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Cold shock proteins aid coupling of transcription and translation in bacteria

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ABSTRACT

Transcription and translation are tightly coupled in bacterial cells. However, the transcription machinery and ribosomes generally occupy different subcellular regions in bacteria such as Escherichia coli *and* Bacillus subtilis*, indicating the need for (a) mechanism(s) coupling these processes. A prime function of this mechanism(s) would be ensuring the transfer of unfolded mRNA from the nucleoid to ribosomes, which require linear mRNA for the initiation of translation. During conditions of a sudden decrease in temperature (cold shock), secondary structures in mRNA would pose an even greater problem for the initiation process. Two conserved classes of proteins, cold shock proteins (CSPs) and cold induced RNA helicases (CSHs), appear to be major players in the prevention of secondary mRNA structures and in transcription/translation coupling. CSPs are general mRNA-binding proteins, and like CSH-type RNA helicases, the presence of at least one* csp *gene in the cell is essential for viability. Members of both protein families have recently been shown to interact, suggesting that a two-step process achieves the coupling process, removal of secondary mRNA structures through CSHs and prevention of reformation through CSPs.*

Keywords: *cold shock, cold shock proteins, transcription, translation, mRNA, ribosomes, nucleoid*

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Coping with cold!!

Exposure to low temperatures is a frequent event that is encountered by different bacterial species in various situations. A striking aspect in nature is the estimation that the temperature of more than 80% of biosphere is below 5°C[1]. Food-related bacteria are particularly and repeatedly exposed to low temperatures during food handling and storage[2]. This exposure to cold temperatures has increased over last decades because of the extended use of refrigeration and freezing in food preservation. While low temperatures inhibit the growth of some food spoilage and pathogenic bacteria, they enrich the numbers of others. Among the latter are serious pathogens such as *Listeria monocytogenes* that causes spontaneous abortion in pregnant women or the birth of a severely sick baby and may affect the bloodstream and the central nervous system in humans[3]. Chilling and freezing weather temperatures in different areas of the world also represent cold stress to bacteria residing in soil and on the skin of animals and plants.

There has been a growing trend in research to study bacterial response to low temperatures over the last few decades. Focus has been particularly placed on the cell's response to the sudden decline in temperature (cold shock). This response is generally considered as a model for the cell's adaptive behavior during cold stress. It is currently appreciated that most bacterial species can respond to cold shock by transient induction of arrays of specific proteins, termed cold-induced proteins (CIPs), and repression of other proteins synthesized during active growth or on exposure to other stressful conditions such as heat shock[4,5]. Such a response is presumed to aid cells overcoming physiological stress generated by cold shock.

The negative influence of exposure to cold stress stems mainly from the physical effect of low temperature on the cell's structures and enzymatic reactions. For instance, low temperature lowers the fluidity of the cytoplasmic membrane and induces the formation of nonfunctional mRNA secondary structures (for a comprehensive review see Phadtare *et al.*[5]). Low temperatures also decrease the rate of enzymatic reactions due to a reduction in molecular dynamics[6]. These structural and metabolic changes bring about difficulties in central functions in cells involving membrane transport, transcription and translation. Coping with cold stress thus requires the synthesis of diverse mechanisms that enable cells to keep surviving under this hardship.

A prime cold-adaptive mechanism is the synthesis of membrane fatty acid desaturases. Low temperatures modify the physical state of the cytoplasmic membrane from the normal semi-liquid crystalline phase into more rigid gel phase. *Escherichia coli*, *Bacillus subtilis* and cyanobacteria overcome this change by increasing the desaturation rate of membrane fatty acids. This increases the proportion of unsaturated fatty acids in the membrane phospholipids, which lowers their melting points and confers an extent of fluidity to the membrane under cold temperatures[4]. *Listeria monocytogenes* does not, however, desaturate fatty acids in the membrane, but changes the branching pattern of fatty acids from *iso* into *anteiso*[7]. The changes in membrane fatty acids and phospholipids are generally associated with modifying the activity of the membrane integral proteins involved in nutrient and ion transport[8]. This could be of adaptive value to cells since it was shown that the transport of compatible solutes such as betaine increased 15 times at 7°C compared with 30°C and that the presence of betaine stimulated the growth of *L. monocytogenes* at this cold temperature[9]. While alterations in membrane fatty acids and phospholipids are presumed to be highly conserved among bacteria, their relative role in adaptation to cold stress varies in different bacteria. It was found that the deficiency of membrane-bound fatty acid desaturases caused cold vulnerability in cynaobcteria[10], yet it did not affect the ability of *B. sublitis* to survive cold stress[5].

Another cold adaptive response is the increase in DNA negative supercoiling that is reported to occur in *E. coli* and *B. subtilis*[11,12]. This is due to the activity of DNA gyrase, whose expression increases upon exposure to cold temperatures. While *E. coli* showed increases in DNA negative supercoiling on cold shock, this effect disappeared when cells were pretreated with nalidixic acid, which inhibited DNA gyrase activity[11]. It is presumed that the increase in DNA negative supercoiling induces the expression of cold adaptive genes whose promoters are sensitive to DNA twist. These genes may include those encoding membrane fatty acid desaturases. It was reported that the presence of novobiocin, another inhibitor of DNA gyrase, inhibited the desaturation of fatty acids normally induced following cold stress[13].

As mentioned above, low temperatures increase the tendency of mRNA molecules to form secondary structures, which may inhibit the initiation of translation and limit bacterial growth and/or survival[4]. To counteract such a difficulty, cells produce RNA chaperones, which are proteins that bind to RNA to retard the

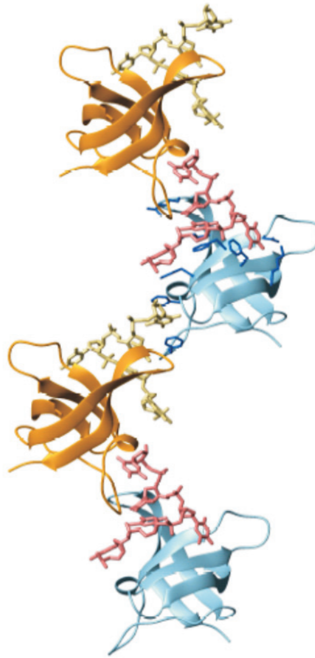

Fig. 1. *Crystal structure of CspB from* Bacillus subtilis *in complex with ssDNA. Intriguingly, although the short ssDNA molecules (yellow and pink) are not connected (each CspB binds to one short oligonucleotide), the CspB molecules (orange and light blue) line up in the crystal, and this way could form an extended filament on a longer mRNA, keeping the nucleic acid in an unfolded and accessible conformation. Aromatic residues on CspB that mediate the interaction with ssDNA are in dark blue. The barrel structure of CspB is well visible. Image courtesy of Klaas E.A. Max and Udo Heinemann.*

formation of secondary structures (misfolding) and/or repair misfolded RNA molecules (Figure 1). These chaperones include cold shock induced DEAD box RNA helicases (CSHs) and the so-called cold shock proteins (CSPs). While CSHs repair RNA misfolding by destabilizing RNA duplexes, CSPs bind to single stranded RNA to prevent or lower the formation of secondary structures[6].

Cold shock proteins

CSPs are a subset of CIPs, which are induced on exposure to cold shock (see above). CSPs are small (7.4 kDa) and mostly acidic proteins that have been detected in many bacterial species[4]. They appear to be ubiquitous and conserved since the early stages of

microbial evolution. This is evidenced by their wide distribution in various bacterial species and existence in ancient bacterial branches of *Aquifex* and *Thermotoga*. However, homologues of CSP genes have not been observed in the genomes of *Helicobacter pylori*, *Campylobacter jejuni* and *Mycoplasma genitalium*[2,4], all of which are human pathogens. CSPs are expressed by both Gram-positive and Gram-negative bacteria, in which they show a sequence similarity of >45%[2,4]. Furthermore, it was reported that several nucleic acid binding proteins in eukaryotic cells contain a domain that is highly similar in sequence to CSPs, and which was named CSD for cold shock domain. The CSD is among the most highly conserved domains found so far, with >45% sequence identity between pro-and eukaryotes. In eukaryotic proteins, the CSD has been shown to confer binding to RNA, and to ssDNA[14]. Because of this high degree of conservation, it is not surprising that CSPs are also ssDNA and RNA-binding proteins (see below). However, it is surprising and amazing that a eukaryotic CSD-containing protein has recently been shown to complement the function of bacterial CSPs, which depended on a functional CSD[14], further emphasizing the high degree of evolutional conservation of CSD proteins.

CSPs normally exist in families of 2–9 members in bacterial cells, because of frequent gene duplications[2]. For example, *E. coli* contains nine CSPs and *B. subtilis* 3[1,2]. CSPs serve regulatory roles in diverse cellular processes including transcription antitermination, DNA recombination and supercoiling, ribosome assembly and mRNA binding, which ultimately aids adaptation to cold stress[2]. However, the regulatory effects of CSPs may also extend to cell physiology during normal growth and stationary phase[1,4]. It should be noted that not all CSPs are induced by low temperatures, which suggests that such non-cold-inducible proteins are mainly involved in growth under normal conditions. For instance, only three out of nine CSPs synthesized by *E. coli* are induced by cold shock, while three of the other six proteins (CspC, CspD and CspE) are involved in DNA replication and chromosome condensation at optimal growth temperature[15,16]. It should be kept in mind that CSPs are defined on the basis of their amino acid sequence rather than their being induced by cold shock or not.

The presence of at least one CSP out of three is essential for viability in *B. subtilis*, and the loss of two CSPs renders cells cold sensitive[17]. Similarly, loss of four *csp* genes in *E. coli* leads to cold sensitivity[18]. Lowering the intracellular level of CSPs in *B. subtilis* results in a strong decrease of global protein synthesis, showing

that CSPs serve a general function in transcription or, more likely, translation in bacteria. A CSP has been isolated from nascent mRNA in *E. coli*[19], suggesting that mRNA-binding may be a major function for CSPs.

RNA binding by CSPs

The structure of CSPs involves five β-sheet strands forming a barrel[4] (Figure 1). With the exception of CspF and CspH in *E. coli*, CSPs contain two RNA-binding motifs, RNP1 and RNP2, existing in many RNA-binding proteins (RNP stands for ribonucleoprotein). Both motifs are situated on two adjacent β-strands, and several aromatic residues are extending into the solvent and are essential for ssDNA and RNA binding. These residues intercalate between the bases of ssDNA in the solution structure, such that extensive stacking between aromates from the protein and from the bases of nucleic acid (plus some hydrogen bonds) achieve the relatively non-specific binding properties of CSPs[20,21]. Several positively charged residues surround the RNP1/2 surface, and contribute to nucleic acid binding, while the remaining surface of most CSPs (note that not all CSPs are acidic) is highly negatively charged. This binding mode allows for cooperative association with ssDNA at almost diffusion controlled rates, with KD values in the micro- to nanomolar range[20,21]. Figure 1 shows a cocrystal structure of CspB from *B. subtilis* in complex with ssDNA, and illustrates how CSPs could bind to mRNA like beads on a string and essentially keep the nucleic acid in a linear form.

Because of their ability to bind to single stranded DNA and RNA, CSPs were first presumed to act as activators of the transcription of cold-adaptive genes by presumably stabilizing open-complex formation by RNA polymerase[22]. However, further research showed that CSPs are more likely to serve as RNA chaperones weakening the formation of secondary structures in mRNA[23,24]. It is interesting to note that while extensively folded mRNA is non-functional in translation, a degree of folding may be necessary for protecting mRNA molecules from hydrolysis by RNase. This could be evidenced by the observation that the presence of CspA sensitized mRNA to RNase degradation *in vitro*[24]. The idea that CSPs destabilize mRNA structures is further supported by the finding that *E. coli* CSPs act as transcription antiterminators *in vitro* and *in vivo*[25] and by the finding that a eukaryotic CSD containing protein can destabilize dsDNA *in vitro* and also act as antiterminator protein in a bacterium[14].

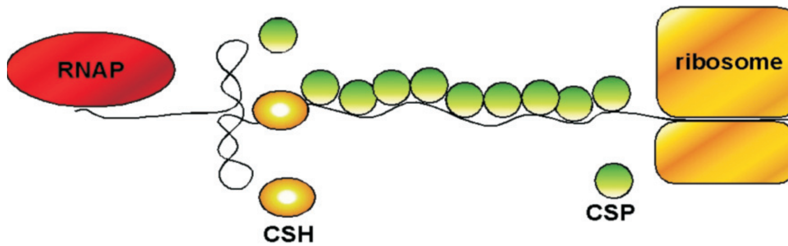

Fig. 2. Proposed mechanism for coupling or transcription and translation through CSPs and CSHs. Newly synthesized RNA that leaves the RNA polymerase complex readily forms secondary structures, which appear to be resolved through CSHs. Cooperative binding of CSPs to linearized mRNA and interaction with CSHs may keep mRNA in a linear form, such that initiation of translation can occur.

Interestingly, RNA binding by CSPs appears to be of mutual benefit to both kinds of molecules since the stability of CSPs was found to be increased *in vitro* by binding to RNA[26]. The mRNA/CSPs binding reaction is non-specific, moderate and requires unfolded mRNA[4,24,27]. These characteristics led to the proposal of an interesting model suggesting that CSPs may aid coupling transcription and translation in bacteria[1]. In this model, CSPs are suggested to bind to nascent mRNA molecules preventing the formation of secondary structures. This binding remains until the delivery of mRNA to ribosomes where CSPs are displaced by ribosomes allowing proper initiation of translation (Figure 2).

Coupling transcription and translation in bacteria

The relevance of the above hypothesis was probably reappraised following the emergence of striking data suggesting that the transcription and translation machineries are located in different cellular compartments. This was reported in both *B. subtilis*[28] and *E. coli*[29]. It was found that RNA polymerase (RNAP) is predominately located on the nucleoid, which contains the chromosome and is present within the core of the cell, while most ribosomes were found outside the nucleoid being predominantly concentrated towards the cell poles, where DNA is absent. These observations were generated using GFP-fluorescence-based methods that allow proper localization of cell components. However, such findings contradicted a previous paradigm suggesting that translation and transcription are tightly coupled in bacteria. This view was based

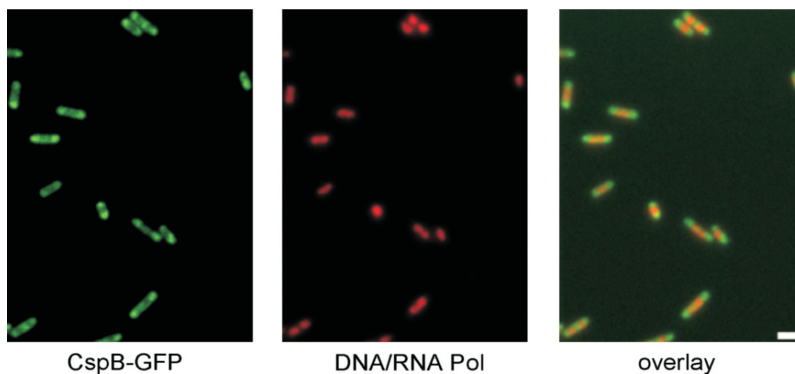

Fig. 3. *Fluorescence microscopy of* Bacillus subtilis *cells expressing CspB-GFP (CspB is the major cold shock protein from* B. subtilis*). RNA polymerase localizes to the nucleoids that are stained by DAPI (DNA panel), while CspB localizes to sites surrounding the nucleoids. White bar 2 μm.*

on classical experiments, in which DNA–RNAP–RNA–ribosomes complexes were isolated from bacterial cells[30]. While the fluorescence-based data did not exclude the coupling of translation and transcription, *i.e*. mRNA is being translated while it is still being transcribed, these data suggested that a cellular mechanism(s) to spatially couple the two processes and ensure the safe delivery of mRNA to ribosomes has to exist.

Subsequent experimental work, also employing fluorescence-based procedures, has provided strands of evidence that the expression of CSPs may fulfil such a mechanism in *B. subtilis*. It was found that CSPs exist within the area between the nucleoid periphery and spaces occupied by ribosomes (Figure 3) and that CSPs colocalized with ribosomes[31]. This supported the idea that CSPs may spatially couple transcription and translation. It also indicated that a kind of interaction between ribosomes and CSPs may occur to presumably facilitate the initiation of translation. This interaction may occur on the handing of mRNA from CSPs to ribosomes. These conclusions are somewhat analogous to an established function of certain CSD-containing Y-box proteins in eukaryotic cells. For instance, FRGY2 protein binds to mRNA in the nucleus of frog oocytes, which – once transported into the cytoplasm – is sequestered by FRGY2 and thus prevented from being translated, until mRNA is released at a certain time during oocyte development[32]. The idea that CSPs bind to mRNA and keep the molecule linear until initiation of translation has also been supported by the finding that the essential function of CSPs in *B. subtilis* can be complemented by translation initiation factor 1 (IF1)

from *E. coli*, which has a very similar overall fold as CSPs and the CSD[33].

A striking observation was that the specific localization of CSPs and ribosomes was found to depend on active transcription. Inhibition of transcription by the addition of antibiotic inhibitors was associated with an even distribution of CSPs and ribosomes throughout the cells[31,33]. Inhibition of translation did not, however, have this effect. This may suggest that the specific cellular distribution of CSPs and ribosomes is induced by the emergence of mRNA molecules from the nucleoid. It could be simply proposed that the diffusion of mRNA molecules into the cytoplasm may force the bigger ribosomal particles towards the cell poles, while small CSPs and ribosomal subunits may co-distribute with mRNA in the area between the nucleoid periphery and cell poles. Lewis *et al.*[28] reported that a majority of ribosomal particles (>80%), comprising bigger polysomes and 70S units, are concentrated towards cell poles, while smaller ribosomal subunits may be scattered in the cytoplasm. Whatever the mechanism of specific localization of ribosomes, this arrangement may facilitate the spatial organisation of the cells such that DNA replication/segregation and transcription can run separate from translation, *i.e.* to compensate for the absence of a nuclear membrane in prokaryotic cells.

CSPs interact with essential RNA helicases

A second class of proteins that is able to render secondary mRNA structures accessible to ribosomes are RNA helicases. Unlike eukaryotic ribosomes, bacterial ribosomes do not appear to be directly associated with RNA helicases, but several cold-induced RNA helicases have been identified in the past in archea and in bacteria[34,35]. It has been recently shown that two helicase-like proteins (CshA and CshB) perform a redundant essential function in *B. subtilis*. CshA and CshB were found in a genome wide analysis of the changes in transcription after cold shock in *B. subtilis*, and shown to be considerably cold-induced[36]. It was only possible to delete both genes in the presence of one extra copy at an ectopic site on the chromosome, showing that the presence of at least one gene is essential for viability. Interestingly, the deletion of a *csp* gene and of the *cshA* gene led to a pronounced cold sensitivity, indicating that CSPs and RNA helicases perform interdependent functions during cold adaptation.

Like ribosomes and CSPs, CshA and CshB localize to sites surrounding the nucleoid, and inhibition of transcription abolishes this specific localization pattern[6]. Using the fluorescence resonance energy transfer (FRET) technique, it was shown that CSPs and CshA interact within the polar sites. This technique allows *in vivo* visualization of cellular molecules that are very close to each other, typically within a distance of 1–5 nm. It depends on labelling the examined molecules with a pair of compatible fluorescent dyes. If the tagged molecules are within the above distance, the fluorescence emitted by one dye (donor) would excite the other dye (acceptor) to produce fluorescence signals. Interestingly, the FRET signal between CSPs and CSHs was strongly reduced after inhibition of transcription[6]. These findings suggest that CSPs and the putative RNA helicases cooperate during the binding to their substrate, most likely mRNA. CSPs could keep mRNA in a linear form after the helicases have unwound any stem loop structures, and this interaction may present the spatial bridge between transcription and translation (Figure 2).

Conclusions and future prospects

Given the seemingly opposing findings of tight coupling between transcription and translation, and the general spatial separation of both processes in bacteria, it is surprising that it is still unclear how the processes are coupled in case the transcript is too far away from the nearest ribosome to be kept linear. Preventing and/or resolving extensive secondary mRNA structures on exposure to cold has been recently suggested to be carried out by cooperative performance of CSPs and CSHs[6]. It is proposed that CSHs destabilize double-stranded RNA and that CSPs prevent refolding until translation is initiated at the ribosome. Such a cooperative relationship is indicated by the close proximity of CSPs and CSHs on their presumed substrate, mRNA. However, this model awaits hard proof, in the form of biochemical data, *e.g. in vitro* translation with CSPs and CSHs added, and studies on the interaction of CSPs, CSHs with ribosomes, mRNA and other possible interacting factors.

The observation that CSPs and CSHs displayed their cooperative performance under both optimal and cold shock conditions supports the view that essential knowledge of cell physiology can be gained through research on bacterial stress responses. The induction of stress-responsive proteins under adverse environmental conditions intensifies their existence and thus allows

proper monitoring of their functions, which may be performed to a lesser extent under normal conditions. Pursuing research on stress responses of bacteria is thus important for understanding a number of yet-unresolved physiological mysteries.

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