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

Sooyon Song, Dong-Won Bae, Kwangseki Lim, Mansel W. Griffiths, Sejong Oh

**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 (*cspC*, *cspL*, and *cspP*) 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 (*cspP* and *cspL*) were up-regulated 1.4 (*cspP*) and 1.2 (*cspL*) times compared to the control. However, *cspC* 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.

**Keywords:**

*Lactobacillus plantarum* L67  
Cold stress response  
*cspC,*  
*cspL,*  
*cspP,*

**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 tract 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* NRC 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
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 (cspa/cspB and cspC/cspD) (Wouters et al., 2000a, 2000b). Small heat shock (sbs) genes are also induced by cold in L. plantarum, and a role for the sfsPs in preventing damage by low temperature has been suggested and L. plantarum strains overproducing Hsp18.5, Hsp18.55 and Hsp19.3 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 theGI tract. In the case of L. plantarum, cold stress genes such as cspC, cspL, and cspP 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, 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 (OD600 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 (Ishin 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/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 WSFC1 rpoD (Kleerebezem et al., 2003; Duany 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−(ΔCt−SΔCt) (Livak and Schmittgen, 2001).

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotides used for real-time PCR in this study.</th>
<th>Gene</th>
<th>Sequence of PCR primers (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cspP Forward: 5′-CTCAAGGAGGCTTACGCTTT-3′</td>
<td>reverse: 5′-GTTGTTGAACGTTCGTTGCT-3′</td>
<td></td>
</tr>
<tr>
<td>cspC Forward: 5′-ATCAGCTGCCAAAGGCTTG-3′</td>
<td>reverse: 5′-CCACATCGTCTTCCTCACCACG-3′</td>
<td></td>
</tr>
<tr>
<td>cspL Forward: 5′-TACTTGGAAAGTGGCGG-3′</td>
<td>reverse: 5′-CAGACACGTTTAGCAGCGG-3′</td>
<td></td>
</tr>
<tr>
<td>rpoD Forward: 5′-CTGGAAGGGGAGGTTCCACG-3′</td>
<td>reverse: 5′-GGTATTCAAGGACCATCCACG-3′</td>
<td></td>
</tr>
</tbody>
</table>
2.6. Protein extraction of L. plantarum L67

For the proteomic study, 200 ml of the mid-exponential phase (OD_{600} of 0.6) cultures was obtained as described above at 37 °C. For the cold shock experiments, the cells were cultivated at 37 °C at the mid-exponential phase (OD_{600} of 0.6) and subjected to 1, 4, or 6 h (respectively) of cold shock treatment at 5 °C. The cell pellets were collected by centrifugation (12,000 × g, 4 °C). The collected cell pellets were washed 3 times using 0.85% NaCl. The soluble protein fractions were extracted twice with phenol (Sigma Chemical Co, St. Louis, USA) (Kim et al., 2008). Then, 2 ml aliquots of each individual extract were transferred to 15 ml polypropylene tubes. One millilitre of phenol (Sigma Chemical Co.) was then added. The sample was vortexed after and was heated at 70 °C for 10 min. The sample was cooled on ice for 5 min, and the phases were separated by centrifugation at 12,000 × g for 20 min. The top aqueous phase was discarded and replaced by 1 ml of distilled H2O. The sample was then vortexed and heated at 70 °C for 10 min. The sample was cooled on ice for 5 min, and the phases were separated again by centrifugation, as before. Then, the top aqueous phase was discarded. The proteins were precipitated by the addition of cold acetone at 4 °C for 2 h. After centrifugation, the supernatant was discarded, and cold acetone was added. The pellet was disrupted by vigorous vortexing, and the precipitated proteins were pelleted by a final centrifugation of 12,000 × g for 20 min. The resulting pellet was air-dried for 2 h under a hood. The protein concentrations were measured by a Bradford assay (Bradford, 1976) using the Bio-Rad Protein Assay (Bio-Rad, California, USA).

2.7. Two-dimensional gel electrophoresis

Protein loads of 1 mg/ml in 315 μl of rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 10% isopropanol, 5% glycerol, 0.5% ampholytes at pH of 3 to 10, and a trace of bromophenol blue) were used to load the Ready Strip IEF Strips (17 cm, pH 3 to 10, Gnomeine, Pohang, Korea) under mineral oil at 20 °C for 15 h. Isoelectric focusing (IEF) was performed using a PROTEAN IEF cell (Bio-Rad, California, USA) according to the manufacturer’s instructions. The two dimensional gel electrophoresis (2D SDS-PAGE) was performed at 150 constant volts using 12.5% SDS-PAGE gels. The separation was conducted using a PROTEAN II xi system (Bio-Rad, California, USA) with 10 mA per gel for 1 h and thereafter with 20 mA per gel at 4 °C. The protein spots were visualized by Coomassie blue staining (Candido et al., 2004). The stained gels were scanned with a densitometer scanner (800 by 1600 dpi; UTI 2100XL, UMAX, Techville Inc., Dallas, TX), and the spots were analyzed with PD Quest software (Bio-Rad, California, USA).

2.8. Analysis of matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS)

The protein spots of interest were manually excised. The proteolytic digestion was performed overnight at 37 °C using trypsin (20 μg/ml; Promega, WI, USA) in the presence of 25 mM ammonium bicarbonate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was employed using the method described by Kim et al. (2008) for protein identification. The obtained spectra from the Ettan MALDI-TOF (Amersham Pharmacia Biotech, Uppsala, Sweden) were subjected to analysis by Mascot software.

2.9. Crude enzyme extract of L. plantarum L67 for the ATPase activity assay

The L. plantarum L67 cultures at the mid-exponential phase of growth (OD_{600} of 0.6) at 37 °C were harvested by centrifugation at 7000 × g for 10 min. Then, they were washed three times at room temperature in sterile water. The cells were suspended in Tris HCl buffer (pH 7.5, 75 mM) containing 0.4 mM succrose and 2 mM MgCl2 and were disrupted by grinding with 0.1 mm glass beads using the Micro Mini Bead beater (BioSpec Products, Seoul, Korea). After the addition of DNase and RNase (10 μg/ml), the solutions were maintained at room temperature for 45 min. The unbroken cells and debris were subsequently removed by centrifugation at 15,000 × g for 20 min. The supernatant was further centrifuged at 20,000 × g for 20 min, and the ATPase activity in the supernatant was determined.

2.10. ATPase activity assay

The ATPase activity was estimated from the release of inorganic phosphate, as measured according to the method of Fiske and Subbarow (1925). The membrane protein was incubated at 37 °C for 10 min in Tris maleate buffer (pH 7.5, 50 mM) containing 10 mM MgCl2 and 5 mM ATP (Sigma Chemical Co, St. Louis, USA). The reaction was stopped by addition of 1 ml of sterile water containing 3.5 N sulfuric acid, 1 ml of 3.5% ammonium molybdate and 1 ml of 2.1% sodium bisulfite with 0.7% Developer (Kodak Korea, Seoul, Korea) subsequently added. After 20 min, the absorbance at 660 nm was measured. One unit of ATPase activity was defined as the amount of enzyme that 1 μmol of inorganic phosphate released in 1 min. The calibration was performed with a series of P, standards.

2.11. Statistical analysis

All the experiments were performed in triplicate. The data were analyzed using Duncan’s multiple range test (Duncan, 1955). A P value of < 0.05 in all replicate experiments represented statistical significance.

3. Results

3.1. Effect of temperature on L. plantarum L67 growth

As a first step to understanding the cold stress response in L. plantarum L67, we constructed a growth curve for L. plantarum L67 at 5 °C, 37 °C and under cold stress at 5 °C following growth to the mid-exponential phase at 37 °C. The cell viability at 37 °C after cold shock treatment for 1, 4 or 6 h was also determined. Fig. 1 shows the growth curve of L. plantarum L67 at 37 °C and 5 °C. The L. plantarum on the MRS broth at 37 °C reached an OD of 1.2 (OD_{600}) after 6 h, whereas the L. plantarum cells did not grow at 5 °C.

The effect of prior cold stress on the growth kinetics of L. plantarum L67 at 37 °C is shown in Fig. 2. The cell density reached 1.2 (OD_{600}) at

![Fig. 1. Growth curve of L. plantarum L67 at 37 °C (□), 5 °C (*) and cold stress at 5 °C (△) after growth at 37 °C until reaching an OD_{600} of 0.6. The arrow (→) indicates a temperature change shift to 5 °C. The cells were inoculated in 1% of 10 ml of overnight culture in 10 ml of fresh MRS broth and incubated at the appropriate temperature (37 °C, 5 °C and 5 °C). For the cold stress experiments, when the cultures reached 0.6 (OD_{600}) at 37 °C, they were transferred to incubation at 5 °C. The optical density (OD) was measured with a Synergy HT multimode plate reader (BioTek, Winsoski, VT). The results are the means of triplicated trials, and the error bars indicate the standard errors.](image-url)
37 °C in 6 h, whereas it reached only 0.6 after prior exposure to 5 °C for 1 h. However, after 9 h of pre-exposure at the lowest temperature, the cells grew at a similar rate to those of the control. The fastest resumption of growth occurred for the cells that had been subjected to cold stress for 6 h.

3.2. Freeze–thaw challenge of L. plantarum L67

To measure the freezing tolerance, the freeze and thaw cycle was repeated for 4 cycles. The survival ratio of the cells after the freeze–thaw was then determined and is shown in Fig. 3. Only 56% of the control cells without cold shock survived after 4 consecutive freeze–thaw cycles. However, the cells adapted for 4 and 6 h at 5 °C exhibited increased survival (up to 69 and 78%, respectively). The cells exposed to cold stress for 6 h exhibited an almost constant level of survival after freeze–thaw following an initial reduction in count. This result indicates that cryotolerance is improved by subjecting the cells to prior cold stress.

3.3. Storage stability of dried L. plantarum L67

The viabilities of the L. plantarum L67 cultures were initially 3.8 × 10^{11} CFU/g and 2.21 × 10^{11} CFU/g for the cold-stressed cultures and the controls, respectively. At 5 °C, the cell population of the cold-stressed L. plantarum L67 remained stable at 1.05 × 10^{10} CFU/g, while the cell numbers in the control samples declined (1.82 × 10^{9} CFU/g). L. plantarum L67 was stored for 60 days.

At 25 and 35 °C storage, dramatic losses in cell count were observed in the freeze dried cultures of the probiotic, with levels of L. plantarum L67 cells that were subjected to prior cold-stress decreasing to 4.6 × 10^{8} CFU/g (at 25 °C) and 5.4 × 10^{8} CFU/g (at 35 °C), whereas the...
cell counts in the control sample fell to $8.7 \times 10^7$ CFU/g (at 25 °C) and $9.8 \times 10^7$ CFU/g (at 35 °C) following 60 days of storage (Fig. 4).

3.4. mRNA expression and ATPase activity under cold stress

RT-qPCR was performed to determine the expression of the cold-shock genes in *L. plantarum* L67 (Fig. 5). After applying the cold stress, two genes (*cspP* and *cspL*) were significantly expressed, but *cspC* was not expressed at 6 h compared to the control.
The ATPase activity of *L. plantarum* L67 subjected to cold stress is shown in Fig. 6. The ATPase activity of *L. plantarum* L67 grown at 37 °C was 8.5 unit enzymes (UE). The ATPase activity in the cells that were cold stressed for 1 h at 5 °C was 7.2 UE, while following cold stress for 4 h, the enzyme activity reached the maximum observed level of 9.2 UE. The degree of ATPase activity (8.7 UE) after cold stress for 6 h was slightly lower compared to the levels in cells that underwent cold stress for 4 h. In the early stages of cold stress, ATPase activity was decreased, but after 4 h, the ATPase activity of the cells returned to normal levels.

### 3.5. Inductive protein by cold stress in *L. plantarum* L67

Fig. 7 displays the 2D SDS-PAGE gels of the CBB stained proteins extracted from *L. plantarum* L67 following growth at 37 °C (OD_{600} of 0.6) and subjected to cold stress for 6 h, which resulted in the highest survival rate of cells after the freeze–thaw cycles. Under cold stress, 24 spots were significantly overexpressed compared with the control. The proteins identified by MALDI-TOF/MS were classified according to their functions (Table 2). The thirteen identified proteins were classified into six groups: cell growth (31%), unknown function (23%), energy (15%), stress response (15%), signal transduction (8%) and transcription (8%) (Fig. 8).

### 4. Discussion

*L. plantarum* is important within the food industry as a starter strain for the fermentation of food. When foods are processed, bacteria are exposed to low temperature stresses. Hence, it is important to understand the cold shock response and cold adaptation phenomenon of bacteria such as *L. plantarum* L67. As a first step to understanding the cold stress response in *L. plantarum* L67, we determined the *L. plantarum* L67 viability after cold shock treatment for 1, 4 and 6 h before incubation again at the optimal temperature by measuring the growth kinetics. The results of the study assumed that the cells might have a tolerance against freezing upon preadaptation to cold stress due to the expression of stress proteins such as CSPP and CSPL (Fig. 3). The relevance of cold shock and cryotolerance was first reported in *B. subtilis*, where it was determined that the removal of the cold shock protein B resulted in increased sensitivity to freezing (Willinsky et al., 1992). In many bacteria, survival to cold shock prior to freezing increases survival because cold adaptation likely results in a greater proportion of unsaturated fatty acids in the plasma membrane of the affected cells compared to untreated cultures. However, the exact function of cold shock proteins in relation to cryotolerance is still unknown (El kest and Marth, 1992).

In a recent report (Derzelle et al., 2000), it was shown that the overproduction of each CSP causes distinct phenotypic effects in *L. plantarum*. It is speculated that the different CSP proteins may antagonize the transcription of the three *csp* genes of *L. plantarum*, thus allowing the cell to adequately respond to stressful environmental challenges. In this study, RT-qPCR was performed to measure the expression of cold-shock genes. After cold stress, two genes (*cspP* and *cspL*) were significantly expressed, but *cspC* was not significantly expressed at 6 h compared to the control. Recent reports have shown that cold inducible CSP proteins may also be expressed at specific stages of the normal growth cycle (Weber et al., 2001; Graumann and Marahiel, 1999). The highest mRNA level of *cspC* is expressed during early exponentially growing cells at the optimal temperature, while the *cspC* mRNA level declines in the stationary phase (Derzelle et al., 2000). Derzelle et al. (2003) have suggested that *cspC* overproduction improves growth re-umption at the optimal temperature. Although *cspC* is not primarily implicated in cold shock adaptation, it may play a positive role during the acclimation phase of growth at cold temperatures. To better understand the mechanisms involved in this cold response, the role of the *cspC* gene of *L. plantarum* will be further studied in the future. This result is similar to that described by Derzelle et al. (2003). Derzelle et al. (2002) also demonstrate that cold shock has induced the cspL gene. This is a key result of the present study because it demonstrates that csp transcripts may be more stable and more efficiently expressed than other cellular messengers even if their absolute level does not significantly increase during cold exposure. It has been reported that the overproduction of *cspL* after cold shock transiently augments growth, while the overproduction of *cspP* and *cspC* has no effect on growth compared to the control (Derzelle et al., 2003). Additionally in this study, as the mRNA levels of *cspC* increased, the survival ratio of the cells after freeze–thaw also increased. However, we could not observe the CSP protein in the proteomic analysis. While the *csp* gene was overexpressed, the CSP protein was not expressed. It may be because
each CSP protein has a different induction time; for example, the csp gene induction time was different. Although the protein was not expressed under cold stress for 6 h, we observed other stress response proteins such as Hsp and the universal stress protein UspA. The activity of ATPase in the cells exposed to cold stress for 1 h showed a significantly reduced activity compared to the control cells. However, the cells exposed for 4 h showed significantly higher activities compared to the control cells. The results indicated that when the cells were exposed to low temperatures, ATPase activity decreased, whereas the ATPase activity of the adapted cells increased. We suggest that ATPase activity is related to the maintenance of membrane fluidity. When the growth temperature decreases, there is an increase of unsaturated fatty acids and methyl branching. Thus, the decrease of average chain length improves the membrane fluidity (Casanueva et al., 2010).

For the proteomic analysis, we performed 2D SDS-PAGE (Fig. 7). Under cold stress, twenty four spots were overexpressed and thirteen spots were identified. The proteins were classified into six groups: energy metabolism, cell growth, unknown function, signal transduction, stress response and transcription (Fig. 8). Thirty-one percent of the overexpressed proteins were related to cell growth. We reported that spot 4 and spot 18 were related to pantothenate kinase and synthetase. Pantothenic acid is used in the synthesis of coenzyme A (CoA). CoA is important to energy metabolism for

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession number</th>
<th>Homology</th>
<th>% coverage</th>
<th>Matched peptides</th>
<th>Mascot score</th>
<th>Mr value</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>F4XMM6</td>
<td>Transposase</td>
<td>20</td>
<td>11</td>
<td>59</td>
<td>23509</td>
<td>Lyngbya majuscula</td>
</tr>
<tr>
<td>3</td>
<td>A8S6V7</td>
<td>Putative uncharacterized protein</td>
<td>17</td>
<td>10</td>
<td>59</td>
<td>68556</td>
<td>Facalibacterium prausnitzii</td>
</tr>
<tr>
<td>4</td>
<td>B3X7G0</td>
<td>Pantothenate kinase</td>
<td>12</td>
<td>13</td>
<td>61</td>
<td>38641</td>
<td>Shigella dysenteriae</td>
</tr>
<tr>
<td>5</td>
<td>C3KZR4</td>
<td>Putative Yqaj</td>
<td>19</td>
<td>12</td>
<td>59</td>
<td>41066</td>
<td>Clostridium botulinum</td>
</tr>
<tr>
<td>6</td>
<td>gi</td>
<td>167622455</td>
<td>hypothetical protein Shal_0515</td>
<td>10</td>
<td>7</td>
<td>60</td>
<td>34112</td>
</tr>
<tr>
<td>7</td>
<td>F7MRE9</td>
<td>Arsena reductase-like protein</td>
<td>13</td>
<td>4</td>
<td>61</td>
<td>5412</td>
<td>Clostridium botulinum C str. Stockholm</td>
</tr>
<tr>
<td>11</td>
<td>C1H41</td>
<td>Predicted protein</td>
<td>21</td>
<td>8</td>
<td>59</td>
<td>15350</td>
<td>Micromonas sp. RCC299</td>
</tr>
<tr>
<td>15</td>
<td>UPI0001E29AA2</td>
<td>phage integrase family protein</td>
<td>21</td>
<td>9</td>
<td>59</td>
<td>10913</td>
<td>Pseudomonas syringae pv. syringae</td>
</tr>
<tr>
<td>18</td>
<td>D3HCG0</td>
<td>Pantothenate synthetase</td>
<td>54</td>
<td>9</td>
<td>54</td>
<td>31481</td>
<td>Streptococcus galgaloticus subsp. galgaloticus</td>
</tr>
<tr>
<td>19</td>
<td>Q2JAH4</td>
<td>Heat shock protein 101</td>
<td>99</td>
<td>5</td>
<td>52</td>
<td>4285</td>
<td>Triticum monococcum</td>
</tr>
<tr>
<td>21</td>
<td>A8TV77</td>
<td>Universal stress protein UspA and related nucleotide-binding protein</td>
<td>46</td>
<td>10</td>
<td>55</td>
<td>28634</td>
<td>Alpha proteobacterium</td>
</tr>
<tr>
<td>22</td>
<td>F0S3W5</td>
<td>Multi-sensor signal transduction histidine kinase</td>
<td>29</td>
<td>11</td>
<td>57</td>
<td>74423</td>
<td>Desulfurobacterium thermolithotrophum DSM</td>
</tr>
<tr>
<td>24</td>
<td>F3KX52</td>
<td>Regulatory protein, LysR:LysR, substrate-binding</td>
<td>38</td>
<td>10</td>
<td>56</td>
<td>36076</td>
<td>Hylemonella gracilis ATCC 19624</td>
</tr>
</tbody>
</table>

**Table 2**
Differentially expressed proteins in *L. plantarum* L67 with cold shock for 6 h at 5 °C. The proteins were identified by MALDI-TOF/MS.

**Fig. 8.** Functional classification of the identified proteins from *L. plantarum* L67 analyzed by MALDI-TOF/MS using Map Man ontology as described by Beven et al. (1998).
pyruvate to enter the tricarboxylic acid cycle (TCA cycle) as acetyl-CoA and for α-ketoglutarate to be transformed to succinyl-CoA in the TCA cycle (Gropper et al., 2008). CoA is also important for the biosynthesis of many important compounds such as fatty acids, cholesterol, and acetylcholine (Gropper et al., 2008). Spot 2 had homology with a transposase protein, which is an enzyme that binds to the ends of a transposon and catalyzes the movement of the transposon to another part of the genome by a cut and paste mechanism or through a replicative transposition mechanism (Jones et al., 2011). Spot 5 had homology with a putative protein, YqjY. The function of this protein domain is to digest DNA for recombination. This recombination is crucial to viral replication (Vellani and Myers, 2003). DNA exonucleases play roles in DNA metabolism, e.g., in replication, repair, and recombination. Spot 15 was associated with the homology phage integrase family protein. Spots 2, 5, and 15 were related to chromosome structuring.

The proteins related to energy metabolism were identified as spots 3 and 7. Spot 3 was recognized as a protein involved in electron transport (Sudarsanam et al., 2007). Spot 7 was related to arsenate reductases family of cold shock proteins in L. plantarum L67 requires further study.

Spot 22 had homology with multi-sensor signal transduction histidine kinase. Histidine kinases (HK) are multifunctional and typically transmembrane; they are proteins of the transference class that plays a role in signal transduction across the cellular membrane (Wolanin et al., 2002). Spot 24 was a regulatory protein involved in regulated transcription (Chen et al., 2011).

Among the identified proteins, spots 6 and 11 have yet to be identified according to their function (Worden et al., 2009). Many L. plantarum L67 proteins were overexpressed in the cells exposed to low temperatures, and these proteins may interact with each other, resulting in cryotolerance.

Cold stress induced proteins play roles in a variety of cellular processes such as fatty acid metabolism, chromosome structuring, transcription, translation, general metabolism, energy metabolism, and stress response (Graumann et al., 1997; Wouters et al., 2000a, 2000b; Yamanaka et al., 1998). Additionally, the induced proteins in this study were related to energy metabolism, cell growth, signal transduction, stress response and transcription.

In conclusion, we suggest that L. plantarum L67 adapts to low temperatures by a constitutive expression of the potentially cryoprotective cspA gene and proteins related to metabolism and stress response. In a bio-preservation strategy, the protective strains are generally cultivated at their optimum temperatures, eventually freeze-dried, and then directly inoculated into products that are stored at chilled temperatures. Cold acclimation thus constitutes a demonstrable advantage in bacterial competition against spoilage and pathogenic psychrotrophic bacteria in terms of food preservation. This property would enable cells to multiply at optimal temperatures and to be successfully inoculated into chilled foods without delaying the food preservative effect.

Acknowledgments

This research was supported by IPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (112121-2).

References


Derzelle, S., Hallet, B., Francis, K.P., Ferain, T., Delcour, J., Hols, P., 2000. Changes in cspL, cspA promoter sequences and adaptation of translational apparatus in different stress-regulative networks (Capozzi et al., 2011). The universal stress protein UspA-related nucleotide-binding protein was induced (spot 21). The levels of UspA have been evaluated in the response to the cell status of E. coli under stressful conditions (Gustavsson et al., 2002; Kvint et al., 2003). The UspA protein of E. coli has a role in resisting damage to DNA. Based on our conclusions, the induced expression of the nucleotide-binding protein UspA may be associated with protecting the DNA of cells during cold stress. However, the exact physiological role of UspA in L. plantarum L67 requires further study.

Spot 22 had homology with multi-sensor signal transduction histidine kinase. Histidine kinases (HK) are multifunctional and typically transmembrane; they are proteins of the transference class that plays a role in signal transduction across the cellular membrane (Wolanin et al., 2002). Spot 24 was a regulatory protein involved in regulated transcription (Chen et al., 2011).

Among the identified proteins, spots 6 and 11 have yet to be identified according to their function (Worden et al., 2009). Many L. plantarum L67 proteins were overexpressed in the cells exposed to low temperatures, and these proteins may interact with each other, resulting in cryotolerance.

Cold stress induced proteins play roles in a variety of cellular processes such as fatty acid metabolism, chromosome structuring, transcription, translation, general metabolism, energy metabolism, and stress response (Graumann et al., 1997; Wouters et al., 2000a, 2000b; Yamanaka et al., 1998). Additionally, the induced proteins in this study were related to energy metabolism, cell growth, signal transduction, stress response and transcription.

In conclusion, we suggest that L. plantarum L67 adapts to low temperatures by a constitutive expression of the potentially cryoprotective cspA gene and proteins related to metabolism and stress response. In a bio-preservation strategy, the protective strains are generally cultivated at their optimum temperatures, eventually freeze-dried, and then directly inoculated into products that are stored at chilled temperatures. Cold acclimation thus constitutes a demonstrable advantage in bacterial competition against spoilage and pathogenic psychrotrophic bacteria in terms of food preservation. This property would enable cells to multiply at optimal temperatures and to be successfully inoculated into chilled foods without delaying the food preservative effect.

Acknowledgments

This research was supported by IPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (112121-2).

References


