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Full Length Research Paper

Development of immunoassay for the identification of cold shock proteins from diversified microflora

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Cold shock response in various organisms is induced by an abrupt downshift in temperature and leads to a dramatic increase in production of a homologous class of cold shock proteins. These proteins are essential for low temperature survival of bacteria. To identify CSP from diversified microflora, immunoassay was developed. A small 14 kDa protein from cold tolerant mutant, $CRPF_8$ of *Pseudomonas fluorescens* was concentrated and fractionated by HPLC and antisera was raised. Specificity of anti-$CRPF_8$ was checked using western blot analysis and further confirmed by Immunoelectron Microscopy. Bacterial strains from various habitats were isolated and their crude protein was purified. CSPs were characterized from crude extract using anti-$CRPF_8$. Expression of CSPs was observed only in bacterial strains isolated from temperate region and negligible or no expression was observed in bacterial strains isolated from arid zones. Therefore this anti-$CRPF_8$ can be used as immunological tool for the identification of CSP from diversified microorganisms.

Key words: Cold shock proteins, psychrophiles, *Pseudomonas fluorescens.*

INTRODUCTION

Temperature has profound effect as major environment factor on cellular process of all organism(s). Due to their high surface to volume ratio, prokaryotes have stringently cope with temperature changes and adapt to a given temperature regime. Bacteria have occupied all life supporting ecological niches and have adapted to the respective temperature allowing them to be classified as psychrophiliic, mesophillic, or thermophillic microorganism (Nedwell and Rutter, 1994). Microorganisms respond to abrupt temperature shift by rapid accumulation of cold shock protein (CSPs) (Phadtare et al., 1999) which were found in a wide range of gram positive and gram negative bacteria including psychrophiliic, mesophillic, and thermophillic strains and can be assumed as evolutionary old class of protein(s) (Graumann and Marahiel, 1996). CSPs were also found in *Aquifex aeolicius* and *Thermatoga maritima* indicating their presence at the origin of bacterial divergence and can be assumed as evolutionary old class of protein(s) (Graumann and Marahiel, 1996). These were discovered originally because they are strongly induced in response to cold shock and were assumed to be important for adaptation at low temperature (Graumann et al., 1997).

For the microbial gene expression associated with cold shock and identification of CSPs, different methods have been used including selective capture of transcribed sequence (SCOTS) (Liu et al., 2002). Cloning and sequencing of genes which encode the enzymes that are up regulated at lower temperature (Janiyani and Ray, 2002) and immunological assay that depend on specificity of antibodies raised against CSPs (Stamm et al., 1999) have been employed.

In the present study, we focused on development of immunoassay for the identification of cold shock proteins (CSPs) in verified microbial population two standard microbial population. Two standard strains i.e. *Pseudomonas fluorescens* MTCC 103 and MTCC665 which were originally from different climatic zones were used as reference strains for CSPs identification. Furthermore,

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Table 1. Bacterial strains used in study.

Bacterial strains	Characteristics
Pseudomonas fluorescens MTCC 103, *Pseudomonas fluorescens* MTCC 665	Mesophillic strain Antarctic strain (Procured from IMTECH Chandigarh)
$CRPF_1$ $CRPF_3$ $CRPF_4$ $CRPF_5$ $CRPF_7$ $CRPF_8$ CRM	Cold tolerant mutants (Kumar et al., 2002; Katiyar and Goel, 2003)
PRS_9 & GRS_1	Departmental Culture Collection
Soil Isolates	Isolated from arid, Semi arid and Temperate region

antibodies raised against cold shock protein of $CRPF_8$ were used as immunological tool for detection of CSPs in unknown microbial population.

MATERIAL AND METHOD

Bacterial strains used in study has been summarized in Table 1.

Media

Ps. fluorescens MTCC 665 and its mutants were incubated on trypticase soya peptone (TSP) broth composed of 0.5% soya peptone, 1.5% peptone, and 0.5% NaCl (pH-7.0) or TSP agar (TSP broth + 2% w/v agar). All other strains were incubated on nutrient broth composed of 0.5% peptone and 0.3% beef extract or nutrient agar (nutrient broth + 2% agar).

Isolation of strains from various habitat

Soil was taken from different climatic zones; Bikaner and Rajkot in arid, Etawah and Jhansi in semi-arid, and Munsiari in temperate zones. Serial dilution was done and plated at 10^{-4} and 10^{-6} dilution on nutrient agar and incubated at 30°C. Colonies were picked up and purified by subculturing.

Protein extraction

The method for isolation and purification of cold shock proteins was followed as described earlier (Tyagi and Goel, 1999). However, for the isolation of cold shock protein (CSP), mutant $CRPF_8$ was grown at 10°C for 72 h in medium. Rest of the protocol remained same.

Isolation of soluble proteins from mutants grown at different temperatures

The mutant $CRPF_8$ was grown at different temperatures; 4, 10, 20, 25, 30 and 37°C in trypticase soya broth. After 2-4 days of incubation, the cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The pellet was washed with normal saline solution (0.85% NaCl, pH 7) twice and suspended in 1 ml of phosphate buffer saline (0.1 M, pH 7.0). Cells were then sonicated for 3 min and PMSF was added to the final concentration of 1 mM. Cells were harvested by centrifugation at 10,000 rpm for 45 min. Supernatant was separated and kept at –20°C.

Raising of anti sera

Monospecific, polyclonal antibodies were raised against purified proteins conjugated with Freunds adjuvant (Khan et al., 2003) These conjugated proteins were injected in white New Zealand rabbits at an intervals of 10 days occurring five times and were bled after 7days of last booster**.**

Enzyme linked immunosorbent assay (ELISA)

Protocol for ELISA was used according to Boer et al. (1994) with modification. 1 ng of protein was coated on wells and 1:1000 dilutions for primary and horse reddish peroxidase goat anti rabbit IgG were used as anti bodies.

Immunoblotting

Total protein (20 µg) was loaded onto a gel for immunoblotting with rabbit anti-CRP (14 kDa) antibodies. The protein was blotted on a nitrocellulose membrane by using transfer buffer (Goel et al., 1993) and incubated for 5 h with anti-CSP antibodies. The membrane was treated with goat anti-rabbit I gG and stained with alkaline phosphatase colour substrate (BCIP/NBT, Genei Banglore). Prestained markers (Bio Rad) were used for determination of molecular mass of proteins.

Immuno electron microscopy

For proteins localization cell were harvested and fixed with 0.5% gluteraldehyde and 2% *p*-formaldehyde in 0.1 M PBS (pH 7.2). Pellets were washed with 0.05% between 20 and 0.5% glycine. Dehydration of samples were done through a graded ethanol series (30-100% ethanol), then infiltrated with L.R. white resin (Sigma) and polymerized at 60-65°C for 36 h. Sections were cut (Leica ultra cut) and blocking was done with 1% BSA in PBS (pH 7.2). Grids were then incubated in primary antibodies (1:50) for 4 h. The grids were washed three times for 5 min in blocking solution before the addition of the immunogold conjugate in a 1:20 of 10 nm colloidal

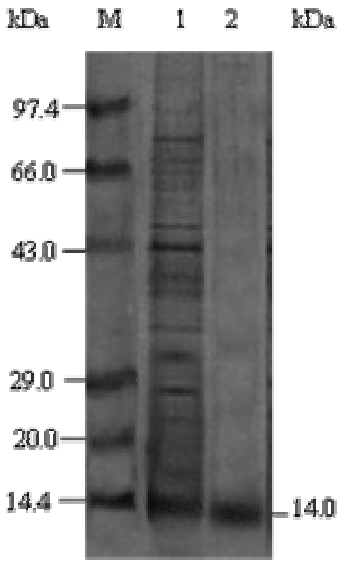

Figure 1. SDS-PAGE for characterization of soluble cold shock proteins using silver staining. M, Markers; lane 1, whole cell soluble protein of $CRPF_8$; and lane 2, purified 14 kDa protein.

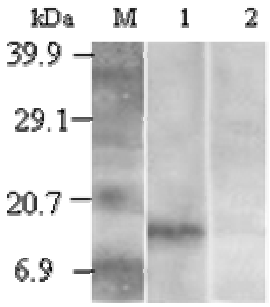

Figure 2. Cross reactivities detection using Anti -$CRPF_8$ antibodies. M, Prestained Markers(Biorad); lane 1, Cold resistant proteins of $CRPF_8$ (14 kda); and lane 2, Crude protein of *Ps. fluorescens* (wild type).

gold-labeled goat anti-rabbit 1gG. The grids were washed further 3 times in 0.1 M PBS (pH 7.2) containing tween-20 and 3 times for 1 min in triple distilled water before staining with 30% uranyl acetate and 70% lead acetate. The sections were visualized with Phillips technia-12, an acceleration voltage of 80 kV.

RESULTS

Purification of cold shock proteins

The cold shock protein from $CRPF_8$ (MW 14 kDa) was isolated and purified to homogeneity by HPLC. The molecular weight and purity was verified by SDS-PAGE analysis (Figure 1).

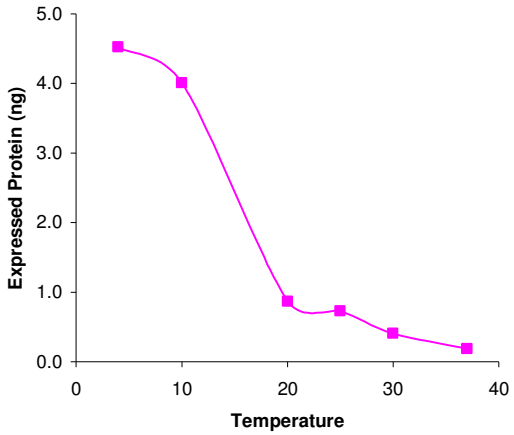

Figure 3. Effect of temperature on cold resistant protein expression of $CRPF_8$ mutant using anti-$CRPF_8$.

Effect of temperature on cold shock proteins expression

Polyclonal antiserum raised against CSP was used to detect protein expression in western immunoblot analysis, which indicated that the antibodies are highly specific, and reacts with respective protein only (Figure 2). To study the expression level of cold shock proteins in $CRPF_8$ mutant the culture was grown at different temperature; 4, 10, 20, 30 and 37°C and crude proteins was isolated. Expression profile of cold shock protein of $CRPF_8$ by ELISA showed that with the decrease in temperature expression level of CSPs increases. At 37°C expression was negligible but at 10°C optimum expression was observed (Figure 3).

To confirm the effect of temperature on expression of cold shock proteins, localization was checked using immunogold electron microscopy. The localization of CSPs was observed only in $CRPF_8$ growing at low temperature, but no localization was observed in wild type and $CRPF_8$ at 28°C. Total number of gold particles per cell was 20±1.45 and 45±1.75, respectively, in $CRPF_8$ mutant growing at 20 and 10°C, which further confirmed that with the decrease in temperature expression of cold shock protein increases (Figure 4).

Proteins expression profile of wild type and their respective mutants using anti -CSPs

A total of 10 strains (cold tolerant mutants) were grown at two different temperatures; 10 and 30°C, and their crude protein was purified. Expression of CSP in all strains was checked using ELISA. At 10°C, maximum expression was observed in all the mutants which decreased at 37°C. Furthermore, it was observed that expression level of wild types; GRS1 and PRS9 was negligible (Figure 5).

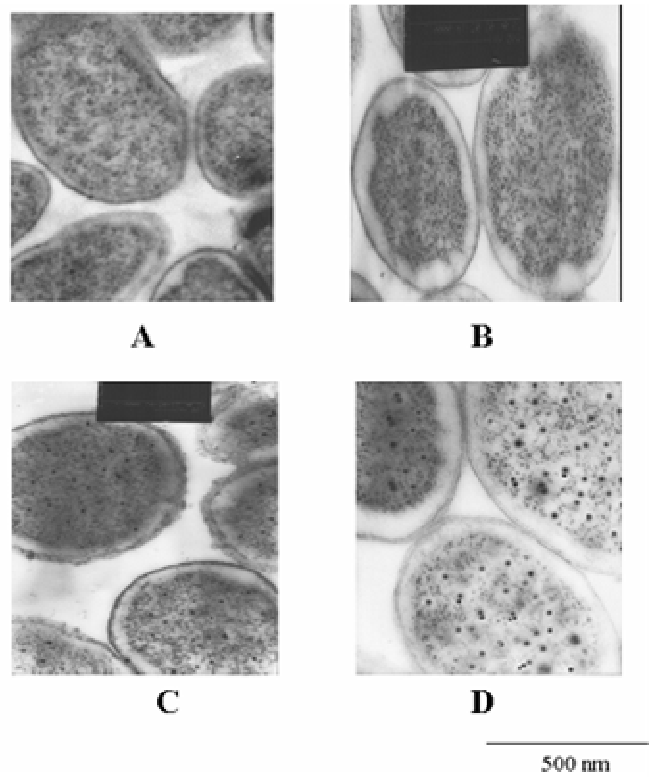

Figure 4. Immunogold electron micrographs of *Ps. fluorescens* showing localization of CSP. **A**, *Ps. fluorescens* wild strain; **B**, *Ps. fluorescens* mutant $CRPF_8$ at 28°C; **C**, *Ps. fluorescens* mutant $CRPF_8$ at 20°C; and **D**, *Ps. fluorescens* mutant $CRPF_8$ at 10°C.

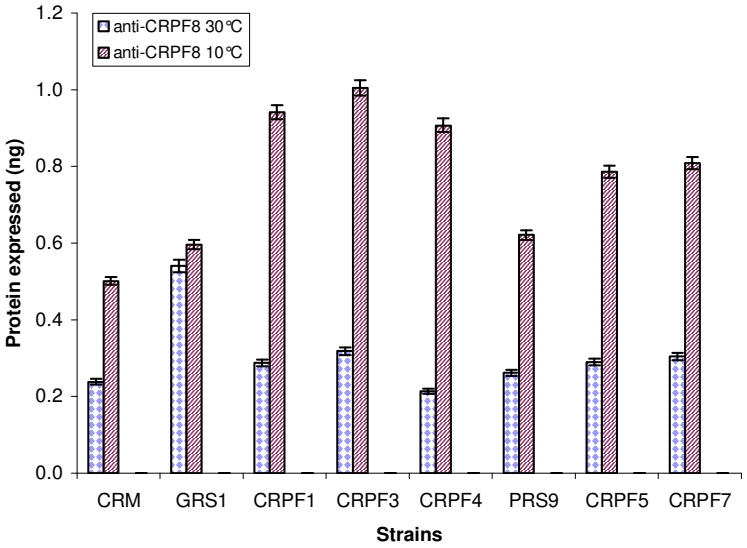

Figure 5. Cold shock protein expression profile in wild type and their mutant at two different temperature using ELISA with anti-$CRPF_8$.

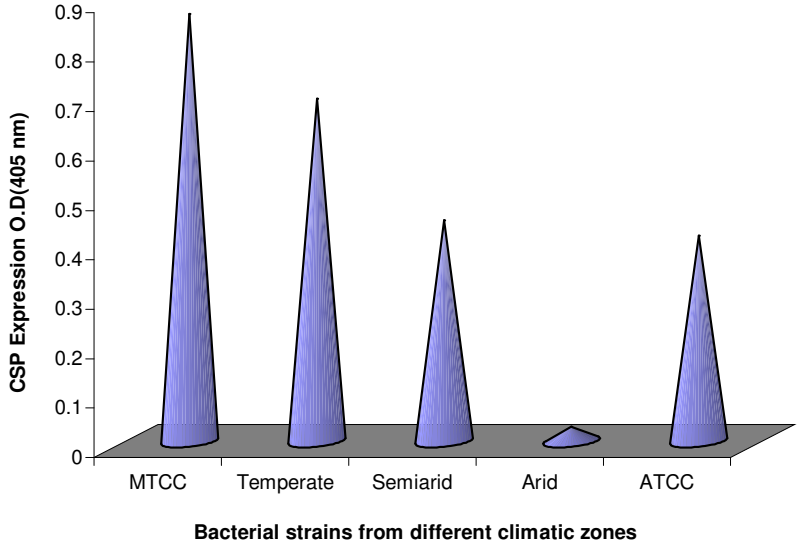

Figure 6. Detection of cold shock proteins in bacterial strains from different climatic zones using ELISA.

Immunodetection of CSP from diversified microflora

Bacterial strains from different habitats were grown at30°C and their protein was isolated and cold shock proteins were detected using antibodies raised against CSPs by ELISA. *Ps. fluorescens* MTCC 103 and MTCC 665 were used as reference strains for two habitats i.e., semi arid and Antarctic, respectively. Expression of CSP was detected in *Ps. fluorescens* MTCC 103 and bacterial strains isolated from temperate and semi arid zones but no expression of CSP was detected in strains isolated from arid zones (Figure 6). Furthermore, it was also observed that expression level of CSP was in accordance with the climatic conditions of bacterial strains.

DISCUSSION

Bacteria are able to adapt to temperatures far below their optimum growth temperatures, and a set of 7-15 kDa proteins (named cold shock proteins [CSPs]) is strongly induced in response to a rapid decrease in growth temperature. CSPs are found in a wide variety of gram-positive and gram-negative bacteria such as *Escherichia coli*, *Bacillus subtilis* and *Lactococcus lactis*. In cold tolerant mutant of *Ps. fluorescens*, expression studies of CSP showed that with the decrease in temperature, expression of CSPs increases, which suggested higher expression of *CSP* gene at low temperature as reported earlier (Weber et al., 2002). According to Xia et al. (2001), *E. coli* cells growing at low temperature contain nearly two millions CSP molecules per cell, which protects *E.coli* from cold stress. These CspA homologous interacts with nascent transcripts and preventing the formation of hairpin structure, which is essential for termination. One dimension gel electrophoresis analysis of protein performed after a cold shock in *L. monocytogenes*, showed that 2 h after the cold shock, expression level of 7 kDa protein increased. Western blot analysis performed with anti-CspB of *B. subtilis* indicated that the level of CSPs increases after the cold shock (Henrik et al, 2002).

Kim et al. (2005) quantitatively monitored and analyzed the proteomic response to a temperature downshift from 37 to 20°C during fed-batch cultivation of *E. coli* K-12, by using two-dimensional electrophoresis. When the temperature of exponentially growing *E. coli* K-12 culture was downshifted to 20°C, the synthesis level of 57 intracellular proteins showed significant changes for a prolonged period of time, compared to the fed-batch culture controlled at 37°C. Thus, these proteins are regarded as important stress proteins responsive to cold shock, which were analyzed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and identified using the *E. coli* SWISS-2DPAGE database.

The reactivity of these antibodies with the proteins of other fluorescent pseudomonad and their mutants was due to the presence of some similar epitops. Hebraud et al. (1994) reported cross reactivities of antibodies with different proteins and suggested that similar epitops existed in all the proteins**.**

The localization of CSPs only in cold tolerant mutant of *Ps. fluorescens* further confirms the specificity of antibodies. Giangrossi et al. (2001) studied the *in vivo* localization of two CSPs of *E. coli* using GFP fusion; CspA-GFP and CspD-GFP. The localization of CSP was found to be consistent with the finding that this protein is

required during transition from steady state to resumption of growth and from growth at optimal temperature to cold shock conditions.

Webber et al. (2001) demonstrated that in growing cells of *B. subtilis* CSP specifically localized to cytosolic region surrounding the nucleoid. After inhibition of translation, protein was still present around the nucleoid in a manner similar to that in cold shocked cells, indicating that specific localization of CspB depends on active transcription and is not due to simple exclusion from nucleoid.

Higher expression of CSP in bacterial strains isolated from temperate regions and no expression in bacterial strains isolated from arid zones is evident that anti-CSP are capable to identify the cold shock proteins in diversified bacteria isolated from different climatic zones. These evidences are strongly supported by other findings. Sato (1995) reported homologue of CSPs from thermophilic *B. caldotylicu*s that grows at 70^{o}C and hyperthermophile *Thermotogo maritima*, which grows at temperatures up to 90^{o}C.

Therefore these results clearly show the presence of unique epitops in antibodies raised against CSPs and their specificity. Furthermore the use of these antibodies as immunological tool for the identification of CSP in the microbial flora isolated from various habitats can be suggested with this development of immunoassay for the identification of cold shock proteins.

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