariello et al., 2001; Castaldo et al., 2006) and the regulation of aerobic metabolism in both *L. plantarum* (Lorquet et al., 2004) and *L. lactis* (Gaudu et al., 2003; Lopez de Felipe and Gaudu, 2009), and it regulates the expression of the class I heat shock response operons in *L. plantarum* (Castaldo et al., 2006). Aerobic growth under respiration conditions was able to improve the tolerance of several technologically relevant stresses (heat, oxidative stress, and starvation) in lactic acid bacteria (LAB; Lechardeur et al., 2011) and then CcpA can perform the role of stress response regulation and aerobic metabolism.

The CcpA directly or indirectly controls the transcription of a large number of genes (>300 in *Bacillus subtilis*, ≥237 in *L. lactis*) involved in both energy and

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¹Corresponding author: liboliu@126.com

nitrogen metabolism. The role of CcpA in metabolism regulation has been studied in several LAB, including *L. lactis* (Zomer et al., 2007; Lopez de Felipe and Gaudu, 2009), *Lactobacillus casei* (Gosalbes et al., 1999), *Lactobacillus pentosus* (Mahr et al., 2000), and *L. plantarum* (Castaldo et al., 2006; Rud et al., 2011).

In this study, the CcpA gene knockout mutant of *L. bulgaricus* was constructed using homologous recombination technology to study the effect of the inactivation of CcpA and aerobic growth on metabolite production, enzyme activities, and stress tolerance.

MATERIALS AND METHODS

Materials

Escherichia coli DH5 α was used as the host for cloning. *Escherichia coli* strains were cultivated aerobically at 37°C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). *Lactobacillus bulgaricus* ATCC11842 strain was purchased from American Type Culture Collection (Manassas, VA). Its mutant (Δ *ccpA tet*) was obtained in this experiment. *Lactobacillus bulgaricus* and its mutant were cultivated at 37°C, under aerobic or anaerobic conditions, in de Man, Rogosa, Sharpe (MRS) broth. The plasmid pBR322 harboring the gene for the tetracycline resistance (Tet) gene, pBackZero-T Vector, DNA polymerase and restriction enzyme *Eco*RI and *Pst*II, T₄ DNA ligase, and agarose gel DNA purification kit were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The DNA sequencing and primer synthesis were performed by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). The PCR primers were designed using the Primer Premier software program (version 6; Premier Biosoft, Palo Alto, CA).

Cloning of the CcpA Gene

The whole CcpA gene (NCBI accession number 4084515) was amplified by PCR using *L. bulgaricus* genomic DNA as a template. CcpA-F: 5'-TTTA-AGGCTGGAGTCAGAAGCGTAG-3', CcpA-R: 5'-TTTAAATCGTGATGGCGTCATAGTG-3'. The PCR conditions consisted of 2.5 μ L (50 ng/ μ L) of DNA and 1 μ L of each primer (10 pM) in a 10- μ L 5 $\times$ reaction buffer, 4 μ L of 10 mM dNTP mixture, and 0.5 μ L of 5 U PrimeSTAR HS DNA polymerase in a final volume of 50 μ L in a PCR machine (GeneAmp PCR System 9700, Applied Biosystems Co., Foster City, CA). The PCR was initiated with an incubation step at 94°C, 2 min, followed by 35 cycles of 98°C, 10 s; 55°C, 15 s; 72°C, 3 min, with a final extension step at 72°C, 10 min.

The amplified DNA product was electrophoresed on a 1% (wt/vol) agarose gel. The length of CcpA gene was 2.5 kb; CcpA sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.. The PCR product was purified using a PCR purification kit from Qiagen (Beijing, China) according to the manufacturer's instructions, and was then cloned into pBackZero-T Vector, which was used for constructing the intermediate vector pBackZero-CcpA. The construct was transformed into *E. coli* DH5 α competent cells, which were then used to propagate the plasmid construct. The transformants were selected on Luria-Bertani plates containing 100 μ g/mL of ampicillin, 0.5 mM isopropyl β -D-1-thiogalactopyranoside, and 80 μ g/mL of X-Gal. The white colonies were selected and a mini-scale isolation of the plasmid DNA was used to prepare the recombinant plasmid. pBackZero-CcpA was digested with *Eco*RI and *Pst*II restriction enzyme, generating 2 fragments of 5.1 and 0.7 kb.

Cloning of tet Gene

For the selection of knockout colonies, the tetracycline resistance gene (National Center for Biotechnology Information accession number J01749.1) was used for the transformant selection. Briefly, the Tet gene was amplified by PCR with the 5'-primer containing an *Eco*RI restriction site (Tet-F: 5'-CCGGAATTCTTCTCAT-GTTTGACAGCTTATC ATC-3') and 5'-primer containing a *Pst*II restriction site (Tet-R: 5'-AACTGCAGTAATAGATATGTTCTGCCAAGGGTT-3'). The PCR conditions were the same as those for the CcpA gene. The PCR was initiated with an incubation step at 94°C, 2 min, followed by 30 cycles of 98°C, 10 s; 55°C, 15 s; 72°C, 1.5 min, with a final extension step at 72°C, 10 min. The following cloning procedures were the same as that of CcpA gene. The length of the Tet gene was 1.4 kb. The Tet sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.. The PCR product was cloned into pBackZero, generating pBackZero-Tet. pBackZero-Tet was digested with *Eco*RI and *Pst*II restriction enzyme generating 2 fragments of 3.2 and 1.4 kb. Details on CcpA, mutation, and tet sequencing are in the Supplemental Data (<http://dx.doi.org/10.3168/jds.2015-10321>).

Preparation of an Insert for Homologous Recombination

Two constructs (pBackZero-CcpA and pBackZero-Tet) were digested with 2 restriction enzymes, *Eco*RI and *Pst*II, and the Tet gene was inserted into the *Eco*RI + *Pst*II-site of pBackZero-CcpA (0.6-kb fragment of CcpA gene was exchanged with 1.4-kb Tet gene),

generating pCT. A linear DNA fragment (CcpA-Tet-CcpA: 3.3 kb) was produced by PCR amplification of pCT and used as an insert (50 ng/ μ L) for homologous recombination. The insert sequencing were performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd..

Gene Disruption by Homologous Recombination

Five milliliters of the *L. bulgaricus* stock culture stored at -80°C was inoculated into 30 mL of MRS broth containing a final concentration of 1% glycine from Solarbio (Beijing, China). Cells were cultivated to an optical density at 600 nm (OD_{600}) of approximately 0.3, chilled on ice for 15 min, centrifuged at $7,000 \times g$ for 10 min at 4°C . The supernatant was removed and the pellet was concentrated and washed 3 times with ice-cold sterile distilled water. Wash water was removed and cells were resuspended in an equal volume of cold sterile PEG-8000 (30%, wt/vol) and served as a source of competent cells. Eighty microliters of the electro-competent cells were transformed with 4 μ L (50 ng) of insert DNA (CcpA-Tet-CcpA) and 1 μ L (50 ng) of vector pCT by using an electroporation system (Gene Pulser Xcell, Bio-Rad Co., Hercules, CA) at 2.3 kV, 200 Ω , and 25 μ F. Knockout mutants were selected on MRS plates containing 10 $\mu\text{g}/\text{mL}$ of tetracyclines at 37°C , 72 h.

PCR Verification

The structure of mutant was analyzed by PCR. Genomic DNA from a mutant colony was prepared (50 ng/ μ L) and used as a template. Primers CcpA-F and Tet-F were used for PCR 1, and CcpA-R and Tet-R were used for PCR 2.

Measure of the Strain Phenotype

Both strains were observed based on their colony morphology in MRS agar medium without tetracycline, including the size of the colony, color, and edge appearance. Growth of both strains were observed in MRS agar medium with 10 $\mu\text{g}/\text{mL}$ of tetracycline.

Growth, Chemical, and Biochemical Analyses

The growth of cell was measured by spectrophotometry. One hundred microliters of culture containing about 10^8 cfu/mL was transferred to 5 mL of MRS broth and cultivated at 37°C under aerobic or anaerobic conditions. The absorbance of the culture was monitored at 600 nm in a spectrophotometer (DU-800; Beckman Coulter Co., Fullerton, CA) every 2 h. The

growth curve was drawn with culture time as abscissa and OD_{600} as ordinate.

High-performance liquid chromatography was used for the measurement of glucose, DL-lactic acid, and acetate concentrations in culture supernatants (Sánchez et al., 2004). Studies were performed with buffered cell suspensions, which greatly facilitate product analysis. Five milliliters of *L. bulgaricus* cultures was collected in the stationary growth phase by centrifugation at $10,000 \times g$ for 10 min at 4°C . The pellet was washed twice with 33 mM potassium phosphate buffer, pH 7.0 (PB7), and resuspended in 5 mL of 33 mM potassium phosphate buffer, pH 5.6, containing 25 mM glucose. The suspension was incubated with constant mild stirring for 4 h. Cells were removed from the suspension by centrifugation at $7,311 \times g$ for 10 min and the supernatant was analyzed by HPLC to quantify the glucose, DL-lactic acid, and acetic acid levels. The Alliance HPLC 2695 series (Waters, Palo, Alto, CA) was used. Ten microliters of samples was separated at a flow rate of 0.5 mL/min at 65°C on a 300×7.8 mm HPX-87H Aminex ion-exchange column protected by a cation Ht Microguard cartridge (Bio-Rad Co.), using 5 mM sulfuric acid as the mobile phase. The Waters 2487 Dual λ Absorbance detector was used for the quantification of organic acids at 210 nm. Glucose was analyzed using a Waters 2414 Differential Refractometer detector.

Enzyme Activity

The BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine protein concentrations. The LDH kit was used to determine lactate dehydrogenase (LDH). The PFK kit was used to determine phosphate fructose kinase (PFK). The PK kit was used to determine pyruvate kinase (PK). The PDH kit was used to determine pyruvic dehydrogenase (PDH). These enzyme kits were purchased from Comin Biotechnology Co., Ltd. (Suzhou, China).

Stress Tolerance

The tolerance to heat, oxidative, and cold stress was evaluated on stationary phase cells (24 h), which were recovered by centrifugation at $10,000 \times g$ for 5 min at 4°C and washed twice in 20 mmol per liter PB7 and resuspended in PB7 to 600 nm of 1.0. For heat stress, standardized cells were rapidly added to pre-warmed (55°C) PB7 in tubes in a circulation water bath (van Kranenburg et al., 1997). Survival was measured by counting the colony-forming units per milliliter (37°C , 48 h) in MRS agar at 0, 10, 20, 30, and 40 min. Oxidative stress tolerance was tested by spotting 2 μ L of 1.0%

H₂O₂ on the top layer (7 mL) of MRS agar (containing 1.5% wt/wt bacteriological agar) inoculated with 100 µL of standardized cell suspension, on a 10-mL bottom layer of sterile MRS agar. The area of the inhibition zone was measured with a caliper after incubation at 37°C, 48 h. Tolerance to cold storage was evaluated by storing standardized cell suspensions at 4°C and survival was measured by counting the colony-forming units per milliliter in MRS agar at 0, 5, 10, 15, and 20 d.

Statistical Methods

Excel 2003 software (Microsoft Corp., Redmond, WA) was used for statistical analysis, and the test was repeated 3 times. The results were the average $\pm$ standard deviation from 3 independent tests.

RESULTS

PCR Verification of Genomic DNA from a Mutant Colony

The sequencing results were tested by using BLAST and compared with the corresponding subtypes. The results showed that similarity degrees of CcpA, Tet, and CcpA mutation sequence were 99%. The expected sizes and targets of 2 PCR are represented in Figure 1A. In the 2 PCR for the verification of mutated genomic structure, 2 (2.0 and 2.7 kb) PCR products were produced as shown in Figure 1B, indicating the correct structure was generated.

The Strain Phenotype

Lactobacillus bulgaricus and its mutant were cultivated at 37°C in MRS agar medium without tetracycline. The CcpA gene deletion mutant and wild type strains have no obvious phenotypic difference on colony morphology, colonies were white, translucent, with luster and a jagged edge, and the colony was shaped like a snowflake. The colony morphology is shown in Figure 2 (A: wild type; B: mutant).

Lactobacillus bulgaricus and its mutant were cultivated at 37°C in MRS agar medium with 10 µg/mL of tetracycline. The wild type strain could not grow, whereas the growth condition of CcpA gene deletion mutant was normal.

Growth and Metabolite Production

The growth in aerobiosis and anaerobiosis of *L. bulgaricus* and of its mutant was compared. The growth curve is shown in Figure 3, whereas kinetic parameters

Figure 1. The results of the PCR amplification of genomic DNA from a mutant colony. (A) Diagram of PCR verification for genomic structure. The expected sizes and targets of 2 PCR (1–2) were indicated. (B) The result of the PCR verification. One 2.0 kb (PCR 1: lane 1) and one 2.7 kb (PCR 2: lane 2) of PCR products were produced. Catabolite control protein A (CcpA).

and metabolite concentrations are reported in Table 1. The mean $\pm$ standard error are for 3 replicate fermentations.

The growth curve was lower in anaerobiosis for both strains after 12 h and was lower for the mutant strain than *L. bulgaricus*. Inactivation of CcpA severely impaired growth. Estimated maximal growth rate ($\mu_{\max}$) values are shown in Table 1, indicating differences between $\mu_{\max}$ of *L. bulgaricus* and its mutant were significant ($P < 0.05$). Total lactic acid concentration was significantly lower in aerobiosis for both

Figure 2. Colony morphology for (A) the wild type *Lactobacillus bulgaricus* and (B) the mutant. Color version available online.

Table 1. Kinetic parameters (μ = growth rate) and metabolite concentrations (means $\pm$ SD)

Strain	Culture condition	μ (1/h)	Glucose (g/L)	DL-Lactic acid (g/L)	Acetic acid (g/L)
Wild type	N ₂	0.583 $\pm$ 0.011 ^b	2.451 $\pm$ 0.033 ^c	17.508 $\pm$ 0.027 ^a	0.965 $\pm$ 0.038 ^c
	Air	0.645 $\pm$ 0.005 ^a	1.508 $\pm$ 0.012 ^d	14.126 $\pm$ 0.100 ^c	4.164 $\pm$ 0.048 ^b
Mutant	N ₂	0.405 $\pm$ 0.007 ^d	4.792 $\pm$ 0.041 ^a	14.376 $\pm$ 0.025 ^b	0.931 $\pm$ 0.004 ^c
	Air	0.467 $\pm$ 0.007 ^c	2.875 $\pm$ 0.027 ^b	9.584 $\pm$ 0.015 ^d	6.246 $\pm$ 0.035 ^a

^{a-d}Values in each column with different superscripts show significant differences between the group and other groups ($P < 0.05$).

strains after 24 h (stationary phase) and was lower for the mutant strain than *L. bulgaricus*. The yield of lactic acid was always lower than that predicted for homo-lactic fermentation (2 mol lactic acid per mol glucose). Acetic acid production was higher in aerobiosis than anaerobiosis and for the mutant strain other than *L. bulgaricus*.

Enzyme Activity

The enzyme activities in aerobiosis and anaerobiosis of *L. bulgaricus* and its mutant were compared. The protein concentrations and key glycolytic enzymes are reported in Table 2. Protein concentrations are shown in Table 2, indicating that differences between *L. bulgaricus* and its mutant were significant ($P < 0.05$). Enzyme activities (LDH, PK, PFK, PDH) were significantly lower in the mutant strain than *L. bulgaricus*. For *L. bulgaricus*, enzyme activities (LDH, PK, PFK) were significantly lower in aerobiosis than anaerobiosis, but it was not significant for the mutant strain. For *L. bulgaricus* and its mutant strain, enzyme activity (PDH) was not significant in either aerobiosis or anaerobiosis.

Stress Tolerance

The tolerance of stationary phase cells to heat, oxidative, and cold stresses was assayed. The curve of heat inactivation is shown in Figure 4. Inactivation of CcpA impaired heat tolerance. For both strains, cells grown in aerobiosis were more tolerant of heat compared with anaerobiosis ($P < 0.05$).

Oxidative stress tolerance was assayed using a spot-on-lawn method (Zotta et al., 2012). The diameters of the inhibition zone caused by 2 μ L of a 1.0% H₂O₂ solution were measured with a vernier caliper. The diameters of the inhibition zone were 13.59 $\pm$ 0.02 mm and 9.76 $\pm$ 0.02 mm for *L. bulgaricus* in anaerobiosis and aerobiosis, respectively; and 8.12 $\pm$ 0.02 mm and 7.38 $\pm$ 0.02 mm for the mutant in anaerobiosis and aerobiosis, respectively.

The above results showed that the mutant was more tolerant than the wild type for oxidative stress, regard-

less of whether it was grown in aerobiosis or anaerobiosis. Moreover, no matter which strain, aerobically grown cells were significantly more tolerant.

The survival of stationary cells after cold storage in potassium phosphate buffer (pH 7) was evaluated. The results of plate counts on MRS agar for stationary phase cells are shown in Figure 5. Cells grown in aerobiosis were significantly more resistant. The difference in survival for aerobic grown cells was negligible for both strains, whereas *L. bulgaricus* cells grown in anaerobiosis were significantly more tolerant than mutant cells grown in the same conditions.

DISCUSSION

Inactivation of CcpA, and to some extent incubation in aerobiosis, significantly affected growth and its metabolites in *L. bulgaricus*. The results were in agreement with what has been found for other microorganisms (Titgemeyer and Hillen, 2002; Esteban et al., 2004), although the growth differences of some species have not been observed in CcpA-disrupted mutants (Asanuma et al., 2004). Aeration did not significantly affect the growth rate in spite of increasing ATP production, which would be expected in the mutant grown

Figure 3. Growth curve of representative fermentations measured as optical density at 600 nm (OD₆₀₀). *Lactobacillus bulgaricus* (Δ, ▲) and its mutant (□, ■) were controlled at temperature (37°C) in de Man, Rogosa, Sharpe broth with anaerobiosis (open symbols) or aerobiosis (closed symbols). Error bars indicate SD.

Table 2. Protein concentration and enzyme activity (means $\pm$ SD)¹

Strain	Culture condition	Protein (mg/mL)	LDH (U/mg)	PFK (U/mg)	PK (U/mg)	PDH (U/mg)
Wild type	N ₂	2.255 $\pm$ 0.089 ^b	50.980 $\pm$ 0.502 ^a	50.089 $\pm$ 0.586 ^a	90.028 $\pm$ 0.717 ^a	35.906 $\pm$ 0.382 ^a
	Air	2.808 $\pm$ 0.079 ^a	23.316 $\pm$ 0.542 ^b	28.977 $\pm$ 0.797 ^b	37.792 $\pm$ 0.310 ^b	34.103 $\pm$ 0.336 ^a
Mutant	N ₂	0.845 $\pm$ 0.081 ^c	14.159 $\pm$ 0.513 ^c	20.006 $\pm$ 0.615 ^c	26.490 $\pm$ 0.893 ^c	16.472 $\pm$ 0.711 ^b
	Air	0.820 $\pm$ 0.066 ^c	14.747 $\pm$ 0.400 ^c	10.757 $\pm$ 0.586 ^c	26.767 $\pm$ 0.594 ^c	17.540 $\pm$ 0.079 ^b

^{a-c}Values in each column with different superscripts show significant differences between the group and other groups ($P < 0.05$).

¹LDH = lactate dehydrogenase; PFK = phosphate fructose kinase; PK = pyruvate kinase; PDH = pyruvic dehydrogenase.

in aerobiosis because of pyruvate oxidase and acetate kinase activity (Quatravaux et al., 2006). On the other hand, aeration did not reduce the growth rate in the exponential phase (Murphy and Condon, 1984b). The expression of enzymes (LDH, PFK, PK) was reduced in the mutant compared with the wild type, in agreement with what has been found for *L. lactis* (Ramos et al., 2004), and this might account for the reduced growth rate of the mutant in agreement with what has been found for other species (Luesink et al., 1998; Ludwig et al., 2001). No data are available to show that CcpA might be a key protein controlling PDH metabolism in the citric acid cycle. The expression of enzymes (PDH) was reduced in the mutant compared with the wild type, which might be caused by the reduced growth rate of the mutant.

However, the largest differences between the 2 strains were found in aerobiosis. The yield of lactic acid at 24 h was lower than in anaerobiosis, whereas more acetic acid was produced, as expected by the role of CcpA in the control of the expression of *Pox* genes (Lorquet et al., 2004; Goffin et al., 2006), which is confirmed by the increased and anticipated acetic acid production in the mutant. Oxygen and CCR play an important role in

the presence of *Pox*, *Nox*, and *Npr* genes (Murphy and Condon, 1984a).

The higher stress tolerance of stationary phase cells is in agreement with the literature for *L. lactis* (Esteban et al., 2004) and is probably due to a generalized stress response. Although aeration significantly affected growth and stress tolerance for *L. plantarum* (Zotta et al., 2012), no data are available in the literature on the effect of aerobic growth of *L. bulgaricus* on stress tolerance. The increasing expression of some heat-stress-related proteins (GrpE, ClpL) of *L. bulgaricus* grown in aerobiosis is the key cause of increasing heat stress resistance and can also be one of the indirect causes of increasing tolerance to oxidative stress, which may improve recovery of sub-lethally damaged cells (Zotta et al., 2012). However, growth in aerobiosis can promote the formation of an electron transport chain, which has been found to significantly increase tolerance to oxidative stress (Gaudu et al., 2002).

However, the mutant strain seemed to be more responsive to the oxidative environment, which implied that a larger number of proteins was involved in the adaptation mechanism (Mazzeo et al., 2012). Hydrogen peroxide has been found to affect the transcription of a

Figure 4. The curve of stationary phase cells to heat. *Lactobacillus bulgaricus* (Δ , $\blacktriangle$) and its mutant ($\square$, $\blacksquare$) were controlled at temperature (37°C) in de Man, Rogosa, Sharpe (MRS) broth with no air (open symbols) or air sparging (closed symbols) following exposure to 55°C in 20 mmol/L potassium phosphate buffer, pH 7.0. Error bars indicate SD.

Figure 5. The curve of stationary phase cells to cold. *Lactobacillus bulgaricus* (Δ , $\blacktriangle$) and its mutant ($\square$, $\blacksquare$) were grown in de Man, Rogosa, Sharpe (MRS) broth at controlled temperature (37°C) with no air (open symbols) or air sparging (closed symbols). Number of survivors was enumerated on MRS. Average of 3 replicates for a single fermentation with standard deviation bars.

large number of genes, and the transcription levels of 2 stress-related proteins, Asp1 and Asp2, increased with the addition of H₂O₂ (Serrano et al., 2007).

In our experimental strategy, the temperature of sample adjusted to 4°C (close to the minimum temperature for growth for *L. bulgaricus*) and storage in a nutrient-depleted buffer is a cold stress condition. Starvation and temperature downshift are key factors that cause physiological changes of bacteria in viable but abnormal growth status (Keer and Birch, 2003). Survival during cold is usually attributed to the capacity of maintaining an active metabolic state and of avoiding energy depletion (Van de Guchte et al., 2002). The higher survival of cells may be due to a generalized stress response and to lower initial metabolic activity, whereas survival of aerobically grown exponential phase cells is higher, and at least after 15 d may be due to higher oxidative stress tolerance (Gaudu et al., 2002). Although CcpA regulates some key gene expression in carbon and nitrogen metabolism in gram-positive bacteria, it is different between these strains and other LAB reported on the roles of CcpA. *Lactobacillus plantarum* cells grown in aerobiosis were significantly less tolerant than mutant cells grown in the same conditions (Zotta et al., 2012), whereas the difference in survival for aerobic grown cells was negligible for both strains in this study. This study found that enzyme activities (LDH, PK, PFK) were significantly lower in aerobiosis than anaerobiosis for the wild type, but it was not significant for the mutant strain, which implies that CcpA may participate in cellular respiration.

CONCLUSIONS

The lower activities in the mutant strain correlate with decreased gene expression. After CcpA gene deletion, enzyme activities (LDH, PK, PFK) were significantly lower, which resulted in reduction of the glycolytic pathway, and caused reduction of the utilization ratio of glucose and decrease of growth rate. It is known that aerobic growth promote the presence of a respiratory chain, which is related to stress tolerance of some important LAB in the food industry. In our study, results showed that aerobic growth can significantly enhance heat and oxidative stress tolerance of *L. bulgaricus*. Furthermore, our team confirmed the role of the pleiotropic regulator CcpA in stress resistance and aerobic metabolism of *L. bulgaricus*. Some stress response proteins (DnaK, GroEL, GrpE, ClpL, ClpE), detoxification of reactive oxygen species (Pox, Nox, Npr), and some other oxidoreductases or proteins related to stress (Hsp1, Asp1, Asp2) were affected by aerobic and anaerobic growth and by CcpA inactivation. Further work is needed to find these proteins in *L.*

bulgaricus and clarify the regulation of the expression and their role in stress response.

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