

Recent Developments in Bacterial Cold-Shock Response

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Abstract

In response to temperature downshift, a number of changes occur in cellular physiology such as, (i) decrease in membrane fluidity, (ii) stabilization of secondary structures of nucleic acids leading to reduced efficiency of mRNA translation and transcription, (iii) inefficient folding of some proteins, and (iv) hampered ribosome function. Cold-shock response and adaptation has been quite extensively studied in *Escherichia coli* and *Bacillus subtilis*. A number of cold shock proteins are induced to counteract these harmful effects of temperature downshift. General principles of cold-shock response along with recent findings on desaturase system, RNA chaperone and transcription antitermination function of CspA homologues, cold shock induction of chaperones and synthesis of trehalose, CspA homologues from hyperthermophilic bacteria and possible multiple roles of cold shock proteins in other stress responses of bacteria are discussed.

Introduction

Temperature is one of the major stresses that all the living organisms have to face. Heat-shock response from bacteria to human has been extensively studied, while cold-shock response has caught attention of researchers relatively

recently. A major reason why heat shock is extensively studied is because it causes well-defined damage to the cells, i.e. unfolding or denaturation of proteins. Heat-shock-induced proteins, chaperones, assist in protein folding. In contrast, cold shock does not cause such well-defined cellular damage. Cold-shock response is classically exhibited when an exponentially growing culture is shifted from its optimum growth temperature to a lower temperature. In case of majority of bacteria such as *Escherichia coli*, upon temperature downshift, there is a transient arrest of cell growth, during which general protein synthesis is severely inhibited. However, synthesis of a number of proteins, called cold-shock proteins, is induced under these conditions. Eventually, the synthesis of these proteins decreases, cells become acclimated to low temperature and growth resumes (Jones *et al.*, 1987). The effect of cold shock is seen at multiple levels such as; (i) decrease in the membrane fluidity affecting the membrane-associated functions such as active transport and protein secretion, (ii) stabilization of the secondary structures of RNA and DNA, leading to reduced efficiency of mRNA translation and transcription, (iii) slow or inefficient folding of some proteins and (iv) ribosomes need to be cold-adapted to function properly at low temperature.

The study of cold-shock response is now in limelight because of its commercial and health implications. Table 1 illustrates how studies on bacterial cold-shock response are useful in avoiding potentially disastrous situations in various industries. Understanding cold-shock response of food-borne pathogens such as *Listeria* is imperative as refrigeration is a commonly used method of food storage. Cells, which are cold-shocked prior to freezing, exhibit better cryotolerance. Therefore, food-spoilage bacteria can be sensitized to damage caused by cold temperatures through direct freezing of the food articles (Willimsky *et al.*,

Table 1. Commercial significance of study of cold-shock response

Industry	Concern	Solution
Food	Spoilage of refrigerated food and infections due to food-borne pathogens	Reduce efficiency of cold-shock response of food-borne pathogens
	Instability of lactic acid bacteria starter cultures due to temperature changes during fermentations in dairy industry	Modify starter cultures to improve cold adaptation and cryotolerance
Agriculture	Reduced efficiency of 'biofertilizers' due to low temperatures	Use of cold-adapted Rhizobial cultures
	Great economic losses due to low tolerance of plants to low temperatures	Enhance chilling resistance of plants using gene transfer from bacteria to plants
Research	Inefficient expression of proteins due to temperature sensitivity or proteolysis	Cold-inducible expression systems for production of these proteins
	Reduced efficiency of certain processes using biocatalysts at low temperatures	Use of cold-adapted enzymes

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1992). In the same token, cryotolerance of useful bacteria such as lactic acid bacteria can be improved, so that economic losses resulting from reduced starter culture's activity/viability due to harsh temperature changes in the fermentation processes are minimized. Recently, the *desC* gene for the acyl-lipid desaturase from the thermophilic cyanobacterium *Synechococcus vulcanus* was introduced into *Nicotiana tabacum*. As a result, the lipid content, the extent of fatty acid unsaturation and chilling tolerance of these plants significantly increased. Seeds of plants that expressed the *desC* gene also demonstrated higher chilling tolerance than those of the control plants (Ishizaki-Nishizawa *et al.*, 1996; Orlova *et al.*, 2003). Certain proteins are not produced efficiently at 37 °C. These can be overproduced in large quantities at low temperatures using cold-inducible promoters, for example, the promoter of *cspA*, encoding the major cold shock protein of *E. coli*. This will prove to be a very powerful tool in biotechnology (Baneyx and Mujacic, 2003; Vasina and Baneyx, 1997). This will be useful in not only producing proteins for structural determination, but also proteins of medical importance.

Recently, certain aspects of bacterial cold-shock response such as regulation of expression of CspA homologues have been extensively reviewed (for review see Ermolenko and Makhatadze, 2002; Gualerzi *et al.*, 2003; Phadtare *et al.*, 1999; Phadtare, 2000; Weber and Marahiel, 2003; Yamanaka *et al.*, 1998). Aside from summarizing the general principles underlying this response, this article will mainly focus on the recent developments in cold shock response, with emphasis on *E. coli*.

General principles of cold-shock response

Bacteria sense the change in temperature mainly at level of cell membrane, nucleic acid and ribosomes (Phadtare, 2000). The changes in cell membrane are discussed in detail later in this article. Various environmental factors such as changes in temperature or osmolarity, exposure to chemicals can change the extent of DNA supercoiling, which in turn affects the expression of various genes. The supercoiling of DNA is presumed to act as a thermosensor and its regulation is important in order to maintain the DNA related functions, such as replication, transcription and recombination (Drlica, 1992; Higgins *et al.*, 1988). The usual negative supercoiling state of DNA transiently increases after the temperature downshift (Krispin and Allmansberger, 1995; Mizushima *et al.*, 1997). The arrangement between the -10 and -35 region of many promoters is affected due to this change, which in turn affects recognition of some σ^{70} promoters, for example, the cold-shock-inducible *E. coli* *recA* promoter is one such twist-sensitive promoter (Wang and Syvanen, 1992). It is hypothesized that organisms sense the changes in temperature also at the level of ribosomes (VanBogelen and Neidhardt, 1990). It was shown that artificially induced high levels of the guanosine 5' triphosphate-3'diphosphate (pppGpp) and guanosine 5' diphosphate-3'diphosphate (ppGpp) (collectively abbreviated as (p)ppGpp) diminish the expression of cold-shock proteins, while low concentration increases their production. Thus, (p)ppGpp affect the magnitude of the cold-shock response (Jones *et al.*, 1992).

Cold shock proteins

One of the most prominent responses of the microorganisms to cold shock is induction of cold shock proteins. All the three groups of microorganisms, i. e. psychrophiles, mesophiles and thermophiles, synthesize cold shock proteins to counteract the effect of temperature downshift. The cold-shock response and cold shock proteins have been studied in detail using *E. coli* and *Bacillus subtilis* as model systems (for review see Ermolenko and Makhatadze, 2002; Phadtare *et al.*, 1999; Phadtare, 2000; Weber and Marahiel, 2003; Yamanaka *et al.*, 1998).

The cold shock proteins of *E. coli* include; group I: CspA (Goldstein *et al.*, 1990), CspB (Lee *et al.*, 1994), CspG (Nakashima *et al.*, 1996), CspI (Wang *et al.*, 1999), CsdA (Toone *et al.*, 1991), RbfA (Dammel and Noller, 1995), NusA (Friedman *et al.*, 1984), PNP (Donovan and Kushner, 1986), and group II: RecA (Walker, 1984), IF-2 (Gualerzi and Pon, 1990), H-NS (Dersch *et al.*, 1994), GyrA (Sugino *et al.*, 1977), Hsc66, HscB (Lelivelt and Kawula, 1995), dihydrolipoamide transferase and pyruvate dehydrogenase (Jones and Inouye, 1994). The induction levels of these proteins vary, proteins belonging to group I being more dramatically induced than those from group II. CspA, CspB, CspG and CspI have been proposed to function as RNA chaperones; CsdA is a ribosomal associated protein with RNA unwinding activity. RbfA is a ribosomal binding factor, recently shown to be involved in maturation of ribosomes at low temperatures (Xia *et al.*, 2003). NusA is involved in termination and antitermination of transcription and PNP is a ribonuclease. PNP selectively degrades *cspA* mRNA at 15 °C and represses production of CspA homologues at the end of the lag phase (Neuhaus *et al.*, 2000; Yamanaka and Inouye, 2001a). A recent DNA microarray analysis of a PNP-deficient mutant showed that this enzyme may have a complex role in controlling cold adaptation of cells. Along with *csp* genes, genes previously not reported to be associated with cold shock response such as *rpoE* and *rseA* increased upon cold shock in this mutant (Polissi *et al.*, 2003). In the case of *Pseudomonas putida*, deletion of the *pnp* gene did not lead to cold sensitivity and the transcription pattern of *pnp* upon cold shock in this organism was markedly different from that in *E. coli*. It thus appears that regulation of expression of *pnp* and its physiological role in cold shock response may be different in different bacterial species (Favaro and Deho, 2003). RecA and IF2 are recombination and initiation factors, respectively. H-NS is a nucleoid-associated, DNA-binding protein and GyrA is the subunit of topoisomerase DNA gyrase. Recently, more cold-shock-inducible proteins have been reported. A trigger factor (TF) enhances viability of cells at low temperature and is induced after a lag period of 2-3 h upon cold shock (Kandror and Goldberg, 1997). It presumably helps protein synthesis and folding to continue at low temperature, and may also help to maintain preexisting proteins in a functional form by promoting refolding of cold-damaged proteins. A ribosome-associated protein of *E. coli*, pY is also induced by temperature downshift. Ribosomes from bacterial cells growing at a normal physiological temperature do not contain pY, whereas a temperature downshift results in the appearance of this protein in ribosomes. It also appears in the ribosomes of those

cells that have reached the stationary phase of growth at a physiological temperature. It inhibits translation at the elongation stage by blocking binding of aminoacyl-tRNA to the ribosomal A site (Agafonov *et al.*, 2001). In addition, trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) are also induced by cold shock in *E. coli* (Kandror *et al.*, 2002).

In case of *B. subtilis*, three CspA homologues CspB, CspC and CspD were shown to be essential for efficient growth at optimal temperature, for efficient adaptation to low temperatures and survival during stationary phase (Graumann *et al.*, 1997). In addition to these, other proteins that are induced after temperature downshift include: CheY (chemotaxis), Hpr (sugar uptake), ribosomal proteins S6 and L7/ L12 (translation), peptidyl propyl cis/trans isomerase (protein folding), cysteine synthase, ketol-acid reductoisomerase, glyceraldehyde dehydrogenase, and triosephosphate isomerase (general metabolism) (Graumann *et al.*, 1996; Graumann and Marahiel, 1999).

CspA family of cold shock proteins

The first cold-shock protein, CspA, was reported from *E. coli* and its homologues have been reported from a number of gram-positive and gram-negative bacteria, but not from archaea and cyanobacteria. The CspA family of *E. coli* consists of nine homologous proteins, CspA to CspI, but among them only CspA, CspB, CspG and CspI are cold-shock inducible. The functions of the CspA family members overlap since they are able to substitute for each other during cold acclimation. *E. coli* cells harboring double or triple deletions of the *csp* genes ($\Delta cspA\Delta cspB$, $\Delta cspA\Delta cspG$, $\Delta cspB\Delta cspG$, $\Delta cspA\Delta cspI$ or $\Delta cspA\Delta cspB\Delta cspG$) are not cold sensitive, and in the triple deletion strain, CspE is overproduced at low temperatures. On the other hand, a quadruple deletion strain ($\Delta cspA\Delta cspB\Delta cspG\Delta cspE$) is cold-sensitive, and this defect can be complemented by overproduction of any one of CspA homologues except CspD (Xia *et al.*, 2001a). In contrast, in the case of *B. subtilis*, however, a *cspB/cspC/cspD* triple deletion mutation is lethal, indicating that at least one CspA homologue is essential for the survival of the organism, CspB being the most important of the three proteins (Graumann *et al.*, 1997).

Regulation of expression of cold-shock induction of CspA and its homologues occurs at levels of transcription, mRNA stability and translation and has been a topic of extensive studies. The cold-shock induction of *cspA* does not need any additional transcription factors in contrast to heat-shock induction of proteins. One of the unique features of *cspA*, *cspB*, *cspG* and *cspI* is the unusually long 5' untranslated region (5'-UTR). The 5'-UTR contains a highly conserved unique 11-base sequence called the cold box (Jiang *et al.*, 1996; Xia *et al.*, 2001b). It is a presumed transcriptional pausing site and is involved in the repression of *cspA* expression. Based on deletion analysis, *cspA* 5'-UTR is presumed to be responsible for the extreme instability of *cspA* mRNA at 37 °C, and has positive effect on mRNA stabilization at low temperature (Mitta *et al.*, 1997). *CspA* mRNA is dramatically but transiently stabilized (half-life more than 20 min at 15 °C as compared to half life of 12 s at 37 °C) immediately following cold shock. Its promoter is active at 37 °C, but

due to instability of its mRNA, CspA is hardly detected at 37 °C. Interestingly, CspA is also produced at 37 °C during early exponential growth phase and its mRNA becomes unstable by mid- to late-exponential growth phase (Brandi *et al.*, 1999). This expression is attributed to the position of *cspA* near *oriC* resulting in higher gene dosage effect, and high concentration of its transcription activator Fis and higher stability of its mRNA due to lower RNase activity. With increasing cell density, Fis is diluted out, while, a transcriptional repressor, H-NS, accumulates leading to decline in level of *cspA* transcript. This is followed by rapid disappearance of the *cspA* mRNA due to increased rate of its degradation at 37 °C (Brandi *et al.*, 1999). Later it was shown that production of CspA at 37 °C during early exponential growth phase was due to nutritional upshift and the induction level of CspA at 37 °C was one-sixth of its cold shock induction level (Yamanaka and Inouye, 2001b).

The *cspA* mRNA contains a unique sequence located 14-bases downstream of the initiation codon. This element is also present in CspB, CspG, CspI, CsdA and RbfA and presumed to enhance translation initiation in cold shock mRNAs. It was originally termed as the downstream box (DB). It is complementary to a region in the penultimate stem of 16S rRNA and was initially thought to enhance translation initiation by facilitating the formation of translation preinitiation complex through binding to 16S rRNA, however this view is disputed and the exact mechanism of the enhancing effect on translation initiation by DB is unknown at present (Mitta *et al.*, 1997; Moll *et al.*, 2001).

Psychrophilic bacteria

The cold-shock response of psychrophiles is distinct from that of mesophiles (Hebraud and Potier, 1999). Mesophilic bacteria, with the exception of *Enterococcus faecalis* do not produce Caps (cold acclimation proteins) in response to continuous growth at low temperature (Panoff *et al.*, 1997). In psychrophilic bacteria, the synthesis of housekeeping proteins is not repressed following an abrupt temperature downshift, a large number of cold shock proteins are synthesized at relatively moderate levels and synthesis of Caps is prolonged. Two psychrophiles, namely *Arthrobacter globiformis* (Berger *et al.*, 1996; Berger *et al.*, 1997) and *P. fragi* (Michel *et al.*, 1997) are well studied with respect to their cold-shock response. A low-temperature-specific proteolytic system has been described for *A. globiformis* that presumably eliminates denatured proteins whose accumulation can be detrimental to the cells.

Cyanobacteria

Cyanobacteria, the photosynthetic bacteria lack CspA homologues, but they have a family of cold-inducible, RNA-binding proteins (Rbp) (Sato, 1995). These are similar to eukaryotic RNA-binding proteins. These belong to the RBD (RNA-binding domain) family of proteins, and are structurally distinct from the CSD (cold-shock domain) family of proteins. In addition, Clp proteins, S21 protein in the small subunit of ribosome and CrhC, an RNA helicase are also induced by temperature downshift in cyanobacteria

(Chamot *et al.*, 1999; Chamot and Owtrim, 2000; Los and Murata, 1999; Schelin *et al.*, 2002). A membrane-bound histidine kinase, Hik33, was identified as a cold sensor in *Synechocystis* and it was postulated that Hik33 might detect decrease in temperature by sensing the rigidification of membrane lipids (Suzuki *et al.*, 2000). A 'knockout' library of cells that lacked the activities of individual histidine kinases was constructed by systematically disrupting all the putative genes for histidine kinases in *Synechocystis*. One of the 41 histidine kinases was then identified as a cold sensor. Mutation of the gene for this histidine kinase, Hik33, diminished the extent of induction of several cold-inducible genes, such as the *desD* gene and the *crh* gene for RNA helicase. This suggested role of Hik33 in perceiving and transducing cold signal to regulate the expression of cold-inducible genes. The desaturase system of cyanobacteria is discussed in detail later in this article.

Current issues in cold-shock response

Desaturase system

The desaturase system of *Bacillus* and cyanobacteria has been reviewed and it is suggested that the expression of cold-inducible genes is regulated by a two-component system and the cold sensors are membrane-bound histidine kinases (Sakamoto and Murata, 2002).

Desaturase system of *Bacillus*

The elastic liquid crystalline nature of the cell membrane changes to a gel-phase state upon temperature downshift. Bacteria adapt to this change by either increasing the proportion of unsaturated fatty acids (UFAs) in the membrane lipids, shortening the fatty acid chain length or by altering fatty acid branching from iso to anteiso. The increase in UFA is achieved by two distinct mechanisms. In *E. coli* the enzyme β -ketoacyl-acyl carrier protein (ACP) synthase II, that is activated (but not induced) upon cold shock, converts palmitoleic acid to *cis*-vaccenic acid (Garwin and Cronan, 1980; Garwin *et al.*, 1980). On the other hand, the desaturation system in *B. subtilis* is cold inducible. *Bacillus* is the only non-photosynthetic bacterium in which the presence and cold induction of desaturase is reported (Aguilar *et al.*, 1998; Aguilar *et al.*, 2001). It has also been shown that in *B. subtilis* anteiso-branched fatty acids are prominent after cold shock. After shift to a lower temperature, the ratio of anteiso- to iso-branched fatty acids is dramatically changed, the change being dependent on the presence of isoleucine or precursors of anteiso-branched fatty acids. Anteiso- fatty acids have lower melting point than iso- fatty acids (Kaneda, 1967, 1991). Similar system was also reported from *L. monocytogenes* (Annous *et al.*, 1997).

As mentioned above, *Bacillus* is the only non-photosynthetic bacterium in which the presence and cold induction of desaturase has been reported (Aguilar *et al.*, 1998; Aguilar *et al.*, 2001). The *des* transcript is hardly detected at 37 °C and its synthesis is transiently induced (10-15-fold) 4 h after cold shock. Deletion of *des* gene does not cause cold-sensitivity in *Bacillus* growing in a rich medium (Aguilar *et al.*, 1998). However, its deletion causes a severe cold-sensitive phenotype in the absence

of isoleucine. The four UFA species of different lengths, branching patterns and positions of the double bond that are found in the wild-type strain are not synthesized in the *des* deletion mutant. Also, the deletion mutant exhibits significantly altered saturated fatty acid profile at the onset of the stationary phase in the presence of exogenous isoleucine sources. It was reported that during cold shock adaptation, *des* expression can completely replace the isoleucine-dependent fatty acid branching adaptation mechanism (Weber *et al.*, 2001).

A two-component signal transduction system consisting of a sensor kinase DesK and a response regulator DesR is responsible for the cold shock induction of *des* gene. DesR binds to a DNA segment from -28 to -77 positions relative to the start site of *des* gene. UFAs act as negative signaling molecules of *des* transcription (Aguilar *et al.*, 2001). DesK assumes different signaling states in response to a temperature-induced change in membrane fluidity. This is accomplished by regulating the ratio of kinase to phosphatase activity such that at 37 °C, when membrane lipids are disordered, a phosphatase- dominant state is present. A kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature downshift to 25 °C. DesK has four transmembrane domains, and one or more of these domains may propagate a conformational change across the membrane that is sufficient to significantly alter its activity. This conformational change can be governed by the physical state of the membrane lipid bilayer. The liquid crystalline state of membranes changes to a gel phase state when upon temperature downshift. This change may cause activation of the autokinase activity, resulting in autophosphorylation of a conserved histidine present in the transmitter domain of DesK. The phosphoryl group of histidine can be directly transferred to DesR, which activates transcription of *des*, resulting in synthesis of Des. The newly synthesized UFAs inhibit *des* transcription either by favoring DesK dephosphorylation of DesR-P or by causing dissociation of DesR-P from its binding site (Aguilar *et al.*, 2001). It was also shown that exogenous isoleucine sources, as well as its alpha-keto acid derivative, which is a branched-chain fatty acid precursor, negatively regulate the expression of the *des* gene at 37 °C. Isoleucine affects the signaling state of the DesK sensor protein by dramatically increasing the incorporation of anteiso-branched-chain fatty acids into membrane phospholipids. It was proposed that both a decrease in membrane fluidity at constant temperature and a temperature downshift induce *des* by the same mechanism. Therefore, the Des pathway may provide a novel mechanism to optimize membrane lipid fluidity at a constant temperature (Cybulski *et al.*, 2002). Recently, two groups have published genome-wide transcriptional analysis of cold-shock response in *B. subtilis* using DNA microarrays (Beckerling *et al.*, 2002; Kaan *et al.*, 2002). Both analyses reveal that *des* is the strongest cold-inducible gene, however the DesKR system is not the cold-triggered regulatory system of global relevance. These studies also confirmed the cold induction of classical cold-shock genes *cspB*, *cspC* and *cspD* along with identification of possible new factors involved in cold shock adaptation of *B. subtilis*, namely elongation factor homologue *yIaG* and the σ^+ -dependent transcriptional activator homologue *ypIP* (Beckerling *et al.*, 2002; Kaan *et al.*, 2002).

Desaturase system of cyanobacteria

In case of cyanobacteria, the change in membrane composition in response to temperature downshift is achieved with help of desaturases. These are acyl-lipid desaturases that introduce double bonds into fatty acids that have been esterified to glycerolipids and are bound to the thylakoid membrane in these bacteria (Murata and Wada, 1995). In the case of *Synechococcus* sp., the two of the desaturase genes, *desA* and *desB*, are induced after temperature downshift from 38 °C to 22 °C. Their expression is tightly controlled by a combination of mRNA synthesis and stabilization at low temperature (Sakamoto and Bryant, 1997). Deletion of *des* causes cold sensitivity, as in these organisms desaturation of lipids is correlated with acclimatization of photosynthetic activity at low temperature. On the other hand, introduction of the *desA* gene into the chilling-sensitive cyanobacterium *Anacystis nidulans* increased the cold resistance of this organism (Wada *et al.*, 1990). These results suggest that desaturases are essential for the cold-shock adaptation of cyanobacteria. It has also been shown that transcription of *desA* is supported by the energy produced by photosynthesis. Thus, the low temperature-induced desaturation of membrane lipids occurs only in the light, and polyunsaturated fatty acids are important for growth and the ability to tolerate photoinhibition of photosynthesis at low temperature (Gombos *et al.*, 1992, 1994).

Recently, it was reported that disruption of genes for fatty acid desaturases in *Synechocystis* leads to rigidification of membrane lipids and enhances cold inducibility of gene expression. DNA microarray analysis of wild type and *desA*/*desD* cells revealed that three types of cold-inducible genes are evident based on degree of enhancement in cold inducibility, high, moderate and none. Genes such as heat shock genes, sulfate transport system subunit genes and a histidine kinase gene belong to the first category, while those encoding RNA helicase belong to third category. Hik33, a cold-sensing histidine kinase, regulated the expression of most genes in the second and third group, but of only few genes in the first group, suggesting that the first group genes are regulated by an unidentified cold sensor (Inaba *et al.*, 2003).

CspA homologues as RNA chaperones and transcription antiterminators

At low temperatures, the secondary structures of RNA stabilize, which presumably slows down (i) transcription elongation and (ii) ribosomal movement on RNA and thus translation. *CspA* homologues are speculated to function as 'RNA chaperones' as they can destabilize the secondary structures in RNA and thus presumably facilitate transcription and translation (Figure 1). Increased levels of *CspA* homologues after cold shock may be important for

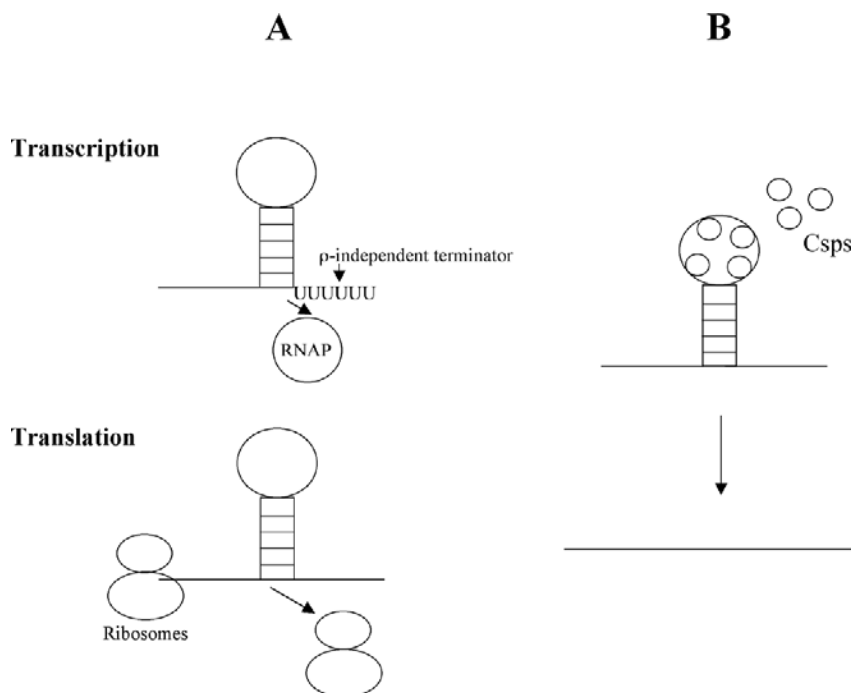


Figure 1. RNA chaperone function of *CspA* homologues.

A. Schematic representation of inhibition of transcription and translation due to stabilization of secondary structures in RNA upon cold shock. Transcription is terminated when RNA polymerase (RNAP) encounters ρ -independent terminator-hairpin loop preceding U-tract and ribosomes fall off RNA when they encounter stem-loop structure, hindering translation. B. Schematic representation of melting of the secondary structure in RNA by *CspA* homologues. *Csp* molecules are shown by small circles. The structures are not drawn to scale.

compensating for higher stability of secondary structures in RNA at low temperatures.

The three-dimensional structure of CspA from *E. coli* and CspB from *B. subtilis* has been resolved by X-ray crystallography and NMR-analysis (Feng *et al.*, 1998; Newkirk *et al.*, 1994; Schindelin *et al.*, 1993; Schindelin *et al.*, 1994; Schnuchel *et al.*, 1993). The protein consists of five antiparallel β -strands, β 1 to β 5, forming a β -barrel structure with two β -sheets. Two evolutionarily conserved RNA-binding motifs, RNP1 and RNP2, are located on the β 2 and β 3 strands. The RNP1 W¹¹, F¹⁸ and F²⁰, and RNP2 F³¹, H³³ and F³⁴ form a compact surface-exposed aromatic patch on the three-dimensional structure of *E. coli* CspA. Presumably, the aromatic patch residues contribute to nucleic acid binding by intercalating between DNA or RNA bases. Recently, the binding of CspB from *B. subtilis* to a 25-mer ssDNA was characterized using heteronuclear 2D NMR spectroscopy, which supported the involvement of aromatic patch residues in DNA binding (Zeeb and Balbach, 2003). Several studies involving site-directed mutagenesis of aromatic patch residues have been performed. The results of these studies suggest that substitution of these amino acids with leucine or serine residues destabilize the protein and diminish its RNA binding (Hillier *et al.*, 1998; Schroder *et al.*, 1995). For example, mutations of three phenylalanine residues from the aromatic cluster adversely affect the RNA binding in the case of CspA from *E. coli* (Hillier *et al.*, 1998). Similarly, in the case of CspB from *B. subtilis*, the nucleic acid binding as well as the protein stability is abolished by the mutations in the two RNP sites (Schindler *et al.*, 1998; Schroder *et al.*, 1995).

CspA from *E. coli* binds RNA without apparent sequence specificity and with low binding affinity (Jiang *et al.*, 1997). CspB, CspC and CspE from *E. coli* are able to more selectively bind RNA/single stranded (ss) DNA (the preferred sequences being UUUUU, AGGGAGGGA and AU-rich regions, respectively), while CspB from *B. subtilis* binds to T-rich regions preferentially, however the magnitude of this selectivity is small (Lopez *et al.*, 2001; Phadtare and Inouye, 1999). The non-specific and weak binding of CspA homologues to RNA/ssDNA is also important for the chaperone function, as binding of the protein would not hamper ribosome movement on mRNA. Hence, heat-shock proteins function as protein chaperones at high temperatures, while CspA homologues function as RNA chaperones at low temperatures (Graumann and Marahiel, 1998; Jiang *et al.*, 1997).

E. coli CspE is a homologue of CspA and is mainly produced constitutively at 37 °C. In a strain of *E. coli* in which *cspA*, *cspB* and *cspG* are deleted, CspE level increases upon cold shock and the quadruple deletion strain ($\Delta cspA\Delta cspB\Delta cspG\Delta cspE$) is cold sensitive (Xia *et al.*, 2001a). CspE interacts with the nascent RNA in transcription elongation complexes and interferes with Q-mediated transcription antitermination (Hanna and Liu, 1998). The link between function of CspA homologues and transcription regulation was further strengthened by the demonstration that several CspA homologues, including CspC and CspE can act as transcription antiterminators *in vivo* and *in vitro*. During cold shock, the expression of several promoter-distal genes of the *metY-rpsO* operon whose products such as, NusA, IF2, RbfA, and PNP are

involved in cold acclimation of cells, is increased at the level of transcription antitermination. Overproduction of either CspA, CspE or CspC leads to similar increase in promoter-distal *metY-rpsO* operon gene expression even at 37 °C (Bae *et al.*, 2000). This result provided evidence that transcription antitermination function of cold shock-induced CspA homologues is relevant. In addition to reducing the efficiency of transcription termination on ρ -independent terminators, CspA homologues also reduce hairpin-induced transcription pausing (Bae *et al.*, 2000).

Current results demonstrate that nucleic acid melting function of CspA homologues is necessary for cellular adaptation to cold, and are consistent with the idea that their transcription antitermination function is linked to their cold shock function (Phadtare *et al.*, 2002a). In *E. coli* CspE, the aromatic patch residues, Trp¹⁰, Phe¹⁷, Phe¹⁹, Phe³⁰, His³² and Phe³³ were individually substituted with Arg residue (Phadtare *et al.*, 2002a; Phadtare *et al.*, 2002b). Only the mutant proteins harboring substitutions of Phe¹⁷, Phe³⁰, and His³² residues located in the center of the aromatic patch, were unable to melt nucleic acids. Since, all three mutants bound RNA as efficiently as the wild-type CspE, the results directly implicated Phe¹⁷, Phe³⁰ and His³² in the melting process. The primary interaction between the protein and RNA is probably due to a set of positively charged Lys residues (K⁴, K¹⁰, K¹⁶, K²⁸, K⁴³ and K⁶⁰) that surround the aromatic patch. This primary electrostatic interaction must position CspE such that Phe¹⁷, Phe³⁰ and His³² residues can intercalate between the bases and thus initiate separation of the nucleic acid strands. In case of each of the above three mutants, the inability to melt nucleic acids resulted in the inability of CspE mutants to antiterminate transcription and to function in cold acclimation, further strengthening the link between nucleic acid melting activity of CspA homologues, transcription antitermination and cold acclimation (Phadtare *et al.*, 2002a; Phadtare *et al.*, 2002b). Further, our results show that binding of multiple CspE molecules in the single-stranded loop region "triggers" the melting of the stem and there are two intermediates of the melting pathway. Phe¹⁷ and Phe³⁰ act at the earliest stages of melting, while His³² acts later and is necessary for the propagation of melting (unpublished data).

Low temperature protein chaperones

Misfolding of proteins and aggregation of misfolded peptides are major problems at high temperatures. Cells have heat-shock-inducible systems to synthesize heat-shock proteins. Some of them act as molecular chaperones by assisting in correct protein folding and proteolysis of abnormally folded polypeptides. GroEL is one of the major molecular chaperones, which acts in association with other chaperones such as GroES. In contrast, protein misfolding was previously not considered a major problem upon cold shock. But recent reports suggest that proper folding of proteins as well as refolding of cold-damaged proteins is important after cold shock (Kandror and Goldberg, 1997). A peptidyl prolyl isomerase (trigger factor-TF), that catalyzes the *cis/trans* isomerization of peptide bonds N-terminal to the proline residue was identified in *E. coli*. This enzyme is induced upon cold shock at a modest level after a growth

lag period of 2-3 h. Similar to other cold shock proteins, its synthesis is induced after temperature downshift from 37 °C to 10 °C or exposure to chloramphenicol. Cells with reduced levels of TF show reduced viability during storage at 4 °C; on the other hand, its overexpression leads to enhanced viability. It was suggested that TF has a 'maintenance and repair' function as it helps protein synthesis and folding to continue at low temperature (Kandror and Goldberg, 1997). It accelerates proline-limited steps in protein folding with a very high efficiency. It associates with ribosomes and influences the folding of newly formed protein chains (Maier *et al.*, 2003). It has been proposed to be the first chaperone to interact with the nascent polypeptide chain as it emerges from the tunnel of the 70S ribosome and thus probably plays an important role in co-translational protein folding (Blaha *et al.*, 2003). Its cooperation with other chaperone systems, such as GroEL also strengthens its role as a molecular chaperone (Kandror and Goldberg, 1997). Similarly, a trigger factor was identified in *B. subtilis*, which is involved in protein folding at low temperature (Graumann *et al.*, 1996).

Caseinolytic proteases (Clps) represent a new family of bacterial molecular chaperones that includes proteases that are expressed constitutively in some cases and induced by stress in others (Kessel *et al.*, 1995; Thompson and Maurizi, 1994). These are induced in cyanobacteria after temperature downshift. ClpP1 is induced by cold shock and exposure to UV. Its amount increases 15-fold within 24 h of the temperature downshift (Porankiewicz *et al.*, 1998). Deletion of its gene leads to severe impairment of growth at low temperatures. ClpB in *Synechococcus* sp. PCC 7942 was identified initially as a heat-inducible molecular chaperone that is essential for the acquisition of thermotolerance. However, its synthesis is also strongly induced upon cold shock. It has been suggested that ClpB may renature and solubilize aggregated proteins at low temperatures at which translation is repressed (Porankiewicz and Clarke, 1997).

CspA homologues from hyperthermophilic bacteria

The hyperthermophilic bacterium *Thermotoga maritima* has an optimum growth temperature of 80 °C. As this bacterium is capable of metabolizing cellulose and xylan, it is important for generating combustible fuels using renewable carbon and energy sources. *T. maritima* also has an evolutionary significance as according to phylogenetic analysis based on small subunit ribosomal RNA (SSU rRNA), it is located at one of the deepest and most slowly evolving lineages of Eubacteria (Achenbach-Richter *et al.*, 1987). Analysis of the genomic sequence of *T. maritima* MSB8 revealed that almost one quarter of the genome is archeal in nature (Nelson *et al.*, 1999). The similarity between *T. maritima* and Archaea is speculated to be due to the shared ancestry of portions of the genome resulting from extensive lateral gene transfer between these organisms, which inhabit similar and unique ecological niche. However, archeal genomes so far sequenced do not show presence of CspA homologues, while *T. maritima*, has two CspA homologues: *TmCspB* and *TmCspL* (Nelson *et al.*, 1999). These are the most thermostable CspA homologues known to date with T_m values above 80 °C (Wassenberg *et al.*, 1999).

Recently, *TmCspB* has been extensively studied, mainly from the structural point of view (Frankenberg *et al.*, 1999; Kremer *et al.*, 2001; Martin *et al.*, 2001; Perl *et al.*, 1998; Perl and Schmid, 2001; Wassenberg *et al.*, 1999; Welker *et al.*, 1999). It has a β -barrel structure similar to that of *E. coli* CspA and *B. subtilis* CspB. It was suggested that Arg² in *TmCspB* forms an ion cluster with several centrally and C-terminally located residues (Kremer *et al.*, 2001). Since an arginine residue in the penultimate N-terminal position is found exclusively in CspA homologues from thermophilic bacteria, it was suggested that the thermostability of *TmCspB* is based on this ion cluster (Kremer *et al.*, 2001).

As discussed above, in the case of some of *E. coli* *csp* genes, an unusually long 5'-untranslated region of their mRNAs (5'-UTR) were shown to be essential for regulation of expression during cold-shock response. For example, the 5'-UTR of *E. coli* *cspA* influences the stability of its own mRNA, and increased transcription of this region led to a prolonged cold-shock response (Mitta *et al.*, 1997). Interestingly, *TmCspB* also contains a long 5'-UTR and thus, it was suggested that *TmCspB* may be cold-inducible (Welker *et al.*, 1999). In a recent study it was shown that both *TmCspB* and *TmCspL* are able to perform essential functions of *E. coli* CspE *in vitro* and *in vivo*, at conditions 50 to 65 °C below the temperature optimum of *T. maritima*. They are also able to help cold-acclimatization of *E. coli* cells and in this aspect, resemble their mesophilic counterparts suggesting that their functions in *T. maritima* may be similar and/or identical. Most importantly, they can antiterminate transcription with *T. maritima* RNA polymerase at 55 °C, the lower limit of temperature range for growth of *T. maritima*. While additional physiological studies will be necessary to determine when the cold-shock response becomes operational in *T. maritima*, these results suggest that these properties of *TmCsp*s are physiologically relevant and these may be important for adaptation of *T. maritima* to physiologically low temperatures (Phadtare *et al.*, 2003).

Trehalose synthesis upon cold shock

A protective role of the sugar trehalose in heat and osmotic stress is well established. Recently, its important role in cold adaptation of *E. coli* has been reported (Kandror *et al.*, 2002). Mutant cells deficient in trehalose production show decreased viability at 4 °C, which is restored after mutants are transformed with *otsA* and *otsB*, genes involved in trehalose synthesis. Upon temperature downshift from 37 °C to 16 °C, cellular level of trehalose increases 8-fold. The induction of OtsA and OtsB is dependent on RpoS. Their mRNAs show enhanced stability at 16 °C and contain a 'cold box', characteristic of cold shock mRNAs (Kandror *et al.*, 2002). Although, it is not clear at present exactly how trehalose protects cells against cold shock, various mechanisms are suggested based on its mode of action in other stresses such as heat shock, oxidative damage etc. These include: (i) it prevents denaturation and aggregation of proteins, (ii) it functions as a free radical scavenger *in vivo* and thus protects against oxidative damage, (iii) it stabilizes cell membranes (Kandror *et al.*, 2002). The elucidation of mechanism of protection by trehalose may

have broad biological significance, as it is present in variety of organisms including *Drosophila*, *Caenorhabditis elegans* and yeasts.

Cold-shock response and global stress response network of cell

The cellular stress response network is complicated and one system may respond to more than one stress and on the other hand, more than one system may be involved in protecting the cells from a particular stress. Although the cold-shock response machinery seems to be mainly targeted towards reversing the adverse effects of temperature downshift, involvement of CspA homologues and other cold shock proteins in stresses other than cold shock suggests that, regulation and functions of these proteins are intricate. For example, CspC and CspE from *E. coli* regulate the expression of number of RpoS-regulated stress proteins such as OsmY, Dps, ProP and KatG, possibly thorough regulation of RpoS itself. These proteins are induced in response to osmotic stress, oxidative stress, or upon stationary phase. CspE and CspC also regulate expression of Universal protein A, UspA, a protein responding to numerous stresses. These findings implicate CspA homologues in the regulation of expression of stress proteins in the complex stress response network of *E. coli* (Phadtare and Inouye, 2001). In addition, CspE has been implicated in number of cellular functions such as, downregulation of poly(A)-mediated 3' to 5' exonucleolytic decay by PNP (Feng *et al.*, 2001), camphor resistance and chromosome condensation (Hu *et al.*, 1996; Sand *et al.*, 2003), and downregulation of λ Q-mediated transcription antitermination (Hanna and Liu, 1998). The mechanism(s) by which CspE performs these diverse functions are not defined. In addition, CspA homologues are involved in diverse phenomena such as, response to freezing conditions, stationary phase, osmotic stress, starvation, antibiotic biosynthesis, resistance to antimicrobial peptides, inhibition of replication, heat resistance of the spores, UV sensitivity etc. (Becker *et al.*, 2000; Derzelle *et al.*, 2003; Katzif *et al.*, 2003; Leblanc *et al.*, 2003; Mangoli *et al.*, 2001; Martinez-Costa *et al.*, 2003; Movahedi and Waites, 2002; Porankiewicz *et al.*, 1998; Yamanaka and Inouye, 1997; Yamanaka *et al.*, 2001).

Conclusions and future perspectives

Cold-shock response is manifested at various levels such as cell membrane, transcription, translation, metabolism etc. The systems such as desaturases, proteins chaperones, trehalose-synthesizing machinery and cold shock proteins that protect the organism from detrimental effects of cold shock are being extensively studied. These studies have made it possible to understand many, though not all, the events that take place inside the cell upon temperature downshift. Cold-shock response system is probably one of the complex stress response systems of bacteria, as in spite of wealth of knowledge accumulated in recent years regarding different aspects of cold-shock response, a number of questions are yet unanswered. For example, it is still not clear why many bacteria have multiple CspA homologues, for example, *E. coli* has nine CspA

homologues. As minimum of four *E. coli* csp genes had to be deleted before cold sensitivity was manifested, it will be interesting to analyze genome-wide transcriptional profile of cold-shock response of this quadruple deletion strain. The regulation of CspA upon temperature downshift has been a topic of extensive research and debates. Even though cold shock proteins are implicated in number of functions, elaborate research is essential for thorough elucidation of their cellular roles.

Recently, a more general aspect of post-transcriptional control was proposed, which previously was mostly restricted to regulators acting at a single target. According to this concept, global regulators act at the post-transcriptional level and as a result are involved in regulation of number of genes. It was also suggested that the CspA family of cold shock proteins have potential to be global post-transcriptional regulators as they are able to destabilize mRNA secondary structures at low temperatures and thereby allow efficient translation (Nogueira and Springer, 2000). This concept is also supported by involvement of some CspA homologues in regulating other global regulators (see above). However, identification and detail studies of exact cellular targets of these proteins are required before any conclusion can be drawn regarding global regulator status of these proteins.

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