Effect of Growth Temperature and Culture Medium on the Cryotolerance of Permafrost *Exiguobacterium Sibiricum* 255-15 by Proteome-Wide Mass Mapping

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Abstract: *Exiguobacterium sibiricum* 255-15 has shown significantly improved cryotolerance after liquid broth growth at 4°C and agar surface growth at both 4°C and 25°C compared with liquid broth growth at 25°C. The ability to survive freeze-thaw stress is expected to depend on the physiological state and protein composition of cells prior to freezing. Using 2-D liquid separation and an ESI-TOF MS-based mass mapping technique, we examined the differences in the proteomic profiles of the permafrost bacterium *E. sibiricum* 255-15 grown at two temperatures (4°C and 25°C) and two media (liquid broth and agar surface) before freeze-thawing treatments. In this study, a total of 330 proteins were identified. The cells cultured under the growth conditions associated with the improved cryotolerance have revealed a general down-regulation of enzymes involved in major metabolic processes (glycolysis, anaerobic respiration, ATP synthesis, fermentation, electron transport, and sugar metabolism) as well as in the metabolism of lipids, amino acids, nucleotides and nucleic acids. In addition, eight proteins (2'-5' RNA ligase, hypoxanthine phosphoribosyl transferase, FeS assembly ATPase SufC, thioredoxin reductase and four hypothetical proteins) were observed to be up-regulated. This suggests these eight proteins might have a potential role to induce the improved cryotolerance.

Key Words: Bacterial cryotolerance, *Exiguobacterium sibiricum*, 2-D mass mapping, ESI-TOF MS, MALDI-TOF MS, MALDI-QIT-TOF MS.

INTRODUCTION

There has been growing interests in the survival mechanism of psychrophilic bacteria at repeated freeze-thaw challenge largely due to the fact that the processes of freezing and thawing are common processes in nature and 80% of the earth's surface is cold [1]. Bacteria in environments which experience seasonal temperature fluctuations are expected to be adapted to repetitive freeze-thaw cycles. On the other hand, bacteria in stable subfreezing environments are expected to be adapted to low temperature since these bacteria do not experience any repetitive freeze-thaw cycles in their native habitat. The example of a stable subfreezing environment is permafrost. Permafrost, which is defined as a subsurface frozen layer, primarily soil or rock that remains frozen for more than two years, makes up more than 20% of the land surface of the earth, including 82% of Alaska, 50% of Russia and Canada, 20% of China, and most of the surface of Antarctica [2-4]. Within the buried Siberian permafrost soils, high numbers of viable microorganisms have been discovered [5-9]. The presence of the microorganisms there is surprising, not only because of the constant subzero temperature of permafrost soils, averaging from -10°C to -12°C,

but also because of the length of time the permafrost soils have been frozen, which ranges from a few thousand years up to 2-3 million years. These organisms may well be the only living cells that have survived for a geologically significant period of time. The microorganisms in the permafrost may be viewed as the result of a continuous process of selection for those capable of withstanding prolonged exposure to subzero temperatures. Even more significant is the fact that before the bacteria became trapped within the permafrost they were the outcome of cyclic freeze-thaw preselection in the original "active" tundra layers where they were exposed to warmer growth periods in summer and colder surface temperatures in winter.

Cryotolerance, which has been defined as the ability of cells to recover their activity after freezing, has been studied using serial freeze-thaw treatments of five *Exiguobacterium* strains including *E. sibiricum* 255-15 [10]. The strain *E. sibiricum* 255-15 has been isolated from a 2-3 million year old Siberian permafrost sediment [11]. *Exiguobacterium* spp. are low G+C Gram-positive non-spore-forming bacteria. Eurypsychrophilic *E. sibiricum* 255-15 was found to grow in a temperature range from -6°C to 40°C [10, 12]. Our previous study [10] has shown that cryotolerance of *Exiguobacterium* strains is significantly influenced by low temperature (4 and -6°C) and by surface- (agar) –associated growth. Molecular mechanisms underlying such improved cryotolerance of *Exiguobacterium* spp. remain unexplored.

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The objective of this study was to compare proteomic profiles of *E. sibiricum* 255-15 grown at the different temperatures and media, and to identify proteins differentially expressed at conditions under which the bacteria developed improved cryotolerance, using a 2-D liquid separation and mass spectrometry-based mass mapping technique. The application of this technique has recently been evaluated during studying the low temperature adaptations in *E. sibiricum* 255-15 [13]. PMF by MALDI-TOF MS and peptide sequencing using MALDI-QIT-TOF MS/MS have proved to be a powerful combination of techniques in protein identification. In the present work, with the aid of MALDI-QIT-TOF MS/MS, the fraction of proteins identified using both PMF and peptide sequencing has been improved to over 60%. In addition, the recently sequenced genome of *E. sibiricum* 255-15 (http://genome.ornl.gov/microbial/exig/, accession number CP001022-CP001024) has greatly facilitated the characterization of proteomic profiles. The current study both confirmed and significantly extended our previous studies.

MATERIALS AND METHODOLOGY

E. sibiricum 255-15 Cell Culture and Cell Lysis

E. sibiricum 255-15 cell pellets were obtained from the Department of the Food Science at North Carolina State University. All cells were cultured in tryptic soy broth (TSB, Difco, BD Diagnostics Systems, Franklin Lakes, NJ) with 7% yeast extract (Difco, TSB-YE) for liquid broth growth and tryptic soy agar (TSA-YE) consisting of TSB-YE with 1.2% agar (Difco) for agar surface growth. For liquid broth growth, bacteria were grown in TSB-YE to the mid-log phase (O.D. = 0.7) at 4°C for 7 days and at 25°C overnight. For growth on agar surface, bacteria were grown on TSA-YE at 4°C for 14 days and at 25°C overnight. Cells grown on agar medium were transferred with a sterile swab in TSB-YE to approximately 5 x 10^8 cell/mL (O.D. = 0.7). Cells grown at 4°C were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C, while cells grown at 25°C were pelleted by centrifugation at 4,000 rpm for 10 min at room temperature. Bacterial cells were lyzed as described before [13]. The supernatants from whole cell lysates were then desalted by a PD-10 Sephadex G-25 gel filtration column (Amersham Biosciences, Piscataway, NJ) and the protein concentrations were determined by the Bradford-based protein assay using a commercial kit (Bio-Rad, Hercules, CA). The samples were quantified in triplicate at 595 nm with bovine serum albumin, BSA (Sigma-Aldrich, St. Louis, MO) as a standard. The protein concentrations of the samples were between 1.0-2.0 mg/mL.

Freeze-Thawing

The cells grown on agar medium were collected in TSB-YE and frozen at -20°C while the cells grown in TSB-YE were frozen in the growth medium at -20°C. For the freeze-thaw experiment, every two days the cells were thawed completely in a water bath at the room temperature and then refrozen at -20°C. The CFU (colony forming units) of viable cells before and after 2, 6, 9, 12, 16, 20 cycles of freeze-thaw were determined on 1/10 TSA. The plates were incubated at 24°C for 48 h and the resulting colonies were counted using Protos Plus Colony Counter (Synoptics Ltd., Cambridge,

UK). The results are the mean values of three independent replicate assays done in duplicate.

2-D Liquid Phase Separation

A 2-D liquid phase mass mapping method has been developed [13] to profile protein expression in *Exiguobacterium sibiricum* 255-15 (Fig. **1**). Proteins extracted from *E. sibiricum* 255-15 were first fractionated by CF using a Beckman Gold HPLC equipped with model 127S pump and model 166 detector (Beckman Coulter, Fullerton, CA) on an HPCF-1D column (250 × 2.1 mm) (Eprogen Inc., Darien, IL). The pH gradient was generated using a start buffer (25 mM bis-tris propane, pH 8.5, Sigma-Aldrich) and an elute buffer (3% v/v poly-buffer 74, 7% v/v poly-buffer 96, pH 4.0) (Riedel de Haen, Seelze, Germany). Both buffers were prepared in 6 M urea (Sigma-Aldrich) and 0.1% *n*-octyl-β-D-glucopyranoside, OG (Sigma-Aldrich) with pH adjusted by adding iminodiacetic acid (Sigma-Aldrich). A total of 5 mg of protein from each sample was loaded on the CF column which was equilibrated with the start buffer for 2 hours at 0.2 mL/min followed by elution at the same flow rate using the elute buffer. The pH was monitored online by a pH electrode (Lazar Research Laboratories, Inc. Los Angeles, CA) and the separation was detected at 280 nm. Effluent from the CF separation was collected from pH 8.5 to 4.0 every 0.3 pH unit intervals. After running the pH gradient, the column was washed with 1 M NaCl solution (Sigma-Aldrich) to elute proteins with p*I* lower than 4.0.

NPS-RP-HPLC separation was performed on a 33 × 4.6 mm ODS III column packed with 1.5 μm nonporous silica beads (Eprogen Inc.) at a flow rate of 0.5 mL/min using the same HPLC system as used in the CF fractionation. To improve the resolution and speed of the separation, the column temperature was maintained at 60°C using a Model 7971 column heater (Jones Chromatography, Resolution Systems, Holland, MI). The NPS-RP-HPLC separation was performed using gradient elution with a water (solvent A) and acetonitrile HPLC grade (Sigma-Aldrich) (solvent B) gradient, both of which were prepared in 0.1% trifluoroacetic acid, TFA (Sigma-Aldrich). The water was purified using a Milli-Q water filtration system (Millipore, Inc., Bedford, MA). The gradient profile used was as follows: (1) 5 to 26% B in 1 min; (2) 26 to 35% B in 3.5 min; (3) 35 to 40% B in 9.5 min; (4) 40 to 50% B in 13 min; (5) 50 to 58% B in 4 min; (6) 58 to 75% B in 1 min; (7) 75-100% B in 1 min; (8) 100 to 5% B in 1 min.

Intact Protein *M*r Measurement and Interlysate Quantification by ESI-TOF MS

Half of the effluent from NPS-RP-HPLC was collected for further MS-based identification and the other half was directly injected into ESI-TOF MS for quantification (LCT, Micromass, Manchester, UK). ESI-TOF MS was externally calibrated by directly infusing NaI-CsI standard solution and normalized internally by the peak area of 1 μg of insulin (Sigma-Aldrich) added as an internal standard. The intact *M*r values were obtained from the deconvolution of the combined ESI-TOF spectra by MaxEnt I software (MicroMass) using a target mass range of 4-95 kDa, resolution of 1 Da, peak width of 0.75 Da, and peak height value of 65% as fixed parameters.
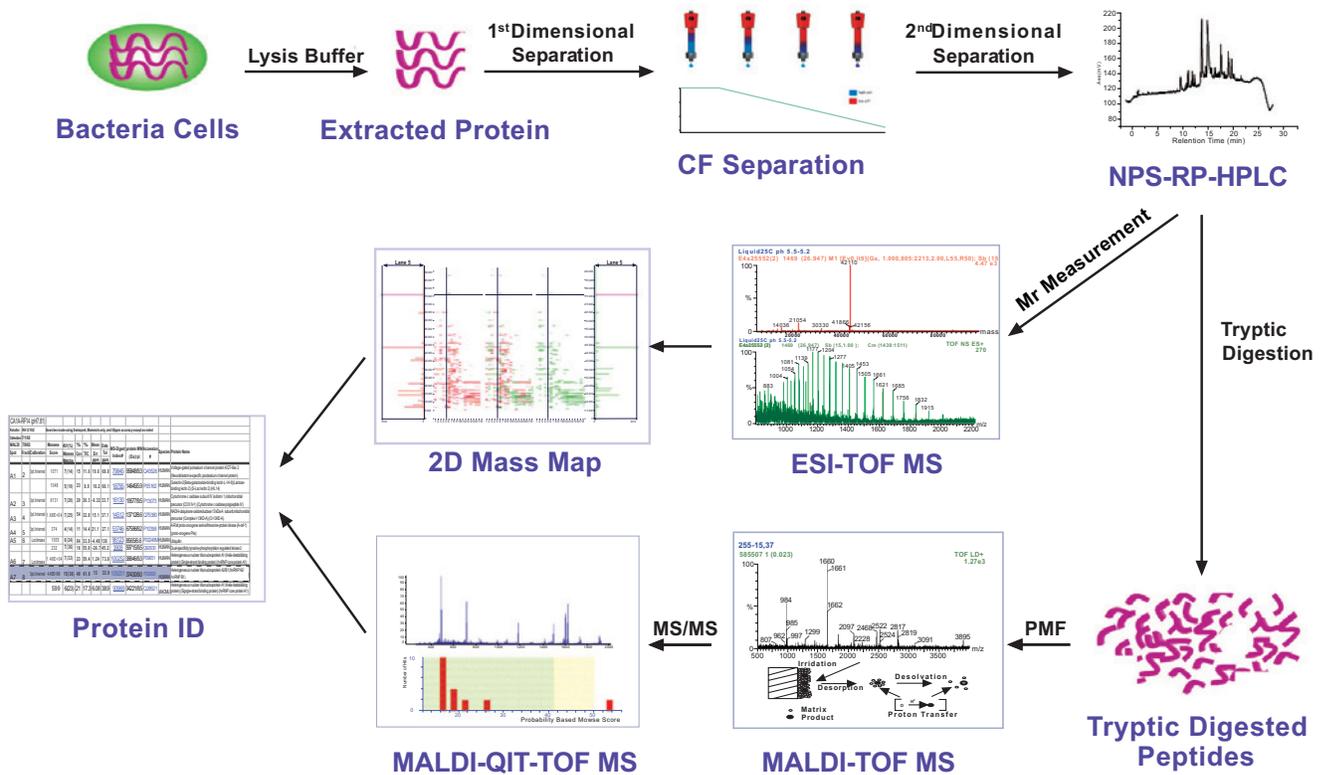
Fig. (1). Experimental overview of 2-D liquid phase separation combined with MS for proteomic analysis of cryotolerance in *E. sibiricum* 255-15.

Quantification of the separated proteins was achieved by integration of the molecular ion peak obtained from deconvolution of multiple charged mass spectra using protein Trawler (BioAnalyte Inc., South Portland, ME) in conjugation with MassLynx 4.0 (MicroMass). A total ion chromatogram (TIC) was automatically examined for multi-charged protein spectra, which were deconvoluted and merged to provide accurate integration of the molecular ion peak for every protein. Quantification of each run was normalized with the intensity of 0.5 μg bovine insulin to eliminate the differences in ESI efficiency among runs. Protein Trawler parameters for raw MS data processing were set as follows: 800-5000 m/z for input mass range, 30 s for TIC combine time, 20% of the base peak for output threshold, 100 for the maximum of number of deconvoluted peaks, 5-80 kDa for output mass range, 2 Da for output resolution. The measured intact molecular weight values and intensities of peaks were summarized into a mass map, which is an analogous to a 2D gel image and was utilized for the proteome comparison between samples.

Tryptic Digestion

Fractions for MS based identification were first reduced to a volume of 80 μL using a SpeedVac concentrator (Centrivap Concentrator, Labconco, Kansas City, Missouri) to remove acetonitrile and TFA. The remaining TFA in each fraction was neutralized by adding 10 μL of 1 M ammonium bicarbonate, NH_4HCO_3 (Sigma-Aldrich) while the proteins in the fraction were reduced by adding 10 μL of 100 mM dithiothreitol, DTT (Sigma-Aldrich). The resulting mixtures

were incubated at 37°C for 20 min, and then 0.5 μg N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) -treated trypsin (Promega, Madison, WI) was added to start the digestion. After being maintained at 37°C for 24 hours, digestion was terminated by adding 2 μL TFA (Sigma-Aldrich). Tryptic digests were then desalted and pre-concentrated in 5 μL of 60% acetonitrile with 0.1% TFA by C18 Zip-Tips (Millipore, Inc.) before MS-based identification.

Protein Identification by PMF and Peptide Sequencing

For PMF by MALDI-TOF MS, peptide mass was measured on a MicroMass TofSpec2E system (MicroMass/Waters, Milford, MA) equipped with a 337 nm nitrogen laser source and delayed extraction. The MALDI-TOF MS was operated in positive ion reflector mode and the final MALDI spectrum was an average of 100-150 spectra and calibrated with an internal standard mixture of Angiotensin I ([M+H]$^+$ 1296.69), human adrenocorticotropic hormone fragment 1-17 (ACTH 1-17 ([M+H]$^+$ 2093.09)), and ACTH 18-39 ([M+H]$^+$ 2465.20) within 50 ppm (the chemicals were purchased from Sigma-Aldrich). The matrix used is α-cyano-hydroxycinnamic acid, α-CHCA (Sigma-Aldrich). The peptide masses were analyzed using MassLynx 4.0 over the range of 800- 4000 Da and then submitted to MS-Fit to search against the NCBInr database (released on Feb 26, 2006) with a mass tolerance of 50 ppm and one missed cleavage as fixed parameters.

Mass spectrometric peptide fragmentation and sequencing was performed on MALDI-QIT-TOF MS (Shimadzu Corporation, Kyoto, Japan and Kratos Analytical, Manches-

ter, UK) in the positive ion mode using an external calibration with a mixture of bradykinin fragment 1-7 ($[M+H]^+$ 757.40), angiotensin II ($[M+H]^+$ 1046.54), P14R ($[M+H]^+$ 1533.86) and ACTH fragment 18-39 ($[M+H]^+$ 2465.20) (the chemicals were purchased from Sigma-Aldrich). The matrix used in this case was 2,5-dihydroxy benzoic acid, DHB (Sigma-Aldrich) solution with a concentration of 10 mg/mL. Data acquisition and processing were controlled by Kompact software (Kratos Analytical Ltd., Manchester, UK). The parent ion mass and the resulting fragment ion masses were searched against the NCBInr database using Mascot 1.8 (Matrix Science, London, UK) setting a peptide tolerance of 1.2 Da, MS/MS tolerance of 0.6 Da, and one missed cleavage site as fixed parameters.

RESULTS

Freeze-Thawing Tolerance

Cell survival was monitored after repeated cycles of freeze-thaw treatments (Fig. **2**). For liquid broth growth at 25^oC, the cell viability was lost by more than 50% after 9 cycles and about 80% after 20 cycles. Cells grown in liquid broth at 4^oC or on agar surface at either 25^oC or 4^oC showed high viability after 20 cycles of freeze-thaw treatments. Bacteria grown in liquid medium at 4^oC tolerate freeze-thawing much better than those grown at 25°C in the same medium. However, when grown on agar, they tolerate freeze-thawing equally well regardless of the growth temperature.
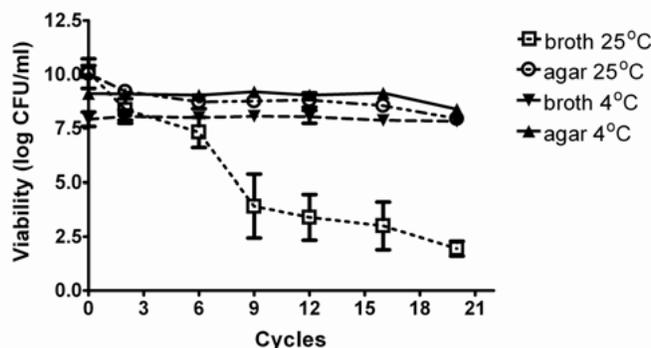
Fig. (2). Viability of *E. sibiricum* 255-15 cells grown in liquid broth or on solid agar medium at both 25^oC and 4^oC after repetitive cycles of freeze-thaw treatments.

Protein Separation and Comparison of 2-D Mass Maps of Protein Expression in Cells Grown Under Different Conditions

Protein extracts from *E. sibiricum* 255-15 were first fractionated by CF according to p*I* in the first dimension and each p*I* fraction was subsequently separated by NPS-RP-HPLC based upon the hydrophobicity before MS-based protein identification. The CF separation was achieved in approximately one hour at a flow rate of 0.2 mL/min. During the pH gradient, the proteins were collected from pH 8.5 to 4.0 at 0.3 pH unit change. For each p*I* fraction, the corresponding chromatogram of NPS-RP-HPLC separation was achieved within 35 minutes, resulting in resolution of ca. 10-100 protein bands.

A mass map was generated by integrating *M*r, p*I* and protein abundance from all the 18 CF fractions (15 pH frac-

tions from pH 8.5 to 4.0, and three NaCl wash fractions) into one single image to compare the protein expression of *E. sibiricum* 255-15 at the different growth temperatures and media. In this study, we focused on the proteins expressed to protect against the freeze-thaw treatments and thus to increase the bacterial cryotolerance. One such map is shown in Fig. (**3A**), which represents the comparison of 2-D mass maps between cells grown in liquid broth at 25° (left) and on agar surface at 25°C (right). The differential map shown in the middle was obtained by point-by-point subtraction. The 18 lanes in the mass map represent all the p*I* fractions from the CF separation, while the vertical axis indicates the *M*r of intact proteins in each p*I* fraction. The mass accuracy in 2-D mass map was determined by the quality of the protein umbrella spectra from ESI-TOF MS analysis and is usually less than 100-200 ppm, which is 200-1000 times better than the 5-10% typically achieved in 2-DE separation. The accurate *M*r values of intact proteins, together with p*I*, provide essential information for protein identification and characterization. The reproducibility of protein profiling by mass mapping is displayed in Fig. (**3B**), which includes two differential maps of all the 18 CF fractions from two duplicate experiments.

Proteins Identified by PMF and Peptide Sequencing

Proteins eluting from HPLC were digested by trypsin and then identified by peptide mass fingerprinting (PMF) and peptide sequencing using MALDI-TOF MS and MALDI-QIT-TOF MS/MS respectively. The identification from PMF was obtained by searching for the best match between the experimentally determined intact masses of the peptides in the peptide map and those calculated by theoretical cleavage of the proteins in a sequence database. The PMF based identification was based on the indication that at least five peptides matched with mass accuracy within 50 ppm and sequence coverage of at least 20% [14], which was further confirmed by protein *M*r and p*I*. In this work, around 42% of the proteins detected by ESI-TOF MS were identified by PMF using MALDI-TOF MS.

MS/MS based peptide sequencing provides a more powerful proteomic technique with higher sensitivity and accuracy in protein identification and was used to confirm these IDs. Protein identification by peptide sequencing is successful when the search score is higher than, or equal to, the homology or identity threshold scores in each search. In this case, multiple peptides are usually found and often all of their fragment spectra are used to correlate to a protein. In both PMF and peptide sequencing, the larger the number of peptides identified the greater the confidence in the protein identification. Small proteins that are difficult to identify by PMF due to an insufficient number of detected peptides (<5) may be identified using peptide sequencing if the peptide signal and its fragment signals are of sufficient intensity. Proteins were identified with confidence when at least two MS/MS sequenced peptides matched a protein above the MASCOT significance level indicating identity or extensive homology. Fig. (**4**) displays two MS/MS spectra of peptides from the digest of hypothetical protein (18.900 kDa, p*I* 5.4). The matched peptide sequences, DDATDETSGASWIDQVK and FIGIFHDESSLHQK, confirmed the protein identification from MALDI-TOF MS based PMF experiment. The discrepancy of 575 Da between the experimental and
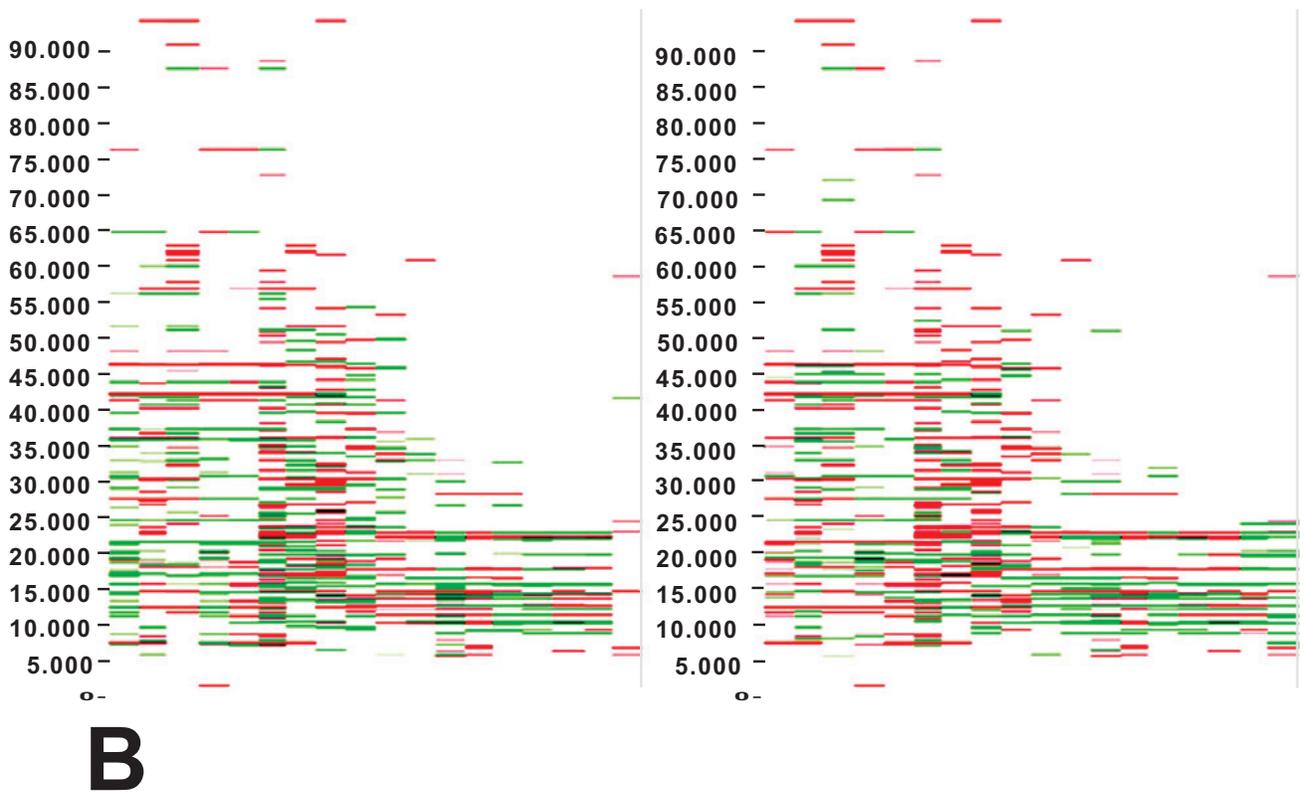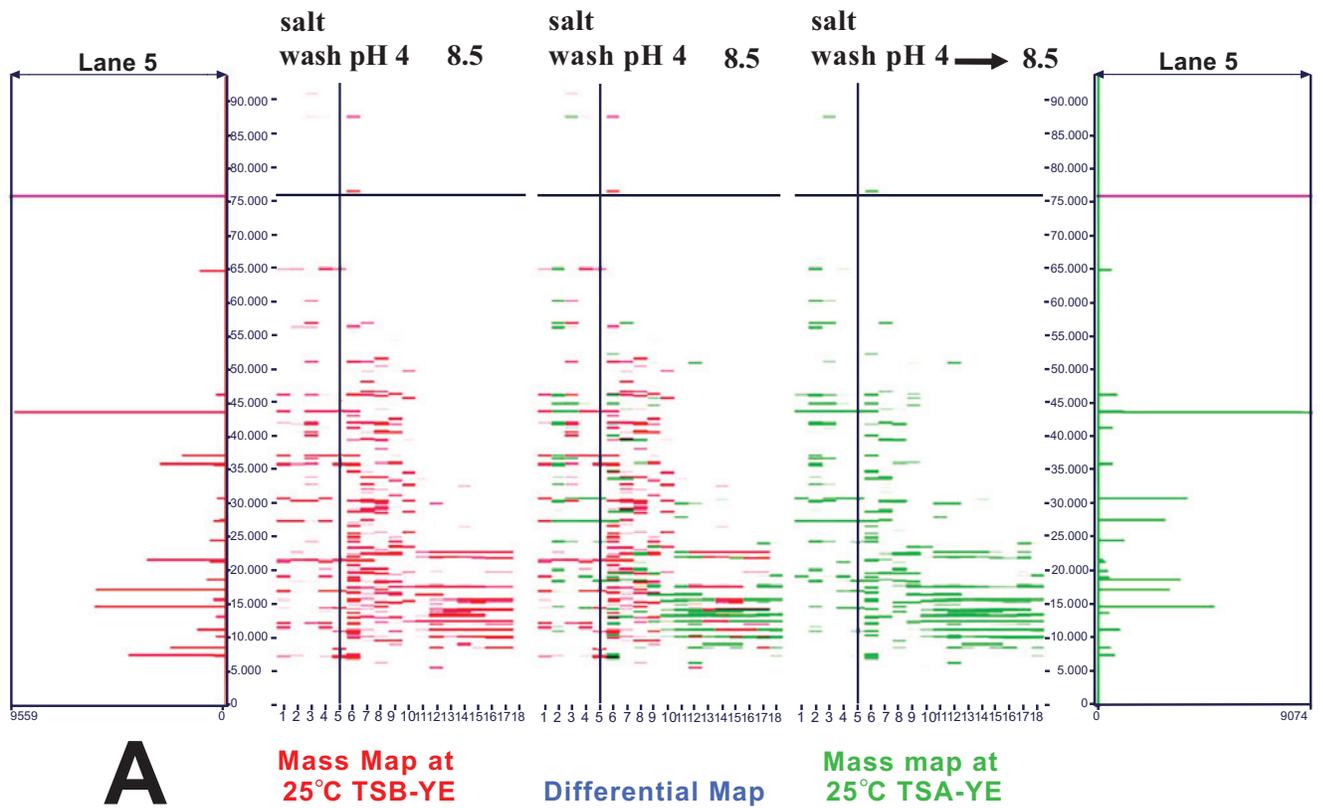
Fig. (3). (**A**) 2-D mass map of *E. sibiricum* 255-15 after 25°C TSB-YE liquid broth growth (left) and 25°C TSA-YE agar surface growth (right) of all CF fractions in *M*r range of 5-95 kDa with the mass map of lane 5 from each sample highlighted on the left and right plot respectively. Differential mass map shown in the middle was generated through point-by-point subtraction. (**B**) Reproducibility demonstration of protein profiling by 2-D mass mapping is generated by two independent CF/NPS-RP-HPLC/ESI-TOF MS experiments with two groups of samples cultured separately. Examples shown in this figure are the comparisons of 2-D mass maps between *E. sibiricum* 255-15 after 25°C (green) and 4°C (red) TSB-YE liquid broth growth.
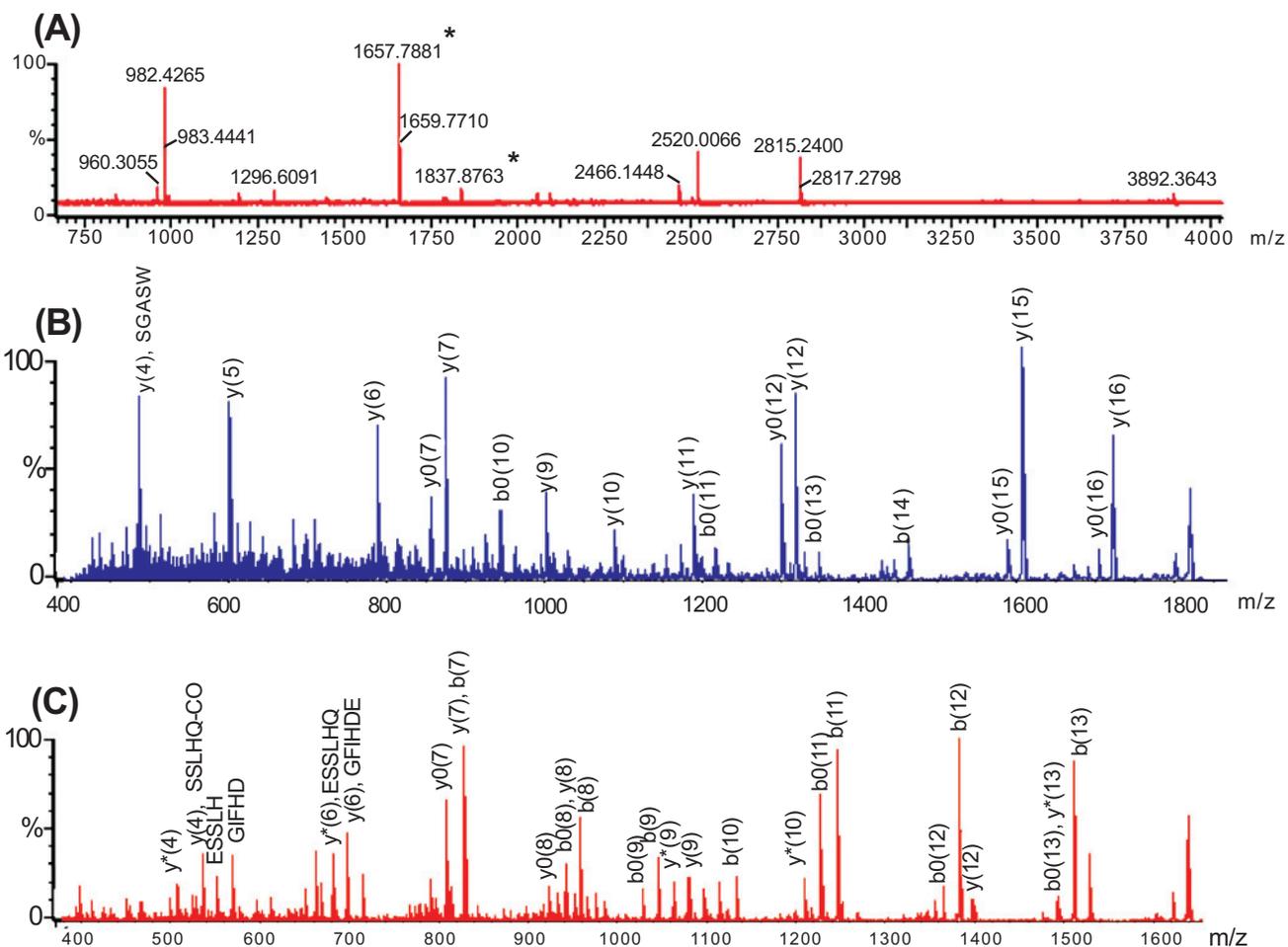
Fig. (4). (**A**) MALDI-TOF MS spectrum of protein (18.900kDa, p*I* 5.44). MALDI-QIT-TOF MS/MS fragmentation of peptide 1837.8 (DDATDETSGASWIDQVK) was shown in (**B**) and peptide 1657.9 (FIGIFHDESSLHQK) in (**C**). Both MS/MS spectra confirm the protein identity. In this case, y*(i)=y(i)–NH$_3$, y0(i)=y(i)–H$_2$O, b0(i)=b(i)–H$_2$O.

theoretical *M*r values of this protein resulted from the loss of the first five amino acids (MVLTM).

Cold Shock Proteins in Inducing Cryotolerance

No consistent differences in the expression of CSPs in the bacteria grown under different conditions were observed (Table **1**), suggesting that there is no direct correlation between cryotolerance and the CSPs expression prior to freezing. The putative CSP of 7.154 kDa and two CSPs of 7.409 kDa with p*I* 4.5 and p*I* 4.4 respectively were found similarly expressed in the same medium regardless of the temperature. Interestingly, they were up-regulated in liquid media compared with solid media.

Table 1.   **Cold Shock Protein Expressed at Different Growth Conditions**

Protein ID (Access. No.)	Exp. *Mr*	Exp. p*I*	Theoretical	Expression Ratio		
			Mr/p*I*	L25/L4 [1]	L25/S25 [1]	L25/S4[1]
Cold shock protein (68055399)	7154	4.0-4.6	7150/4.5	1.4	5.1	4.6
Cold shock protein (68053997)	7413	4.0-4.6	7409/4.5	1.2	45	36
Cold shock protein (68053999)	7413	4.0-4.6	7409/4.4			

[1] In discussion of expression ratio, L25 indicates 25°C TSB-YE liquid broth growth, L4 indicates 4°C TSB-YE liquid broth growth, S25 indicates 25°C TSA-YE agar surface growth, and S4 indicates 4°C TSA-YE agar surface growth. Protein quantification was based on its peak area from ESI-TOF MS analysis. For example the expression ratio L25/S4 was obtained by dividing the amount of protein expressed at 25°C in liquid broth growth with the amount of the same protein expressed at 4°C on agar surface growth.

Variation of Cellular Proteins According to Different Growth Conditions

The identification of about 330 soluble proteins, which represented 60% of the proteins resolved by 2-D mass mapping, was used as a starting point for physiological studies of *E. sibiricum* cells. Observation of the 2-D mass maps of Siberian permafrost bacterium *E. sibiricum* 255-15 revealed some changes in response to the different growth temperatures and media. However, the number of proteins whose relative expression level varied more than two-fold under different growth conditions was low and represented ca. 15% of the total cellular proteins detected by 2-D liquid separation with MS technology.

Down-Regulation of Proteins Associated with the Improved Cryotolerance

When compared with the protein expression in the cells grown in liquid at 25°C, 2-D mass maps revealed down-regulation of 20 identified proteins in liquid growth at 4°C as well as on agar growth at either temperature (Table **2**). We observed a significant suppression of several glycolytic enzymes (phosphoglycerate kinase, enolase, fructose-1, 6-bisphosphate aldolase, pyruvate kinase, glucose-6-phosphate isomerase, pyruvate dehydrogenase) in the cells grown in liquid broth at 4°C and on agar surface at either 25°C or 4°C. Similar suppression was observed with other energy metabolic enzymes such as acetate kinase, NAD+ synthase, ATP synthase F1 beta subunit, iron-containing alcohol dehydrogenase, NADH flavin oxidoreductase and FAD dependent oxidoreductase. Enzymes in metabolism of lipids, amino acids, nucleotides and nucleic acids were also suppressed. Growing the cells on agar enhanced suppression of some enzymes. Glucose-6-phosphate isomerase, pyruvate kinase, and FAD dependent oxidoreductase were not even detected when the cultures were grown on the solid medium. Iron-containing alcohol dehydrogenase was drastically reduced while other proteins, including acetate kinase, were only marginally affected by surface cultivation. Purine nucleoside phosphorylase I was suppressed more during growth at 4°C. The low incubation temperature seemed to have a greater impact when coupled with agar growth for the majority of the 20 listed proteins. Surprisingly, a few enzymes exhibited the greatest suppression when the cells were grown at 25°C on agar (Table **2**), such as enolase, pyruvate kinase, phosphodiesterase, and MECDP synthase. Pyruvate kinase was the only enzyme not detected in any of the cultures grown under conditions that were associated with the increased cryotolerance, and was instead only expressed in cells grown at 25°C in liquid. Porphobilinogen synthase and certain other proteins (enolase, pyruvate dehydrogenase and hypothetical protein 68055276) were similarly expressed in the cells grown in 4°C liquid broth and on agar surface at either 25°C or 4°C (Table **2**).

Up-Regulation of Proteins Associated with the Improved Cryotolerance

At the same time, the consistent up-regulation of eight other proteins was observed (Table **3**). The expression of the putative 2'-5' RNA ligase, hypoxanthine phosphoribosyl transferase, FeS assembly ATPase SufC, and uncharacterized conservative protein (680550340) was especially in-

duced in surface grown bacteria of *E. sibiricum* 255-15 (Table **3**). The putative hypoxanthine phosphoribosyl transferase and thioredoxin reductase were especially induced on agar in the cold (Table **3**). A hypothetical protein of 24460 kDa, which was identified as phage shock protein A (IM30) using the database released in 2005, was also found consistently over-expressed in the cells associated with improved cryotolerance. Comparison of the protein sequence of this hypothetical protein to proteins in the GenBank, RefSeq, PDB, SwissProt, PIR, and PRF databases using the BLAST algorithm identified two highly similar protein sequences (Fig. **5**). PspA (IM30) sequences in *Bacillus cereus* and *Bacillus anthracis* are at least 25% identical and almost 50% similar over their entire length with this hypothetical protein in *E. sibiricum* 255-15.

DISCUSSION

The experiments with *E. sibiricum* 255-15 showed significant impact of growth temperature and solid (structured) medium on cryotolerance [10]. Hence, we assumed that similar cryotolerance mechanism may be involved during both liquid broth growth at cold and surface growth regardless of temperature. Low temperature growth may increase cryotolerance by altering the composition of the cytoplasmic membrane, inducing ice nucleation activity or phenotypically changing the cell formation [15, 16]. On other hand, solid (agar)-medium, which is defined as a complex structured habitat, may develop multiple physical and chemical gradients (stresses) and may promote diverse adaptations including a freeze tolerance [10].

It therefore appears that even short-term cold shock treatments prior to freezing greatly improved the cryotolerance in mesophilic bacteria. Such correlations between cold shock treatment and cryotolerance were first established in mesophilic bacteria *Bacillus subtilis* and *Escherichia coli*. When *B. subtilis* and *E. coli* cultures grown at 37°C were cold shocked at 10°C for 2-6 h, they yielded a significantly higher cell viability following freeze-thawing compared to cultures frozen without the cold shock treatment [17, 18]. Cold shock-induced cryotolerance was also displayed in *Lactococcus lactis* [19-22]. While significant improvement of the bacteria's cryotolerance after the cold shock treatment has been investigated in *E. coli*, *B. subtilis*, and *L. lactis*, little is known about the mechanisms that mediate survival following freezing.

Transient up-regulation of proteins that are defined as cold shock proteins (CSPs) has been shown to occur following an abrupt shift to low temperature. Do CSPs play a role in cryotolerance? Currently, only limited studies that linked CSP level with the protection against freezing have been performed, mainly in *L. lactis*. It is reported that an increased survival rate (~100 fold) upon freezing of cold adapted *L. lactis* coincided with an increase of CSP expression. Also an up-regulation of CspD, CspE, and CspB seemed to increase the survival from freezing 2-10 fold compared to control cells [22, 23]. The above studies provide evidence that the CSPs expression level after freezing is correlated with the cold-induced cryotolerance in *L. lactis*. However, is there a correlation between CSPs expression prior to freezing and cryotolerance? In the current study, we investigated the putative CSPs expressed at different growth temperatures (25°C

Table 2.   **Protein Down-Regulated at 4°C TSB-YE, 4°C TSA-YE and 25°C TSA-YE Growth that are Associated with the Improved Cryotolerance**

Protein ID (Access. No.)	Exp. *Mr*	Exp. p*I*	Theoretical *Mr*/p*I*	Expression Ratio[1]		
				L25/L4 [2]	L25/S25 [2]	L25/S4 [2]
1. INTERMEDIARY METABOLISM						
1.1. Energy Metabolism						
1.1.1. Glycolysis and Glycolysis-Related Pathways						
Enolase (68056418)	46201	4.9-4.6	46329/4.7	2.7	3.1	2.6
Ketose-bisphosphate aldolase, class-II:Fructose-1,6-bisphosphate aldolase, class II (68054326)	30328	4.9-4.0	30458/5.0	3.4	6.2	12
Phosphoglycerate kinase (68056421)	42107	5.2-4.9	42103/5.1	2.2	3.5	22
Pyruvate kinase (68055923)	62770	5.2-4.9	62772/5.2	+++	+++	+++
Glucose-6-phosphate isomerase (68056048)	49322	5.5-5.2	49448/5.2	6.9	+++	+++
Pyruvate dehydrogenase/lipoamide (68056114)	40141	5.2-4.6	40137/5.1	2.7	3.5	2.2
1.1.2. Anaerobic Respiration						
Acetate kinase (68055938)	44146	5.2-4.9	44272/5.1	6.7	22	36
1.1.3. ATP-Proton Motive Force Interconversion						
NAD+ synthase (68056126)	30070	5.8-5.2	30059/5.3	4.2	2.2	14
ATP synthase F1, beta subunit (68054271)	51563	5.5-5.2	51562/4.9	6.8	5.6	+++
1.1.4. Fermentation						
Iron-containing alcohol dehydrogenase (68053559)	94143	6.1-5.8	94287/6.0	2.1	160	190
1.1.5. Electron Transport						
IMP dehydrogenase/GMP reductase:NADH:flavin oxidoreductase/NADH oxidase (68054616)	37115	5.8-5.5	37118/5.5	2.5	2.2	4.9
1.1.6. Metabolism of Sugar						
FAD dependent oxidoreductase (68056283)	61679	5.8-5.5	61681/5.7	10	+++	+++
1.2. Metabolism of Nucleotides and Nucleic Acids						
Inosine guanosine and xanthosine phosphorylase:Purine nucleoside phosphorylase I, inosine and guanosine-specific (68055531)	29500	5.2-4.9	29497/4.9	19	4.9	20
Pyrimidine-nucleoside phosphorylase (68055530)	46094	5.2-4.9	46088/5.0	2.9	2.1	6.7
1.3. Metabolism of Lipid						
[Acyl-carrier protein] phosphodiesterase (68054042)	23640	5.5-5.2	23639/5.1	2.0	6.9	2.3
MECDP synthase (68056360)	17512	5.2-4.6	17540/5.3	2.5	28	13
1.4. Metabolism of Amino Acids and Related Molecules						
Porphobilinogen synthase (68055121)	36246	5.8-5.2	36207/5.3	6.6	7.2	7.3
2. PROTEIN OF UNKNOWN FUNCTION						
Hypothetic protein (68055276)	18346	5.8-5.2	18900/5.4	3.4	2.2	2.5
Conserved hypothetical protein (68055483)	21085	5.2-4.6	21042/5.2	2.9	+++	7.6
Conserved hypothetical protein (68056068)	22599	5.5-4.9	22596/4.8	4.2	22	49

[1] The expression ratios of the proteins that were exclusively expressed at 25°C in TSB-YE liquid medium were shown as "+++".In this case, the ratio is apparently infinite because the denominator could not be distinguished from zero.

[2] L25 indicates 25°C TSB-YE liquid broth growth, L4 indicates 4°C TSB-YE liquid broth growth, S25 indicates 25°C TSA-YE agar surface growth, and S4 indicates 4°C TSA-YE agar surface growth. Protein quantification was based on its peak area from ESI-TOF MS analysis.

Table 3. **Protein Up-Regulated at 4°C TSB-YE, 4°C TSA-YE and 25°C TSA-YE Growth that are Associated with the Improved Cryotolerance**

Protein ID (Access. No.)	Exp. *Mr*	Exp. p*I*	Theoretical *Mr*/p*I*	Expression Ratio		
				L4/L25 [1]	S4/L25 [1]	S25/L25 [1]
Protein of unkown function (68054621)	14582	4.3-4.0	14578/4.3	2.04	4.17	3.23
2',5' RNA ligase (68056138)	19752	5.8-5.2	19743/5.4	4.55	20.4	22.2
Hypothetical protein (68055956)	20237	4.6-4.0	20367/4.7	17.5	35.7	2.08
Hypoxanthine phosphoribosyl transferase (68055907)	20294	5.5-4.9	20292/4.9	5.88	23.3	17.5
Conserved hypothetical protein (68054709)	24467	4.9-4.3	24460/4.9	76.9	6.25	7.14
FeS assembly ATPase SufC (68056341)	28809	5.2-4.9	28807/4.9	2.17	32.3	3.45
Uncharacterized protein conserved in bacteria (680550340)	35710	4.6-4.0	35845/4.5	10.0	116	119
Thioredoxin reductase (68055606)	36634	5.8-5.2	36622/5.3	29.4	90.9	32.7

[1] L25 indicates 25°C TSB-YE liquid broth growth, L4 indicates 4°C TSB-YE liquid broth growth, S25 indicates 25°C TSA-YE agar surface growth, and S4 indicates 4°C TSA-YE agar surface growth. Protein quantification was based on its peak area from ESI-TOF MS analysis.

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E. sibiricum    MKNVFDQLNEFANQMMTEVKKAADQGEA--PAKKLVRHIRTTESDLKEID----RLLERQRTLLAELTQKQDE-----AKEL      71
B. cereus       MKQSLFQRVRDA--ILADLHNVLDEKERKNPIAMLNQYLRDSEREITKIE----KLIIERHKTLKTNFARELEE-----ARYF      71
B. anthracis    MKQSLFQRVRDA--ILADFHNVLDEKERKNPIAMLNQYLRDSEREITKIE----KLIQRHKTLKSNFARELEQ-----ARYF      71
T. erythraeum   M----GI-DHFWRVIRANVNSLISNAED--PEKILEQTIKNMQNDLVKL---------RQVVAEAIATQKQIERQSYQAKST      67
C. perfringens  M----GIFNRISNMIKAKTNSALDEMEN--PVELLDQKIRDMEKSFNEGK----RSSAQIFGNLKDTEKKMNE-----AKEE      67
Y. bercovieri   M----GIFSRFADIVNANINTLLDKAED--PQKLVRLMIQEMEDTLVEIRSTSARALAEKKQLLRRIDHSESQ-----QQEW      71


E. sibiricum    ADKRFAQVEIAKEAGEQELAERA-----------AIESKHYGEQFR--FFEELIVETKRELNQLEREALELKLKLEDLQNK     139
B. cereus       VNKRSKQAIIAQEAGELQLHERA-----------LEEVAYYEGQVA--RLEEMYAGVVEQIDELERRLSEMKNKLKEMNAK     139
B. anthracis    VNKRSKQAIIAQEAGELQLHERA-----------LEEVAYYEGQVT--RLEEMYAGVVEQIDELERRLSEMKNKLKEMHAK     139
T. erythraeum   ADEWYRRAHLALQKGQENLAREA-----------LTRRNSYQETAT--VMKVQMEQQKQVVEKLKQNMKQLEHKVSEAKLK     135
C. perfringens  SAQYDEKVRLAMSKGNEELAKKALKLKLDSDKKFESLKASYEGQRAKADVLKGKLVELEKELDK---TRSYRDEAVARLNNA     146
Y. bercovieri   QDK----AELALRKDKEDLARAA-----------LIEKQKVAALIE--TLTREVATVDETLSR-----------------     117


E. sibiricum    RYEWMMRENVSNLKSKMNQVLER--EPNATIKQEQ     172
B. cereus       RMQLMARENMAHANRRMNTALHKMDESNPFLRFEE     174
B. anthracis    RMELMARENMAHANRRMNTAMHKMDENNPFLRFEE     174
T. erythraeum   KNMYIARARSARASEKLNKMLDLVNTGNTLSTFEE     170
C. perfringens  EASKQINEVIANVQSKSNSINIDDIERSISRKESY     181
Y. bercovieri   -----MKHEITELESKLTETRAR--QQALTLRHQA     145
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Fig. (5). Alignment of hypothetic protein of 24460 Da in *E. sibiricum* 255-15 with putative homologous phage shock protein A from *Bacillus cereus*, *Bacillus anthracis*, *Trichodesmium erythraeum*, *Clostridium perfringens* and *Yersinia bercovieri*. Identical residues are boxed in black and similar residues are boxed in gray.

and 4°C) and media (liquid broth and agar surface). The major homologous CSPs of *E. sibiricum* 255-15 were similarly expressed in the same medium regardless of the temperature, and they were up-regulated in liquid medium compared with agar. Our results suggest that genes for CSPs are turned on continuously to produce "shock" proteins to protect cells from damage during abrupt changes in environmental conditions. However, cold-shock stressed (5h at 4°C) cells of *E. sibiricum* 255-15 (24°C, liquid broth) did not show significantly improved cryotolerance [10]. Therefore, our findings suggest that cold growth (acclimation), rather than cold shock, is required for the enhanced protection to repetitive freeze-thawing stresses of *Exiguobacterium* ssp.

Down-regulation of 20 proteins associated with the improved cryotolerance was observed at different growth tem-

peratures and media. The cells grown at conditions that enhance their cryotolerance display a significant suppression of several glycolytic and energy metabolic enzymes as well as enzymes involved in metabolism of lipids, amino acids, nucleotides and nucleic acids. The temperature dependent changes in metabolic pathways could explain the temperature specific carbon source utilization observed in *E. sibiricum* 255-15 [12]. Contrary to our study low-temperature stress induces the glycolytic activity in lactic acid bacterium *L. lactis* and psychrotrophic *Rhizobium* strains [24, 25]. However, the temperature impact on the regulation of the similar proteins could be various in bacteria from the different genera. Thus, the inhibition of ATPase activity of *E. coli* F1F0 ATP synthase was observed at 15°C compared to 37°C [26], while F1F0 ATP synthase b subunit was up-regulated by low temperature growth in permafrost bacterium *Psy-*

chrobacter cryohalolentis K5 [27]. Growing the cells on agar also enhanced suppression of some enzymes. For instance, glucose-6-phosphate isomerase, pyruvate kinase, and FAD dependent oxidoreductase were not even detected when the cultures were grown on the solid medium. Iron-containing alcohol dehydrogenase was drastically reduced. In bacteria, alcohol dehydrogensase plays an important part in fermentation in which NAD+ is regenerated so that the energy-generating glycolysis can continue. The effect of low temperature or agar growth alone on the suppression of several proteins was not as clearly pronounced as when the low incubation temperature was coupled with agar growth resulting in a greater impact for the majority of the proteins described. A few studies showed that the agar grown cells are more likely to express a phenotype to a much greater extent than cells from broth cultures [28, 29]. Interestingly, pyruvate kinase was expressed in cells grown at 25°C in broth but not in any of the cells grown under conditions that were associated with the increased cryotolerance. Though, the increase of freeze tolerance and oversynthesis of 4 cold induced proteins including pyruvate kinase was observed in cold stressed *Lactobacillus acidophilus* RD758 [30]. Several proteins known as cold inducible proteins in other bacteria were down-regulated in *E. sibiricum* 255-15 at low temperature, nevertheless the cells were resistant to repetitive freeze-thawing. These findings suggest that *E. sibiricum* 255-15 may have evolved unknown protective mechanisms against freezing.

In the course of this study we observed up-regulation of 8 proteins at low temperature or solid (agar) medium. These results suggest these proteins may significantly enhance cryotolerance. What do we know about these proteins and their role in cold adaptation and freeze tolerance? Our previous proteomic analysis of cold adaptation in *E. sibiricum* 255-15 reveals 39 proteins that were preferentially or uniquely expressed at 4°C [13]. The latter and current studies confirmed that enzyme 2'-5' RNA ligase was up-regulated at 4°C as well as agar surface growth regardless of the temperature. Overexpression of the RNA ligase in *E. coli* led to reduction of growth rates and a temperature-sensitive phenotype in both wild-type and RNA ligase knockout strains [31]. The activity of purified RNA ligase was studied *in vitro* and the enzyme was found to be reversible, indicating that this enzyme may perform either cleavage or ligation *in vivo* [31]. Another study has shown the involvement of DNA ligase in recovery of *E. coli* from cold shock [32].

However there are no direct indications that proteins such as hypoxanthine phosphoribosyl transferase (HPRT), FeS assembly ATPase SufC, and thioredoxin reductase play any role in cold adaptation and freeze tolerance in bacteria, while their expression may increase upon other stresses. HPRT is a key enzyme for purine salvage [33, 34]. In living organisms, purine nucleotides needed for cellular metabolism or used as the precursors of DNA and RNA are synthesized either *via* the salvage mechanisms that recycle pre-formed purine bases, or *via de novo* pathway starting from amino acid, carbon dioxide and ammonia. HPRT of distant organisms may share extensive sequence identity [35] and this enzyme is readily released from the bacterial periplasm upon osmotic shock [36, 37]. Although the exact functions of FeS assembly ATPase SufC are still unknown, ATPase was found to be involved in the biosynthesis of Fe-S clusters [38], whereas

SufC revealed the presence of an ABC transporter signature and SufC does indeed bind and hydrolyze ATP [39]. This class of ATPases is mostly found to be associated with membrane proteins, forming a trans-membrane complex [40]. Expression of SufC is increased under oxidative stress for the protection of labile [Fe-S] clusters, which present in various proteins with enzymatic or regulatory functions [38, 41, 42]. Thioredoxin reductase is a member of the family of dimeric flavoenzymes that catalyze the transfer of electrons between pyridine nucleotides and disulfide/dithio compounds and promote catalysis *via* FAD and a redox active disulfide [43-46]. Thioredoxin reductase plays several key roles in maintaining the redox environment of the cell. The role of these proteins in cryoprotection should still be evaluated.

Four up-regulated proteins were hypothetical, uncharacterized or proteins of unknown functions. Conserved hypothetical protein (68054709) was annotated as phage shock protein A (IM30). The apparent conservation of the PspA protein sequence between closely related bacteria (*E. sibiricum*, *B. cereus*, and *B. anthracis*) as well as distantly related bacterial species such as *Yersinia bercovieri*, *Clostridium perfringens*, and *Trichodesmium erythraeum*, suggests an ancient origin for this protein. So far, the exact function of PspA remains elusive. It is shown in previous research that PspA was induced under various stress conditions including severe heat shock, cold shock, osmotic shock, and exposure to ethanol [47-49] that all might lead to the dissipation of the proton-motive force. Since, a portion of PspA was observed to be present in the cytoplasmic protein fraction in *E. coli* [50], it is believed that expression of PspA helps to sustain the proton motive force across the plasma membrane [50-52].

CONCLUSION

In this study, we have presented the proteomic analysis of the effect of the growth temperature and medium on bacterial cryotolerance in *E. sibiricum* 255-15. The use of 2-D liquid separation and MS-based proteomics revealed the growth temperature and medium dependent changes in protein synthesis related to the improved cryotolerance. A total of 28 proteins displayed cryoprotection-related alterations: 8 proteins were up-regulated and 20 proteins were down-regulated. Thus the up-regulated 8 proteins may induce the improved cryotolerance, and the down-regulation of 20 proteins supply additional freeze-thawing protection [53].

The results of this study would be applicable in many technologies for improving cryoprotective characteristics in useful microorganisms and for controlling cold-resistant pathogen bacteria. An understanding of mechanisms contributing to cryotolerance would be also helpful in evaluation of possibilities for life beyond earth, particularly because seven of the eight planets of our solar system, as well as their satellites, comets and asteroids have a cryogenic nature.

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ABBREVIATION

CF	=	Chromatofocusing
p*I*	=	Isoelectric point
NPS-RP-HPLC	=	Nonporous silica reversed phase high performance liquid chromatography
ESI-TOF-MS	=	Electrospray ionization time-of-flight mass spectrometer
*M*r	=	Molecular weight
PMF	=	Peptide mass fingerprinting
MALDI-TOF MS	=	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MALDI-QIT-TOF	=	MSMatrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry

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