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Cold Shock Response of *Leuconostoc mesenteroides* SY1 Isolated from Kimchi

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Abstract Low-temperature adaptation and cryoprotection were studied in *Leuconostoc mesenteroides* SY1, a strain isolated from Kimchi. *L. mesenteroides* SY1 cells grown in exponential growth phase at 30°C were exposed to 15°C, 10°C, and 5°C for 2, 4, and 6 h, respectively, and then frozen at −70°C for 24 h. Survival ratio was measured after the cells were thawed. The freezing-thawing cycles were repeated four times. Preadapted cells survived better than non-adapted control cells, and the highest survival ratio (96%) was observed for cells preadapted for 2 h at 5°C, whereas control cells showed only 22%. The 2D gel showed that two proteins (spots A and B) were induced in cells preadapted at lower temperatures. Spots A and B have the same molecular weight (7 kDa), but the pI was 4.6 for spot A and 4.3 for spot B. The first 29 and 15 amino acid sequences from spots A and B were determined, and they were identical, except for one amino acid. A *csp* gene was cloned, and nucleotide sequencing confirmed that the gene encoded spot A cold shock protein.

Key words: Cold shock, *Leuconostoc mesenteroides*, Kimchi, cold shock proteins, adaptation

Kimchi is a traditional Korean fermented vegetable prepared from Chinese cabbage spiced with brine and other spicy ingredients, such as hot red pepper, garlic, green onion, and ginger [16]. Kimchi fermentation consists of successive ripening processes, and the fermentation temperature significantly influences the type of principal microorganisms. Several lines of evidence show that heterofermentative *Leuconostoc* species dominate during the early and middle phases of Kimchi fermentation, and then more acid-tolerant lactobacilli become dominant at the late stage [2, 11, 15–16]. Lee *et al.* [15] showed that *L. mesenteroides* was a predominant species during the early and middle stage of Kimchi fermentation at 5°C, and *Lactobacillus sake*, *L. brevis*, or *L. plantarum* became dominant at the later stage of fermentation [16]. This succession from *L. mesenteroides* to lactobacilli was fast when the fermentation temperature was increased to 20°C [15]. Since Kimchi fermentation is usually carried out at temperatures lower than 10°C, it is important to understand the physiological adjustments of lactic acid bacteria (LAB) to low temperature. When cells are exposed to low temperature, synthesis of most proteins stops immediately, but that of a special set of proteins such as cold shock proteins (CSP) increases or begins to increase [20]. Small acidic cold shock proteins are known to be highly inducible upon cold shock, and they are found among a wide range of bacteria, such as *E. coli* [27], *Bacillus subtilis* [7], *Lactococcus lactis* [24], and *Streptococcus thermophilus* [26]. Moreover, Francis and Stewart [5] monitored a wide variety of bacteria and observed that *csp* genes were present in all species tested. CSPs may function as RNA chaperones, as they possess a binding site for single-stranded nucleic acid. In this way, they could minimize the secondary folding of mRNA, thereby facilitating the translation process [7, 9]. CspA of *E. coli* also appears to function as a transcriptional activator for two genes whose products, GyrA and H-NS, are both involved in DNA supercoiling [10, 14]. Furthermore, CspB of *B. subtilis* is responsible for freezing tolerance, as shown with a mutant strain in which the *cspB* gene was disrupted [23]. It was noted that many organisms develop an increased ability to survive freezing after a cold shock treatment at sublethal temperature (preadaptation). Maintaining membrane integrity and preventing macromolecule denaturation have been suggested as key factors to increase freeze survival [4, 6, 22]. In this report, the induction of major CSPs from *L. mesenteroides* SY1, a strain isolated from Kimchi, was demonstrated when SY1 cells were exposed to low temperature. Also, it was demonstrated that brief

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exposure to low temperature increased the viability of SY1 cells against following freezing-thawing challenges. Knowledge on the cold shock response of LAB isolated from Kimchi will help toward understanding the growth of LAB during Kimchi ripening, and eventually contribute to developing starters for commercial Kimchi production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Leuconostoc mesenteroides SY1 was isolated from Chinese cabbage Kimchi purchased from a local supermarket. Supernatant obtained by centrifugation of Kimchi (3,000 ×*g*, 5 min) was serially diluted and spread onto MRS plates containing 1% sucrose. Several dextran-producing colonies were obtained, and one of them, SY1, was further examined. For identification of SY1, biochemical properties were first examined using API 50 CHL kit (BioMerieux, France) and finally 16S rDNA sequencing data, as described by Kim *et al.* [13]. *L. mesenteroides* SY1 was grown on MRS broth (Difco Lab., Detroit, U.S.A.) or on MRS plate solidified with 1.5% agar at 30°C. *E. coli* DH5α was used as the host strain for *csp* gene cloning experiments and grown in LB broth with aeration at 37°C. Ampicillin at 50 μg ml^{-1} concentration was included.

Cold Shock Treatment and Freeze-Thaw Challenge

L. mesenteroides SY1 was grown in MRS medium (250 ml) at 30°C until the mid-exponential phase (OD600=0.6), and then 50 ml aliquots of culture were pelleted (10 min at 9,000 ×*g*) and resuspended in 50 ml of precooled medium (15, 10, and 5°C). Cells were further incubated at different temperatures (30, 15, 10, and 5°C) for 24 h, and the OD_{600} was measured. After freeze-thaw challenges, the viability of *L. mesenteroides* SY1 cells was measured as follows. Two-hundred ml of MRS broth was inoculated overnight (1%) and incubated at 30°C. When OD_{600} reached 0.6, 9-ml aliquots were dispensed into 19 tubes. Cold adaptation was done by placing the tubes into circulating water baths where temperature had been respectively adjusted to 5°C, 10°C, and 15°C. Adaptation at lower temperature was continued for 2, 4, and 6 h, and then cells were quickly frozen and stored at −70°C. Control cells were frozen directly without preadaptation. After 24 h of freezing, cells were thawed by placing tubes in a 30°C water bath for 5 min. Aliquots were taken out for viable cell counting, and then cells were frozen again in the −70°C freezer. A total of four cycles of freeze-thaw was carried out. Thawed cells were serially diluted with 1% peptone water, and viable cell counting was done by spreading 100 μl of diluted samples on MRS plates. Five plates were used for each diluted sample and the cell numbers were averaged. Survival ratio was calculated by dividing viable counts obtained after each freeze-thaw cycle with the viable counts before the first freezing.

Two-Dimensional Gel Electrophoresis

Total cellular proteins from *L. mesenteroides* SY1 cells were obtained and subjected to 2D-gel analysis. Cells grown overnight (10 ml) were recovered by centrifugation and resuspended in 1 ml of Tris-HCl buffer (20 mM Tris, 0.1 mM DTT, 10 mM EDTA, pH 7.0). Cells were sonicated for 30 s, followed by cooling on ice for 30 s. A total of four cycles of sonication-cooling was carried out. Disrupted cells were centrifuged, and the supernatant was designated as protein extract. SDS-PAGE of the protein extract was done using a Tricine-Tris buffer system [19]. SE 400 Sturdier Electrophoresis unit (Hoefer Pharmacia Biotech Inc.) with a 16.5% acrylamide gel was used to resolve proteins. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250. Broad range (BioRad) and lower range (Sigma) size markers were used for size estimation. 2D-gel electrophoresis was performed as described previously [18] using a 2D-gel system (BioRad., U.S.A.). Prior to loading samples on the IPG strip, 30–50 μg protein samples were treated with 100 μl lysis solution [8 M urea, 2% CHAPS, 50 mM DTT, 0.2% IPG buffer (pH 3–10), a few grains of bromophenol blue], and the whole sample was loaded onto the acidic end of the first-dimension IPG gel (7 cm) with linear pI ranges from 4 to 7 or from 3 to 10 (Immobiline Dry strips, NO. 17-6001-11, Pharmacia Biotech). For the second dimension, 16% Tricine-SDS-PAGE [18] gels were used to obtain an optimal separation at the low-molecular-mass region. Gels were silver stained after electrophoresis [1].

N-Terminal Sequencing

Proteins were transferred from a 2D gel to a PVDF membrane using a semi-dry blotting apparatus (Hoefer Pharmacia Biotech), and Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) was used for transfer [17]. After staining of the membrane with Coomassie Brilliant Blue G-250, protein spots were excised. The N-terminal amino acid sequence of the corresponding proteins was determined by the Edman procedure using Procise 492 clc protein sequencer (Korea Basic Science Institute in Seoul, Korea). The results were compared for protein identification with the GenBank data.

csp Gene Cloning

PCR was employed to clone a *csp* gene from *L. mesenteroides* SY1. GeneAmp PCR System 2400 (Perkin-Elmer Biosystems, Foster City, U.S.A.) was used with 1 μg genomic DNA as a template. A pair of oligonucleotides (Csp-F2-1: 5'ATGGA-AAAAGGCACAGTAAAGTG-3' and Csp-R2: 5'-TTAAC-CCTTTGTGATGTTTGTT-3') were synthesized (Bionix, Korea), based on the sequences of annotated putative *csp* genes (ZP_00063083) from *L. mesenteroides* ATCC 8293.

PCR was performed in 50 μl reaction volume containing 1 μg of template DNA, 10 pmol of each primer, 0.25 mM of each dNTPs, 2 U of *Ex Taq* (Takara, Japan) DNA polymerase, and 1× *Ex-Taq* DNA polymerase buffer. PCR amplification was carried out under the following conditions; predenaturation for 5 min at 94°C, followed by 25 cycles of amplification (denaturation at 94°C for 45 s; annealing 52°C, 20 s; extension 72°C, 30 s), and a final extension at 72°C for 7 min. PCR products were analyzed on a 2% agarose gel, recovered from the agarose gel using a E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, Doraville, U.S.A.), and ligated to T-vector (pGEM-T Easy Vector, Promega). DNA sequences were determined by the dideoxy-chain termination method using an ABI-PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, U.S.A.) [12]. Standard protocols were followed for the manipulation of DNA and RNA [20].

Nucleotide Sequence Accession Number

The nucleotide sequence of *cspA* from *L. mesenteroides* SY1 was deposited into NCBI GenBank under the accession number AY600963.

Slot-Blot Hybridization Analysis

Total RNA was isolated from *L. mesenteroides* SY1 cells grown on MRS broth (Difico Lab., Detroit, U.S.A.) containing 1% glucose, melibiose, and raffinose using the FastRNA Pro Blue kit (Q.BIO, U.S.A.). Twenty μg of each preparation was heated, chemically denatured, and applied onto a Hybond-XL nylon membrane (Amersham Biosciences) using a slot-blot system (SE646, HoefferPharmacia Biotech.). A 200 bp (base pair) fragment corresponding to the internal region of *cspA* was amplified by PCR and used as a probe. The probe was labeled with ^{32}P-ATP using Rediprime II DNA labeling System (Amersham Biosciences). Prehybridization (1 h) and hybridization (overnight) were performed in ULTRAhyb (ultrasensitive hybridization buffer, Ambion, U.S.A.) at 50°C, and two posthybridization washes were carried out at the same temperature in 2× SSC (1× SSC is

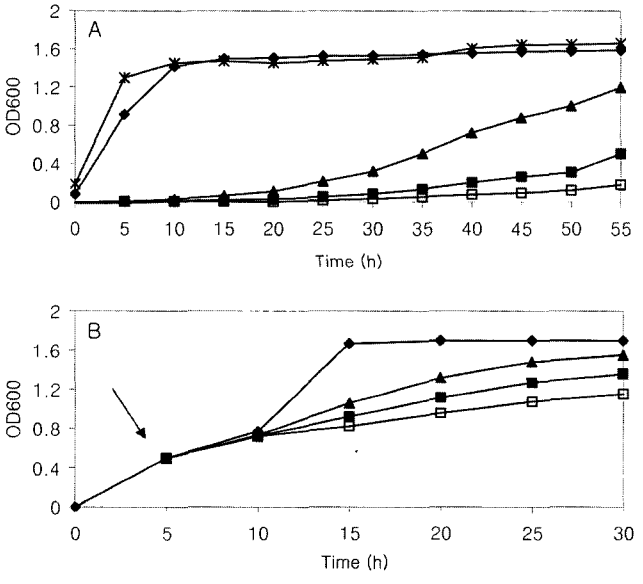

Fig. 1. Growth of *L. mesenteroides* SY1 at different temperatures.
(A) Growth of *L. mesenteroides* SY1 at 37°C (*), 30°C (◆), 15°C (▲), 10°C (■), and 5°C (□). (B) Growth of *L. mesenteroides* SY1 following temperature downshift. SY1 cells were first grown at 30°C and culture at OD_{600}=0.5 was divided into four aliquots. After centrifugation, cells were resuspended in MRS media precooled at 30°C, 15°C, 10°C, and 5°C, and incubated for 24 h. The arrow indicates the time point when temperature downshift was done.

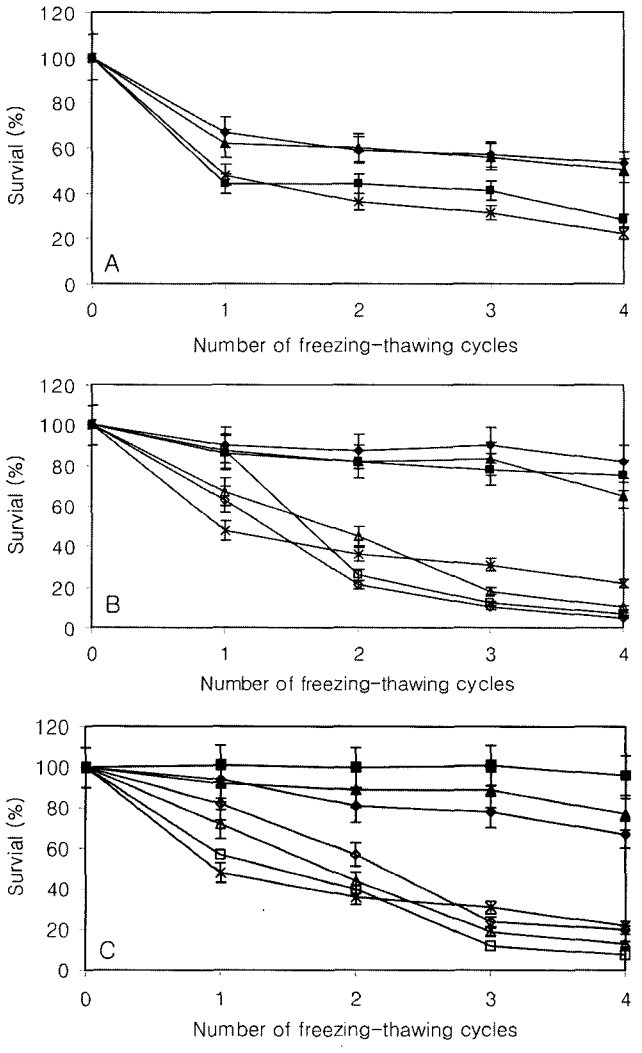

Fig. 2. Survival ratios of *L. mesenteroides* SY1 cells after freeze-thaw cycles.
Survival ratio was calculated as the percentage of survived cells after each freeze-thaw cycle relative to the cell number before freezing (100%). Cm (chloramphenicol) was added at a concentration of 100 μg/ml. A, Cells preadapted at 15°C; B, cells preadapted at 10°C; C, cells preadapted at 5°C. Control cells without preadaptation (*), cells exposed to low temperature for 2 h (■), 4 h (▲), or 6 h (◆), and cells preadapted in the presence of Cm for 2 h (□), 4 h (△), or 6 h (◇).

0.15 M NaCl plus 0.015 sodium citrate)-0.1% sodium dodecyl sulfate prior to film exposure (Hyper film ML).

RESULTS

Effect of Temperature Downshift on the Growth of *L. mesenteroides* SY1

L. mesenteroides SY1 grew optimally at 30°C, and the minimum growth temperature was between 10°C and 5°C, probably slightly higher than 5°C (Fig. 1). The growth rate decreased significantly as temperature dropped. After 55 h of incubation, the OD_{600} of culture was around 0.2 at 5°C and 0.4 at 10°C, however, the value reached above 1.0 at 15°C culture. The final cell number at low temperature increased significantly when SY1 was first grown at 30°C up to the mid-log phase (OD_{600}=0.5), and the temperature was then dropped to 15, 10, or 5°C.

Induction of Cryoprotection in Preadapted Cells

The survival ratio of cells after freezing was determined (Fig. 2). Only 22% of control cells (no preadaptation at lower temperature) survived after four consecutive freeze-thaw cycles. However, cells adapted for 2 h at 5°C or 6 h at 10°C had much higher survival ratio of 96% (±10%) or 82%, respectively. Cells adapted for 4 h at 5°C or 2 h at 10°C showed 77% or 75% viability, respectively. Cells adapted at 15°C showed small increase in viability; 28% (2 h), 50% (4 h), and 53% (6 h). This result indicates that exposing *L. mesenteroides* cells to sublethal low temperature helps the cells to survive through subsequent freezing. This phenomenon of cold inducible protection mechanism(s) has been reported for Gram (−) and (+) bacteria [25–26]. When chloramphenicol (100 μg/ml) was added to the culture during the adaptation period, survival ratio after freezing decreased significantly, less than that of control, indicating that protein synthesis was required for the induction of cold inducible protection mechanism(s) (Fig. 2).

2D-Gel Analysis of CSPs

Protein extracts from *L. mesenteroides* SY1 cells preadapted for 2, 4, and 6 h at 5, 10, and 15°C, respectively, were analyzed by 2D-gel. Two proteins (spots A and B in Fig. 3) were most strongly induced in the adapted cells, whereas

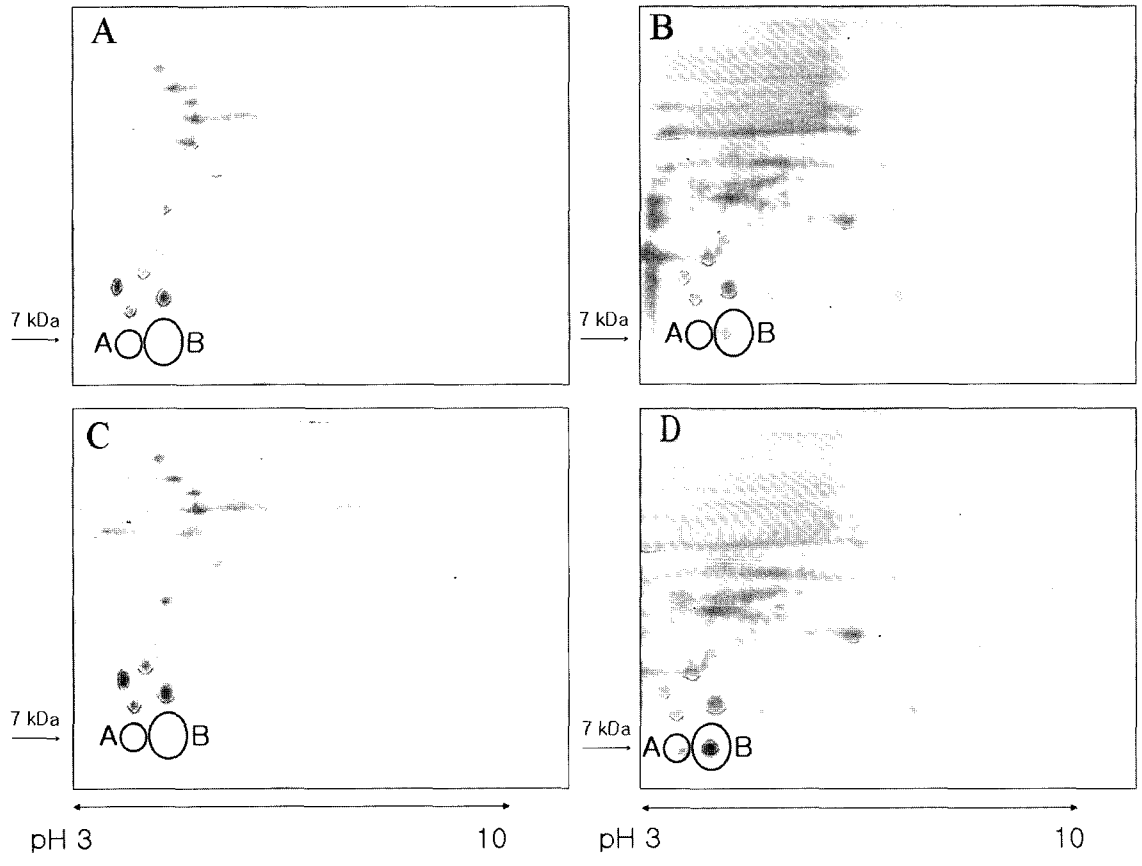

Fig. 3. 2D-gel of protein extracts from *L. mesenteroides* SY1.
A, Total protein extracted from cells grown at 30°C without cold shock (control); B, total protein extracted from cells preadapted for 6 h at 15°C; C, total protein extracted from cells preadapted for 6 h at 10°C; D, total protein extracted from cells preadapted for 2 h at 5°C. Spots induced at low temperature are circled. Arrow on the left indicates the location of CSPs, and a pI scale is given at the bottom.

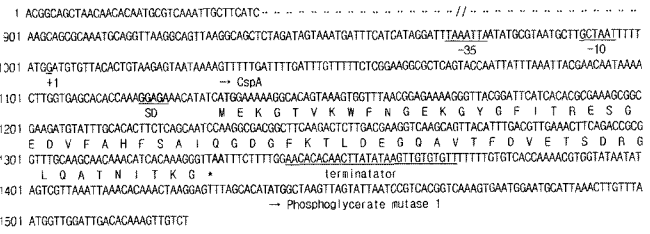

Fig. 4. DNA sequence of the *csp* gene and the deduced amino acid sequence.
The *csp* gene is 201 bp in length and encodes an acidic protein of 67 amino acids. The putative −10 and −35 promoter regions, the putative ribosome binding site (SD), and the transcription terminator are underlined.

there was no induction of corresponding spots in the control. Apparent molecular weights and pI values of these two proteins were 7 kDa for both, and 4.6 and 4.3 for spot A and spot B, respectively.

N-Terminal Amino Acid Sequences

For the spots A and B (Fig. 3), the N-terminal amino acid sequences were determined and shown in Fig. 6A. The first 29 amino acid (aa) sequence of spot A was identical to the translated amino acid sequence from the *csp* gene (Fig. 4). When the amino acid sequences of spot A (29 aa) and spot B (15 aa) were compared, they were identical except for one amino acid: Spot A protein had glycine at the 14th position, whereas it was isoleucine in the spot B protein. Homology was found between the N-terminal sequences of the 7 kDa proteins and the following proteins from the database (Fig. 6): Amino acid sequence of Spot A (29 aa) showed 75% identity with CspC of *Lactobacillus plantarum* (CAC06102.1), CspB of *Lactococcus lactis* (CAA71254.1), CspA of *Lactobacillus casei* (CAD92346.1), CspC of *Bacillus subtilis* (AAC45646.1), and many other CSPs in the database.

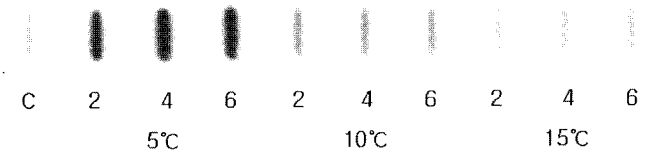

Fig. 5. Quantification of *csp* transcript from *L. mesenteroides* SY1.
Total 20 μg of RNA was spotted onto a Hybond-XL nylon membrane (Amersham Biosciences) and hybridized with a 200 bp radiolabeled *cspA* probe (see the text). C indicates control that was not adapted. Numbers at the bottom (2, 4, and 6) indicate the time (in h) for adaptation at low temperature.

Cloning and Sequencing of the *csp* Gene

Using primers based on the sequences of putative *csp* genes from *L. mesenteroides* ATCC8293 (ZP_00063083), a 1,500 bp fragment was amplified. The fragment was cloned into T-vector, and the whole fragment was sequenced. Sequence analysis showed that the PCR product was 1,526 bp in length and contained a gene homologous to the major *csp* genes. Figure 4 shows the sequences of the *csp* gene and immediate upstream region. Amino acid sequence translated from the DNA sequence matched exactly with the determined N-terminal amino acid sequence of spot A. The gene was accordingly named *cspA*, and the protein product as CspA. Spot B was named CspB. The calculated molecular mass of the CspA was 7,220.91, and the pI value was 4.72. The values agree well with the estimated values for the spot A on 2D gel (Fig. 3). Downstream of *cspA*, a partial ORF was located, and homology search indicated that the ORF might encode a phosphoglycerate mutase I.

Slot-Blot Analysis

Slot-blot hybridization experiment was performed to compare the amount of *csp* transcript at different temperatures.

A

Spot		Amino Acid Sequence
		1 10 20
Spot	A	MEKGTVKWFNGEK G YGFITRESGEDVFAH
Spot	B	MEKGTVKWFNGEK I Y

B

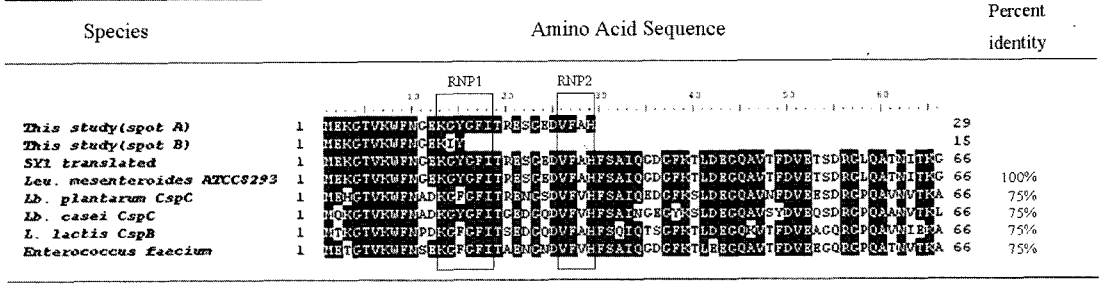

Fig. 6. Alignment of amino acid sequences of the cold shock proteins from *L. mesenteroides* SY1.
A. Determined N-terminal amino acid sequences of CspA and CspB in Fig. 3. B. Alignment of amino acid sequences of the CspA and CspB with amino acid sequences of CSPs from various bacteria. The known RNA-binding motifs RNP1 and RNP2 are boxed.

Figure 5 shows that cells exposed to 5°C produced the strongest hybridization signal, indicating the highest concentration of *cspA* transcript. The intensity of the signal became stronger as the adaptation time increased. After 4 h of exposure, the *cspA* transcript seemed to reach the highest concentration. No difference was observed between cells exposed to 10°C and 15°C, indicating that these temperatures might not cause severe cold shock to *L. mesenteroides* SY1.

DISCUSSION

L. mesenteroides SY1, isolated from Kimchi, grew optimally at 30°C, and its minimal growth temperature was slightly higher than 5°C (see Fig. 1). Since Kimchi ripening occurs at refrigerating temperature, usually below 10°C, the ability of *L. mesenteroides* strains to multiply at this temperature is important for Kimchi ripening. Therefore, it is of interest to ask the question of what the cold shock response or cold adaptation phenomenon is like in *L. mesenteroides* SY1. As a first step to understanding the cold shock response in *L. mesenteroides*, we performed experiments to see if preadaptation to low temperature confers resistance against freezing. The results clearly showed that preadaptation conferred cells with protection against freezing, a lethal challenge. The degree of protection for *L. mesenteroides* SY1 (96% survival in adapted cells versus 22% survival in control) was much higher than that observed for *Lactobacillus paraplantarum* C7 (46.8% versus 22%), another LAB isolated from Kimchi [13]. More importantly, the viability of *L. paraplantarum* C7 cells after freezing was not affected by the presence of chloramphenicol in the media, whereas that of *L. mesenteroides* SY1 decreased dramatically, to less than the control (Fig. 2). Thus, it seems that detailed cold shock responses are different from species to species, although the operation of a general cold shock response is similar among bacteria. For *L. mesenteroides* SY1, protein synthesis is absolutely required for the induction of protection mechanism(s) against freezing. Apparently, newly synthesized proteins, including CspA and CspB, contribute to the development of enhanced resistance against freezing or cause some changes that eventually lead to cryoprotection.

Since 2D-gel analysis revealed that two small proteins with the same molecular weight of 7 kDa were the most strongly induced proteins upon cold shock, it is tempting to speculate that these proteins are directly involved in the protection against freezing. In the case of *Lactococcus lactis* MG1363, overproduction of a 7 kDa *csp* gene, *cspD*, increased the viability of cells after freezing, 2–10 times higher than that of control cells [24]. Willimsky *et al.* [23] showed that deletion of the gene encoding CspB of *B. subtilis* resulted in decreased freeze survival, and the authors suggested a role of CSPs as antifreeze proteins, because of their low molecular mass and their abundant presence [23]. On 2D gel, CspA appeared at both 30°C and lower temperatures (5, 10, and 15°C), but CspB appeared only at lower temperatures. These two proteins have the same molecular weight, 7 kDa, but different pI values; 4.6 for CspA and 4.3 for CspB (Fig. 3). When the first 15 N-terminal amino acid sequences were compared, they were identical, except for the 14th amino acid (glycine for CspA and isoleucine for CspB). Considering all these facts, CspA and CspB appear to be encoded by different genes; however, both genes appear to belong to the major CSP family genes. Both have 75% homology with various CSPs from many different species. CspA has an amino acid sequence identical to a putative CSP from *L. mesenteroides* ATCC8293 (up to 29 amino acid). By PCR, a *csp* homologue from *L. mesenteroides* SY1 was cloned and sequenced. The translated amino acid sequence of the gene matched well with the determined amino acid sequences of CspA. The gene was accordingly designated as *cspA*. *Lactococcus lactis* has five different *csp* genes [24] and *L. plantarum* has three different *csp* genes, *cspL*, *cspP*, and *cspC* [3]. It is possible that *L. mesenteroides* SY1 has additional *csp* genes, gene products of which were not obvious on the 2D gel (Fig. 3) due to a resolution problem. The exact role of each *csp* gene during growth of cells at low or room temperature is still not clear. CspA from *L. mesenteroides* SY1 is synthesized at 30°C, although the synthesis is increased upon temperature downshift. Obviously, more studies are necessary to elucidate how many CSPs are synthesized upon cold shock in *L. mesenteroides* SY1. Because of this small size and unsatisfactory resolution of 2D gel, we could not identify additional larger proteins that might be induced upon cold shock. More extensive and careful 2D-gel analyses are necessary to identify other possible cold shock proteins. The next step will be to find the role of each CSP in the adaptative responses of *L. mesenteroides* SY1 cells at low temperature. To elucidate the function of each CSP, experiments such as *csp* gene cloning, overexpression, and disruption of each *csp* gene appear to be useful. The observed increased survival of this LAB after freezing can be of great importance for the storage of this strain prior to use for Kimchi fermentation processing.

Acknowledgments

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