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Cold stress improves the ability of *Lactobacillus plantarum* L67 to survive freezing

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ABSTRACT

The stress resistance of bacteria is affected by the physiological status of the bacterial cell and environmental factors such as pH, salts and temperature. In this study, we report on the stress response of *Lactobacillus plantarum* L67 after four consecutive freeze–thaw cycles. The cold stress response of the cold-shock protein genes (*csp*C, *csp*L and *csp*P) and ATPase activities were then evaluated. The cold stress was adjusted to 5 °C when the bacteria were growing at the mid-exponential phase. A comparative proteomic analysis was performed with two-dimensional gel electrophoresis (2D SDS-PAGE) and a matrix assisted laser desorption/ionization-mass spectrometer. Only 56% of the *L. plantarum* L67 cells without prior exposure to cold stress survived after four consecutive freeze–thaw cycles. However, 78% of the *L. plantarum* L67 cells that were treated with cold stress at 5 °C for 6 h survived after freeze–thaw conditions. After applying cold stress to the culture for 6 h, the cells were then stored for 60 days at 5 °C, 25 °C and 35 °C separately. The cold-stressed culture of *L. plantarum* L67 showed an 8% higher viability than the control culture. After applying cold stress for 6 h, the transcript levels of two genes (*csp*P and *csp*L) were up-regulated 1.4 (*csp*P) and 1.2 (*csp*L) times compared to the control. However, *csp*C was not up-regulated. A proteomic analysis showed that the proteins increased after a reduction of the incubation temperature to 5 °C. The importance of the expression of 13 other relevant proteins was also determined through the study. The exposure of *L. plantarum* cells to low temperatures aids their ability to survive through subsequent freeze–thaw processes and lyophilization.

1. Introduction

Lactobacilli and bifidobacteria are well known as the most frequently and safely used starter probiotic bacteria, even for children and immunocompromised individuals (Borriello et al., 2003). In particular, *Lactobacillus plantarum* belongs to the facultatively heterofermentative group of lactobacilli and is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. It has a proven ability to survive gastric transit and colonize the intestinal track of humans and other mammals (Sartor, 2004; de Vries et al., 2006).

During the manufacture of lactic acid fermented products, bacterial cells are exposed to various environmental stresses such as extreme temperatures, pH, osmotic pressure, oxygen, high pressure and starvation, which may affect the physiological status and the properties of the cells. However, bacterial cells are naturally equipped with a plethora of defense mechanisms, such as chaperone proteins (Gro ES/GroEL and DnaK/ DnaJ/Grp E), proteases, transport systems and proton pumps to enhance survival in stressful environments (Carcoran et al., 2008). Moreover, many research studies have reported the positive effects of the stress response. For example, to improve the viability of the probiotic strain *Lactobacillus paracasei* NFBC 338 during spray-drying, Desmond et al. (2004) demonstrated that pre-stressing the culture by exposure to 52 °C for 15 min improved the survival of the strain 700-fold (in reconstituted skim milk) during heat stress and 18-fold during spray-drying compared to unadapted cells. Exposure to salt stress also afforded a level of thermo-tolerance. Indeed, following exposure to

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0.3 M NaCl, the survival of the strain improved by 16-fold during spray-drying (Desmond et al., 2001).

Freezing is generally used to preserve a starter culture for an extensive amount of time while still maintaining its viability and acidification activity. After freezing and thawing, many cells are often metabolically damaged or even killed. The freezing resistance of *Lactobacillus* is enhanced by applying cold stress conditions before freezing. In contrast with *Escherichia coli*, *Bacillus subtilis*, and *Lactococcus lactis*, the function of the cold response in *L. plantarum* has not been extensively studied (Barria et al., 2013). Cold-shock response is classically exhibited when an exponentially growing culture is shifted from its optimum growth temperature to a lower temperature. In most bacteria, such as *B. subtilis*, a temperature downshift causes a transient cell growth arrest, during which the general protein synthesis is severely inhibited. However, under these conditions, the synthesis of CIPs is triggered. Eventually, the synthesis of these proteins decreases, the cells become acclimated to the low temperature, and growth resumes (Jones et al., 1987). Recently, it has been established that CSPs might regulate the expression of cold-induced genes such as anti-terminators (Bae et al., 2000). The regulation of *csp* genes takes place at several levels. Additionally, the regulation of CspAE expression seemed to occur transcriptionally and post-transcriptionally through protein stability effectors (Goldenberg et al., 1997). The chromosome of *L. lactis* contains two sets of cold-inducible *csp* genes (*csp*A/*csp*B and *csp*C/*csp*D) (Wouters et al., 2000a, 2000b). Small heat shock (*shs*) genes are also induced by cold in *L. plantarum*, and a role for the sHsps in preventing damage by low temperature has been suggested and *L. plantarum* strains overproducing Hsp 18.5, Hsp 18.55 and Hsp 19.3 have improved growth at low temperature (Spano et al., 2005; Fiocco et al., 2007). *L. plantarum* is usually exposed to harsh, stressful environments during handling and storage. These harsh environments include being either freeze-dried or just subjected to a regular freeze during the food process through the GI tract. In the case of *L. plantarum*, cold stress genes such as *csp*C, *csp*L, and *csp*P have been identified (Mayo et al., 1997; Derzelle et al., 2000).

The purpose of this study was to determine the survival rate of *csp* mRNA levels and the inductive proteins associated with *L. plantarum* L67 under cold stress.

2. Materials and methods

2.1. Bacteria

L. plantarum L67 from infant feces were selected according to their colony characteristics, and underwent a gram stain to be tested for catalase activity. These isolates were further screened for their acid and bile salt tolerance capacities according to Park et al. (2002). For identification of *L. plantarum* L67, the biochemical properties were first examined using an API 50CHL kit (BioMerieux, France) and, finally, 16s rDNA sequencing data, as described by Kim et al. (2004). *L. plantarum* L67 was grown in MRS broth at 37 °C for 18 h (de Man et al., 1960). The bacterial cells were separated by centrifugation at 3000 ×*g* for 15 min at room temperature. Subsequently, the precipitated cells were washed twice with sterile saline (0.85% sodium chloride). One milliliter of 10% skim milk was added to the sediments, and the suspension was stored at −80 °C until use.

2.2. Growth conditions of L. plantarum L67 and the cold stress treatments

The cells were inoculated in 1% of the overnight culture of the strain in 10 ml of fresh MRS broth, incubated at 37 °C and harvested when they reached the mid-exponential phase of growth (OD 600 nm of 0.6). Growth kinetic experiments were performed with MRS broth at 37 °C. For cold shock experiments, the exponentially growing cells were incubated at 5 °C for 1, 4 or 6 h, and then growth kinetic experiments were performed at 37 °C. All experiments were carried out in triplicate.

2.3. Freeze–thaw challenge

Two hundred milliliters of MRS broth was inoculated (1%) and incubated at 37 °C. The cells grown to the mid-exponential phase were cold shocked at 5 °C for 1, 4 and 6 h. Then, the cells were quickly frozen and stored at −70 °C. The control group consisted of cells frozen directly without pre-adaptation for 24 h of freezing. After, the cells were thawed by placing the tubes in a 37 °C water bath for 5 min. Aliquots of each sample were taken out and analyzed for viability before they were frozen again at −70 °C. A total of four freeze–thaw cycles were performed. Each freeze–thaw cycle was performed after 24 h of the previous one. The thawed cells were serially diluted with 1% peptone water, and the viable cells were counted.

2.4. Storage stability of freeze-dried L. plantarum L67

L. plantarum L67 was grown in 1 l of MRS broth at 37 °C. When the growth reached the mid-exponential phase (OD_{600} of 0.6), the cells were subjected to 6 h of cold shock treatment at 5 °C in fresh MRS broth. The cell pellets were collected by centrifugation (5000 ×*g* at 4 °C) and then re-suspended in skim milk before being frozen at −70 °C. The frozen cells were freeze-dried for 48 h at 0.2 mbar with a collecting temperature of −50 °C (IlShin Lab, Yangju, Korea). The cell suspensions were freeze-dried in duplicates. Duplicate samples of each treatment were placed in glass vials (40 ml) and purged with nitrogen gas (10 ml/min) for 10 min. The glass vial lots were stored at 5 °C, 25 °C or 35 °C in the dark for 60 days.

2.5. RNA isolation and RT-qPCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) with the manufacturer's instructions. The cDNA was prepared from 1 μl of total RNA (1 μg/20 μl) using Maxime RT Premix Oligo (dT) for the RT-qPCR kit (Intron, Seongnam, Korea). The primers used in this study were designed by Primer3 software program (www.bioinformatics.nl/primer3plus) based on the genome sequence of *L. plantarum* WCFS1 (GenBank accession number NC_004567). The primers used are shown in Table 1. The expression levels of the respective genes were measured by real-time PCR using 2× Prime QMaster Mix (Kapa Biosystems, Boston, USA) and were analyzed using a CFX96TM Real-Time system (Bio-Rad, California, USA). The reaction parameters for the real-time PCR analysis were 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 60 s. The final elongation step was at 72 °C for 5 min. The sample ΔCt (SΔCt) value was calculated as the difference between the Ct values of the *csp* gene before and after applying the cold shock. The ΔCt value of the undifferentiated *L. plantarum* WFSC1 *rpo*D (Kleerebezem et al., 2003; Duary et al., 2012) gene was used as the control ΔCt (CΔCt) value. The relative gene expression levels between the sample and the control were determined using the formula: $2^{-(S\Delta Ct\ -\ C\Delta Ct)}$ (Livak and Schmittgen, 2001).

Table 1
Oligonucleotides used for real-time PCR in this study.

Gene	Sequence of PCR primers (5′ to 3′)
*csp*P	Forward: 5′GTGAAGACGGTACCGATGTCTT-3′
	Reverse: 5′GTGGTTGAACGTTCGTTGCT-3′
*csp*C	Forward: 5′ATCACTCGCGAAAACGGTAG-3′
	Reverse: 5′CCACGATCGCTTTCTTCAAC-3′
*csp*L	Forward: 5′TACTGGTGAAGATGGCAACG-3′
	Reverse: 5′GAACAACGTTAGCAGCTTGTGG-3′
*rpo*D	Forward: 5′GTGAAGAGGACGATTCACACCT-3′
	Reverse: 5′GGTATCAAGGACACCTTCCAG-3′