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Cold Shock Response in *Escherichia coli*

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Abstract

Sensing a sudden change of the growth temperature, all living organisms produce heat shock proteins or cold shock proteins to adapt to a given temperature. In a heat shock response, the heat shock sigma factor plays a major role in the induction of heat shock proteins including molecular chaperones and proteases, which are well-conserved from bacteria to human. In contrast, no such a sigma factor has been identified for the cold shock response. Instead, RNAs and RNA-binding proteins play a major role in cold shock response. This review describes what happens in the cell upon cold shock, how *E. coli* responds to cold shock, how the expression of cold shock proteins is regulated, and what their functions are.

Introduction

All living organisms have developed sophisticated strategies to respond to several environmental stresses, such as osmolarity, pH, nutrition deprivation and thermal stresses. In terms of thermal stress, heat shock stress (temperature upshift) has been extensively studied and a common strategy how to respond to it has been emerged from bacteria to human (Hendrick and Hartl, 1993; Gottesman *et al*., 1997). In contrast, cold shock stress (temperature downshift) was poorly understood, particularly in higher eukaryotes. To know the strategy how to respond to cold shock stress includes not only biological interests but also economic and health implications. Prevention of the spoilage of foods kept in a refrigerator, which is usually caused by contaminated bacteria, is clearly an important issue in our life.

Cold shock stress causes bacterial cells two major facts; a decrease in membrane fluidity and translational block. The former fact can be overcome by increasing an unsaturated fatty acid, consequently increasing a diunsaturated phospholipid in membrane. A specific set of proteins called cold shock proteins are transiently induced to overcome the translational block. Unlike heat shock proteins, no common cold shock proteins except for CspA-like proteins were identified among bacteria. Most of the free living bacteria possess at least one cold-shock-inducible CspA-like protein, whose function was proposed to be an RNA chaperone (Yamanaka *et al*., 1998; Graumann and Marahiel, 1998). Cyanobacteria and even human were revealed to contain cold-shock-inducible RNA-binding proteins, although they are another type from CspA (Sato and Nakamura, 1998; Nishiyama *et al*., 1997). RNA-binding proteins and RNAs thus play a central role in cell growth under low temperature conditions.

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Cold Shock Response of Membrane Lipid Composition

Membranes are normally in a liquid crystalline form and undergo a reversible transition to a gel phase upon temperature downshift. To compensate for the transition from a fluid state to a nonfluid state, many organisms have developed mechanisms to change the membrane lipid composition, that is, fatty acid composition, by means of one or a combination of the following changes: an increase in fatty acid unsaturation, a decrease in average chain length, an increase in methyl branching, and an increase in the ratio of *anteiso*-branching relative to *iso*-branching.

It was first reported that *E. coli* adjusts its fatty acid composition in response to a lower growth temperature by increasing the amount of *cis*-vaccenic acid and decreasing the amount of palmitic acid incorporated into membrane phospholipid (Marr and Ingraham, 1962). ß-ketoacyl-ACP synthase II, encoded by *fabF*, plays a key role in the change of fatty acid composition and is responsible for elongating palmitoleic acid to *cis*-vaccenic acid (Garwin *et al*., 1980). Thus, upon cold shock, C16:1Δ9 is converted to C18:1Δ11, giving an increase in unsaturated fatty acids, and consequently, diunsaturated phospholipids which lowers the melting point and has a greater degree of flexibility comparing to saturated phospholipids. This type of response is named as homeoviscous adaptation (Sinensky, 1974). It is interesting to notice that the synthesis of ß-ketoacyl-ACP synthase II is not induced upon cold shock, but the enzyme activity is induced at low temperature (Garwin and Cronan Jr., 1980; Garwin *et al*., 1980). Mutants lacking this enzyme are deficient in both *cis*-vaccenic acid synthesis and thermal regulation (Garwin *et al*., 1980), indicating that the changes in membrane lipid composition are critical for bacterial adaptation to low temperatures.

In contrast, *B. subtilis* contains a membrane-bound fatty acid desaturase, encoded by *des*, which desaturates palmitate to *cis*-Δ5-hexadeceonate (Aguilar *et al*., 1998). The *des* expression is regulated at the level of transcription upon cold shock (Aguilar *et al*., 1998). The *des* deletion mutant grew well at both high and low temperatures, but showed severely reduced survival during stationary phase (Aguilar *et al*., 1998). In a cyanobacterium *Synechococcus* sp. PCC7002, expression of *des* genes, *desA*, *desB* and *desC*, is regulated by a combination of mRNA synthesis and stabilization upon cold shock (Sakamoto and Bryant, 1997).

Cold Shock Proteins of *E. coli*

When an *E. coli* culture at 37°C is transferred to 10 or 15°C, a set of proteins called cold shock proteins are transiently induced (Jones *et al*., 1987). These cold shock proteins are conventionally classified into two groups based on their expression patterns (Thieringer *et al*., 1998): Class

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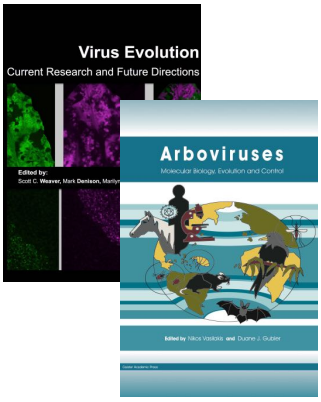

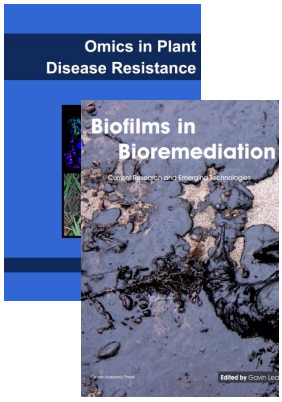

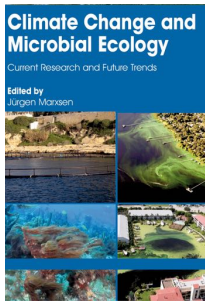

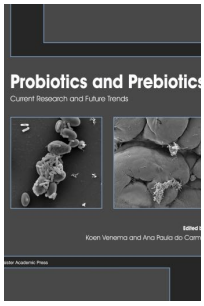

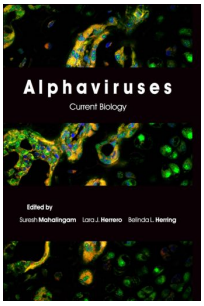

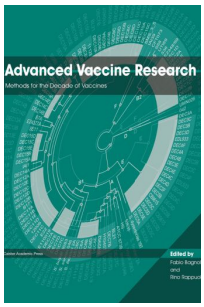

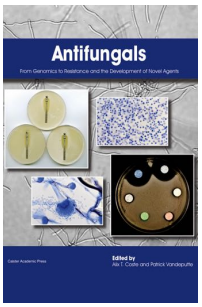

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I cold shock proteins are expressed at an extremely low level at 37°C but are dramatically induced upon cold shock. Class I includes CspA, CspB, CspG, CspI, CsdA, RbfA, NusA, and PNP; ClassII cold shock proteins are expressed at a certain extent at 37°C and are induced moderately upon cold shock. ClassII includes IF-2, H-NS, RecA, α subunit of DNA gyrase, Hsc66, HscB, trigger factor, dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (lipoamide).

Several cold shock proteins have been detected not only in *E. coli* but also in many other bacteria, including mesophilic bacteria, *B. subtilis* (Lottering and Streips, 1995; Graumann *et al*., 1996), *Enterococcus faecalis* (Panoff *et al*., 1997), and *Lactococcus lactis* (Panoff *et al*., 1994), psychrotrophic bacteria, *Pseudomonas fragi* (Hebraud *et al*., 1994; Michel *et al*., 1996; 1997), *Arthrobacter globiformis* (Berger *et al*., 1996), *Bacillus psychrophilus* (Whyte and Inniss, 1992), *Vibrio vulnificus* (McGovern and Oliver, 1995), *Pseudomonas putida* (Gumley and Inniss, 1996) *Listeria monocytogenes* (Phan-Thanh and Gormon, 1995; Bayles *et al*., 1996), and *Rhizobium* (Cloutier *et al*., 1992), and psychrophilic bacterium *Aquaspirillum arcticum* (Roberts and Inniss, 1992). It has yet to be determined how well cold shock proteins are conserved among bacteria.

Energy Generation

Two cold shock proteins, dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (lipoamide), are subunits of pyruvate dehydrogenase complex along with dihydrolipoamide reductase (NAD^+). Pyruvate dehydrogenase complex is essential to convert pyruvate to acetyl CoA, which is an initial, important substrate for the TCA cycle as well as fatty acid synthesis. This suggests that the metabolic pathway to generate energy, ATP, is regulated upon cold shock.

Chromosome Dynamics

Negative supercoiling of plasmid DNA in *E. coli* cells has been shown to transiently increase upon cold shock (Goldstein and Drlica, 1984: Mizushima *et al*, 1997). DNA gyrase and HU protein play an important role in this DNA supercoiling reaction. This notion is consistent with that DNA gyrase α subunit is a cold-shock inducible protein (Jones *et al*., 1992b). Thus, the regulation of chromosomal DNA supercoiling upon cold shock may be important to maintain DNA transactions such as replication, transcription and recombination. It is interesting to note that RecA, which is involved in recombination and repair, and H-NS, which is a nucleoid-associated DNA binding protein and is involved in gene expression and chromosome compaction, are also cold-shock inducible proteins (Jones *et al*., 1987). Growth inhibition at low temperature was observed in *E. coli* strains carrying *hns* mutations (Dersch *et al*, 1994). Expression of *hns* and *gyrA* has been demonstrated to be regulated by CspA in such a way as CspA may help or stabilize an open complex formation for transcription (La Teana *et al*., 1991; Jones *et al*., 1992b; Brandi *et al*., 1994). It is likely that changes in DNA superhelicity upon cold shock might be involved in the induction of cold shock response.

Protein Molecular Chaperones at Low Temperature

Previously protein folding or refolding is not considered to be a major issue at low temperatures. However, it becomes the important issue at present. Hsc66 and HscB, a DnaK and DnaJ homologue, respectively, are specifically induced upon cold shock (Lelivelt and Kawula, 1995). Synthesis of several proteins were affected by an *hsc66* mutation, although cell growth at low temperature was not significantly impaired (Lelivelt and Kawula, 1995). Another cold-shock protein, trigger factor (TF), is a molecular chaperone functioning as a peptidyl-prolyl isomerase (Kandror and Goldberg, 1997; Scholz *et al*., 1997). It associates with nasent polypeptides on ribosomes, binds to GroEL, enhances GroEL's affinity for unfolded proteins, and promotes degradation of certain polypeptides (Kandror *et al*., 1995; 1997; Stoller *et al*., 1995; 1996). TF has been shown to be important for viability at low temperatures. When *E. coli* cells are stored at 4°C, they lose viability at an exponential rate. Cells with reduced TF content die faster, while cells overexpressing TF showed greater viability (Kandror and Goldberg, 1997). These results suggest that the proper protein folding and the refolding of cold-shock-damaged proteins are as important upon cold shock as they are upon heat shock. It should be noticed that as similar to TF, a peptidyl-prolyl *cis-trans* isomerase of *B. subtilis*, encoded by *ppiB*, is cold-shock inducible (Herrler *et al*., 1994; Graumann *et al*., 1996) and is found to be involved in protein folding (Göthel *et al*., 1998).

Adaptation of Translation Factory upon Cold Shock

At low temperature, protein synthesis, especially the translation initiation step, becomes rate limiting for cell growth, resulting in accumulation of 70S ribosomes (Friedman *et al*., 1971; Broeze *et al*., 1978; Farewell and Neidhardt, 1998). The addition of several translation inhibitors such as chloramphenicol has been shown to result in the induction of cold-shock proteins and the addition of other translation inhibitors such as kanamycin induces heat-shock proteins (VanBogelen and Neidhardt, 1990). Thus, it has been proposed that the ribosome is a physiological sensor for thermal stresses, both cold shock and heat shock (VanBogelen and Neidhardt, 1990). The level of (p)ppGpp has also been shown to be involved in the cold-shock response, which reduces the level of (p)ppGpp (Mackow and Chang, 1983; Jones *et al*., 1992a).

Upon cold shock, there is a growth lag period called the acclimation phase before cell growth resumes (Jones *et al*., 1987). During the acclimation phase, most of the cellular protein synthesis is blocked most probably at the translation initiation step. However, the synthesis of cold-shock protein is able to bypass this translational block during this period. The synthesis of non-cold-shock proteins require some translational factors that are induced upon cold shock (Jones and Inouye, 1996). These factors include IF-2, which is a translation initiation factor that lets the initiation tRNA (fMet-tRNA) bind to the 30S subunit (Jones *et al*., 1987), CsdA, which is a DEAD-box protein of helicaces and associates with ribosomes (Jones *et al*., 1996), and RbfA, which is a free 30S ribosome binding factor required for optimal growth particularly at low temperature (Jones and Inouye, 1996). The cold-shock

ribosome adaptation model has been proposed (Jones and Inouye, 1996). In this model, at high temperatures ribosomes are translatable for all cellular mRNAs. Upon cold shock, translation initiation is transiently blocked, resulting in a decrease in polysomes and an increase in 70S, 50S and 30S ribosomes, which are incapable for translation. Cold-shock proteins whose function is related to translation are synthesized to convert cold-sensitive, non-translatable ribosomes to cold-adapted, translatable ribosomes, resulting in the recovery of the cellular protein synthesis and cell growth. As previously proposed that the ribosomes function as the sensor for thermal stresses, the translation is a key factor for adaptation to a given low temperature.

How, then, is the expression of cold-shock genes able to be induced during the acclimation phase upon cold shock, while the synthesis of most of non-cold-shock proteins is blocked? An important feature of the cold shock induction of CspA is the presence of the downstream box (DB) sequence in its mRNA (Mitta *et al*., 1997; Etchegaray and Inouye, 1999b) (Figure 1). The DB sequence was originally proposed to serve as an independent translational signal besides the Shine-Dalgarno (SD) sequence and is located downstream of the initiation codon in the coding sequence of mRNAs (Sprengart *et al*., 1990; 1996). The DB sequence is complementary to the region called the anti-DB sequence of the 16S rRNA. It is speculated that formation of a duplex between the DB sequence of mRNA and the anti-DB sequence of 16S rRNA is responsible for translational enhancement (Sprengart *et al*., 1996). When the DB sequence was deleted from the *cspA* gene, CspA induction upon cold shock was not observed (Mitta *et al*., 1997). Furthermore, when the DB sequence was inserted into the ß-galactosidase gene, it became cold-shock inducible (Mitta *et al*., 1997; Etchegaray and Inouye, 1999b). These results clearly indicate that the DB sequence plays a crucial role in the cold shock induction. It is worth mentioning that not only *cspA* but also other cold-shock genes belonging to the Class I group, whose expression is dramatically induced upon cold shock, contain the DB sequence downstream of the initiation codon in their mRNAs (Mitta *et al*., 1997). The formation of translation initiation complex between mRNA for Class I cold-shock genes and 16S rRNA is likely enhanced by the presence of the DB sequence in concert with the SD sequence (Etchegaray and Inouye, 1999b). The DB sequence is therefore essential for the induction of cold-shock proteins during the acclimation phase, when the translation initiation for non-cold-shock proteins is blocked. In other words, mRNAs for Class I cold-shock genes are efficiently translated without any requirement of cold-shock proteins, whose function is related to translation capacity, such as IF-2, CsdA and RbfA.

It is interesting to note that when a translatable truncated *cspA* mRNA is overexpressed at low temperature, cell growth is completely blocked (Jiang *et al*., 1996b). This effect was termed LACE (low-temperature antibiotic effect of truncated *cspA* expression). It is considered to be caused by the truncated *cspA* mRNA, which still possesses the DB sequence. This mRNA may efficiently form an initiation

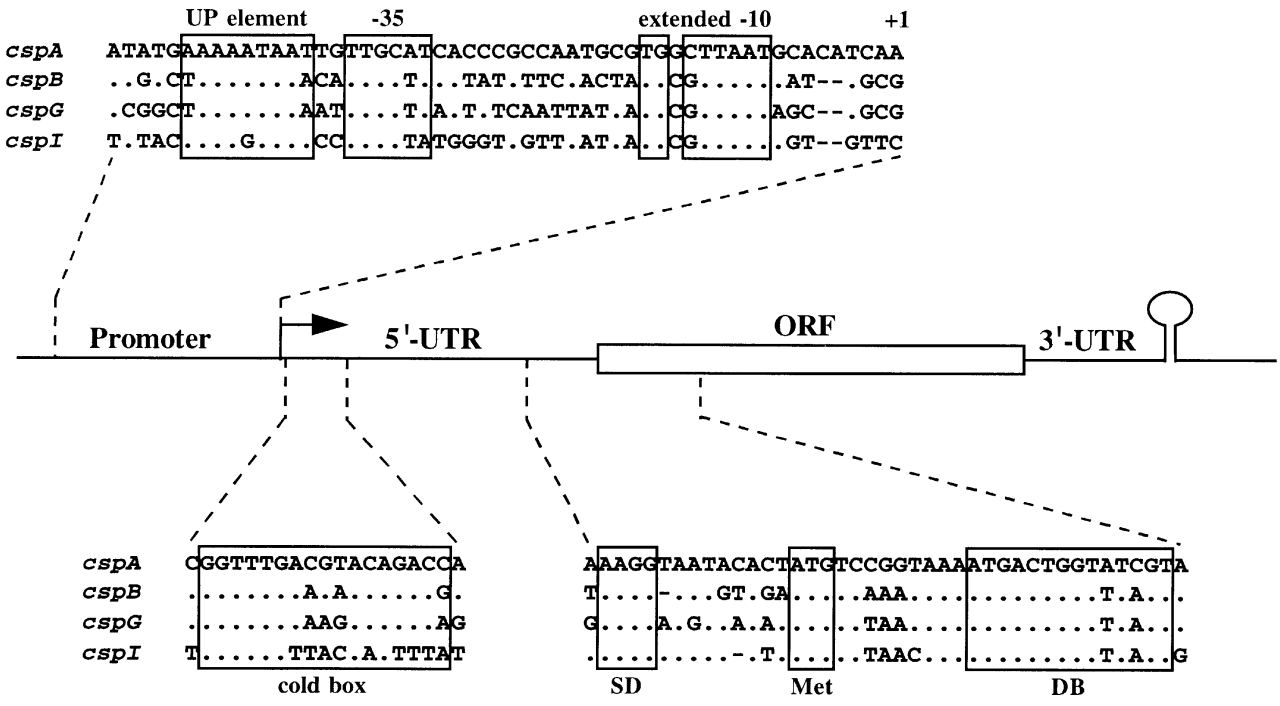

Figure 1. Sequence Comparison of *csp* Genes
Comparison of characteristic regions of *cspA*, *cspB*, *cspG*, and *cspI* genes. Characteristic motifs are boxed. SD, Met and DB represent Shine-Dalgarno sequence, translation initiation codon, and downstream box, respectively. Nucleotides identical to *cspA* are shown as dots and gaps are shown as dashes. At the middle, schematic structure of these genes is presented. See text in details.

complex, in such a way as almost all ribosomes are trapped by the overproduced truncated *cspA* mRNAs. In fact, under LACE, a high polysome profile, which is normally reduced upon cold shock, was retained, resulting in production of only truncated CspA products (Jiang *et al*., 1996b). This also supports the notion that the DB sequence is crucial for efficient translation upon cold shock.

In contrast, it is not well understood how the Class II cold-shock genes are induced. It should be noticed that *hns* and *gyrA* are regulated by CspA at the level of transcription upon cold shock, that is, CspA may help or stabilize the open complex formation for transcription. It is possible that the changes in DNA superhelicity may be involved in cold-shock induction of these genes.

Regulation of *cspA* Expression

The *cspA* gene is located at 80.1 min on the *E. coli* chromosome and it is monocistronically transcribed in a clockwise direction. The most important feature is that the *cspA* mRNA possesses an unusually long 5'-untranslated region (5'-UTR) consisting of 159 bases (Tanabe *et al*., 1992). Since the *cspA* mRNA as well as CspA protein are hardly detected at 37°C, it was suggested that *cspA* expression is regulated at the level of transcription (Tanabe *et al*., 1992). Since then, *cspA* expression has been extensively analyzed, and it is currently considered to be regulated at the levels of transcription, mRNA stability and translation.

The *cspA* gene has a strong promoter equipped with the UP element as well as the extended -10 sequence, which is active at both 37°C and 15°C (Fang *et al*., 1997; Goldenberg *et al*., 1997; Mitta *et al*., 1997) (Figure 1). When the *cspA* promoter was replaced with the *lpp* promoter, a constitutive, strong promoter of a major outer membrane protein, *cspA* expression was still cold-shock inducible, indicating that the cold-shock induction of CspA does not depend on its promoter (Fang *et al*., 1997). However, the expression level at 15°C is higher with the *cspA* promoter than that with the *lpp* promoter (Fang *et al*., 1997), suggesting that the *cspA* promoter is one of the strongest promoter in *E. coli*. The *cspA* promoter is equipped with two unique motifs: one is the presence of an AT rich sequence immediately upstream of the -35 region (Goldenberg *et al*., 1997; Mitta *et al*., 1997). The AT rich sequence, called the UP element, was reported to be directly recognized by the α subunit of RNA polymerase and to confer a strong transcription activity (Ross *et al*., 1993). In fact, when the UP element was deleted from the *cspA* promoter, the promoter activity was almost diminished (Mitta *et al*., 1997). The second unique motif is the presence of a TGn motif immediately upstream of the -10 region. It was reported that this motif together with the -10 region constitute a so called extended -10 region and if a promoter contains the extended -10 region, the -35 region becomes dispensable (Kumar *et al*., 1993). Moreover, the *cspA* promoter itself was shown to be active even at 37°C, at which the CspA protein is hardly detected (Mitta *et al*., 1997). Thus, by virture of these two motifs, the *cspA* promoter has a strong activity both at 37°C and 15°C. It should be mentioned that transcription of the *cspA* gene does not require any *de novo* protein synthesis upon cold shock (Etchegaray and Inouye, 1999a). Note that unlike the heat-shock response, no specific sigma factor responsible for cold shock has been identified among prokaryotes.

It is very important to notice that the *cspA* mRNA has an unusually long 5'-UTR (Tanabe *et al*., 1992) and is extremely unstable at 37°C (half life; <12 sec) (Brandi *et al*., 1996; Goldenberg *et al*., 1996; Fang *et al*., 1997). Immediately upon cold shock, the *cspA* mRNA becomes stable (half life; >20 min) (Brandi *et al*., 1996; Goldenberg *et al*., 1996; Fang *et al*., 1997). Again, this stabilization of the *cspA* mRNA upon cold shock does not require any *de novo* protein synthesis (Etchegaray and Inouye, 1999a). In the 5'-UTR, there is a putative RNaseE cleavage site immediately upstream of the SD sequence. This site is considered to be responsible for the extreme instability of the *cspA* mRNA, since the three-base substitution mutation at this region resulted in dramatic stabilization of the mRNA, allowing a high CspA production even at 37°C (Fang *et al*., 1997). When the region from +26 to +143 of the 5'-UTR was deleted from the translational *cspA-lacZ* fusion construct, high ß-galactosidase activity was obtained even at 37°C (Mitta *et al*., 1997). Thus, it is clear that the mRNA stabilization plays a major role in *cspA* expression upon cold shock and that the unusually long 5'-UTR is responsible for its instability at 37°C, in other words, the 5'-UTR makes the *cspA* mRNA extremely unstable at 37°C. As described above the *cspA* mRNA is constitutively transcribed even at 37°C but can not be translated due to its extreme instability. However, the exact mechanism of the *cspA* mRNA stabilization upon cold shock is not known. RNase, which is responsible for the degradation of the *cspA* mRNA, may be somehow inactivated upon cold shock. Alternatively, the secondary structure of the *cspA* mRNA at 37°C may be highly susceptible to RNase but not be accesible to ribosomes, while the secondary structure of the *cspA* mRNA at low temperature may be changed to be accesible to ribosomes. It is worth mentioning that although the *cspA* mRNA becomes stable and is accumulated at the nonpermissive temperaturte in the temperature-sensitive RNaseE mutant, CspA production was not detected under this condition (Fang *et al*., 1997), suggesting that in addition to the stability of mRNA, the 5'-UTR may have another role. This may be related to the secondary structure of the *cspA* mRNA, its translation efficiency and the transcription attenuation.

The 5'-UTR of the *cspA* mRNA contains a unique sequence called the cold box, which may form a stable stem-loop structure (Jiang *et al*., 1996a) (Figure 1). CspA is transiently produced during acclimation phase upon cold shock. However, when the 5'-UTR of the *cspA* mRNA or the fragment containing the cold box sequence was overexpressed at 15°C, CspA production became no more transient. When CspA was simultaneously overproduced, however, the normal transient expression was resumed (Jiang *et al*., 1996a). Moreover, when the *cspA* gene without the cold box sequence was reintroduced into a *cspA* deletion mutant, CspA production was again poorly repressed at the end of the acclimation phase (Bae *et al*., 1997; Fang *et al*., 1998). These results clearly indicate that the cold-box sequence plays an important role in autoregulation of *cspA* expression.

Very recently, it has been reported that CspA is also transiently induced during early exponential phase after

dilution of overnight culture at 37°C to a level of approximately 100,000 molecules/cell (Brandi *et al*., 1999). Therefore, they claimed that the designation of CspA as a major cold-shock protein is a misnomer. However, upon cold shock CspA is induced at a level of 800,000 to 1,000,000 molecules/cell, 8 to 10 times higher than the transient expression at 37°C (Jiang *et al*., 1997). Furthermore, when the temperature is lowered less than 10°C, CspA and its homologues CspB and CspG are major proteins induced with little production of all the other cellular proteins (Etchegaray *et al*., 1996). It is important to note that well-established heat-shock proteins such as DnaK and GroE are known to be constitutively expressed under normal growth conditions (Yura *et al*., 1993). In addition, heat-shock proteins can be induced without heat shock if other stresses such as alcohol are given. Similarly, it has been shown that CspA can also be induced at 37°C in the presence of chloramphenicol (VanBogelen and Neidhardt 1990; Jiang *et al*., 1993; Etchegaray and Inouye 1999a). Clearly CspA can be termed as a cold-shock protein, and it is the major cold-shock protein in *E. coli*.

Structure and Function of CspA

CspA was originally identified as a major cold-shock protein in *E. coli* and consists of 70 amino acid residues (Goldstein *et al*., 1990). It has been purified and its three-dimensional structure has also been determined by both X-ray crystallography (Schindelin *et al*., 1994) and NMR spectroscopy (Newkirk *et al*., 1994; Feng *et al*., 1998). It exists as a monomer and consists of five anti-parallel ß-strands, ß1 (K5-N13), ß2 (F18-T22), ß3 (D29-H33), ß4 (Q49-E56) and ß5 (A63-L70), forming a ß-barrel structure with two ß-sheets (ß1-ß2-ß3 and ß4-ß5) (Schindelin *et al*., 1994; Newkirk *et al*., 1994; Feng *et al*., 1998). Five of the hydrophobic residues (V9, I21, V30, V32, and V51) form the hydrophobic core in the ß-barrel structure (Feng *et al*., 1998). It is particularly important to mention that the ß-sheet consisting of ß1 to ß3 contains seven out of eight of aromatic residues (W11, F12, F18, F20, F31, F34 and Y42) and two Lys residues (K10 and K16). Furthermore, on this surface there are two RNA-binding motifs, RNP1 ($K_{16}GFGFI_{21}$) on ß2 and RNP2 ($V_{30}FVHF_{34}$) on ß3. CspA has been shown to bind to both single-stranded DNA (ssDNA) and RNA (Jiang *et al*., 1997). Hydrophilic interactions between CspA and nucleic acid occur through aromatic residues on this surface of the CspA molecule. In fact, it has been demonstrated that mutations of Phe residues on this surface severly affected the DNA-binding activity (Hillier *et al*., 1998). In the case of *B. subtilis*, CspB, a major cold shock protein, has been shown to be highly homologous to *E. coli* CspA (Willimsky *et al*., 1992). Its three-dimensional structure has also been resolved to form a similar ß-barrel structure (Schindelin *et al*., 1993; Schnuchel *et al*., 1993). Mutations of Phe residues in the RNA-binding motifs has been shown to abolish the DNA-binding activity (Schröder *et al*., 1995). These results clearly indicate that the ß-sheet of ß1 to ß3, especially RNA-binding motifs, plays a crucial role in the binding of CspA to nucleic acids.

E. coli CspA exists as a monomer (Schindelin *et al*., 1994; Newkirk *et al*., 1994), while *B. subtilis* CspB exists as a dimer, which is formed by intermolecular hydrogen bonds between ß4 strands (Schindelin *et al*., 1993; Schnuchel *et al*., 1993). What could be the determinant for the dimer formation? It is speculated that the ß4 strand in *E. coli* CspA is somehow masked by its N-terminal region, because *E. coli* CspA has an extra 3 amino acid residues at its N-terminal region comparing to that of *B. subtilis* CspB (Newkirk *et al*., 1994). Since a mutant CspA, which is lacking the N-terminal 4 amino acid residues, still exists as a monomer, it is unlikely that the N-terminal region is involved in the inhibition of dimer formation (Wang *et al*., manuscript in preparation). Alternatively, the structural differences, which are observed in the ß4 strand and the loop connecting strand ß4 and ß5, probably preclude dimer formation in *E. coli* CspA (Makhatadze and Marahiel, 1994). Interestingly, CspD, which is a member of the *E. coli* CspA family but is not cold-shock inducible as discussed below, was found to exist as a dimer (Yamanaka and Inouye, unpublished). By analyzing several chimeric proteins between CspA and CspD, it was recently found that only the ß4 strand is enough to determine the dimer formation. When the ß4 strand of CspA was replaced with the ß4 strand of CspD, the resulting chimeric CspA became to be a dimer (Yamanaka and Inouye, unpublished). Whether a critical amino acid residue in the ß4 strand exists or the structure of the ß4 strand is required for dimerization remains to be addressed.

CspA binds cooperatively to RNA and ssDNA without an apparent sequence specificity (Jiang *et al*., 1997). Binding of CspA to RNA renders it more sensitive to RNase. It is thus proposed that CspA functions as an RNA chaperone to facilitate translation at low temperatures by preventing the formation of stable secondary structures in mRNAs (Jiang *et al*., 1997). In fact, in the cell free translation system, which was prepared from cells grown at 37°C, translation of the *cspA* mRNA was shown to be enhanced by the addition of the purified CspA (Brandi et al., 1996). Note that the CspA binding to RNA is rather weak (Jiang *et al*., 1997), so that ribosome movement on mRNA would not be hampered by the CspA binding.

The CspA Family of *E. coli*

The entire genome sequence of *E. coli* has been determined. It possesses totally nine CspA homologues, CspA to CspI (Yamanaka *et al*., 1998). The lengths vary from 69 to 74 amino acid residues and the identities between each two proteins vary from 29 to 83%. The secondary structure analysis by using the Chou-Fassman's method predicts that all of them likely form a ß-barrel structure as shown for CspA (Yamanaka *et al*., 1998). Moreover, the RNA-binding motifs, RNP1 and RNP2, are also well conserved among them except for CspF and CspH, which have S16, K18, Q31, and V34 or I34 instead of K16, F18, F31 and F34 for CspA, respectively, suggesting that the *E. coli* CspA homologues are likely to bind to nucleic acid (Yamanaka *et al*., 1998).

Among nine homologues, only four, CspA (Goldstein *et al*., 1990), CspB (Lee *et al*., 1994; Etchegaray *et al*., 1996), CspG (Nakashima *et al*., 1996), and CspI (Wang *et al*., 1999), are cold-shock inducible. Based on the similarities of their primary amino acid sequences, they may have a similar structure and function as CspA does. It should be noted that although the *cspA* gene has been

shown to be dispensable at both high and low temperatures, the increased production of CspB and CspG was observed upon cold shock in the *cspA* deletion mutant, suggesting that the CspA function may be at least partially complemented by CspB and CspG (Bae *et al*., 1997). Thus, CspA, CspB, CspG, and CspI may have overlapping functions each other, if not the same.

In terms of regulation of gene expression, *cspA*, *cspB*, *cspG*, and *cspI* genes were revealed to share several important features (Figure 1). (i) They all contain the UP element upstream of the -35 region as well as the extended -10 region in their promoter sequences, which are considered to maintain the high promoter activity even at low temperatures (Goldenberg *et al*., 1997; Mitta *et al*., 1997; Wang *et al*., 1999). (ii) They all contain an unusually long 5'-UTR in their mRNAs (159, 161, 156, and 145 bases for *cspA*, *cspB*, *cspG*, and *cspI*, respectively) (Tanabe *et al*., 1992; Etchegaray *et al*., 1996; Nakashima *et al*., 1996; Wang *et al*., 1999). As shown in the case of *cspA*, it is believed that these 5'-UTRs play a crucial role in their cold-shock inducibility. (iii) They all contain the cold box at the 5'-end region of their 5'-UTRs, which plays a role in autoregulation to repress their own gene expression at the end of the acclimation phase (Jiang *et al*., 1996a; Bae *et al*., 1997; Fang *et al*., 1998; Wang *et al*., 1999). (iv) They all contain a DB sequence downstream of the translation initiation codon, which plays an essential role in the cold-shock induction by enhancing translation (Mitta *et al*., 1997; Etchegaray and Inouye, 1999b). Taken altogether, it is likely that expression of all four *csp* genes is regulated essentially in the same manner. It should be mentioned, however, that the optimal temperature ranges for the induction of these genes are different (Etchegaray *et al*., 1996; Wang *et al*., 1999). CspA induction occurs over the broadest temperature range (30°C to 10°C), CspI induction occurs over the narrowest and lowest range (15°C to 10°C), and CspB and CspG occurs at temperatures between the above extremes (20°C to 10°C). The nucleotide sequences of their 5'-UTR are different, suggesting differences in their mRNA secondary structure and therefore in their stabilities. The 5'-UTR of the cold-shock inducible *csp* genes exert a negative effect on their expression at 37°C, affecting their mRNA stabilities and translation initiation efficiencies (Mitta *et al*., 1997). These are likely to be differently modulated upon cold shock, depending upon the secondary structure of each mRNA. A smaller temperature difference woud be enough for *cspA* expression, while a larger temperature difference might be required for *cspI* expression.

CspC and CspE were originally isolated as multicopy suppressors for a temperature-sensitive chromosome partition mutant and are expressed at 37°C (Yamanaka *et al*., 1994). CspE was also shown to be involved in chromosome condensation (Hu *et al*., 1996) and to inhibit the transcription antitermination mediated by Q protein of phage λ (Hanna and Liu, 1998). In a *cspE* deletion strain, the synthesis of a number of proteins at 37°C was found to be altered comparing to the wild-type strain. Interestingly, *cspA* expression was derepressed at 37°C (Bae *et al*., 1999). The derepression of *cspA* in the *cspE* mutant occured at the level of transcription in a promoter-independent manner but was not caused by stabilization of the *cspA* mRNA, which is a major cause of CspA induction upon cold shock (Bae *et al*., 1999). *In vitro* transcription assays demonstrated that CspE may increase the efficiency of transcription pausing through direct binding to either the ssDNA or the nascent RNA of the cold box region of *cspA* (Bae *et al*., 1999). Note that CspE can bind to ssDNA and RNA (Bae *et al*., 1999). Moreover, CspE has been shown to be able to interact with elongation complexes containing RNAs as short as 10 nucleotides through protein-RNA interactions (Hanna and Liu, 1998). These results indicate that CspE functions as a negative regulator for *cspA* expression at 37°C.

cspD expression is not cold-shock inducible but is induced during the stationary phase and upon carbon starvation (Yamanaka and Inouye, 1997). It was also shown to be inversely dependent on growth rate. *cspD* expression was suggested to be regulated at the level of transcription rather than translation. However, it is independent of the stationary sigma factor σ^S (Yamanaka and Inouye, 1997). No significant defect was observed in the *cspD* deletion mutant and purified CspD can bind to ssDNA and RNA but not to dsDNA (Yamanaka and Inouye, unpublished). Interestingly, CspD exists as a dimer and the ß4 strand is found to be a determinant for dimer formation as described above. Cellular function of CspD is yet to be determined. Nothing is known about CspF and CspH.

The chromosomal location of nine genes for the *E. coli* CspA family are as follows: *cspE*, 14.2 min (c); *cspD*, 19.9 min (cc); *cspH*, 22.6 min (cc); *cspG*, 22.6 min (c); *cspI*, 35.3 min (cc); *cspB*, 35.3 min (cc); *cspF*, 35.3 min (c); *cspC*, 41.1 min (cc); and *cspA*, 80.1 min (c), where in parentheses c and cc mean that transcription is occured in clockwise and counter-clockwise direction, respectively (Rudd, 1998; Yamanaka *et al*., 1998). The *cspA* gene located at 80.1 min is unique among the family by being closer to the DNA replication origin (84.6 min), while the others are clustered from 14.2 min to 41.1 min, where the DNA replication termination sites are also clustered. Based on these facts together with sequence similarities, it was proposed that the large CspA family of *E. coli* resulted from several steps of gene duplications (Yamanaka *et al*., 1998). Why did *E. coli* duplicate *csp* genes several times? As mentioned above, the region where eight *csp* genes are located corresponds to the region where the DNA replication termination sites are clustered. The DNA replication termination has to be tightly controlled. This would be the reason the DNA replication termination sites are duplicated several times. If *csp* genes and the DNA replication termination sites are somehow linked, it is possible that these might be co-duplicated. It is interesting to mention that the clusters of *cspE-cspH-cspG* and *cspC-cspF-cspB* are located in a mirror image centered around 28.8 min, where *TerA*, one of the DNA replication termination site, is located (Yamanaka *et al*., 1998).

Evolution of the Cold-Shock Domain

Proteins homologous to CspA are found widely in prokaryotes and to date, more than 90 CspA-like proteins have been identified including Gram-positive and Gram-negative bacteria, and psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria. However, *csp*-like genes have not been found in the archaeal genomes (*Archaeoglobus fulgidus, Methanobacterium thermoautotrophicum, Methanococcus jannaschii,* and

Pyrococcus horikoshii) and most of the parasites (*Borrelia burgdorferi, Chlamydia trachomati, Mycoplasma pneumoniae, Mycoplasma genitalium,* and *Treponema pallidum*), whose entire genomes have been sequenced. It can be considered that CspA-like proteins are not required in the parasitic bacteria, since host cells would possess their own response systems against any stresses. One exception is *Rickettsia prowazekii*, which has one *csp*-like gene (Andersson *et al*., 1998). In addition, cyanobacteria also do not have a *csp*-like gene. However, it has another cold-inducible RNA-binding protein RbpA1, which does not belong to the CspA family but is similar to the eukaryotic RNA-binding proteins, such as U1A snRNP (Sato and Nakamura, 1998; Graumann and Marahiel, 1998). Interestingly, its expression is regulated by several DNA-binding proteins, which bind to the 5'-UTR, at the level of transcription.

Furthermore, eukaryotic Y-box proteins, such as human YB-1 and *Xenopus* FRGY-2, contain a region called cold-shock domain (CSD) with more than 40% identity to *E. coli* CspA (Matsumoto and Wolffe, 1998; Yamanaka *et al*., 1998). These facts indicate that the CSD is well-conserved from prokaryotes to eukaryotes. Cellular functions of the Y-box protein family are diverged and include positive or negative transcriptional regulation (Kashanchi *et al*., 1994; Ohmori *et al*., 1996; Ting *et al*., 1994; Zou *et al*., 1995), translational masking of mRNAs (Ranjan *et al*., 1993), signal transduction pathway (Duh *et al*., 1995; Shinozaki and Yamaguchi-Shinozaki, 1996), cell proliferation (Landomery and Sommerville, 1995; Bargou *et al*., 1997), and development (Moss *et al*., 1997). It should be mentioned that proteins of Y-box protein family are relatively large comparing to those of CspA family by having N- and C-terminal extra domains, which are thought to be responsible for their diverged functions (Matsumoto and Wolffe, 1998; Graumann and Marahiel, 1998).

From the evolutional aspect, it is interesting to note that the archaea, which may be an ancestor for eukaryotic nucleus, does not contain a *csp*-like gene, and that eukaryotes have proteins containing a CSD as mentioned above. Recently, a parasite *Rickettsia prowazekii*, which is considered to be more closely related to eukaryotic mitochondria than is any other microbe studied so far, was revealed to contain a *csp*-like gene (Andersson *et al*., 1998). Thus, it can be speculated that the ancestor of the eukaryotic CSD might come from the ancestor of mitochondria, which may be a bacterial parasite. Later on, the gene for CSD was transferred from mitochondria to nucleus and then diverged, probably by duplication, gene arrangement, insertion and so on. It is also interesting to mention that *Mycobacterium tuberculosis* and *Mycobacterium leprae* possess two *csp*-like genes. One seems to encode a bacterial CspA homologue, while another seems to encode a eukaryotic Y-box protein homologue, since it contains a C-terminal extra domain, which possesses a similar feature as the C-terminal extra domain of human YB-1 protein. It consists of alternating regions of predominantly basic and acidic amino acid residues, which is proposed to function as a charge-zipper to mediate protein-protein interactions. Therefore, the mycobacterial homologue is interesting from aspects of both biological function and evolution of CSD.

Concluding Remarks

All living organisms have developed the mechanisms to respond to environmental stresses, such as temperature fluctuation. In the case of temperature upshift (heat shock response), the heat-shock sigma factor plays a crucial role in induction of heat-shock proteins (Yura *et al*., 1993). Heat-shock proteins, in particularly molecular chaperones and proteases, are well-conserved from bacteria to human (Hendrick and Hartl, 1993; Gottesman *et al*., 1997). This is consistent with the notion that protein folding and refolding and/or degradation are the most important issues upon heat shock. In contrast, in the case of temperature downshift (cold-shock response), no such a sigma factor has been identified. Synthesis of cold-shock proteins seems to be regulated mainly at the post-transcriptional level as described above. Thus, the fate of individual mRNA for each cold-shock protein plays a central role in cold-shock response. Most of the free living bacteria possess at least one cold-shock-inducible CspA homologue, which is likely function as an RNA chaperone (Jiang *et al*., 1997). Cyanobacteria contain another type of cold-shock-inducible RNA-binding protein rather than a CspA-like protein (Sato and Nakamura, 1998; Graumann and Marahiel, 1998). Furthermore, a cold-inducible RNA-binding protein has been identified in human cells (Nishiyama *et al*., 1997). RNA-binding proteins probably with an RNA chaperone activity play a major role in cell proliferation at low temperatures. In fact, it has been demonstrated that at least one out of three CspA homologues is required for cell growth in *B. subtilis* (Graumann *et al*., 1997). In the case of *E. coli*, there are nine CspA homologues and it is considered that their functions may overlap. Simultaneous introduction of mutations of nine genes into one cell seems to be a somewhat challenging task. However, a quadruple deletion mutant (*cspA cspB cspE cspG*) was obtained and did show a cold-sensitive growth (Xia *et al*., unpublished). These results indicate that RNA-binding proteins are essential for bacterial growth. These facts are consistent with the notion that translation block and stable secondary structure formation of DNA and RNA are the major issues upon cold shock.

It is interesting to notice that upon cold shock, expression of heat-shock proteins is repressed (Taura *et al*., 1989). When the heat-shock proteins are artificially produced at low temperatures, cellular survival was lost much faster (Kandror and Goldberg, 1997). These results suggest that cold-shock proteins and heat-shock proteins provide protection against opposite thermal extremes, but these two protectors are tightly controlled not to be existing simultaneously.

Very recently, it has been demonstrated that the *rpoH* mRNA secondary structure at 42°C, which is accessible to ribosomes, is different from that at 30°C, and that the *rpoH* mRNA structure itself is determinant for *rpoH* expression without any other factors (Morita *et al*., 1999). Thus, it was proposed that the *rpoH* mRNA acts as an RNA thermosensor (Morita *et al*., 1999). It was also suggested that *lcrF* mRNA of *Yersinia pestis* and the λ phage cIII mRNA might be other examples for RNA thermosensor (Storz, 1999). CspA is not produced at 37°C because of extreme instability of its mRNA, even though it is transcribed. However, upon cold shock the *cspA* mRNA

becomes stable and translatable with no requirement of any *de novo* protein synthesis. Thus, the *cspA* mRNA is designed to be unstable and non-translatable at high temperatures, and simultaneously it is designed to be stabilized and translatable at low temperatures without any *de novo* protein synthesis. In this regard, mRNAs for cold-shock proteins, at least CspA homologues, can be considered to act as an RNA thermosensor, but in a different manner from above mentioned examples.

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