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REVIEW

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Cold stress response in Archaea

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Abstract We live on a cold planet where more than 80% of the biosphere is permanently below 5°C, and yet comparatively little is known about the genetics and physiology of the microorganisms inhabiting these environments. Based on molecular probe and sequencing studies, it is clear that Archaea are numerically abundant in diverse low-temperature environments throughout the globe. In addition, non-low-temperature-adapted Archaea are commonly exposed to sudden decreases in temperature, as are other microorganisms, animals, and plants. Considering their ubiquity in nature, it is perhaps surprising to find that there is such a lack of knowledge regarding low-temperature adaptation mechanisms in Archaea, particularly in comparison to what is known about archaeal thermophiles and hyperthermophiles and responses to heat shock. This review covers what is presently known about adaptation to cold shock and growth at low temperature, with a particular focus on Antarctic Archaea. The review highlights the similarities and differences that exist between Archaea and Bacteria and eukaryotes, and addresses the potentially important role that protein synthesis plays in adaptation to the cold. By reviewing the present state of the field, a number of important areas for future research are identified.

Key words Cold shock · Low-temperature adaptation · Psychrophile · Adaptive mechanisms · Antarctic Archaea · Gene expression · Protein structure · Review

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Introduction

A lack of knowledge

Archaea are capable of growth from 113°C (Stetter 1996) to below 0°C (Cavicchioli and Thomas 2000; Franzmann 1996); however, knowledge about hyperthermophiles vastly outweighs information about low-temperature-adapted Archaea. It is now recognized that even members of the Crenarchaeota (a kingdom thought to be composed exclusively of hyperthermophiles) may be as prevalent in mesophilic and low-temperature environments as Bacteria (DeLong 1997; Pace 1997). In effect, the application of molecular techniques to a variety of marine and soil environments indicates the abundance and diversity of Archaea in cold environments (Bintrim et al. 1997; DeLong et al. 1994, 1999; Fuhrman et al. 1992; Hershberger et al. 1996; McInerney et al. 1995; Murray et al. 1999). The most complete molecular descriptions of Archaea to date, their genome sequences, are *all* of thermophiles and hyperthermophiles (Bult et al. 1996; Kawarabayasi et al. 1998, 1999; Klenk et al. 1997; Smith et al. 1997). This bias reflects our prejudices rather than the distribution of archaeal life forms.

Although it is recognized that Archaea exist in low-temperature environments, there have been few physiological studies on psychrophilic (low-temperature-adapted) Archaea, and there is an almost complete absence of information describing the effects of cold shock on any Archaea. This review describes the present knowledge in the field, paying particular attention to compare what is known about Archaea with bacterial and eukaryotic systems.

Problems associated with a temperature shift

The heat-shock response appears to be universal, with common mechanisms and protein systems operating in all three domains of life. The crisis caused by heat stress is the unfolding and aggregation of proteins. All cell types respond by inducing molecular chaperone proteins that either stabi-

lize proteins against thermal denaturation or possibly refold denatured proteins. This crisis of protein denaturation is common to many forms of stress, and hence the induction of molecular chaperones is often referred to simply as the "stress response." The cold-shock response appears to be distinct from this more general stress response. The nature of the crisis faced by a cell on temperature reduction is a central theme in cold-shock research.

Our understanding of the cellular response to a sudden drop in temperature lags behind that of heat shock. Studies on bacteria indicate that the key problems that occur after a cold shock are the loss of membrane fluidity, a lowering in the rates of enzymatic and transport processes, the stabilization of nucleic acid secondary structures and their subsequent inhibitory effects on DNA replication, transcription and the translation of mRNA and the formation of crystalline ice and its associated damage to cellular structures if the drop goes below the freezing temperature of the cytoplasm. All these problems are also faced by cells living permanently in cold environments (i.e., not just after "cold shock") and as such must be overcome in the evolutionary process of low-temperature adaptation. In response, microorganisms have evolved various ways to adapt. For example, increases in membrane fluidity may be obtained through a relative increase in polyunsaturated fatty acids, and microorganisms that are restricted to temperature ranges below 15º–20°C tend to be found in environments that are rich in organic substrates to compensate for their less effective uptake and transport systems (Nichols et al. 1995). In addition, structures of cold-active proteins appear to have evolved to produce structurally flexible and catalytically efficient proteins at cold temperatures (Russell 2000). Cellular adaptation to the cold may also involve the induction of synthesis of antifreeze proteins that inhibit the growth of ice-crystals, and these have been characterized in plants (Guy 1999) and fish (Macouzet et al. 1999). They have also been identified in a bacterial species (Xu et al. 1998); however, they have not been readily detected in other low-temperature-adapted bacteria.

Regulation of response

Bacteria and eukaryotes cope with the crises caused by low temperatures via a range of different strategies including upregulation of the expression of cold-shock proteins. Clearly, all life forms must contend with the crises caused by cold shock (see, for example, the symposium on cold shock reported in the *Journal of Molecular Microbiology and Biotechnology*, 1999, Vol. 1, issue 2); however, it is unclear which of the specific cold-shock protein systems (if any) are universal (common to all three domains of life). Thus, the study of the cold-shock response and low-temperature adaptation in Archaea will have broad implications to the understanding of cellular biology and evolution.

Cold-shock proteins are synthesized to enable gene expression and protein synthesis to continue at low temperature (reviewed in Phadtare et al. 1999; Yamanka 1999). In *Escherichia coli,* separate classes of proteins are synthesized throughout cold shock in a two-phase response. Class I proteins are involved in the initial response to the temperature change and include the cold-shock domain proteins, represented by CspA, which functions as a RNA-binding molecular chaperone and possibly as a transcriptional activator, as well as CsdA (a DEAD-box RNA helicase) (Yamanka et al. 1998). Class II proteins are involved in reestablishing cellular function for growth at low temperature and generally require the prior activity of the class I proteins. During the cold shock, synthesis of housekeeping proteins is repressed until cellular adaptation has been completed and synthesis is reestablished, albeit at a lower rate. In a number of psychrotolerant (psychrotrophic) bacteria, the cold-shock response is somewhat different. In these bacteria, some cold-shock proteins are transiently synthesized; however, cold-acclimation proteins are continuously synthesized during growth at low temperature. In addition, the synthesis of housekeeping proteins is not repressed in these psychrotolerant systems.

Clearly, there is potential overlap between cold-shock mechanisms from low-temperature-adapted and non-low-temperature-adapted organisms, as well as mechanisms unique to low-temperature-adapted organisms. In future, it will be fruitful to compare and contrast these mechanisms; however, the current status of the field does not allow such comparisons. Given the paucity of data, in this review we have not separately addressed cold shock from cold acclimation, nor have we discussed low-temperature-adapted Archaea separately from other Archaea. There is a focus on Antarctic Archaea, as these have been studied most extensively with regard to low-temperature adaptation.

This review begins by introducing archaeal species that have been isolated from low-temperature environments and then discusses modes of adaptation of their membranes and proteins. Problems and adaptations associated with protein folding, DNA topology, and RNA composition are then analyzed, and low-temperature gene regulation is discussed via the example of a low-temperature expressed RNA helicase.

Low-temperature-adapted Archaea

The major limitation in the field is that very few free-living, low-temperature-adapted Archaea have been cultivated (Franzmann 1996), despite intensive efforts from established laboratories to isolate them. Three low-temperature-adapted Archaea belonging to the Euryarchaeota have been isolated by Franzmann and colleagues from the lakes in the Vestfold Hills region of Antarctica: two methanogens, *Methanococcoides burtonii* (T_{min}, –2.5°C; T_{opt}, 23°C) and *Methanogenium frigidum* (T_{min}, –10°C; T_{opt}, 15°C), and one halophile, *Halorubrum lacusprofundii* (T_{min}, 2°C; T_{opt}, 33°C) (Franzmann et al. 1988, 1992, 1997). These isolates represent the only pure, cultivatable Archaea that are adapted to growth at low temperature. The two methanogens were isolated from Ace Lake where they were recovered from saline, methane-saturated, anaerobic water at

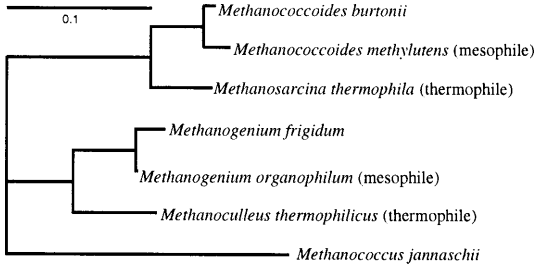

Fig. 1. Phylogenetic tree based on 16S r-RNA sequence of some methanogenic Archaea. The distances were calculated by Kimura distance matrix, and the tree was constructed according to the neighbor-joining method. *Bar* indicates 10 changes per 100 nucleotides

a constant 1°–2°C; they represent metabolically (*Methanococcoides burtonii* utilizes methylamines and methanol whereas *Methanogenium frigidum* grows with H_2:CO_2) and phylogenetically distinct families of methanogens (Fig. 1). This figure shows the phylogenetic relationship between *M. burtonii* and *M. frigidum*, and their respective mesophilic and thermophilic counterparts, with the hyperthermophile *Methanococcus jannaschii* for comparison.

In addition to the low-temperature-adapted, free-living Archaea, a sponge symbiont, *Cenarchaeum symbiosum*, has been isolated from ocean waters off the coast of California (Preston et al. 1996). *C. symbiosum* is a member of the Crenarchaeota, which consists mainly of hyperthermophilic species. This organism can currently be cultivated only in symbiosis with its sponge host, precluding direct physiological studies with respect to low-temperature adaptation. However, through molecular approaches (i.e., sequencing of genomic fragments and recombinant expression of genes), novel insights into the physiology of this organism have been gained (see following). Molecular studies based on direct sequencing of small ribosomal subunit genes have also identified novel members of the Crenarchaeota in cold and moderate temperature environments (DeLong 1997; DeLong et al. 1999; Murray et al. 1999; Pace 1997), however, none of them have been obtained in culture yet. Clearly, a major effort should be taken to obtain more isolates to investigate their physiology and their apparently important ecological role in the environment.

Membrane adaptation

Bacteria adjust the fatty acid composition of membrane phospholipids in response to changes in the growth temperature (reviewed in Russell and Hamamoto 1998). As temperature is decreased, fatty acid side chains in membrane bilayers undergo a change of state from a gel-like, disordered state to a more ordered liquid-crystalline state. To maintain a normal fluid state, low-temperature adaptation involves decreasing the temperature of the ordered-to-disordered transition by altering the fatty acyl composition. This change may be achieved by increasing unsaturation, decreasing the average chain length, increasing methyl branching, increasing the ratio of *anteiso-* relative to *iso-*branching, or isomeric alteration of acyl chains in *sn-1* and *sn-2* positions.

Archaea contain ether-linked lipids, in contrast to ester-linked lipids that are present in Bacteria and eukaryotes. The archaeal lipids may be arranged as diether or membrane-spanning tetraether lipids, macrocyclic diether or hydroxydiether lipids, or cyclic or acyclic dibiphytanyl ether lipids, including various modifications of the lipid glycerol group. The membrane composition of a number of Archaea have been characterized (reviewed in Koga et al. 1993; Sprott 1992), however there is presently no information about low-temperature-induced changes.

Because of their similar size and cellular morphology and the gross architecture of their membranes, it is likely that low-temperature adaptation in Archaea follows similar principles to Bacteria. This adaptation may involve alterations in ether side-chain composition to decrease the temperature of the ordered-to-disordered transition, which may be achieved through side chains that produce a more fluid structure of the membrane. Indirect evidence may be gathered from changes in membrane composition that occur as a result of increasing the growth temperature of thermophilic Archaea. The number of cyclopentane rings in the lipids of *Sulfolobus* spp. has been shown to increase with growth temperature (reviewed in Russell 1984). The shortening of alkyl chains may lead to increased thermal stability at elevated temperatures. In *Methanococcus jannaschii*, the lipid composition changed from predominantly diether lipids at a low growth temperature (47°C) to macrocyclic diether and tetraether lipids at optimal growth temperature (75°C) (Sprott et al. 1991). The cyclization of side chains and increased content of membrane-spanning tetraether lipids at higher temperatures may limit side-chain motion and stabilize the membrane. Conversely, it may be expected that minimizing macrocyclic diether and tetraether lipids with a simultaneous increase in diether lipids may in principle be a means of low-temperature adaptation. Consistent with this concept is the finding that the Antarctic archaeon *Methanococcoides burtonii* contains diether phospholipids but lacks tetraether lipids (Nichols and Franzmann 1992). Bacterial adaptations also include the synthesis of polyunsaturated fatty acids during growth at low temperature and the involvement of desaturase enzymes to increase the desaturation of preexisting fatty acid chains (Russell and Hamamoto 1998). However, these mechanisms of membrane adaptation have not been identified in low-temperature-adapted Archaea.

Protein adaptation

Protein flexibility plays an important role in functional interactions and enzyme catalysis. These processes often involve structural changes at various levels of organization ranging from side-chain movements to domain rotations and quaternary structural changes. Each of these structural changes is effected by thermal fluctuations and hence is

acutely temperature dependent. At lower temperatures, and hence lower mean kinetic energies, these structural changes occur less frequently. For a psychrophilic protein at low temperature to maintain catalytic activity comparable to that of a mesophilic protein at medium temperature, the psychrophilic protein would be expected to show increased flexibility so as to facilitate any functionally required structural changes. To achieve this, there is a decrease in the number of enthalpy-driven interactions and as a result the protein may have a less rigid, more flexible structure (Gerday et al. 1997). Consequently, structural adaptations conferring a more "loose" or flexible structure are expected to be important for cold-active proteins. By comparing the structures of proteins from psychrophiles to those from mesophiles and thermophiles, a number of structural characteristics have been identified in cold-active proteins. These include (1) reduction in the number of salt bridges, (2) reduction of aromatic interactions, (3) reduced hydrophobic clustering, (4) reduced proline content, (5) reduced arginine content, (6) additional loop-structures, and (7) more solvent interaction (Feller et al. 1996). It is important to note that not all these types of changes would be expected to occur in a single protein because the molecular context of the changes is important in altering the stability and activity of the protein. It may generally be expected, however, that cold-active enzymes would have a reduced thermostability and a lower apparent temperature optimum for their activity in comparison to enzymes active at high temperatures. It is important to recognize that while biophysical studies, including X-ray crystal structures, have been gathered for proteins from hyperthermophilic Archaea, only two proteins from low-temperature-adapted Archaea have been studied (described later). As a result, our present understanding of protein adaptation to low temperature is mainly based on studies performed on bacterial and eukaryotic proteins.

Three main approaches may be used to study adaptations in cold-active proteins. The first is to examine the differences between proteins from organisms that are phylogenetically and biochemically as distinct as possible and that include representatives from thermal extremes (e.g., citrate synthase from hyperthermophilic archaea, a pig, and an Antarctic bacterium) (Danson and Hough 1998; Russell et al. 1998). The second is to use a random mutagenesis approach: mutate the protein, screen for a specific trait (e.g., activity at a certain temperature), identify the mutation, and examine the structural change(s) (Feller et al. 1996). The third approach involves choosing proteins from phylogenetically closely related species to minimize the total number of changes that will be present between sequences. This approach has been used for examining lactate dehydrogenase enzymes from psychrophilic and thermophilic *Bacillus* spp. (Zuber 1988). In accordance with this third approach, we have reasoned that the evolution of proteins from organisms growing at different temperature ranges is not significantly different from those that have evolved in environments of the same temperature (Thomas and Cavicchioli 1998). As a result, the amino acid changes that are important for thermal activity are hidden amid the changes produced by genetic drift and all other effectors of natural selection. Thus, choosing a set of closely related proteins from phylogenetically similar organisms assists in identifying changes indicative of thermal adaptation. A suitable set of archaeal microorganisms includes the low-temperature-adapted *Methanococcoides burtonii* and *Methanogenium frigidum* methanogens and their respective mesophilic and thermophilic counterparts (see Fig. 1).

To date, the biochemical properties of only two proteins from low-temperature-adapted Archaea have been studied in any detail; elongation factor 2 (EF-2) from *Methanococcoides burtonii* and DNA polymerase from *Cenarchaeum symbiosum*.

Elongation factor 2

EF-2 was chosen as a model protein for biochemical and structural studies because of its essential cellular function, ubiquity in all known life, and the high level of identify between EF-2 sequences: >77% identity for the EF-2 sequences from *M. burtonii* and related species (Fig. 1) (Thomas and Cavicchioli 1998). Furthermore, ribosomes and associated factors (e.g., initiation and elongation factors) from psychrophilic bacteria (*Pseudomonas* and *Bacillus* spp.) have been shown to be adapted to activity at low temperature in comparison to mesophilic counterparts (reviewed in Russell and Hamamoto 1998), indicating that the stability and activity of the translation apparatus is thermally adapted to the growth temperature of the organism. In addition, the X-ray crystal structure of the *Thermus thermophilus* EF-G and EF-Tu proteins enabled protein homology modeling to be performed (Thomas and Cavicchioli 1998). A comparison of the EF-2 models generated for the related methanogens shows that 15 of the 129 sites of residue variability are likely to be related to cold adaptation. Greater structural flexibility in the *M. burtonii* protein was predicted to occur as a result of fewer salt-bridges, less packed hydrophobic cores, and the reduction of proline residues in loop structures. These changes appear to be similar to those identified in cold-active enzymes from bacteria and eukaryotes.

EF-2 proteins from *M. burtonii* and *M. thermophila* have been overexpressed in *E. coli*, and purified (Thomas and Cavicchioli 2000). Differential scanning calorimetry has been used to assess protein stability while GTPase enzyme assays were used to assess intrinsic activity. Biochemical and biophysical properties that are characteristic of the *M. burtonii* protein include a higher activity at low temperatures caused by a decrease in the activation energy necessary for GTP hydrolysis and a decreased activation energy for the irreversible denaturation of the protein, which indicates a less thermostable structure.

The temperature dependence of the in vitro properties of these proteins does not correspond to the temperature-dependent growth characteristics of the organisms, indicating that additional cytoplasmic factors are likely to be important for the complete thermal adaptation of the proteins in vivo. We have recently found that purified ribo-

somes from the parent microorganisms affect the GTPase activity profiles of the EF-2 proteins, thereby suggesting that interactions with the ribosomes modify the stability of EF-2 (Thomas and Cavicchioli, unpublished results). In addition, the profiles of cytoplasmic solute concentrations appear to depend on temperature, indicating that they may play a role in modifying EF-2 function in vivo (Thomas and Cavicchioli, unpublished results).

DNA polymerase

As part of the analysis of a large genomic contig isolated from *Cenarchaeum symbiosum*, a gene for DNA-polymerase was isolated (Schleper et al. 1997). The polymerase is a member of the family B DNA polymerases (formerly the α-family), which includes bacterial polymerases such as *E. coli* polymerase II, eukaryotic virus- and plasmid-associated polymerases, and the three eukaryotic replicative DNA polymerases (α, β, and ε). The recombinant protein csPol was purified from *E. coli*, and polymerase and exonuclease activity was assessed. The optimal temperature for polymerase activity on activated calf thymus DNA was approximately 42°C, dropping to 10% activity below and above T_{opt} at 24° and 54°C, respectively. The 3′-5′-exonuclease activity had its temperature optimum at 38°C, with no significant activity being detected at 47°C. This temperature profile is distinct from the environmental temperature range for *C. symbiosum*, which is likely to be around 10º–15°C. Preincubation of the archaeal polymerase at elevated temperatures demonstrated that its polymerizing activity was significantly more temperature labile than DNA polymerase I from *E. coli*. Presently, characterization of structural properties that may be important for low-temperature activity is limited by the lack of a three-dimensional structure for any archaeal DNA-polymerase.

The work on EF-2 from *M. burtonii* and DNA polymerase from *C. symbiosum* illustrates the ability to use molecular biology and biochemical methods to examine high molecular weight proteins (EF-2, 80 kDa; DNA polymerase, 96 kDa) from free-living and symbiotic, low-temperature-adapted Archaea. As a result of the development of these methods, it is anticipated that this area of research will rapidly develop in the future.

Protein folding

Cold shock in *E. coli* involves the induction of two classes of cold-shock proteins. Class I includes the cold-shock proteins belonging to the CspA family and the DEAD-box RNA helicase, CsdA (reviewed in Yamanka 1999). Class II includes several members of the molecular chaperone family that bind unfolded and misfolded proteins, including Hsc66, Hsc20, and trigger factor. The cold-shock induction of molecular chaperones suggests that in addition to problems with mRNA structure and stability, difficulties exist in protein folding and stability at low temperatures.

Protein folding and stability are both clearly temperature dependent. In vitro studies have shown that in addition to heat denaturation there is also cold denaturation (Dill et al. 1989; Privalov 1990). Both these processes arise from the temperature dependence of enthalpic and entropic factors that determine protein stability. Although cold denaturation exists, it is unlikely to be a problem in vivo; even proteins from mesophiles do not show cold denaturation until well below 0°C (in the presence of cryoprotective agents). It is possible that proteins in psychrophilic organisms have evolved so as to shift the protein stability curve to lower temperatures, which would further lower the temperature for the onset of cold denaturation.

At present, there is no clear indication that in vivo protein folding differs between psychrophiles and mesophiles. Some of the reasons for this lack of distinction arise from the fact that even in the best studied systems (*E. coli*) the model for protein folding is constantly in flux (Houry et al. 1999; Mclennan and Masters 1998). This variability highlights the need for significant research into the folding pathway for paradigm systems such as *E. coli,* in parallel with those from low-temperature-adapted microorganisms.

A general description of protein folding mediated by chaperones and chaperonins involves the nascent peptide, emerging from the ribosome, being bound by chaperones (e.g., Hsp70/Hsp40 in eukaryotes; DnaK/DnaJ/GrpE in Bacteria) with subsequent delivery of the unfolded, or partially folded, protein to the chaperonin complex (e.g., Cpn60/Cpn10 in eukaryotes; GroEL/GroES in Bacteria) for protein folding. In eukaryotes, the prefolding complex is also required before a protein will interact with the eukaryotic cytoplasmic chaperonins.

There are clear differences between the chaperonins present in Bacteria, in Archaea, and in the eukaryotic cytoplasm. Two types of protein-folding chaperonin machine are found: group I, which is conserved in Bacteria and eukaryotic organelles of bacterial origin, and group II, which is found in Archaea and the eukaryotic cytoplasm (Ellis 1996). The prefolding proteins are associated with group II chaperonins and are found in Archaea and Eukarya; however, they have also been observed in the genomes of thermophilic bacteria (*Aquifex aeolicus*), where they have been proposed to have originated as a result of lateral gene transfer from Archaea (Aravind et al. 1998). Similarly, the group I chaperonin Cpn10 is restricted to Bacteria and eukaryotic organelles of bacterial origin; however, there appears to be a Cpn10 homologue in the genome of *Methanococcus jannaschii* (Koonin et al. 1997). The origin and function of this archaeal Cpn10 is unknown.

The distribution of the Hsp70/Hsp40/GrpE chaperone machinery is even more puzzling than the chaperonin system. It appears to be conserved in all bacteria and to be present in eukaryotes, where it is likely to have originated from the organelles of bacterial origin (reviewed in Macario et al. 1999). The situation in Archaea is more complex. For many years, it was controversial as to whether Archaea had an Hsp70/Hsp40/GrpE chaperone system. The genome sequences of hyperthermophilic archaea do not appear to contain any members of this system (*M. jannaschii,*

Pyrococcus horikoshii, Archaeoglobus fulgidus). However, the genome of the thermophilic methanogen *Methanobacterium thermoautotrophicum* contains a gene locus encoding these proteins in the same gene order as they are observed in many gram-positive bacteria. A similar locus has also been sequenced from *Methanosarcina mazei* and *Methanosarcina thermophila* (reviewed in Macario et al. 1999). Whether this gene locus is archaeal or has arisen via lateral gene transfer remains to be determined.

This variation in the presence of the Hsp70/Hsp40/GrpE chaperones in Archaea raises the question as to whether this system is involved in protein folding at lower temperatures and, in particular, during cold shock. In favor of this concept is the observation that the system is not present in the genomes of hyperthermophilic Archaea. Furthermore, the identification of Hsc66 and Hsc20 (DnaK/DnaJ homologues) as class II cold-shock proteins in *E. coli* (reviewed in Yamanka 1999) may be an indication that this system could also be involved in the low-temperature adaptation and survival of Archaea.

It is not clear why the Hsp70/Hsp40/GrpE chaperones would be involved in cold shock. They are thought to bind to unfolded proteins in an extended conformation, initially preventing folding. As such, they may be coordinating folding with polypeptide synthesis. It has recently been shown that in *E. coli* either DnaK or trigger factor must be present for cell viability, and their role is to interact with newly synthesized polypeptide chains (Deuerling et al. 1999). Thus, one could envisage that, at low temperatures, CspA-like proteins and mRNA chaperones are required to maintain the mRNA message in a translation-competent form on one side of the ribosome while the Hsp70/Hsp40/GrpE or Hsc66/Hsc20 protein chaperone systems maintain the nascent protein in a nonfolding form as it exits the ribosome.

Variation in DNA topology following cold shock

Alterations in nucleic acid stability with temperature are expected to affect transcription because the energy required to melt the DNA double helix depends on temperature. The stability of the double helix at a particular temperature can be altered by variation in GC content and by the state of supercoiling. The latter can only be altered via the action of topoisomerases, which cut one or both strands of DNA and wind or unwind the double helix.

Several lines of evidence indicate that the supercoiled state of DNA is altered on cold shock (and heat shock). The *E. coli* topoisomerase subunit, GyrA, is induced following cold shock, indicating a possible change in supercoiling. Direct studies of archaeal supercoiling have been carried out on plasmids in hyperthermophilic Archaea (Lopez-Garcia and Forterre 1999). These studies show that plasmids in *Sulfolobus* spp. undergo a rapid shift in the DNA linkage number after cold shock (temperature shift from 80° to 65°C). This initial shift in linkage number then slowly returns to near normal-linkage numbers over 24 h. The rapid change indicates that cold shock must induce topoisomerase activity. The return to normal linkage numbers also indicates that there is a mechanism to maintain DNA topology homeostasis. This two-phase response to cold shock is reminiscent to the two-phase bacterial cold-shock response (class I and class II cold-shock proteins).

Thermal adaptations of tRNA and rRNA

Posttranscriptional modification of nucleosides in tRNA may affect the stability of tRNA species. A range of structural modifications have been identified in thermophilic bacteria (Watanabe et al. 1979) and Archaea (Kowalak et al. 1994), with the most extensive types of changes identified in hyperthermophilic Archaea (Edmonds et al. 1991). The modifications increase the rigidity of the tRNA by stabilizing A-type helices, promoting hydrogen bonding, enhancing base stacking, and increasing recalcitrance to nuclease attack (described in Dalluge et al. 1997). In contrast, psychrophilic bacteria appear to lack many of the stabilizing modifications found in thermophiles or hyperthermophiles, have fewer types of modifications overall as compared with thermophiles, but have a molar abundance of the nucleoside dihydrouridine (Dalluge et al. 1997). The presence of dihydrouridine is likely to reduce base stacking, resulting in an inherently more flexible RNA conformation with increased dynamic motion at low temperature. Interestingly, although dihydrouridine is present in tRNA of eukaryotes and Bacteria, it has rarely been found in Archaea (Edmonds et al. 1991). Archaeal species examined to date do not include low-temperature-adapted Archaea, and it will be valuable to determine whether dihydrouridine is present, what kinds of other nucleoside modifications exist, and how their synthesis is regulated in low-temperature-adapted Archaea such as *Methanococcoides burtonii* and *Methanogenium frigidum*.

Other changes in RNA that may be important for low-temperature adaptation include the nucleotide composition of rRNA and the subsequent impact on secondary structure. Based on statistical mechanics calculations of RNA folding, Huynen et al. (Huynen et al. 1997) determined the free energy values for secondary structures of rRNA sequences and the predicted probability of forming each structure for rRNA sequences from a range of Bacteria, eukaryotes, and Archaea that span growth temperature extremes. They concluded that the rRNAs of thermophilic and hyperthermophilic Archaea and Bacteria have a more defined secondary structures than rRNAs from mesophilic or psychrophilic Archaea and Bacteria. In contrast, their analysis showed little correlation between G+C content and average base-pairing probability. This thermodynamic model for RNA folding indicates that adaptation to environmental temperature involves the evolution of rRNA secondary structures to match the kinetic energy of the environment.

Low-temperature gene regulation

Although the basic transcriptional mechanisms in Archaea closely resemble that found in eukaryotes (reviewed in Leigh 1999; Soppa 1999; Thomm 1996), regulation of transcription is still poorly understood. However, eukaryotic-like (Hochheimer et al. 1999; Kruger et al. 1998; Qureshi and Jackson 1998; Thompson and Daniels 1998) and bacterial-like (Cohen-Kuipec et al. 1997; Napoli et al. 1999) regulatory mechanisms have been identified in a number of Archaea. A strategy we have adopted is to determine whether bacteria-like genes involved in the cold-shock response are present and involved in low-temperature adaptation of *Methanococcoides burtonii*. To this extent we have examined DEAD-box RNA helicase genes (e.g., *E. coli csdA*) and cold-shock protein genes (e.g., *E. coli cspA*) in *M. burtonii* (Lim et al. 2000).

Methanococcoides burtonii deaD

Methanococcoides burtonii contains a putative DEAD-box RNA helicase gene, *deaD,* that is low temperature regulated (Lim et al. 2000). The encoded protein, DeaD, is predicted to contain a core element involved in ATP hydrolysis and RNA binding and an unusual C-terminal domain that contains seven perfect, trideca-peptide, direct repeats that may be involved in RNA binding. We have speculated that the predicted high positive charge of the *M. burtonii* DeaD may attract the protein to the negatively charged backbone of RNA, while the central conserved domain directs the protein to specific sequence targets and the C-terminal sheet-coil-sheet structures mediate nonspecific binding to anchor the helicase to RNA.

The expression of the *M. burtonii deaD* at temperatures close to in situ temperatures of 1°–2°C and lack of expression at temperatures producing maximal growth rates in the laboratory (23°C) indicate that expression of the gene may be essential for growth and survival in its native environment, Antarctica. Its presumed role in binding and unwinding duplex RNA may be important in removing cold-stabilized secondary structures in mRNA, thereby allowing translation to occur at low temperature.

Transcriptional regulation of *deaD*

Methanococcoides burtonii cells growing at 4°C contained abundant levels of *deaD* mRNA; however, no mRNA was detected in cells growing at 23°C (Lim et al. 2000). The transcription-initiation site is preceded by an archaeal box-A element (TATA box), and is followed by a long (113 nucleotides) 5′-untranslated region (5′-UTR). Within the 5′-UTR is a bacteria-like cold-box element. Bacterial genes that contain cold-box elements within 5′-UTRs are regulated by cold shock (see reviews of cold shock in Graumann and Marahiel 1998; Panoff et al. 1998; Phadtare et al. 1999; Thieringer et al. 1998; Yamanka 1999; Yamanka et al. 1998). The cold-box elements may function as protein-binding sites to regulate gene expression at the transcriptional or posttranscriptional level.

The conservation of cold-box elements and 5′-UTRs in low-temperature-regulated DEAD-box RNA helicases from *E. coli, Anabaena,* and *M. burtonii*, and in four cold-shock protein (*csp*) genes from *E. coli*, supports the available experimental evidence describing their role in gene regulation. Although the precise role(s) of these structures in bacteria and *M. burtonii* is presently unresolved, this is the first indication that common features of low-temperature gene regulation are present in Bacteria and Archaea.

Translational regulation of *deaD* gene expression

Four base pairs upstream of the ATG initiation codon of the *deaD* gene is a sequence that matches 8 of 11 residues of the canonical ribosome-binding site (5′-AGGAGGTGA TC-3′) for methanogens (Brown et al. 1989). At 35 and 60 nucleotides downstream from the ATG codons are regions with a high level of complementarity to the 3′-end of the 16S rRNA sequence (Fig. 2). The alignment indicates the possibility of simultaneous interactions between the 16S rRNA and the mRNA at the ribosome-binding site, the initiation codon, and at the two downstream elements. Downstream elements (also referred to as downstream boxes) have been identified in the cold-shock-induced genes *cspA, cspG, cspB, cspI, csdA*, and *rbfA* from *E. coli* (reviewed in Phadtare et al. 1999) and *crhC* from *Anabaena*

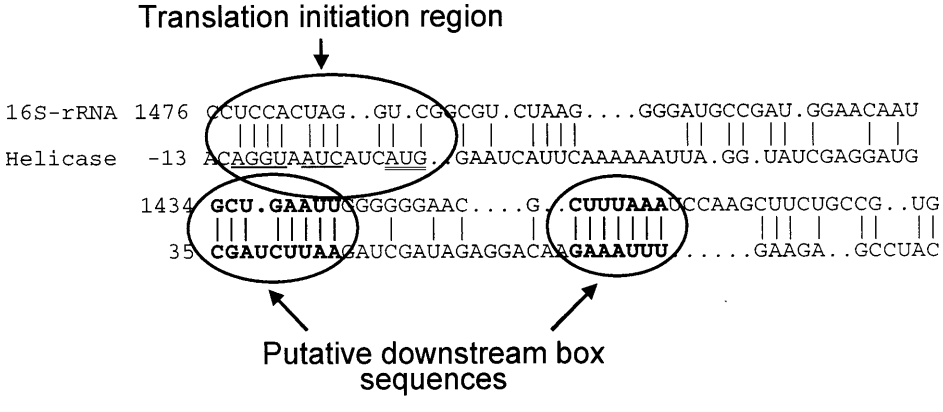

Fig. 2. Potential translation regulatory regions in the *Methanococcoides burtonii deaD* gene. Alignment of the *M. burtonii* 16S rDNA with the *deaD* gene (Lim et al. 2000). *Numbers* are relative to the first nucleotide in the 16S rRNA sequence and relative to the "A" in the ATG initiation codon (*double-underlined sequence* within the translation-initiation region). *Lines* between sequences indicate complementary bases. *Single underlined sequences* within the translation-initiation region indicate identity with the canonical methanogen ribosome-binding site (Brown et al. 1989)

(Chamot et al. 1999). In *E. coli*, the downstream element of *cspA* has been reported to have an important role in enhancing translation and is proposed to allow the formation of a stable initiation complex at low temperature (Etchegaray and Inouye 1999; Mitta et al. 1997). Recently, however, the interpretation of experimental evidence concerning the functional relevance of downstream elements has been debated (Blasi et al. 1999; Etchegaray and Inouye 1999; Yamanka 1999). In view of this debate, and presently in the absence of experimental evidence, it is unclear what role the putative downstream elements in the archaeal *deaD* gene may serve. It is noteworthy, however, that downstream elements have been identified in leaderless archaeal genes. The *deaD* gene is the first example of an archaeal gene with a long 5′-UTR that contains a downstream element and therefore provides an additional transcriptional class for examining the role of downstream elements in regulating translation in Archaea.

Role of cold-shock domain proteins

Cold-shock proteins, such as CspA from *E. coli*, are small (~70 amino acids) nucleic acid-binding proteins (Graumann and Marahiel 1998). Their roles in cold-shock and low-temperature adaptation in bacteria include functioning as RNA chaperones and involvement in regulating gene expression. The *cspA,* and several other *csp* genes from *E. coli,* contain 5′-UTRs and cold-box elements, and are cold-shock induced. The CspA protein is thought to autoregulate expression, and to regulate expression of other cold-induced genes by binding to the 5′-UTR. CspA is also a member of the Y-box family of nucleic acid-binding proteins that are found in mammals and plants (Wolffe 1994). Although highly conserved in some evolutionary branches of life, CspA homologues are not found in *M. burtonii,* or in the genomes of the Archaea *Methanobacterium thermoautotrophicum, Methanococcus jannaschii*, *Archaeoglobus fulgidus, Pyrococcus horikoshii,* and *Pyrobaculum aerophilum*, the yeast *Saccharomyces cerevisiae,* the bacteria *Helicobacter pylori*, *Mycoplasm genitalium*, or the cyanobacterium *Synechocystis* (Lim et al. 2000).

Even though sequences with similarity to CspA are absent in Cyanobacteria, a family of cold-shock-induced RNA-binding proteins (Rbp) (Mulligan et al. 1994; Sato 1995; Sugita and Sugiura 1994) that have very similar tertiary structures to CspA (Graumann and Marahiel 1996, 1998) have been identified. It is possible that cold-shock induction of the *Anabaena* DEAD-box RNA helicase gene *crhC* (Chamot et al. 1999) is regulated by Rbp binding to the cold-box element within the 5′-UTR. Rbp homologues do not appear to be present in the genomes of Archaea. It is possible that *M. burtonii* possesses a class of RNA-binding proteins that are structurally analogous but distinct in sequence to CspA and Rbp, and that regulate expression of *deaD* by interacting with the 5′-UTR. In this regard, it is noteworthy that the crystal structure of the C-terminal domain of the translation-initiation factor 5A (IF-5A) from *Pyrobaculum aerophilum* has been found to be most similar to CspA, even though the primary amino-acid sequences are distinct (Peat et al. 1998).

Recently, DeLong identified a gene from the low-temperature-adapted sponge symbiont *Cenarchaeum symbiosum* with similarity to *cspA* (E. DeLong, personal communication). The presence of a CspA homologue in this sponge symbiont, but its absence in other Archaea, indicates that at least two classes of functionally related genes have evolved in Archaea. It will be interesting to determine whether the sponge host contains a similar gene, as this might suggest lateral gene transfer between host and symbiont.

Closing comments

The current research on cold-shock response and low-temperature adaptation focuses on translation and the ribosome plus the associated machinery for protein synthesis. This focus is in keeping with earlier observations that led to the proposal that the ribosome was the thermal sensor for bacterial cold shock (VanBogelen and Neidhardt 1990). Almost all the proteins discussed in this review are connected with ensuring successful translation of mRNA into functional proteins at low temperatures. This mechanism is also a consistent theme for the proteins involved in the cold-shock response for organisms from the other domains, in particular, Bacteria. A major problem caused by cold shock may be that it halts protein synthesis, and hence acts in the same manner as the most potent bacterial toxins (diphtheria toxin, botulinum toxin) or antibiotics (chloramphenicol). This shutdown of protein synthesis may be the ultimate crisis for a cell. The cold-shock response works to reverse the adverse effects of low temperature on protein synthesis. It is a multipronged attack that focuses on the state of the incoming mRNA, the ribosomal machinery itself, and the status of the exiting nascent polypeptide chain.

Metabolic pathways and their adaptation to low temperature have been less thoroughly investigated. Studies on the cytosolic enzymes aspartate aminotransferase (Birolo et al. 2000), triose phosphate isomerase (Alvarez et al. 1998), and citrate synthase (Gerike et al. 1997) from psychrophilic bacteria have shown that their catalytic efficiencies appear not to be higher at low temperature when compared to their mesophilic or thermophilic counterparts. These findings imply that these enzymes have not been exposed to strong selective pressure to function at low temperature, and therefore the respective metabolic pathways controlled by these enzymes might not constitute rate-limiting steps. In this context, one might speculate that for a given organism a limited number of cellular processes may be crucial to adapting to growth in extreme environments. For low-temperature adaptation, these mechanisms may include protein synthesis in addition to a limited number of fundamental metabolic processes. It may therefore be expected that Archaea featuring novel metabolic pathways (e.g., methanogenesis) might also have evolved unique ways of

adapting to low temperature that are not found in Bacteria or Eukarya.

Our understanding of the genetics and physiology of archaeal responses to the cold is still very primitive. There are large gaps to be filled. The major advance is likely to come from the sequencing of several genomes from mesophilic and psychrophilic archaea, some of which are currently under way. These genomic sequences will allow us to determine likely candidates involved in cold survival; inferred as homologues of bacterial or eukaryotic systems or by absence from genomes of thermophilic and hyperthermophilic archaea. Functional studies targeting specific genes, or examining global gene expression using microarrays for mRNA levels and proteomics for protein levels, will dramatically enhance this understanding. In addition, this knowledge can be coupled with biophysical studies to determine structural and functional properties of proteins important for activity at low temperature. Clearly, once these studies on Archaea reach a significant stage, we will gain a much more complete and enhanced view of the general adaptation of life to low temperature.

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