

Expression of CspE by a Psychrotrophic Bacterium *Enterobacter ludwigii* PAS1, Isolated from Indian Himalayan Soil and *In silico* Protein Modelling, Prediction of Conserved Residues and Active Sites

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Abstract Proteome analysis of *Enterobacter ludwigii* PAS1 provide a powerful set of tool to study the cold shock proteins along with that combination of bioinformatics is useful for interpretation of comparative results from many species. There is a considerable interest in the use of psychrotrophic bacteria for nitrogen fixation, especially at hilly regions, thus better understanding of cold adaptation mechanisms too. The psychrotrophic *E. ludwigii* PAS1 grown at 30 and 4 °C, isolated from Himalaya soil was undertaken for proteomic responses during optimal and cold shock conditions. Comparative proteomic analyses using two-dimensional gel electrophoresis (2-DE) and MALDI-TOF/TOF MS revealed the presence of Cold shock protein E (CspE). Three-dimensional structure of CspE of *E. ludwigii* PAS1 divulge the presence of five antiparallel β -sheets forming a β -barrel structure with surface exposed aromatic and basic residues that were responsible for nucleic acid binding and also reveals the presence of highly conserved nucleic acid-binding motifs RNP1 and RNP2 in Csp family.

Introduction

Of all the natural stress conditions on our planet and solar system, cold is arguably the most widespread, at least from the perspective of mesophilic and thermophilic organisms. About 90 % of the Earth's oceans have a temperature of 5 °C or less [24]. Cold loving microorganisms are widely distributed in nature at temperatures around 0 °C or below. In recent past, there has been a growing interest in microorganisms isolated from extreme environments. Cold stress drastically modifies all physical and chemical parameters of a living cell. Formation of stable secondary structures in DNA and RNA may inhibit transcription and translation; association of an inhibitory factor with ribosome and increase in DNA supercoiling. This also lead to hinder the genetic information processing and decreased membrane fluidity may hamper vital membrane functions such as transport and protein movement [16]. Normal proteins of organism are decreased under cold stress while some special proteins [Cold Shock Protein (Csp)] are increased until adaptation [18]. Although some of these proteins are known to participate in metabolism, transcription, translation and protein folding processes that are affected by cold stress and it has not yet been identified which proteins sense the temperature downshift [17]. To date, the multiple-homologue nature and functional variability of Csps are mostly derived from studies conducted with *Escherichia coli* (CspA–CspI) and *Bacillus subtilis* (CspA, CspB and CspD). Among these, CspA, CspB, CspE, CspG and CspI have been linked to modulation of cold adaptation functions. The CspC and CspE proteins have been implicated in chromosomal condensation and cell division and in the regulation of RpoS and UspA stress response proteins [25]. Several studies have been using the 2D-PAGE for studying the various changes in protein

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expression under heat shock [29], cold shock [11], pH stress [3], phosphate limitation [27, 30] or the presence of copper [19], respectively. Genomics coupling with proteomics, which is relied on the integration of significant advances recently achieved in two-dimensional (2D) electrophoretic separation of proteins and mass spectrometry (MS). These are important high throughput techniques for qualifying and analysing gene and protein expression, discovering new gene or protein products and understanding of gene and protein functions including post-genomic study. However, 2-DE in combination with mass spectrometry is the most widely used technology for comparative bacterial proteomics analysis [11].

Bioinformatics is very useful for searching the unknown sequence including gene function and allow the identification of proteins. It is also useful for further characterisation ranging from the calculation of basic physiochemical properties to three-dimensional structures. Structure knowledge is essential for all areas of protein research such as enzyme kinetics, ligand–protein binding studies, gene characterisation and construction, structure-based molecule design and rational designing of proteins. Homology modelling can provide a useful model which guides biochemical experiments far beyond the range of experimental determined structure.

The present study was planned to analyse principal cold-induced proteins detected between the optimal growth of *Enterobacter ludwigii* PAS1 at 30 °C and growth after cold shock at 4 °C using 2-DE in pairing with Mass spectrometry. Furthermore, *in silico* analysis of identified protein was carried out using the databases NCBI BLAST, Mult-align tool, GORIV tool for secondary structure prediction and SWISS-MODEL tool for modelling of three-dimensional structure of the protein.

Materials and Methods

Bacterial Strain and Growth Conditions

The bacterial strain *E. ludwigii* PAS1 used in this study was obtained from departmental culture collections (Department of Microbiology, G.B. Pant University of Agriculture and Technology, Pantnagar, India), originally isolated from Himalaya soil. Further, sub culturing was performed in 10-ml nutrient broth for 20 h at an optimal temperature (30 °C) and glycerol stocks were prepared and kept at –80° C for future use. The characteristic details of cultures used in this work are given in Table 1.

Protein Extraction

For the proteomic study, cells were cultivated at 30 °C in 50 ml of nutrient broth until mid-exponential-phase (OD_{620}

of 0.4), and then cultures were submitted to 20 h of cold shock treatment at 4 °C. Under each condition (normal and cold shock), cytosoluble proteins were extracted by centrifugation at 10,000 rpm for 10 min, and pellet was washed twice with 0.85 % normal saline solution (NSS), then resuspended in 4 ml of 0.1 M chilled Phosphate-buffered saline (PBS). Cells were broken by ultrasonic treatment (6 cycles of 30 s interval), and cell debris were removed by centrifugation at 10,000 rpm for 45 min. Pellet was discarded and supernatant were stored at –80 °C for further analysis. The extracted protein concentrations were determined by the method of [7] using bovine serum albumin as the standard.

Two-Dimensional Electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed as described by O'Farrel [20] using a 2-DE system (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were precipitated in 10 % (w/v) cold TCA in acetone containing 20 mM DTT and washed three times with acetone to eliminate contaminants like nucleic acid and salts. The dry pellet was solubilised in rehydration buffer (8 M urea, 4 % w/v CHAPS, 100 mM DTT, 0.5 % v/v biolyte (3–8) and 40 mM Tris) by vortexing for 10 min and centrifuged to remove insoluble material. Dry IPG Strips, pI 4–7, 17 cm (Bio-Rad) were loaded with 1.0 mg of protein and allowed to rehydrate for 18–22 h. IEF was performed at 20 °C using the Protean IEF cell (Bio-Rad) according to manufacturer's instructions. After IEF, strip was equilibrated in solution A (0.375 M Tris, pH 8.8 containing 6 M urea, 2 % SDS, 20 % glycerol, 2 % w/v DTT) and B (solution A without DTT, but with 2.5 % w/v Iodoacetamide) for 20 min each at room temperature, the strips were inserted into 12 % SDS-PAGE gels and sealed with 1 % agarose. Electrophoresis was performed at 16 mA/gel for initial 30 min and then at 24 mA/gel until the running dye reached the bottom. The gel was stained with 0.1 % Coomassie Brilliant Blue R-250 and gel images were acquired by gel imaging and spot picking system (InvestigatorTM ProPic, Genomic solution, USA).

Mass Spectrometry Identification and Characterisation of Proteins

Differentially expressed protein spots were excised from gel and destained by washing twice with 100 mM ammonium bicarbonate (ABC)/50 % acetonitrile for 30 min each. Gel pieces were dehydrated with 100 % acetonitrile, dried in vacuum concentrator and incubated with 200 ng/ml Trypsin Gold (Promega) in 25 mM ABC for 3 h at 37 °C. After the digestion, tryptic peptides were aspirated and extracted with 50 % acetonitrile, 2.5 % TFA.

Table 1 Cultural characteristic of *Enterobacter ludwigii* PAS1 isolated from Himalaya soil

Bacterial culture	Acc. no.	Organism	Sampling site	Longitude/latitude	Elevation (m)	Cell morphology	Grams reaction	Growth on N ₂ free medium	
								Optimum temp. (30 °C)	Low temp. (4 °C)
PAS1	HQ222368	<i>Enterobacter ludwigii</i>	Patalbhuneswar, India	31.01°N78.45°E	1,350	Medium oval	–ve	+ve	+ve

Extracts were combined and evaporated to 5 µl of final volume. Samples were spotted onto a MALDI target and overlaid with 0.5 µl of 5 mg/ml MALDI matrix (α -Cyano-4-hydroxycinnamic acid). Mass spectrometric data were collected using an ABI 4800 MALDI-TOF/TOF (Applied Biosystems, Foster City; CA). Mass data were acquired in reflector mode in a mass range of 600–4,000 Daltons. The peak list was generated from raw data based on signal-to-noise filtering, exclusion list and de-isotoping parameters using GPS Explorer software (Applied Biosystems, Foster City; CA). The resulting peak list was then searched and compared against existing data bases in NCBI using Mascot search engine (Matrix Science, Boston; MA).

In silico Analysis

BLAST (www.ncbi.nlm.nih.gov/BLAST/Bethesda, MD, USA) [1, 2] algorithm was used for homology searches against the entries of Csp databases. Physicochemical properties of Csp was calculated using ProtParam tool and Multalign tool was used to detect conserved as well as variable patterns of amino acids. The secondary structure prediction was performed using GORIV tool for revealing the predominance of coils and helices.

The three-dimensional structure was predicted using SWISS-MODEL, an automated protein homology modelling server [26]. The amino acid sequence of Csp was submitted to SWISS-MODEL server and following steps were performed: domain annotation, template identification, automated modelling and structure assessment. Domain annotation determined the super family to which respective protein belonged as well as secondary structure elements of the target protein. Template identification predicted the possible templates for target sequence on the basis of target-template sequence similarity. Three-dimensional structure was determined using automated modelling mode and the predicted models were evaluated using Structure Assessment tool of SWISS-MODEL server [6].

Multiple sequence alignment was carried out and a Neighbor Joining (N-J) Phylogenetic tree with 1,000 bootstrap was then constructed using Clustal X [28].

Results and Discussion

Proteomic Analysis

Cold stress and adaptation response mechanisms in several bacterial species have been extensively studied at the transcriptional level [8] while proteomic studies are still not conclusive. We applied expression proteomics to gain insight into key cellular events that allow *E. ludwigii* PAS1 to survive and multiply even at refrigeration temperatures. The 2-DE maps of *E. ludwigii* PAS1 proteome extracted from cells grown at 30 and 4 °C showed almost a similar pattern of protein expressions. In general, most of the proteins were present in same proportions at the two temperatures. However, some qualitative and quantitative differences appeared for few proteins. Differential protein patterns showed within the pI range of 4–7 and low molecular mass less than 14 kDa were also observed (Fig. 1). The induced Csp was detected only in the downshifted temperature of 4 °C, while none of the protein spot was detected in the control (30 °C) at this pH range.

It was significant that protein spot designated as spot A1 was expressed in acidic range below 7 kDa from *E. ludwigii* PAS1. The molecular mass (Mr) and apparent pI values of the protein A1 was 7,276 and 5.67, respectively. The peptide masses of protein A1 obtained from MALDI-TOF/TOF MS was identified as CspE (Table 2) by similarity searching in the protein database (Fig. 2). The results reveals that the absence of a major cold-induced protein of 7-kDa Csp family which has been reported from many bacterial species. We are also able to identify this non-cold-inducible 7-kDa Csp-E. This result is in accordance with the findings of Wouters et al. [31] reported that the constitutive expression of 7-kDa CspE, probably involved in cryoprotection of *Lactococcus lactis*. Furthermore, CspE from *E. coli* possess a role in cell division, possibly in chromosome condensation, in the regulation of RpoS and UspA stress response proteins and in transcription anti-termination activity [4, 13, 15, 22, 23, 32]. Besides CspA, which influences transcriptional processes, the homologues CspE and CspC are also involved in transcription anti-termination since purified CspE inhibits

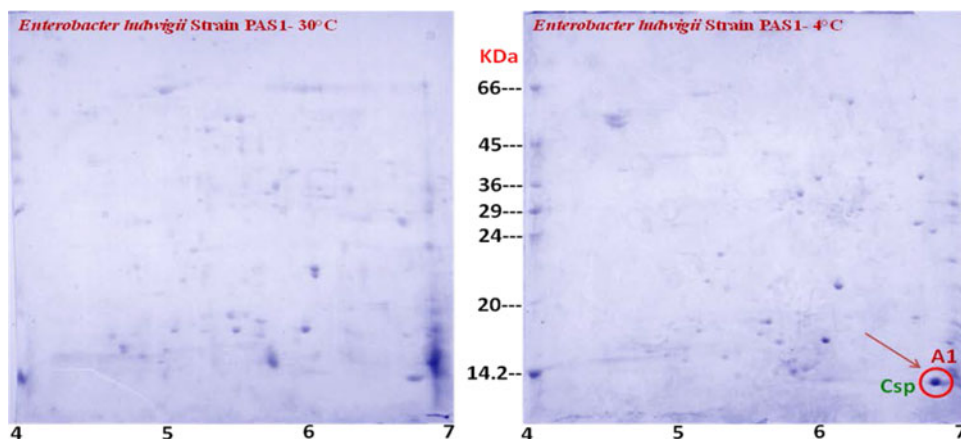


Fig. 1 2-DE analysis of protein expressed at low temperature in *Enterobacter ludwigii* strain PAS1, where the Csp spot expressed in acidic range. The pH gradient of the first-dimension electrophoresis

was shown on bottom of the gel and migration of molecular mass markers for SDS-PAGE in the second dimension was shown on the side

the Q-mediated transcription anti-termination (Bae et al. 1999) [15]. CspE binds to the polyA tail at the 3' end of mRNAs, and interferes with the degradation of both polynucleotide phosphorylase (PNPase) and RNase E at 310 K [10]. Using double-stranded model substrates, it was shown that CspE induces melting of the stem region [22].

Protein Annotation

Annotated protein and two-dimensional electrophoresis is a database in the bioinformatics core of proteome research. In this paper, SWISS-MODEL was used to predict structure of CspE and CspE sequence of *E. ludwigii* PAS1 was used as target. BLASTp a protein sequence similarity search engine enabled to locate 21 homologous sequences from bacterial species with matching cold shock functions. These sequences were used for conducting multiple sequence alignment (Multalign tool) which enabled to detect conserved and variable patterns of amino acids among the aligned 21 homologous sequences. The results revealed the presence of RNA-binding motifs, RNP1 and RNP2 which are reported to be highly conserved in the CSD family [22]. The predominant amino acid sequences for these motifs are KGFGF for RNP1 and VVVFH for RNP2. CspE from *E. ludwigii* PAS1 also showed similar amino acid sequences for these motifs (Fig. 3). The protein-nucleic acid interaction is performed by the moderately well-conserved canonical RNP1 (K/R-G-F/Y-G/

A-F-V/I-X-F/Y) and RNP2 (L/I-F/Y-V/I-G/K-N/G-L) motifs [9] that are present with small variations in all Csp. These RNA-binding motifs facilitate nucleic acid recognition/binding and prevents RNA secondary structure formation, thereby enhancing translation at low temperature and serve as transcription antiterminator [21]. The conserved functional site which is responsible for cold shock is predominantly positively charged amino acids Arg, Lys along with those other amino acid patterns. Physicochemical properties of Csp protein was elucidated by PROT-PARAM tool and showed that the molecular weight and Theoretical pI were 7.28 and 5.68, respectively.

3-D Modelling of CspE Protein

Structure knowledge is essential for all areas of protein research such as enzyme kinetics, ligand– protein binding studies, gene characterisation and construction, structure based molecule design and rational designing of proteins. Structure analysis of the predicted protein was carried out using computational tool in which secondary structure predicted using GOR IV tool revealed predominance of coils followed by helices (nearly 64 %). Coils are essential in imparting structural stability for protein at times of challenges such as cold shock. Furthermore, 3-D modelling of CspE protein was also performed. Homology modelling can afford a valuable model which guides biochemical experiments far beyond the range of experimental

Table 2 Summary of MALDI-TOF/TOF MS data obtained from 2-DE spot from *Enterobacter ludwigii* PAS1 and identification of Csp protein

Culture name	Spot no.	Protein name	UniProt KB ID no.	pI	Mr (Da)	Score	Sequence coverage (%)	Protein sequences
<i>Enterobacter ludwigii</i> PAS1	A1	CspE	P63237	5.67	7,276	106	26	⁹ K.WFNESK.G ¹⁴ ¹⁵ K.GFGFITPEDGSK.D ²⁶

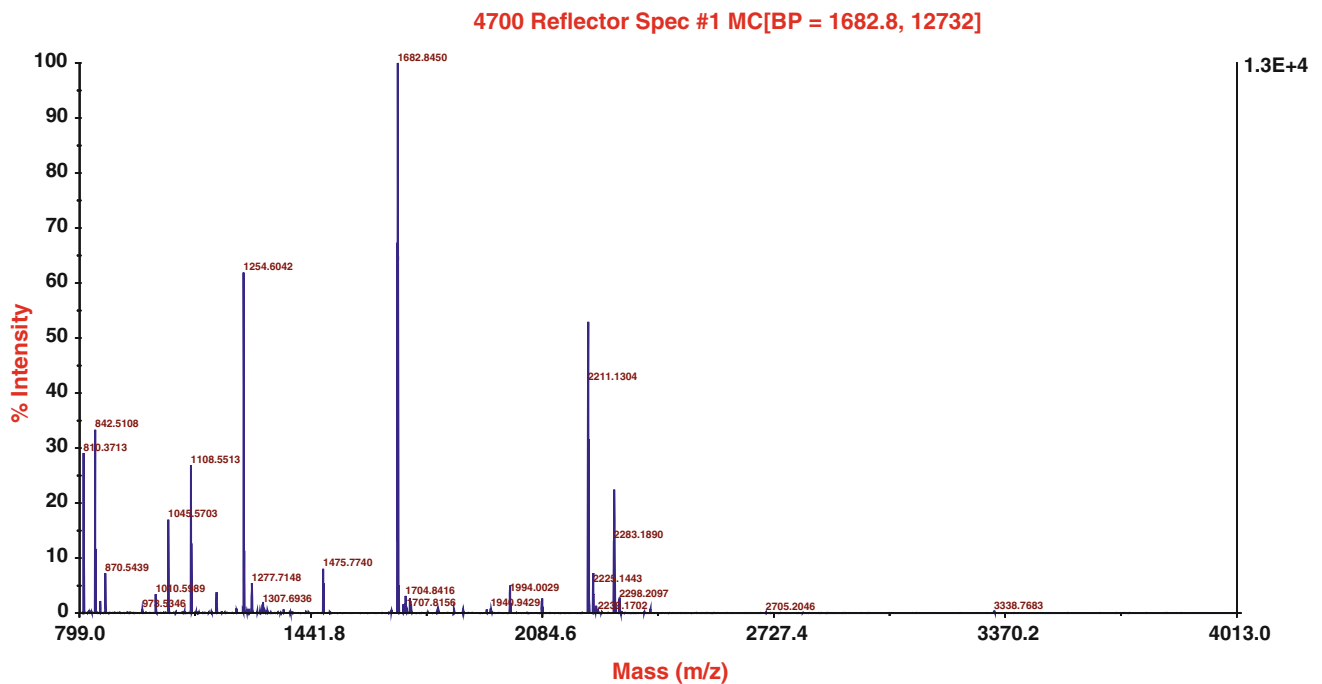


Fig. 2 Identification of protein A1 expressed in 2-DE using MALDI-TOF/TOF MS spectrum of tryptic peptides. After database searching, protein score is found to be significant i.e. ($P < 0.05$)

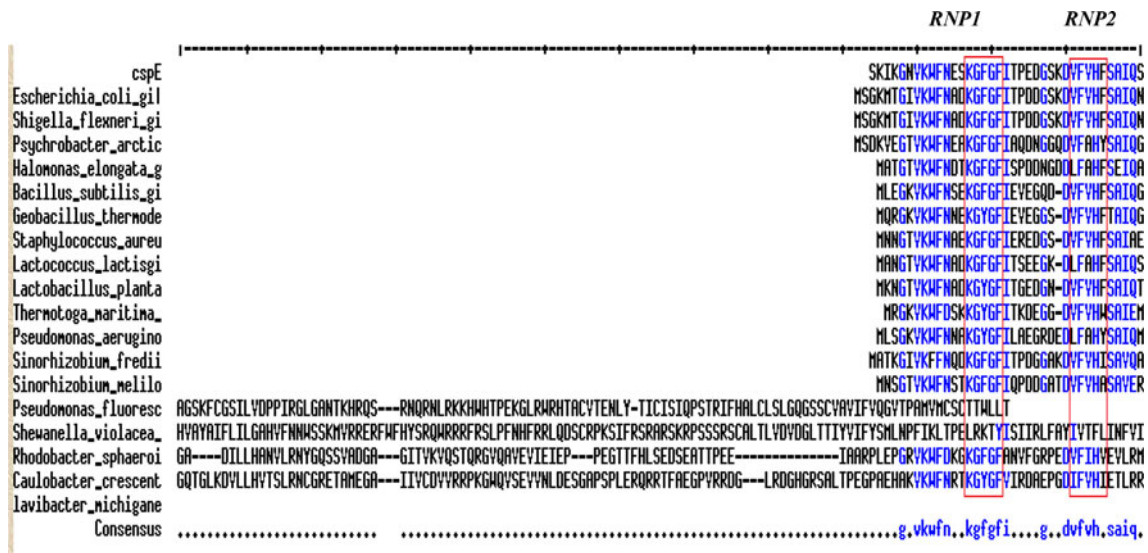


Fig. 3 Alignment of sequences of Cold shock protein by Multalign tool showing consensus regions (RNP1 and RNP2)

determined structure. For which, template identification searched templates for the query sequences on the basis of significant sequence similarity. CspE (PDB ID 3i2z), from *Salmonella typhimurium*, was identified as template showing 92 % of sequence similarity score with the given protein sequence. Three-dimensional structure of modelled CspE reveals that, like other Csp proteins, it also consists of five antiparallel β -strands that fold into β -barrels built of

two β -sheets (Fig. 4). One of the β -sheet consisting strand β_1 - β_2 - β_3 with the highly conserved ribonucleoprotein consensus RNP1 and RNP2 on its surface, and other sheet consisting β_3 - β_4 strands. In addition, CspE covers β_1 residues 5–13 (IKGNVKWFN); β_2 residues 18–23 (FGFITP); β_3 residues 30–33 (VVFH); β_4 residues 50–56 (SVEFEIT) and β_5 residues 63–69 (SAANVIS). Aromatic amino acid residues W11, F12, F18, F20, F31 and H34, which are

Fig. 4 Three dimensional structure of CspE Protein of *Enterobacter ludwigii* PAS1 and its RNA-binding sites RNP1 (grey colour) and RNP2 (blue colour) (Color figure online)

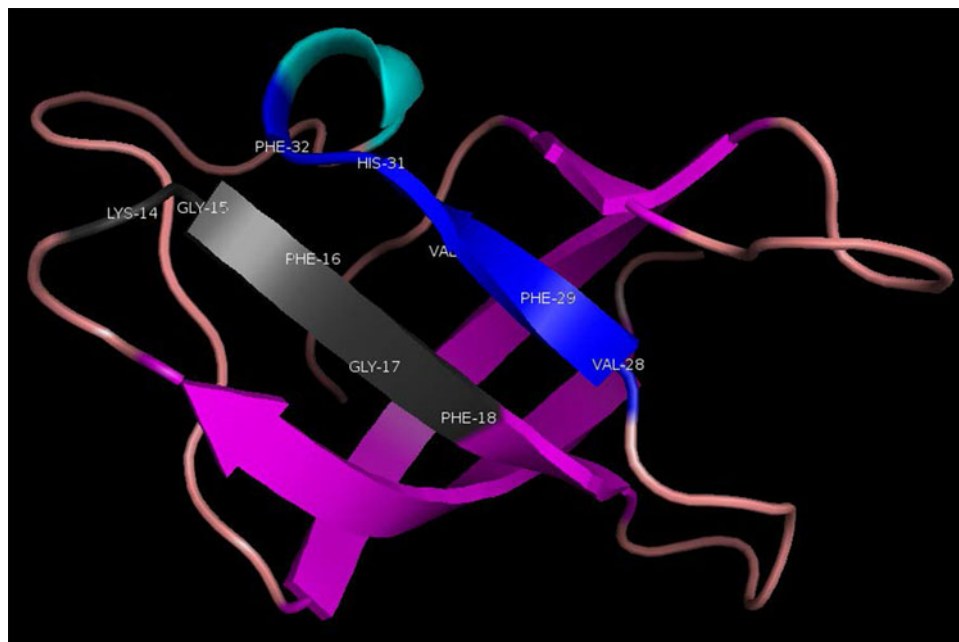
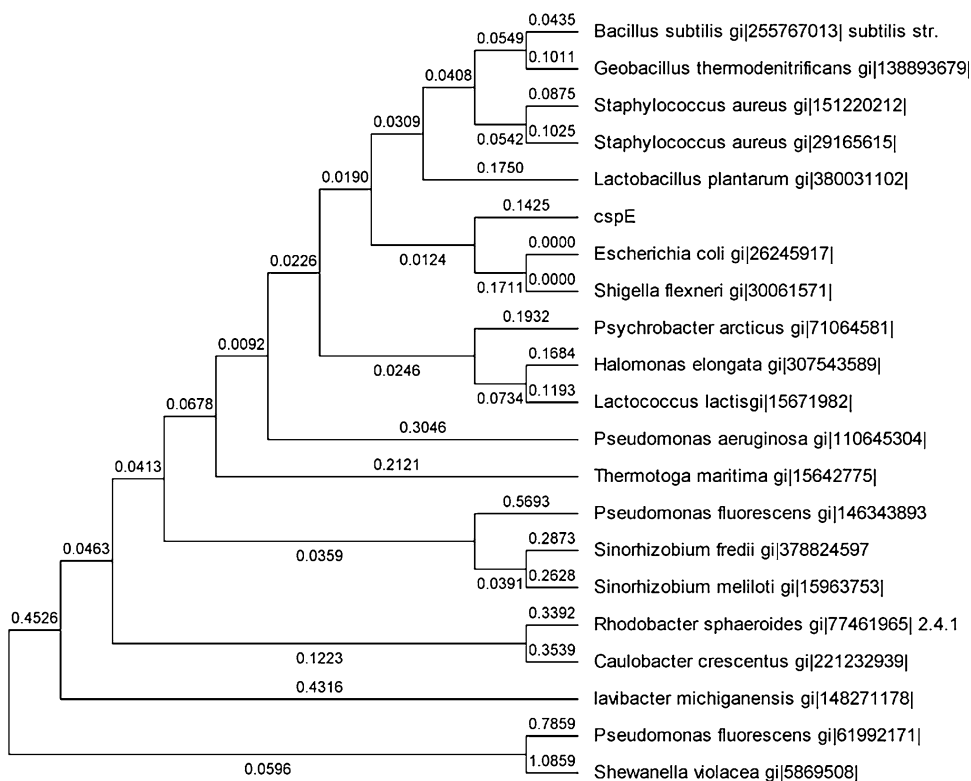


Fig. 5 Phylogenetic tree showing the relationship among CspE gene sequence from *Enterobacter ludwigii* PAS1, with reference sequences retrieved from GenBank through BLAST analysis



essential for single strand nucleic acid binding and lysine residues K10 and K16, located on the surface were also revealed in the present study.

Recently, a new measure QMEAN Z-score has been introduced to determine the closeness of the computationally predicted models with the experimentally validated structures [5]. In order to calculate the QMEAN Z-score of

a predicted model, the normalised raw scores of the model were compared to the scores obtained for a representative set of high resolution X-ray structures of similar size (number of residues of query protein $\pm 10\%$). The output was obtained in the form of a model quality plot in which the query model was marked on normalised QMEAN score data obtained from high resolution structures of similar

size. The QMEAN Z-score for CspE was predicted to be greater than 1 but less than 2 which was within the defined limits (Fig. 5) of the good quality model.

Phylogenetic Analysis

Neighbor Joining (N-J) phylogenetic tree was built to identify the closely related cultured relatives of *E. ludwigii* PAS1 using Csp homologous sequences retrieved from NCBI GenBank. Based on the position in phylogenetic tree, CspE of *E. ludwigii* PAS1 was close to the genus *Escherichia coli*. The best studied Csps were from the mesophilic bacterium *E. coli*. According to Gualerzi et al. [12], a constitutive expression (although at lower levels) seems to exist also for the other Csps. This view was also confirmed by multiple deletion experiments of Csps in *E. coli* like CspE, which shares 69 % identity and is transiently induced at physiological temperatures, is able to rescue the cold adaptive phenotype cells with a triple deletion of inducible Csps (Δ cspA Δ cspB Δ cspG) [14].

Conclusion

This study suggests that *E. ludwigii* PAS1 adapts quickly to low temperatures by a constitutive expression of the potentially cryoprotective CspE. The predicted proteins indicate that this pattern of gene expression is perhaps requisite for optimal adaptation to the lower temperature. Moreover, these genes encoding cold shock proteins could be transferred to promising plant growth promoting rhizobacteria (PGPRs) which failed to grow at low temperatures. The interest in the use of biological approaches to replace chemical agents in fertilising soils is the need of this hour in view of organic and sustainable agriculture in Himalayan regions.

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