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Bacterial cold shock responses

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As a measure for molecular motion, temperature is one of the most important environmental factors for life as it directly influences structural and hence functional properties of cellular components. After a sudden increase in ambient temperature, which is termed heat shock, bacteria respond by expressing a specific set of genes whose protein products are designed to mainly cope with heat-induced alterations of protein conformation. This heat shock response comprises the expression of protein chaperones and proteases, and is under central control of an alternative sigma factor (σ^{32}) which acts as a master regulator that specifically directs RNA polymerase to transcribe from the heat shock promotors. In a similar manner, bacteria express a well-defined set of proteins after a rapid decrease in temperature, which is termed cold shock. This protein set, however, is different from that expressed under heat shock conditions and predominantly comprises proteins such as helicases, nucleases, and ribosome-associated components

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that directly or indirectly interact with the biological information molecules DNA and RNA. Interestingly, in contrast to the heat shock response, to date no cold-specific sigma factor has been identified. Rather, it appears that the cold shock response is organized as a complex stimulon in which post-transcriptional events play an important role. In this review, we present a summary of research results that have been acquired in recent years by examinations of bacterial cold shock responses. Important processes such as cold signal perception, membrane adaptation, and the modification of the translation apparatus are discussed together with many other cold-relevant aspects of bacterial physiology and first attempts are made to dissect the cold shock stimulon into less complex regulatory subunits. Special emphasis is placed on findings concerning the nucleic acid-binding cold shock proteins which play a fundamental role not only during cold shock adaptation but also under optimal growth conditions.

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Investigation of low temperature effects on bacteria – what is it good for?

In recent years, the analysis of temperature-dependent gene expression at the molecular level has become of central importance to our understanding of several cellular functions. One of the aspects relevant in this context is bacterial pathogenicity. In many cases, the production of bacterial virulence factors required to successfully interact with and/or invade a host cell is temperature-controlled[1,2]. Interestingly, it appears that pathogenic bacteria can be classified into two general groups depending on the direction of temperature change that triggers virulence factor expression. While human pathogens like *Listeria* and *Yersinia* or more generally speaking pathogens that selectively affect warm-blooded organisms usually experience an increase in ambient temperature upon contact with their host, many plant pathogens like *Erwinia* and *Pseudomonas* have been shown to activate virulence factor expression upon low temperature exposure thereby causing the typical "cold-weather" diseases[3]. For example, it has been demonstrated that during nutrient deprivation *P. syringae* induces the production of INPs if exposed to cold shock[4]. In contrast to AFPs that prevent or delay ice-crystal growth[5], INAs promote the formation of ice-crystals[6] and like AFPs

have so far been identified in a number of organisms that have adapted to survive in cold environments, including bacteria[7,8]. In case of *P. syringae*, INPs appear to induce frost damage in the leaves of the host plant resulting in the release of nutrients from the plant cell which thereafter can be utilized by the bacterium to promote growth.

It is clear that a detailed understanding of cold-induced molecular mechanisms employed by pathogens to negatively affect nutrient plants is of considerable public interest. However, in addition to pathogenicity-related analyses of low-temperature effects on gene expression there is a broad number of important applications and many other aspects that make investigations of bacterial CSRs interesting and useful. These comprise the development of cold-inducible expression systems for heat- or proteolysis-sensitive proteins[9], the identification, isolation, and over-expression of cold-adapted enzymes relevant to industrial processes and bioremediation[10–12], the genetic engineering of plant-growth-promoting rhizosphere bacteria[8], and the development of new techniques for food production and preservation – to give only a few examples. Most importantly, however, analyses of CSRs at the molecular level provide us with valuable insights into the complex organization of gene regulatory networks and fundamental mechanisms for stress adaptation. In many cases, the explosion of information accumulated by analysis of CSRs during the past years had a profound impact on our general understanding of cell function even under non-stress conditions.

In this review, we will give a brief overview on our current understanding of how bacteria adapt to a sudden drop in temperature. First some general principles and definitions will be introduced that are accompanied by a phenomenological description of the typical characteristics of cold shocked bacterial cells. After that we will discuss some of the known bacterial cold signal perception systems and finally summarize some selected examples of the cold-relevant regulatory and functional molecular interconnections that have been discovered so far. Emphasis will be placed on the structure, function, and regulation of the bacterial CSPs that play an important role during cold adaptation and, at least in *B. subtilis*[13] and *A. globiformis*[14], have been demonstrated to be essential for cell survival even under optimal growth conditions.

Some principles and definitions – a three stages model for bacterial cold adaptation

In all organisms, a sharp temperature drop, which is termed cold shock, has a profound impact on cell function at various levels[14–21].

Interestingly, this phenomenon does not necessarily require the formation of extra- and/or intracellular ice-crystals but is readily evident at temperatures well above the freezing point of water as reflected by the transient growth arrest that has been described not only for cold shocked *E. coli*[22] but even psychrotolerant *Y. enterocolitica* cells under such conditions[23]. Consequently, although cold-induced expression of certain genes may occur only below a defined threshold temperature[24], for global analysis of the cold shock response (CSR), which is defined as the sum of all cellular reactions required for efficient adaptation to a sudden decrease in environmental temperature, the difference ΔT between the initial growth temperature and the temperature reached after cold shock is of importance rather than its final value post-shock[25]. The more dramatic this ΔT difference, the more challenging the demand for adaptational power and hence the observable effects that can be expected. Therefore, to uncover functional and regulatory events taking place in mesophilic bacteria in response to cold exposure, many laboratories have standardized their experimental conditions to base on an abrupt temperature down-shift from 37°C to 15°C.

In fact, all cellular effects resulting from a decrease in temperature can in principle be reduced to one main cause which is the immediate reduction of molecular dynamics that lead to lowered diffusion rates and conformational alterations of molecular structures. Such temperature-dependent conformational changes may perturb proper function of the complex enzymatic reaction network, membrane integrity (transport, cell division, energy production), processes involving the biological information molecules DNA and RNA (transcription, translation, replication, nucleoid packing, mRNA folding), and are also the basis for rapid molecular thermosensing as will be discussed below. According to the Arrhenius equation that describes the effect of temperature on chemical reactions, any decrease in temperature will result in an exponential reduction of the molecular reaction rate, the extent of which depends on the value of the reaction's activation energy. It is for this reason that temperature is one of the most important environmental factors for life as it affects most cellular processes by directly influencing biochemical reactions. If the velocities of all enzymatic reactions within a cell would uniformly decrease but remain functional upon temperature lowering, it would be expected that cellular growth continues – albeit at a reduced rate that is dictated by temperature. Indeed, within a certain species-dependent temperature range, bacterial growth rates can be described according to Arrhenius's law. However, since individual enzymes are often differently optimized such that some of them possibly func-

tion within only a narrow temperature range, it seems reasonable to assume that the overall coordinated function of the cell's complex enzymatic network will be compromised after a temperature reduction and may therefore finally collapse. To counteract this, cells are capable of reprogramming gene expression within a certain range in order to adjust the relative amounts of enzymes already present or to even add novel protein functions where necessary. In addition to maintaining active metabolism by modulating the enzymatic network, further adaptational mechanisms designed to cope with cold-induced conformational alterations of other cell components such as *e.g.* nucleic acids, the translation machinery, and the cell membrane(s) play important roles after low temperature exposure. Therefore, cellular survival after a sudden temperature drop requires the capability to (i) timely detect the incoming ΔT stimulus and (ii) activate an appropriate regulatory signaling cascade that results in (iii) a functionally effective adaptation of protein synthesis to replace or modify those cellular components that are impaired in the cold.

Although it is difficult to globally generalize with respect to the extraordinary bacterial diversity known to date, growth measurements and proteomic studies of several species such as *A. globiformis*[26,27], *E. coli*[22], *B. cereus*[28], *B. subtilis*[29–31], *B. stearothermophilus*[32], *L. monocytogenes*[33–37], *E. faecalis*[38], *P. fragi*[39,40], *M. smegmatis*[41], *S. aureofaciens*[42], *S. thermophilus*[43], and many more indicate that the response of exponentially growing cells to non-lethal cold shock conditions above the minimal growth temperature can roughly be separated into three different stages (Figure 1). Stage I represents the initial but transient shock response (acclimatization phase) that immediately follows cold exposure and, depending on the organism, may take up to several hours during which a profound reduction of the growth rate as well as marked reprogramming of protein synthesis is observed. During stage II, termed recovery phase, cells of many bacteria grow significantly faster and show further modification of their protein profile compared to stage I. These cells are considered cold-adapted and enter stage III as soon as they reach stationary growth phase where gene expression is altered again. So far, most studies have focused on the examination of stage I and, to a lesser extent, on stage II. Interestingly, to our knowledge, there are no systematic analyses available that compare the possible differences between exponentially and stationary growing cells exposed to cold shock, even though in their natural environment, bacteria most likely exist in stationary phase. Investigations of time-resolved protein profiles utilizing two-dimensional gel electrophoresis allowed for the identification of a large number of proteins whose expression levels

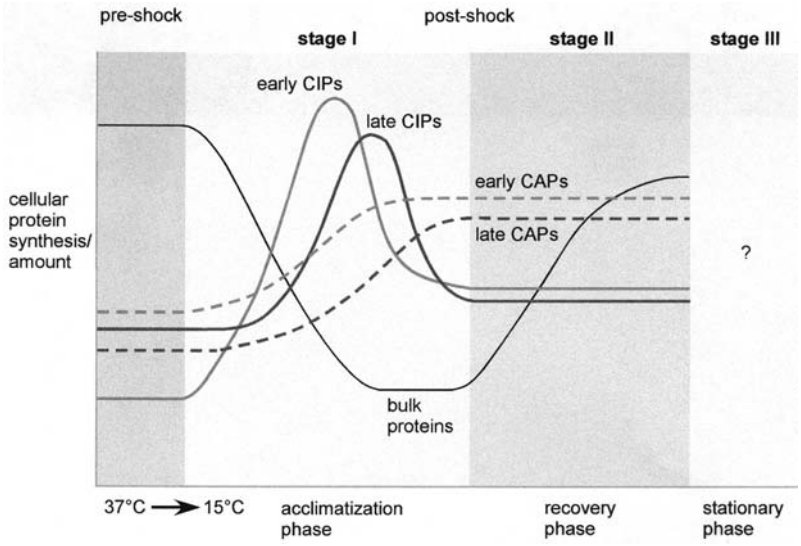

Fig. 1 *A qualitative three stages model for the physiology of bacterial cold adaptation. For details see text.*

are modulated after cold shock[22,30]. The upregulated fraction of these proteins can be assigned to two distinct classes defined as cold-induced proteins (CIPs), and cold acclimatization proteins (CAPs) (Figure 1). While CIPs are transiently induced, CAPs are permanently present at significantly elevated levels in cold shocked cells. Depending on the time-point of cold-induction, members of both of these groups can be further subdivided into early and late CIPs and CAPs. A third class, designated cold shock proteins (CSPs), represents a conserved family of widespread proteins containing the nucleic acid-binding cold shock domain (CSD) of which many, but not all, can be classified as CIPs and/or CAPs. It is tempting to speculate that the specific time-dependent up- and downregulation of individual proteins reflects a highly organized regulatory network with precisely defined function, however, much more investigations are required to substantiate this notion.

In spite of the fact that, according to the protein synthesis pattern detectable after cold exposure, psychrophilic/psychrotrophic (cold-loving), mesophilic, thermophilic (heat-loving), and hyperthermophilic prokaryotes show subtle species-dependent differences in their response to cold shock[14,17,19,44], they all appear to have the dominating requirement in common to adjust their membrane composition and to add proteins that modulate structure and/or function of nucleic acids and the translation machinery.

Low-temperature perception and signal transduction

As stated, a decrease in ambient temperature is accompanied by reduced molecular motion which results in conformational changes that affect structure and hence function of proteins, nucleic acids, and other molecules such as the membrane constituting lipids. On the basis of this physical principle, conformational alterations represent excellent candidates for incorporation into rapid responding temperature detection systems. In fact, for each of the mentioned cell components examples for specific functions in temperature sensing have been identified and described[45].

Membrane-associated signal transduction systems

Since it is the cellular envelope that is initially opposed by a decrease in temperature, it appears reasonable to expect primary sensors of cold to reside in the bacterial membrane. In fact, FT-IR, EPR and many other techniques used to examine model lipid bilayer and biological membranes have revealed that the cold-induced reduction of lipid molecular dynamics results in a decrease of membrane fluidity which, depending on the lipid composition and magnitude of cold shock, may result in a phase transition from a liquid-crystalline to a more rigid gel-like state[46–52]. Such membrane rigidification affects membrane-coupled processes and might initially occur in specialized lipid microdomains surrounding appropriate sensor proteins such as two-component system sensor kinases. Indeed, the cold-induced expression of the FA-D-encoding *desA* gene in the completely sequenced unicellular cyanobacterium *Synechocystis* sp. PCC6803[53,54] can be triggered under isothermal conditions upon reducing the membrane fluidity by palladium-catalyzed hydrogenation of the UFA content of the membrane constituting lipids[55]. This effect was abolished when RNAP was inhibited by rifampicin supplementation[55]. These results suggest that membrane fluidity serves as a primary signal for cold perception in cyanobacteria that is transduced to specifically induce transcription of *desA*. Systematic disruption of putative genes for histidine kinases and random mutagenesis of almost all the genes in this bacterium subsequently allowed for the identification of two histidine kinases (Hik33, Hik19) and a response regulator (Rer1) as components of the pathway for perception and transduction of low temperature signals[56]. Comparative DNA microarray analyses of the wild type and the corresponding *hik33* deletion strain finally demonstrated that the potentially membrane-bound Hik33 kinase indeed controls expression of a number of cold-regulated genes[57]. In summary, the current work hypothesis assumes that a

decrease in temperature is accompanied by a reduction in lipid dynamics which results in rigidification of the membrane fluidity. This in turn appears to affect the conformation of lipid-embedded Hik33 thereby activating its kinase function that leads to an activation of the cytosolic histidine kinase Hik19 which might act as a checkpoint regulator for phosphoryl group transfer to a variety of response regulators including Rer1. Rer1 finally mediates cold-induced expression of at least *desB* (Figure 2A). It is important to note, however, that the DNA microarray analyses also revealed that additional sensor system(s) for cold are likely to exist in *Synechocystis* sp. PCC6803[57].

A similar membrane-associated mechanism for cold signal perception has recently been discovered in *B. subtilis*[58]. In this fully sequenced Gram-positive soil bacterium[59] one of the 35 two-component signal transduction systems present[60] is encoded by the *desKR* operon (formerly *yocFG*[59]). In contrast to the cyanobacterial *hik33* gene[56], this operon was shown to be non-essential under standard conditions[61] and is located directly downstream of the cold-inducible FA-D gene *des* (formerly *yocE*[59,62]). The predicted protein products of *desK* and *desR* exhibit sequence similarity to membrane-bound histidine kinases containing four transmembrane segments (*E. coli* NarL type) and cytosolic response regulators, respectively[60]. DesKR has been demonstrated to be responsible for transient cold-induced expression of *des* regulated at the transcriptional level[62–64] via specific recognition of a dyad-symmetry-containing sequence element located upstream of the *des* -10 promotor region by action of response regulator DesR[58]. According to the current work hypothesis (Figure 2B), DesK is proposed to possess dual functionality acting either as kinase or as phosphatase to phosphorylate or dephosphorylate DesR in a temperature-dependent manner, respectively[58]. It is assumed that at 37°C DesK acts as a phosphatase to keep DesR in an inactive dephosphorylated form. After cold shock, DesK activates DesR by phosphorylation to recognize and bind to the *des* promotor region and initiate transcription which, depending on the experimental conditions, reaches a maximum around 30 min[63] or 70 min post-shock[64]. Production of Des finally results in conversion of lipid-incorporated SFAs to UFAs which in turn shut down *des* expression by converting DesK back to its phosphatase state rather than directly interacting with DesR[58]. It seems reasonable to assume that UFA-mediated readjustment of membrane fluidity results in a conformational change in membrane-bound DesK thereby triggering the kinase to phosphatase conversion. One of the most exciting questions is of course whether the DesKR thermosensor system serves as a global regulatory system for cold adaptation in *B. subtilis*. Our comparative DNA macro-

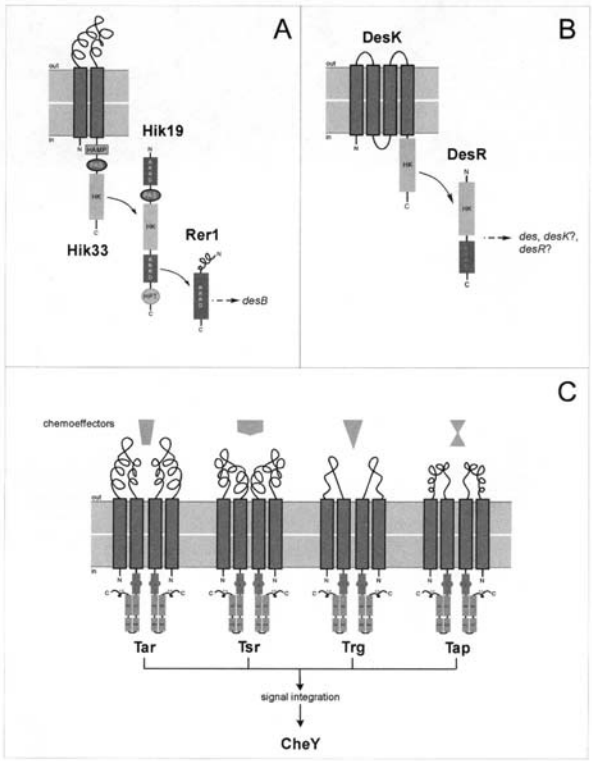

Fig. 2 *Membrane-associated bacterial cold signal perception and transduction systems. (A) The Hik33-Hik19-Rer1 pathway identified in* Synechocystis *sp. PCC6803[56,57]. (B) Control of fatty acid desaturase gene expression by the DesK-DesR two-component system in* Bacillus subtilis*[58]. (C) Role of the* Escherichia coli *methyl-accepting chemotaxis proteins (MCPs) Tsr, Tar, Trg, and Tap in temperature-dependent modulation of bacterial motility[67–69]. The indicated protein domain signatures HAMP, PAS, HK (histidine kinase domain), HPT, and RRRD (response regulator receiver domain) were identified by comparing the relevant protein sequences with the InterPro database entries[291] using the InterProScan search engine available at ExPASy[292], whereas the L (linker), SD (signaling domain), AH (adaptation helices), and TrT (transferase tail) regions were assigned as described earlier[76]. Protein and gene names appear in bold and italic fonts, respectively. Solid arrows represent phosphoryl group transfers, while dashed arrows symbolize response regulator-mediated gene activation processes. Note that the individual protein domains, transmembrane helices (black boxed bars filled in dark gray),* etc. *are not drawn to scale and that squiggles do not necessarily represent alpha helices but rather regions connecting individual protein domains. The amino- and carboxy-terminal ends of the proteins are labeled with N and C, respectively.*

array studies of *B. subtilis* JH642 and a *desK* deletion strain, however, revealed that upon cold shock DesKR appears to exclusively regulate the cold-induced expression of *des* and possibly its own two components[64]. This is in contrast to what was found in cyanobacteria where expression of several other genes was affected by a mutation in Hik33[57]. In this context it is interesting to note that our comparisons of the Hik33 histidine kinase responsible for cold shock temperature perception in *Synechocystis*[56] with the ORFs predicted from the *B. subtilis* genome suggest that *resDE*, *phoPR*, and most importantly the essential two-component system enoded by the *yycFG* operon[60,61] rather than *desKR* might represent primary candidates for a global temperature perception machinery in *B. subtilis*. Existence of such additional membrane-bound temperature sensor systems is also supported by the fact that overproduction of the DesR response regulator in the absence of DesK kinase allows for expression of *des*[58] which indicates cross-activation of DesR possibly by another kinase.

Early examinations of the Gram-negative enterobacterium *E. coli* revealed that its swimming pattern is altered upon temperature changes[65]. This dynamic response is characterized by a temporary increase in the frequency of tumbling upon cold shock and a temporary suppression of tumbling after a sudden temperature upshift. Interestingly, this phenomenon was not observed in various nonchemotactic strains suggesting that the chemotaxis apparatus is involved in molecular thermosensing[65,66]. Indeed, several detailed studies demonstrated that each of the four classical methyl-accepting chemotaxis proteins (MCPs) Tsr, Tar, Trg, and Tap represents a thermosensor in *E. coli*[67–69]. MCPs have been shown to accumulate at the cell poles[70] and function as apparently further aggregated homodimers[71–73] that form a ternary complex with a homodimer of the cytoplasmic autokinase CheA and two molecules of the adaptor protein CheW[74]. However, the exact functional stochiometry of these signal transduction system components is still subject to controverse discussion[75]. Each receptor monomer consists of a periplasmic ligand-binding domain, a C-terminal cytoplasmic signaling domain, and two membrane-spanning segments[76,75]. In case of chemotaxis, interaction of chemoeffectors such as *e.g.* Ni^{2+} ions (repellent) or maltose (attractant) with the periplasmic domains either directly or via specific binding proteins triggers some structural change within the receptor dimer. This structural change leads to repellent-induced activation or attractant-induced inactivation of ATP-dependent histidine autophosphorylation of CheA and phosphotransfer from CheA to either of two cytosolic aspartate autokinases involved in signal output (CheY) and sensor adaptation (CheB). Phosphorylated CheY binds

to FliM at the cytosolic face of the flagellar motor's switch complex and induces clockwise rotation (CW: repellent) of the motor (causing tumbling of the cell), which otherwise rotates counterclockwise (CCW: attractant) (causing smooth swimming). Phosphorylated methylesterase CheB acts as an antagonist to the S-adenosylmethionine-dependent methyltransferase CheR to remove methyl groups previously attached by CheR to the 4–6 glutamate residues present in the so-called MCP adaptation helices. This reversible methylation machinery appears to counteract the respective structural changes induced in the cytoplasmic signaling domain by binding of a chemoeffector to the periplasmic ligand-binding domain and is required to reset the signaling (kinase modulating) properties of the receptor[76,75].

Similar to the interaction of a chemoeffector with the periplasmic MCP ligand-binding domain, a sudden temperature change appears to induce a conformational switch in MCPs that allows for the generation of CW or CCW signals upon cold or warmth exposure, respectively. Like chemoeffector-mediated signaling this temperature-triggered signal is transient and has been shown to be accompanied by reversible MCP methylation that is responsible for adaptation[68]. Although the thermosensing mechanism is thought to involve temperature-dependent changes in the structure of the receptor-CheW-CheA ternary complex and/or in the interactions among multiple ternary complexes[66,77–79,73], the exact nature of temperature-dependent alteration of the MCP signalling state remains to be elucidated. It is interesting to note, however, that repellents such as ethylene glycol and glycerol have been proposed to affect MCP structure via perturbation of membrane organization rather than by binding to specific sites located in the periplasmic ligand-binding domain[80,81]. Therefore, it seems possible that temperature-dependent alterations of membrane fluidity might cause the observed signaling states. In fact, mutation analyses of the transmembrane segment (TM2) connecting the periplasmatic ligand-binding domain to the cytosolic signaling domain have shown that structural alterations of this region affect thermosensing properties[82].

Since chemical as well as thermal stimuli are perceived and transduced by the same membrane-bound sensor system, it appears reasonable that warmth and cold are intracellularly interpreted as attractant and repellent stimuli, respectively. It is worth noting that although MCPs have been generally classified into warmth sensors (Tsr, Tar, Trg) and cold sensors (Tap)[78] the respective signal generated by each *E. coli* MCP type upon temperature alteration may differ depending on at least the individual MCP chemoeffector occupancy and degree of receptor methylation[69,66–68,79]. Therefore, the final

overall signal output must be regarded as a result of a complex signal integration (Figure 2C). Interestingly, as part of the chemotaxis machinery CheY has been identified as one of the cold-induced proteins in *B. subtilis*[30]. This observation and the fact that homologs of MCPs are well conserved among different bacterial species suggests that MCP-dependent cold perception and signal transduction might reflect a more common mechanism and it remains to be elucidated whether cross-talk between chemotaxis and other regulatory systems as reported for *E. coli*[83] might contribute to bacterial cold shock responses.

In addition to the classical two-component sensor transduction systems, other membrane-associated mechanisms for cold sensing have been identified in higher organisms that, although yet undiscovered, might also exist in bacteria[84,85]. These comprise cation channel proteins that are activated[85] or whose activities are modulated[84] upon cold shock. Interestingly, in case of the mammalian ion channel TRPM8, cold shock stimulates calcium influx[85]. This phenomenon is also of importance during the CSR of plants[16,86] where Ca^{2+} influx and Ca^{2+}-dependent phosphorylation has been shown to be involved in cold stress signal transduction. In this context, it is interesting to note that the cold shock protein CspB appears to belong to one out of a group of four calcium responsive proteins in *B. subtilis*[87]. To our knowledge, so far none of the numerous studies carried out on members of the CSP protein family have been performed in the presence of Ca^{2+} ions. Future studies will clarify whether Ca^{2+} might be involved in functional modulation of CSPs *in vitro* and *in vivo*.

Translation apparatus and stringent factors RelA/SpoT

Since the cold insulating properties of one or even two of the typical bacterial phospholipid bilayer membranes can be expected insufficient to prevent quick cytosolic progression of temperature reduction, bacteria which harbor additional alternative temperature sensing and signalling capabilities would have a considerable advantage for survival. In fact, several lines of evidence support the idea that other membrane-independent principles for molecular thermosensing may exist in bacteria that appear to operate in parallel to those mentioned above. Most importantly, it has been discovered that a cold shock-like response in both *E. coli* and *B. subtilis* can be induced under isothermal growth conditions upon partial blocking of the ribosome by antibiotics such as chloramphenicol[13,88]. This observation suggests that a sudden reduction of translational capacity might be interpreted as a cellular signal triggering the CSR that, under physiological conditions, correlates with but is not necessarily dependent on the actual

temperature. Hence, the ribosome itself, possibly in concert with one of its many associated components, appears to participate as a temperature perception system. However, it remains unclear whether intrinsic properties of the ribosome or mRNAs or both simply allow for a preferential translation of specific mRNAs encoding cold-induced proteins or whether in addition to this a yet undetected ribosomal regulatory signal is generated.

Importantly, in amino acid starved *E. coli* cells, the ratio of amino acid-charged to uncharged tRNAs decreases dramatically and finally results in the occupation of the ribosomal A-site with an uncharged tRNA. Under such conditions, where protein synthesis arrests, stringent factor RelA is thought to hop from one blocked ribosome to another in order to synthesize the alarmone (p)ppGpp that binds to key enzymes such as RNA polymerase and modulates protein function(s) and gene expression[89,90]. This process, termed stringent response, is strictly dependent on the presence of uncharged tRNA in the A-site and appears to be mediated by ribosomal protein L11[90]. As a result, genes encoding metabolic enzymes, especially those involved in amino acid biosynthesis, are upregulated whereas genes associated with the translation apparatus are transcriptionally repressed[89,91]. Since chloramphenicol, in contrast to amino acid starvation, leaves the ribosomal A-site occupied with a charged tRNA[88], after addition of the antibiotic or upon cold exposure, a significantly reduced frequency of cellular (p)ppGpp basal synthesis would be expected due to a decreased probability that uncharged tRNA enters the ribosomal A-site. Indeed, at low temperature as well as in the presence of chloramphenicol, the (p)ppGpp level has been demonstrated to decline in *E. coli*[92–94]. Strikingly, induction of (p)ppGpp synthesis in *E. coli* immediately prior to cold shock results in reduced synthesis of CIPs and a delayed adaptation to low temperature whereas a *relA*/*spoT* mutant showed increased production of CIPs and faster cold adaptation[25]. These results clearly indicate that a decrease in cellular (p)ppGpp level following a temperature downshift plays a physiological role in the regulation of gene expressen and adaptation to growth at low temperature. It should be noted that, except for a few differences between Gram-positive and Gram-negative bacteria[91,95], the global principle underlying the stringent response as described for *E. coli*[89] appears to apply in other bacteria as well[91,95,96]. Hence, the increased (p)ppGpp level reported for cold-stressed *B. subtilis*[97] most likely results from an artifakt caused by unphysiological cooling from 37 to 0°C which is associated with cell lysis.

Consequently, the following globalized model for regulation of gene expression under optimal, stringent, and cold shock conditions

is proposed and incorporates (p)ppGpp as one of the regulatory elements being part of the cold shock stimulon. On the basis of the fact that the protein pattern in *B. subtilis* growing under optimal conditions is dramatically altered in a *relA*/*spoT* deletion mutant compared to the wild type[96], we conclude that the cellular (p)ppGpp basal level is of crucial importance for regulation of gene expression even during vegetative cell growth. We further assume that a change in the (p)ppGpp level in either of the two possible directions alters the population ratio of (p)ppGpp-modified to non-modified RNA polymerase (RNAP) which in turn results in a different transcription profile, possibly due to differentially altered promotor affinities of modified compared to non-modified RNAP. Strikingly, our recently published DNA array analysis of cold-shocked *B. subtilis* cells[64] in comparison to the transcription profile observed after induction of the stringent response[91] suggests that significant parts of the CSR resemble an inverted stringent response. Many of the genes induced or repressed during the stringent reponse are repressed or induced after cold shock, respectively. These findings might be explained by assuming the RNAP-(p)ppGpp population model introduced above and strongly suggest that (p)ppGpp controls at least in part a good fraction of the genes that constitute the cold shock stimulon. It should be noted that (p)ppGpp used as a collective term for pppGpp or ppGpp or both might allow for more than just one type of modified RNAP. Differences between the expression pattern predicted by this model of an inverted stringent response and the actual experimental measurements may be attributed to additional constraints defined by: (i) temperature; (ii) altered mRNA stabilities/turnover rates; (iii) altered nucleoid structure; (iv) differences in strains; (v) additional regulatory factors, and the complexity of interacting regulatory circuits as detailed below in terms of a multiple filter model. It will be one of the many challenging tasks of the future to precisely dissect and evaluate the validity of this model in different bacteria.

DNA, DNA-topology modulating proteins, and nucleoid re-organization

A third temperature perception system capable of directly affecting gene expression is the DNA molecule itself, most likely in concert with proteins modulating its complex structure such as DNA gyrase. In *E. coli*, genomic DNA consists out of a large closed circular polymer of approx. 4.6 Mbp in size that is compacted more than 1,000-fold to form an irregular dispersed lobular shape containing many clefts[98]. This microscopically distinct structure, termed nucleoid,

occupies less than half of the apparent intracellular space and, as was found for chromosomes from diverse biological sources including eukaryotes, is thought to be organized in topologically independent domains covering around 100 kbp each. Although size and shape of *B. subtilis* cells do not change detectably after cold shock, microscopic analyses have revealed that low temperature exposure results in nucleoid compaction[99]. Such an observation could generally be interpreted in terms of (i) temperature-dependent conformational alteration of the DNA polymer itself, (ii) changed activity and/or amounts of DNA structuring proteins such as DNA gyrase[100], bacterial histone-like proteins (H-NS[101,102], HU[103,104]/HBsu, IHF), SASPs, topoisomerase I, *etc*., and/or (iii) impaired synthesis of proteins that are directed towards the membrane which may result in condensation of the nucleoid due to reduced expansion forces normally mediated by coupling transcription and translation to membrane insertion[105]. In fact, it appears that all of the three suggested mechanisms are involved in a complex interplay after cold shock.

Upon heating, the DNA polymer tends to denature which results in decreasing local twist (Tw) of both strands around each other. For a covalently closed DNA molecule having constant linking number, compensatory coiling of the helix in space, which is termed writhe (Wr), is generated ($\Delta Lk = \Delta Tw + \Delta Wr$). Consequently, after cold shock DNA would physically tend to overtwist and, correspondingly, create negative supercoiling ($\Delta Wr<0$). Since changes in DNA topology may significantly modulate the location of –35 and –10 promotor regions relative to each other this principle provides a powerful mean of gene expression after cold shock. However, it seems unlikely that the changes observed under the microscope can be attributed solely to physical distortion of the molecule itself, especially since DNA exists in association with several structure stabilizing proteins[98]. Therefore, it appears reasonable to assume that modulator proteins play an important additional role. In fact, investigations of *B. subtilis* and *E. coli* demonstrated a cold shock-induced increase in negative supercoiling of DNA that was abolished by the addition of DNA gyrase inhibitors but occurred even under conditions that block protein *de novo* synthesis[63,106–108]. Furthermore, this effect was less apparent in an *E. coli* mutant deficient in expression of histone-like protein HU[108] which suggests that pre-existing amounts of DNA gyrase and HU might function in a cold-activated cooperative manner to enhance negative supercoiling after cold shock. Consequently, these proteins in concert with DNA appear to serve as an additional bacterial temperature perception system and may define a further cold-relevant regulatory element.

It is interesting to note that increased negative supercoiling facilitates processes involving DNA duplex opening such as transcription and replication. Hence, the ATP-dependent introduction of negative supercoils immediately following cold shock by DNA gyrase and HU may as well be interpreted in terms of utilizing the DNA molecule to store energy. While detailed analyses of HU null mutants revealed a cold-sensitive phenotype with dramatically reduced cell viability for such *E. coli* strains[103], a recent study indicates that during cold shock the expression of the HU β-subunit is preferentially stimulated whereas that of the HU α-subunit is repressed. These findings suggest that an altered HUα to HUβ ratio resulting in an increase of HUα/HUβ heterodimers and/or $(HU\beta)_2$ homodimers might play an important role during cold adaptation[104]. The importance of histone-like proteins at low temperature is further underlined by the finding that in other bacteria such as *M. smegmatis* a histone-like protein has been identified as one of the major cold-induced proteins whose removal results in the inability to resume metabolic activity such as ATP synthesis after cold shock[41]. Moreover, H-NS is known to compact the nucleoid when overproduced[109] and represents one of the CIPs in *E. coli*[101] that plays an important role during cold adaptation[102]. To our knowledge, no clear cold-relevant roles have yet been assigned to other DNA structuring proteins although a recent DNA array study of cold-shocked *B. subtilis* cells demonstrated an approx. 3.3-fold higher abundance of the SASP-encoding *sspF* gene[64] whose product does not appear to have a specific function during sporulation[110]. It might be interesting to investigate whether it is of relevance to the cold shock response, especially in conjunction with HBsu which was shown to modulate effects of SASPs at least *in vitro*[111].

Finally, the dramatic reduction in translation capacity reported for cold-shocked *E. coli* and other bacteria as well[112–116] strongly suggests that DNA structure-affecting expansion forces mediated by coupling transcription and translation to membrane insertion[105] could be significantly reduced under such conditions and might therefore very well account for additional cold-induced nucleoid structure alterations. Hence, it appears that at least three factors determine the status of the nucleoid in the cold.

Function of the cold shock response – some selected aspects

Although it appears clear that bacterial cold shock stimulons are controlled by at least three different types of temperature perception

systems which reside in the membrane, the ribosome, and the DNA, the precise molecular organization of this complex signaling network requires much further investigation. However, a second question of central importance is what individual functionalities are required after cold shock.

Adaptation of the cellular membrane(s)

For all organisms, maintenance of functional cell membranes is a limiting factor for survival. As introduced above, upon cold shock the physical status of biological membranes is altered from being fluid to becoming rigid which provides the basis for molecular thermosensing. Such perturbations affect membrane-coupled processes such as transport, energy generation, and cell division, to mention only a few. Therefore, in a process generally termed homeoviscous adaptation[117], with decreasing temperature, bacteria incorporate fatty acids (FAs) of lower melting points into lipids in a species-specific mode to restore membrane integrity and hence function. So far, three general strategies have been discovered that are utilized to counteract rigidification of membrane fluidity at low temperature. In principle all of these focus on a reduction of van der Waals interactions between adjacent lipid acyl chains and include FA acyl chain length shortening, FA acyl chain branching, and/or the introduction of usually *cis*-configured carbon double bonds[118–120]. While shortening and methyl-branching of FAs have so far been reported to be strictly dependent on *de novo* biosynthesis[121], introduction of double bonds into acyl chains can either be achieved anaerobically by at least two distinct mechanisms during FA synthesis as demonstrated for *E. coli* and *S. pneumoniae*[122,123] or aerobically by modification of readily synthesized FAs through fatty acid desaturase enzymes as shown for cyanobacteria[17] and bacilli[120,124].

In *E. coli*, adaptation of the cytoplasmic membrane fluidity during cold shock occurs by increasing one of the two UFA species that are synthesized at 37°C[122]. At low temperature (25°C), *cis*-vaccenic acid is produced in significantly higher amounts than at 42°C. This regulatory response is due to the intrinsic properties of β-ketoacyl-ACP synthase II that converts palmitoleic (*cis*-16:1Δ9) to *cis*-vaccenic (*cis*-18:1Δ11) acid. This enzyme is present at all temperatures but is more active in the cold and represents a key checkpoint in thermal adaptation of the cytoplasmic membrane. In contrast to *E. coli* and Gram-negative bacteria in general, lipids of Gram-positive bacilli contain a high proportion of iso- and anteiso-branched FA species. These molecules are derived from valine, leucine, and isoleucine precursors resulting in iso-branched-even-chain, iso-branched-odd-chain, and anteiso-branched-odd-chain

FA species[125,126] respectively. A fact that reflects a tight coupling of amino acid metabolism and FA biosynthesis.

B. subtilis has been shown to utilize a dual strategy to restore membrane fluidity after cold shock. In parallel to initiating *de novo* synthesis of saturated anteiso-branched C-15 and C-17 fatty acids for long-term adaptation[127], this Gram-positive bacterium transiently induces the unique fatty acid desaturase gene *des*[62,63,128] whose presence is essential for synthesis of all four unsaturated fatty acid species so far detected in this organism[129]. Although earlier investigations assigned *B. subtilis* desaturase activity to a membrane-associated enzyme of the acyl-CoA-desaturase type[130], it has now become clear that this enzyme is a lipid-desaturase[131]. As such it modifies the lipids present in the membrane directly at the time of cold shock thereby providing a rapid responding system for modulation of membrane fluidity. Interestingly, in the absence of exogenous isoleucine sources, deletion of *des* in a *B. subtilis* strain JH642 background results in a cold-sensitive growth phenotype indicating an important role for this enzyme during cold adaptation[129]. In such a mutant, artifical expression of *des in trans* does not only cure the observed phenotype but demonstrates that Des possesses cryoprotective properties which significantly increase cold-tolerance of *B. subtilis*[129]. Although it was reported that UFAs are exclusively synthesized upon cold shock[62,63], recent investigations revealed that in the absence of isoleucine, *B. subtilis* produces detectable amounts of UFAs even at 37°C[129]. This finding suggested for the first time that isoleucine itself might be involved as a modulator molecule. In fact, analyses of a transcriptional *des-lacZ* fusion integrated at the original chromosomal location of *des* revealed that transcription of the desaturase gene was abolished in the presence of isoleucine at 37°C and it was demonstrated that this isoleucine-dependent control of *des* expression at 37°C is mediated by the DesKR two-component regulator system in a membrane fluidity-dependent manner[132] similar to what has been described for cold shock[58]. The addition of sufficient amounts of the branched-chain amino acid isoleucine to the growth medium leads to elevated levels of lipid-incorporated anteiso-branched fatty acids. As a consequence, membrane fluidity increases under isothermal conditions which is sensed by DesK and results in shutting down the transcription of *des* as summarized above. In this context, the fact that membrane composition in *B. subtilis* is nutrient-dependent and therefore dramatically altered at the transition to stationary growth phase[129] together with the observation that a *des* null mutant showed a reduced viability after prolonged growth[62] leads us to propose that additional *des*/*desKR*-dependent

regulatory effects might be observed in stationary compared to exponentially growing cells under optimal growth conditions as well as after cold shock. This notion is of importance since the comparative expression analyses of *B. subtilis* JH642 and *des*/*desKR* null mutant strains available so far are restricted to examinations of the exponential growth phase[64]. It cannot be excluded that UFA species possibly released from the membrane at a later growth stage might have regulatory properties and that their absence accounts for the growth defect reported for a *des* null mutant during stationary growth.

Several antarctic marine psychrophilic bacteria as well as cyanobacteria such as *Synechocystis* sp. PCC6803 contain PUFAs[17,133]. In spite of the fact that the introduction of more than two double bonds has little additional effect on further lowering the liquid-crystalline to gel phase transition temperature, it creates a structural element which influences the acyl-chain packing order and possibly prevents non-bilayer phase formation in the cold. Although the exact functional significance of PUFAs in psychrophiles is still subject to discussion[133], it seems that in certain cyanobacterial strains PUFAs are required for proper function of photosynthesis at low temperature[134,135]. To synthesize lipids containing PUFAs, in *Synechocystis* sp. PCC6803 the individual enzymes of a family of four lipid-desaturases successively introduce a double bond into their respective substrate lipids with high positional specificity[17]. In surprising contrast to this, the production of all four mono-UFAs which appear to differ in the position of double bonds in *B. subtilis* is strictly dependent on the presence of a single desaturase gene[129]. This suggests that either the corresponding enzyme, Des, represents a novel class of lipid-desaturase enzymes with a rather broad product specificity or that some of the detected UFA species might be the products of a yet unknown alternative biosynthesis/isomerization/degradation pathway that is strictly dependent on Des. However, although it has become clear now that *B. subtilis* produces more than just one UFA species[129], it should be noted that the assignment of the double bond position was derived using gas chromatography in conjunction with a computerized microbial FA identification system. Since in the case of UFA double bond assignment these systems are sometimes suspected of not being accurate enough, it would be useful if the reported double bond positions could be re-examined by a more detailed investigation employing mass spectrometry.

Although a wealth of information is available concerning cold adaptation of the cytoplasmic membrane of diverse bacterial species, comparably little is known about the impact of low temperature on the outer membrane of Gram-negatives[136]. One of the characteristic

features of *E. coli*, however, is the presence of a surface-exposed glycolipid which is termed lipopolysaccharide (LPS). LPS is a major component of the outer leaflet of the outer membrane of Gram-negative bacteria that is of significant immunological importance and forms a protecting barrier around the cell[136]. It consists of three building blocks: (i) a highly variable surface-exposed O-antigen polysaccharide which is involved in establishing infections in higher organisms; (ii) a hydrophobic membrane anchor known as lipid A whose presence is essential for normal growth; and (iii) a core oligosaccharide region that connects the O-antigen polymer to lipid A[137]. For *Salmonella* strains as well as for *E. coli*, it has been demonstrated that *de novo* synthesis of the lipid A component of LPS is altered upon cold shock[138,139]. In *E coli* cells growing at optimal temperature, the two SFA species laurate and myristate are successively incorporated into the lipid A precursor Kdo_2-lipid IV_A by action of the two acyltransferases LpxL and LpxM, respectively. After cold shock, the palmitoleoyl-ACP-dependent acyltransferase LpxP is induced and replaces the LpxL-mediated incorporation of laurate by incorporation of the UFA palmitoleate[139]. Since the melting point of *cis*-9-palmitoleic acid is significantly lower than that of lauric acid, it has been suggested that this exchange of SFA building blocks by UFA moieties during LPS biosynthesis might be required to re-adjust the outer membrane fluidity after cold shock[139]. In fact, *lpxP* deletion strains selectively show a dramatically increased sensitivity to antibiotics in the cold indicating that the barrier function of the outer membrane is severely compromised[140,141]. LpxP has been reported to represent the first example of a protein in *E. coli* specifically induced by cold shock whose activity has effects on the outer membrane lipid composition that are beneficial to the cell[140].

With respect to the fact that diffusion rates are reduced at low temperature, it is interesting to note that, independent of the growth phase examined, *E. coli* outer membrane diffusion pore proteins (porins) OmpF and OmpC are thermoregulated in an inversely related manner. While the total number of these porins in the membrane remains constant at approx. 100.000 copies per cell, the larger of the two pores, formed by OmpF, is expressed at an elevated level in the cold compared to optimal growth temperature. At the same time the amount of OmpC is reduced. Thermoregulation of *ompF* expression is at least dependent on EnvY[142] and a non-coding RNA known as *micF*[143,144]. It therefore appears that, in order to counterbalance reduced diffusion rates, EnvY and *micF* determine that larger channels are present in cells growing at low temperature. The exact role of EnvY, however, remains to be elucidated.

Structure, function, and evolution of cold shock proteins

One of the most extensively studied protein classes relevant to bacterial cold shock responses is the widespread family of small (approx. 70 amino acids), nucleic acid-binding cold shock proteins (CSPs)[145] whose members typically represent the most prominently cold-induced polypeptides known to date[30,146]. With only a few exceptions, genes encoding these proteins have been identified in the majority of psychrotrophic, mesophilic, thermophilic, and even hyperthermophilic bacteria so far examined[147] including the earliest diverging bacterial branches *Thermotoga* and *Aquifex* which indicates an ancient origin[148]. In a given bacterial species, CSPs are often found in multiple gene copies that are differentially regulated and code for protein products of identities/similarities ranging between 29%/46% and 83%/91%[149]. Strikingly, based on their virtually ubiquitous presence in bacteria, a recent study revealed that CSPs may serve as elicitors of the plant immunological defense response directed against microbial infections[150]. In this context, it is interesting to note that CSPs constitute the prototype of the cold shock domain (CSD)[151,152] which is conserved from bacteria to man[153,154]. Apart from its identification as isolated ORFs (termed CSPs) in bacteria[146], archaea[155], and eukaryotes[156,157], CSDs have also been discovered as integral components of larger proteins in mycobacteria[147] and eukaryotes[158,157] (Figure 3). The presence of extended mycobacterial CSP variants containing an additional, yet functionally and structurally uncharacterized C-terminal domain of approx. 70 amino acids might reflect a first step during evolution of simple CSPs towards more complex CSD-containing proteins such as the eukaryotic Y-box factors. With respect to the immunological relevance of bacterial CSPs to plants[150] and the fact that the first eukaryotic CSP was discovered as a fungal allergen[156] it is tempting to speculate

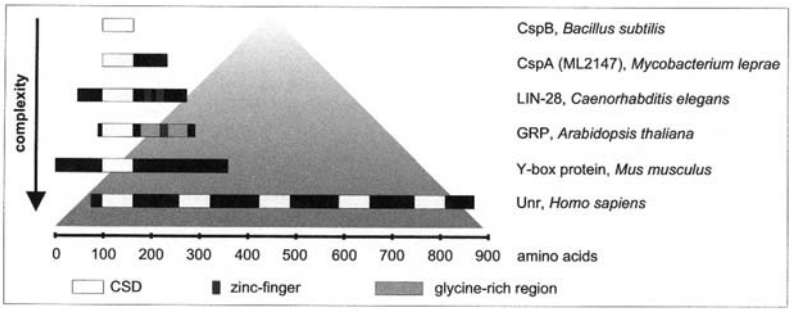

Fig. 3 *Conservation of the cold shock domain (CSD) throughout evolution and its appearance in different protein types of varying complexity[147].*

whether these so far unique proteins could be utilized to design novel vaccines addressing pathogens like *M. tuberculosis* and *M. leprae*.

Importantly, the CSD harbors the nucleic acid binding motifs RNP-1 and RNP-2[159] and hitherto CspB from *B. subtilis*[151,152], CspA from *E. coli*[160,161], CspB from thermophile *B. caldolyticus*[162], TmCsp from hyperthermophile *T. maritima*[163], and the CSD from human Y-box protein YB-1[164] have been structurally determined using crystallography and/or NMR techniques. Based on the high degree of sequence conservation, the available experimental structure data were used in a large scale comparative molecular modelling approach to generate computer models for each of the CSD-containing proteins known[147]. With approximately 70 amino acids in length, CSPs share a highly similar overall fold consisting of five antiparallel β-sheets that form a β-barrel structure harboring surface-exposed aromatic and basic residues (RNP-1 and RNP-2 motifs on β-strands two and three, respectively) which are involved in nucleic acid binding[164–166]. These amino acids are arranged on one side of the molecule and, surprisingly, some of the surface protruding aromatic residues that participate in nucleic acid interaction also contribute to protein stability and play a role in the rate-limiting step of CSP folding[167–169]. In this context, it should be noted that CSPs fold extremely rapidly[168,170,171] and, depending on whether they originate from mesophilic or thermophilic bacteria, possess a low or high thermodynamic stability *in vitro*, respectively[172–174]. In the presence of nucleic acids, however, purified CSPs become less sensitive to proteolytic degradation, suggesting that their significantly improved stability *in vivo* is mediated by nucleic acid binding[172].

Interestingly, some CSPs form dimers *in vitro*[28,151,175,176]. Although it is yet unclear whether dimerization plays a role in living cells, it has been suggested that the shape of a *B. subtilis* CspB dimer resembles a protein mimic of one of the two strands forming a DNA duplex in which the presumed nucleic acid binding sites adopt a twinned position relative to each other while the CSP dimer's back is negatively charged in a manner similar to the DNA phosphate backbone[21]. This is an interesting notion regarding the fact that CSPs bind to single-stranded rather than to double-stranded nucleic acids with individually different binding affinities and sequence selectivities[13,166,177,178–183,]. It should be noted, however, that *E. coli* CspA, which apparently exists exclusively as a monomer[160,161], was also shown to bind to dsDNA fragments containing the *gyrA* promotor[100] or the promotor of *hns*[101] and plays a role as transcriptional activator. Nevertheless, since binding of purified CspA to the promotor of *hns* is much weaker than that of CspA in crude cell extracts[184], it seems

possible that dsDNA binding by CspA is mediated by additional proteins or even requires RNA polymerase for DNA duplex opening *in vivo*. In fact, other studies employing different DNA probes failed to demonstrate any affinity of CspA to dsDNA[178,185] unless the loop connecting β-strands three and four in CspA was replaced by the corresponding region from the human Y-box protein YB-1[185]. Therefore, this loop appears to play an important functional role which is in line with the results of a recent NMR study demonstrating that, in addition to the RNP-1 and RNP-2 motifs, it is indeed involved in nucleic acid interaction even in CspB from *B. subtilis*[166]. Thus, except for the first eukaryotic CSP Cla h 8 found in the mould *Cladosporium herbarum* which appears to bind to both ssDNA and dsDNA[156], the remaining CSPs examined to date preferentially bind to single-stranded nucleic acids. Interestingly, the affinity of Cla h 8 to dsDNA increases with decreasing temperature and is paralleled by an increase in the β-sheet content of this protein[156]. Moreover, similar to CspB from *B. subtilis*, the stability of Cla h 8 is enhanced upon addition of nucleic acid ligands *in vitro*.

In *B. subtilis*, three cold-inducible CSPs have been identified (CspB-D)[13,30,186] while in *E. coli* only four (CspA[146], CspB[187], CspG[188], and CspI[189]) out of a total of nine CSPs (CspA-I) are cold-induced[18]. In spite of the very similar overall fold, the reported individual differences in CSP:CSP and CSP:RNA/DNA interactions may also reflect different functions *in vivo*. As shown in Figure 4, construction of a phylogenetic tree from the two CSP families discovered in *E. coli* and *B. subtilis* allows for a clear definition of five distinct CSP subgroups. Interestingly, these groups cluster according to *csp* origin and expression pattern and comprise (i) the highly homologous *B. subtilis* CSPs CspB-CspD, (ii) the cold-inducible CSPs CspA, CspB, CspG, and CspI, (iii) constitutively expressed CspC and CspE[190], (iv) stationary phase and nutritional starvation induced CspD[191], and (v) the so far uncharacterized, highly basic CSPs CspF and CspH from *E. coli*. In addition to some functional overlap concerning properties such as transcriptional antitermination[192] and during suppression of a cold sensitive phenotype in a multiple *csp* deletion strain[193], in several cases, this assignment of individual CSPs to distinct subgroups is also reflected by different functions, as far as has yet been determined. Apart from its properties as a transcriptional activator[101,184], CspA from *E. coli* has also been shown to act as a RNA chaperone *in vitro*[178] and possesses transcriptional antiterminator functions like its homologs CspC and CspE[192]. Furthermore, CspC and CspE were identified as high-copy suppressors of mutations in the chromosome partition gene *mukB*[190] and are involved in regulat-

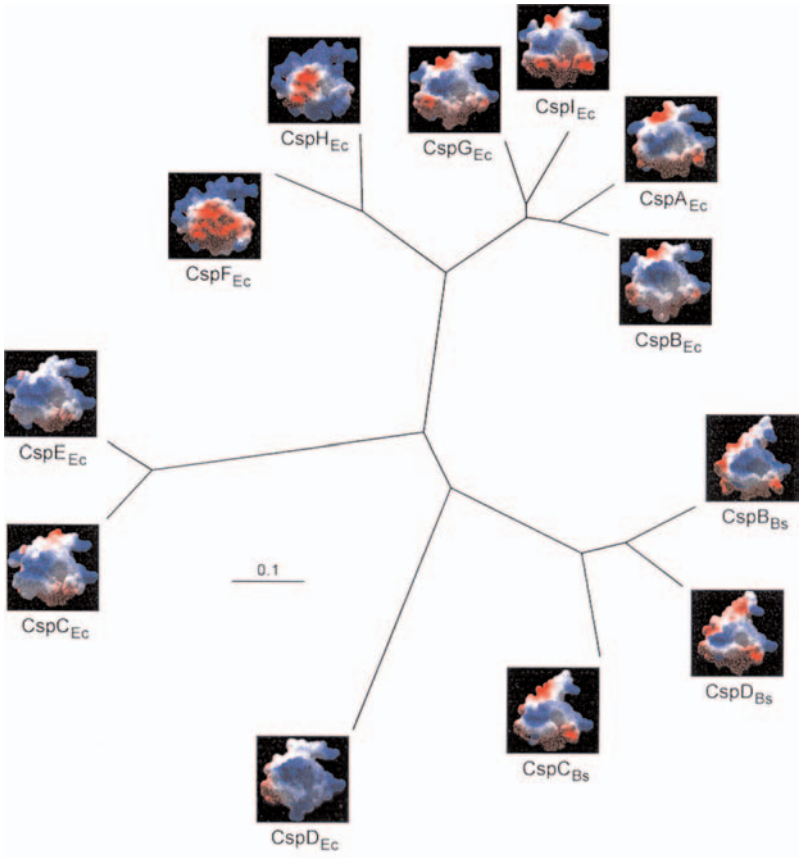

Fig. 4 *The cold shock protein families (CSPs) of* B. subtilis *and* E. coli. *The presented phylogenetic tree includes all CSP family members identified in* B. subtilis *(Bs) and* E. coli *(Ec) and was essentially constructed as described[21] except that in this case the neighbor-joining method[293] was applied. Using the computational molecular modelling results extracted from CSDBase[147], for each CSP a surface charge distribution model was generated as detailed[21]. Note that $CspB_{Bs}$ and $CspA_{Ec}$ are represented by the experimental crystal structure coordinates deposited in PDB database under accession # 1CSP[151] and 1MJC[161], respectively. The dimer formation reported for $CspB_{Bs}$[151,21] and $CspD_{Ec}$[176] was not considered.*

ing the expression of the stationary growth phase sigma factor RpoS and the universal stress protein UspA[194]. Additionally, overproduction of CspE leads to resistance against camphor treatment indicating a role in promoting or protecting chromosome folding[195]. In striking contrast to this, the homodimeric, nucleoid-localizing[196] CspD has been shown to inhibit replication[176] and it seems that (p)ppGpp is

one of the positive factors responsible for the regulation of *cspD* expression[191].

Although no attempt has yet been made to completely remove all nine CSP-encoding genes present in *E. coli*, combined deletion of four of them results in severe physiological defects[193]. Similar to *B. subtilis* in which a *cspB/cspC/cspD* triple deletion mutation is lethal even under optimal growth conditions[13], a unique *cspA*-like gene copy appears to be essential for survival of the psychrotroph *Arthrobacter globiformis*[14]. This suggests a more general role of CSPs than their sole implication in cold acclimatization processes. In fact, analysis of a *B. subtilis cspB/cspC* double deletion mutant revealed that removal of these two *csp* genes results in: (i) pleiotropic alteration of protein synthesis; (ii) cell lysis during entry into stationary growth phase; (iii) alteration of nucleoid structure; and (iv) the inability to differentiate to endospores[13,99,197]. Consequently, although the three *B. subtilis* CSPs genes can functionally complement each other to allow for survival under optimal growth conditions, inactivation of only one of them readily results in detectable physiological perturbations[30,186] while the deletion of two *csp* genes severely affects proper function of a multitude of fundamental cellular processes. This is consistent with a hierarchy of importance in which CspB appears to dominate followed by either CspD or CspC depending on the growth conditions examined[13]. In addition to induction upon cold shock, CspB and CspC have also been reported to represent major stationary phase-induced proteins[198]. Therefore, lysis of the corresponding double mutant under these conditions highlights once again important functions of CSPs independent of cold shock. Interestingly, among the processes which cold shock and stationary phase have in common at least in *E. coli* is rapid inactivation of ribosomal function[115,199] which in both *E. coli* and *B. subtilis* is discussed as one of the possible signals triggering CSP induction[13,88,200]. Only recently, on the basis of the fact that CSPs and IF1 share a highly similar fold, heterologous expression of translation initiation factor IF1 from *E. coli* in a *B. subtilis cspB/cspC* double deletion strain was shown to cure both the growth and sporulation defects observed for this mutant, suggesting that IF1 and CSPs have at least in part overlapping cellular function(s)[197]. Since the initiation step in bacterial protein biosynthesis requires translation initiation factor IF1 which binds directly to the 30S subunit of the ribosome as demonstrated recently by co-crystallization experiments[201], these data might suggest a potential connection between CSPs and the initiation of translation which is believed to represent the bottle-neck of bacterial adaptation during cold shock[112–116]. Interestingly, for *B. subtilis* as well as for

B. stearothermophilus, expression of an IF1 protein homolog has not yet been reported[202]. It is therefore tempting to speculate whether CSPs in Gram-positive bacteria might act as general or at least alternative translation initiation factors under certain stress conditions. In fact, *in vivo* localization studies using immunofluorescence techniques and a fusion of GFP to the C-terminus of CspB revealed that CSPs co-localize with ribosomes and that this localization depends on active transcription in *B. subtilis*[99,203]. This is in line with the observations reported for *Streptomyces aureofaciens* in which CSPs were co-purified with ribosomes[42]. However, since the resolution of fluorescence microscopy is rather low and the reported co-purification results might also be explained by interaction of CSPs with ribosome-bound mRNA rather than by direct CSP:ribosome interaction, at this stage alternative explanations should be considered.

In an early work, apart from its evident binding to ribosomal decoding site during the translation initiation process, IF1 was shown to alter the structure of various oligonucleotides in terms of disrupting nucleic acid interactions *in vitro*[204]. These data may be interpreted as a putative chaperone activity as shown *in vitro* for CspA from *E. coli*[178] and as suggested for *B. subtilis* CSPs[13]. Furthermore, crosslinking studies have shown that IF1 binds to mRNA *in vivo*, although it is unclear whether this interaction occurs during the translation initiation step or reflects actvities of non-ribosomal associated IF1 comparable to RNA chaperones[205,206]. In conclusion, the data presented above can be interpreted in accordance with a previously proposed RNA chaperone model for CSP function[13], or alternatively, give rise to a novel hypothesis of CSPs acting as translation initiation-like factors in *B. subtilis* and possibly other bacteria as well[197]. In this context, it is important to note that certain *E. coli* CSPs could also be functionally replaced by over-expression of a protein fragment representing the S1 domain of PNPase[193] which together with IF1 and CSPs belongs to the OB-fold protein family[207,208] but in contrast to IF1 is not involved in translation. Therefore, on the basis of the fact that after cold shock exclusively CSPs are produced under conditions that totally block protein synthesis (100 µg/mL kanamycin or 200 µg/mL chloramphenicol)[200], a two-step model could be imagined where cold-inactivated ribosomes first exclusively synthesize CSPs (*e.g.* due to intrinsic properties of their mRNAs) which then either alter ribosome conformation to enable synthesis of additional CIPs such as CsdA and RbfA that finally allow for resumption of synthesis of bulk protein[21] or act as transcriptional antiterminators to up-regulate expression of CIP-encoding genes at the transcriptional level[192,209,210], or both. In either

of the two cases, a similar overall result would be achieved, albeit by fundamentally different mechanisms.

Modulation of the translation apparatus, RNA folding, and coupling of transcription to translation

While studies of psychrotrophic, mesophilic, and thermophilic microorganisms suggest that crossing the lower temperature limit for growth creates a situation where an organism is no longer able to import the growth rate-limiting nutrient due to loss of affinity for that substrate[211,212], other investigations support the idea that cold-dependent cessation of bacterial growth is rather linked to an inhibition of protein synthesis[112–114]. Of course, it could simply be argued that the inhibition of protein synthesis itself might result from running out of nutrients, however, it has been demonstrated for several bacterial species, in particular Gram-negatives, that even shifts to temperatures well above the minimal growth temperature lead to growth arrest – albeit only transiently[22,23]. Importantly, comparison of the results reported for analyses of the ribosomal fraction of *E. coli* cells shifted from 37°C to temperatures below (5°C)[114] or above (15°C)[115] the minimal growth temperature (approx. 8°C) reveals that both ribosome profiles are similarly characterized by a severe reduction in polysomes with a concomitant increase in monosomes and 70S, 50S, and 30S particles (Figure 5). These data suggest that the initiation step of translation is impaired after cold shock and that re-activation of the translation machinery might represent a bottle neck during cold adpatation[112–116]. In fact, numerous studies employing two-dimensional gel electrophoresis have shown that a dominating fraction of those proteins induced after cold exposure comprises ribosomal proteins, translation factors, or components which specifically associate with the ribosome in order to facilitate its function and/or biogenesis at low temperature[22,30,213,214,115].

It seems reasonable to assume that a cold-induced block in translation initiation may be attributed to intrinsic properties of both the ribosome (including its auxiliary components) and the mRNA pool even though only few experimental data are available on temperature-dependent kinetics and thermodynamics of possible structural re-arrangements that affect the ribosome, the translation process as a whole, and/or RNA folding. Since according to Arrhenius the transcription rate is expected to be reduced at low temperature, more time would be available for the establishment of mRNA secondary structures within the 5′UTR before the ribosomal binding site (rbs) becomes accessible for ribosome binding. Therefore, at 15°C mRNAs containing a long 5′UTR might form additional/different and/or

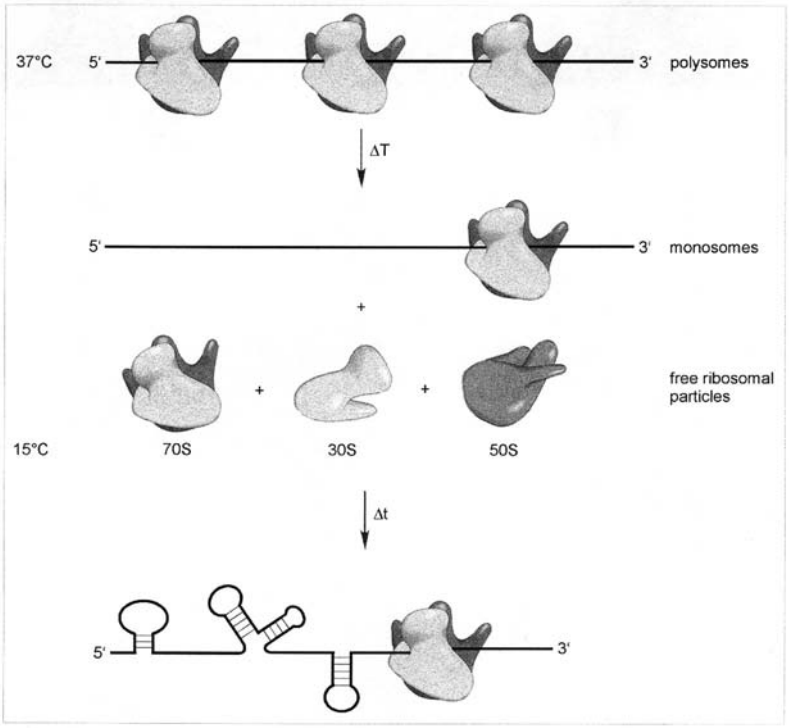

Fig. 5 *Temperature-dependent changes in the cellular ribosome profile. Upon cold shock (ΔT<0), the number of polysomes existing at optimal temperature (upper panel) decreases in favor of increasing amounts of monosomes, 70S particles, and free ribosomal 30S and 50S subunits[114,115] (central panel). This effect has been suggested to result from a cold-induced block in translation initiation[113–115] and is likely to lead to decoupling transcription from translation. As a consequence, additional mRNA structuring may occur (lower panel) which would further complicate protein biosynthesis at low temperature.*

more stable secondary structures compared to 37°C that could impair or entirely prevent the ribosome from recognizing the mRNA rbs as a substrate (Figure 6A). In a similar manner, lowered translation rates may lead to the formation of secondary structures inbetween ribosomes translating the same mRNA thereby affecting translation elongation (Figure 6B). Finally, even the 3′UTR can contain elements that might selectively interfere with proper termination of translation in the cold, for example by masking the stop codon (Figure 6C). Although rarely considered, a similar effect would be expected even in the absence of a structured 3′UTR provided that low temperature impairs the processes of peptide release and recycling of the 70S

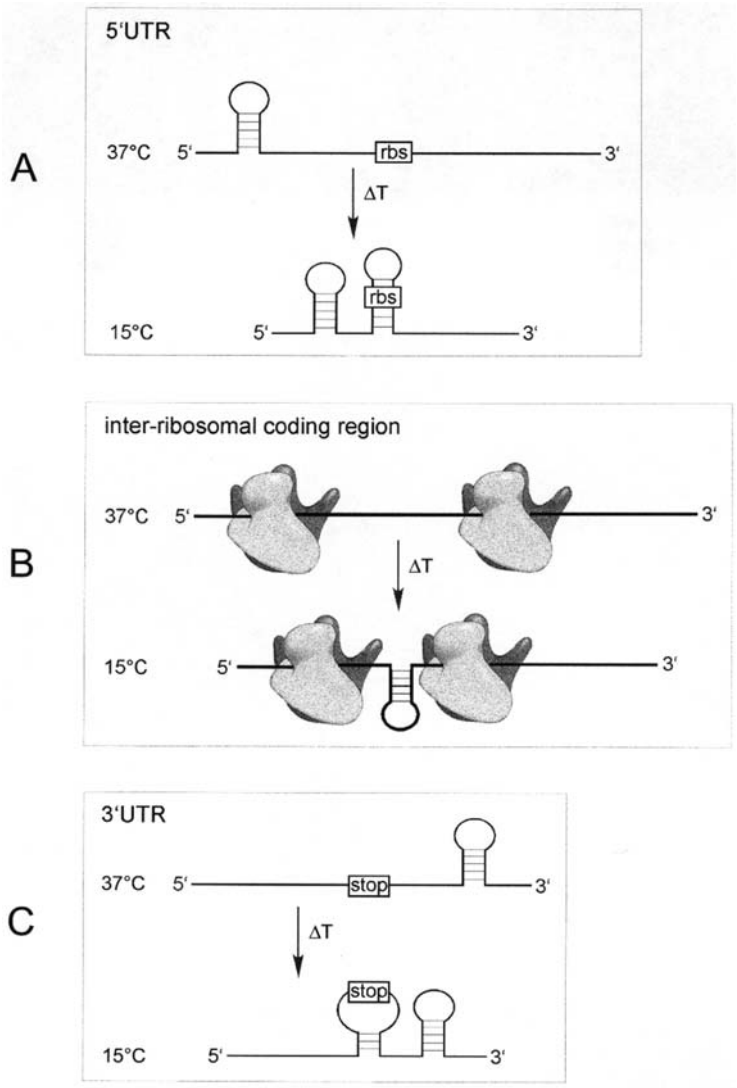

Fig. 6 *Temperature-dependent alteration of mRNA structures affecting ribosomal protein synthesis. (A) Impairment of translation initiation by establishment of mRNA secondary structures involving the 5′ untranslated region (5′UTR) that mask the ribosomal binding site (rbs). (B) Formation of inter-ribosomal mRNA secondary structures interfering with efficient elongation. (C) Perturbation of proper translation termination caused by mRNA secondary structures masking the stop codon. Note that any trapping of translating ribosomes would be accompanied by a reduced availability of free 30S subunits which are required for translation initiation. Consequently, impaired elongation and termination processes may in turn affect translation initiation.*

ribosome which is mediated by the peptide chain release factors and ribosome recycling factor, respectively[215]. However, apart from the fact that the *B. subtilis* mRNA encoding the peptide chain release factor 2, *prfB*, is slightly induced upon cold shock while that coding for the ribosome recycling factor, *frr*, is repressed[64], so far no further experimental data appear to be available concerning this subject. In conclusion, any event which causes trapping of the ribosome during translation elongation or termination may as well affect translation initiation *in vivo*, since under such conditions the amount of initiation-required free 30S subunits would be reduced. In addition to the possible alterations of mRNA secondary structures and a reduction of the available 30S subunit concentration due to a yet hypothetical impairment of 70S ribosome recycling, at least two other major events may cause a cold-dependent perturbation of translation initiation. These are structural re-arrangements of the readily assembled ribosome (including its auxiliary components) and/or kinetic trapping of an unfavourable intermediate that occurs during standard biogenesis of the translation apparatus.

How are these multiple problems affecting translation at low temperature coped with? Firstly, to counteract the deleterious influence of cold-induced mRNA structuring, bacteria employ two general mechanisms that are designed to either prevent mRNA secondary structure formation and/or to actively resolve those structures that have already established. While the former mechanism is thought to base on a cooperative binding of CSPs to nascent mRNA transcripts[13], the latter involves cold-induced RNA helicases that have been identified in *E. coli*[214] and the cyanobacterium *Anabaena* PCC7120[216–218]. Importantly, RNA helicases not only remove RNA secondary structures but simultaneously protect mRNA from RNase-mediated degradation, in particular under conditions where transcription and translation is uncoupled as a result of inefficient translation initation[219]. Secondly, the observed increase in synthesis of proteins constituting the standard translation apparatus[22,30,214,115] might generally be interpreted in terms of: (i) replacement of cold-critical ribosomal components; (ii) specific requirement for non-ribosomal functions of individual ribosomal proteins; and/or (iii) *de novo* assembly of a newly synthesized ribosome fraction. Supporting the latter notion as well as the concept detailed above in which a significant fraction of the CSR might represent an inverted stringent response, *S. meliloti* has been reported to transiently induce rRNA operon expression upon cold shock[220,221]. Interestingly, the temporal pattern and magnitude of cold-induced *rrnA* accumulation as well as some structural features of the three 16S rRNA promotors *rrnA*,

rrnB, and *rrnC* (AT-rich UP element[222], display striking similarity to an operon encoding an *E. coli* CspA homolog and ribosomal protein S21 that has been identified only recently in this organism[223]. Finally, apart from the standard ribosomal components, a number of additional ribosome-interacting factors such as CsdA[214] and RbfA[213,115] have been discovered in *E. coli* that are cold-induced. While CsdA (formerly DeaD) represents a homolog of the DEAD-box RNA helicase protein family that appears to associate with 30S, 50S, and 70S ribosome particles[214] and, like ribosomal protein S1[224], has been shown to destabilize double-stranded RNA in an ATP-independent manner *in vitro*[214], RbfA was originally identified as a multicopy suppressor of a cold-sensitive 16S rRNA mutation and binds exclusively to free ribosomal 30S subunits, but not 70S particles or polysomes[213]. Inactivation of the corresponding genes results in severe perturbation of cellular physiology at low temperature which is characterized by growth defects, formation of filamentous cells, impaired resumption of bulk protein synthesis at the end of the cold acclimatization phase, and constitutive expression of CIPs[115,213,214]. Importantly, the phenotype of the original cold-sensitive 16S rRNA mutation is strikingly similar to that of the *rbfA* mutant[115,213], whereas RbfA over-expression allows for significantly quicker adaptation to cold compared to the wild type[115]. Since cold-shocked *E. coli* cells lacking RbfA constitutively express CIPs and appear to be trapped in the process of trying to adapt the ribosome to low temperature, it has been suggested that RbfA plays an important role during ribosome biogenesis and/or translation initiation, particularly in the cold[213,115]. In this context, it is interesting to note that CsdA was isolated as a high-copy suppressor of a temperature sensitive mutant in the *rpsB* gene encoding ribosomal protein S2[225]. At the non-permissive temperature, this mutant is unable to recruit ribosomal proteins S2 and, as a consequence, S1 (essential in *E. coli*) to the translation apparatus, however, overproduction of CsdA restores both proteins on the ribosome[226]. Therefore, in addition to its suggested role in destabilizing mRNA secondary structures as a ribosome-bound RNA helicase[214] and protecting mRNAs from degradation[219], CsdA like RbfA is most likely involved in the biogenesis of ribosomes[226] as has been reported for other homologs of this protein family[227,228]. It should be noted that, compared to optimal growth temperature, cold-exposed *B. subtilis* cells contain significantly increased amounts of mRNAs encoding homologs of both CsdA and RbfA[64,229] which suggests that the concept presented above is not necessarily limited to *E. coli*.

Taken together, upon cold shock in several bacterial species protein synthesis appears to be severely compromised at the level of

translation initiation. Consequently, in addition to the expression of proteins designed to combat excessive mRNA structuring such as CSPs and RNA helicases, bacteria initiate *de novo* synthesis of ribosomes and specialized proteins that assist during biogenesis of newly assembled ribosomal subunits probably in order to overcome unfavourable cold-specific intermediates or represent auxiliary factors required to re-activate non-functional ribosomes. Importantly, mRNAs encoding early induced CIPs and CAPs are either capable of effectively bypassing the requirement for cold-adapted ribosomes by a yet unknown mechanism or are preferentially translated by a non-compromised ribosomal subfraction for which at present no evidence exists. Strikingly, in support of the former notion, synthesis of *E. coli* CSPs is highly efficient even in the presence of antibiotics that virtually entirely block bulk protein production[200]. At present, exploring the molecular basis of how cold- or antibiotic-inactivated ribosomes are still able to specifically select and translate these mRNAs is one of the most exciting tasks in this research field. It has been suggested that *B. subtilis csp* mRNAs might utilize a conserved element located near the 5′ end of their mRNAs which is complementary to an at least partly single-stranded region in 16S rRNA as an additional ribosomal interaction site[21]. This would enable the small ribosomal subunit to preferentially select *csp* transcripts from the bulk mRNA pool and/or might as well result in a conformational alteration of the 30S subunit to facilitate initiation of translation of these mRNAs. However, to our knowledge, no data have yet been reported that could support or disprove this idea experimentally.

Interestingly, so far cold-dependent inactivation of ribosomal function has always been assumed to passively result from kinetic and/or thermodynamic constraints caused by temperature reduction. However, it cannot be excluded that this blocking of the translation apparatus might be part of a protective program that is actively initiated by the cell. Only recently, a cold-induced protein has been identified in *E. coli* that binds to the ribosomal 30S subunit and to 70S ribosomes and is located at the interface between the 30S and 50S subunits in 70S ribosomes[230,231]. This protein, termed pY (encoded by *yfiA* and also known as YfiA, protein Y, or RaiA), stabilizes 70S ribosomes against dissociation especially under conditions of low magnesium concentration[230] and specifically inhibits translation by blocking the binding of aminoacyl-tRNA to the ribosomal A site[231]. Importantly, while pY is present throughout the cold acclimatization phase during which *E. coli* is subject to growth arrest, disappearance of this protein at the transition to recovery phase is paralleled by growth resumption[231]. Only recently, the structures of pY and that of

the corresponding *H. influenzae* homolog HI0257 were solved by NMR analyses[232,233] and revealed a two domain organization consisting of an N-terminal dsRBD-like fold and a highly flexible C-terminus. Except for the fact that pY is induced upon cold shock and during stationary growth[231], so far no further details are known about the regulation of its expression or a possible phenotype of an appropriately constructed deletion strain. However, it cannot be excluded that the unstructured C-terminus represents an interaction site[233] which adopts a defined structure only upon binding of a putatively regulatory molecule. Although it might be a coincidence, in this context we recognized that all completely sequenced bacterial genomes that were reported to lack a pY homolog[230] also do not contain ORFs encoding CSPs. Since *E. coli* CSPs like pY are downregulated at the time of growth resumption, it is tempting to ask whether these proteins are regulatory and/or functionally correlated or might even physically interact *in vivo*. It should be recalled that in ribosomal fractions prepared from *S. aureofaciens* CSPs could be detected[42] while *in vivo* localization experiments in *B. subtilis* revealed that CSPs and ribosomes appear to co-localize[203,99]. Anyhow, it is worth to emphasize the fact that, in contrast to IF3 which rather prevents the association of 30S and 50S ribosomal subunits, pY stabilizes 70S ribosomes. This is in agreement with the observed increase in 70S particle concentration after cold shock although such accumulation of 70S ribosomes could also result from reduced amounts or activity of ribosome recycling factor[215] which, at least in *B. subtilis*, is cold-repressed in terms of a lowered *frr* mRNA abundance[64].

Interestingly, 70S ribosomes can efficiently initiate translation of so-called leaderless mRNAs but barely that of canonical mRNAs[234]. Leaderless mRNAs are present in all kingdoms of life and represent protein-encoding transcripts that harbor a 5′ proximal start codon and therefore lack any 5′UTR including a SD[235]. While at optimal growth temperature, translational efficiency of canonical mRNAs by far outperforms that of their leaderless counterparts, at low temperature leaderless mRNAs were found to be translated comparatively better[236]. In addition to this inverse correlation between temperature and translational efficiency characteristic for these two mRNA classes, leaderless mRNAs can bypass the inhibitory effect of antibiotics affecting translation initiation such as kasugamycin[237]. This is a feature these transcripts have in common with those encoding CSPs[200]. Finally, it has been shown that the ratio of IF2 to IF3 and the availability of a functional ribosomal protein S1 determines the efficiency by which ribosomes can translate leaderless mRNAs[238,239]. In

presence of elevated levels of IF2 such as evident after cold shock in *E. coli*[22] and under conditions where the activity of IF3 and/or S1 is reduced, translation of leaderless mRNAs is enhanced[240]. Importantly, in addition to the fact that Gram-positive bacteria such as *B. subtilis* usually contain more ORFs encoding leaderless mRNAs than Gram-negatives do[235], these species typically lack a functional full-sized ribosomal protein S1 and although *B. subtilis* IF2 and IF1 have not yet been resolved on two-dimensional gels due to their basic pI, the corresponding mRNAs *infA* and *infB* are present at elevated levels after cold shock, while that of *infC* is induced to a significantly lower extent[64,229]. Therefore, since leaderless and canonical mRNAs co-exist in the same cell, environmental conditions such as cold shock might differentially modulate the translational efficiency of the two classes of transcripts. Hence, on the basis of the fact that genes encoding leaderless mRNAs are usually not identified during standard computational genome annotations, it seems possible that under such ideal conditions, translation of yet unrecognized genes encoding leaderless mRNAs might play an important role in adaptation to low temperature that awaits to be further explored experimentally.

Metabolism – insights from the first global gene expression profiles

Surprisingly, although bacterial cold shock responses have been under close examination for more than 15 years now, only little information has been accumulated concerning the impact of cold stress on cellular metabolism. To our knowledge, neither detailed temperature-dependent measurements of the cellular ATP/ADP ratio as a simplified term for cellular energy load nor determinations of GTP levels have yet been reported. Both values might serve well as metabolic signals for gene regulation and modulation of protein activities as *e.g.* discussed for gyrase. It is clear that lowered diffusion rates, conformationally altered cellular structures, and significantly reduced specific substrate affinities as for example evident for utilization of nitrogen sources in a broad variety of psychrotolerant, mesophilic, and thermotolerant bacteria[212] are likely to create conditions under which the complex metabolic network might be perturbed and growth is impaired. Therefore, irrespective of the organism, it may be expected that expression of a number of genes encoding metabolically important proteins requires significant re-adjustment in the cold. However, under optimal growth conditions, bacteria are programmed for rapid cell division and hence contain the appropriate amounts of enzymes, nutrients and metabolites required to fulfill this task. Consequently, a sudden drop in temperature which, according to the Arrhenius

equation, quickly delays global reaction rates could, at least initially, create a situation of metabolite excess. Despite the few experimental data available and in agreement with this notion, cold-shocked *E. coli* cells contain reduced amounts of (p)ppGpp[93,94] which appears indicative of an excess of amino acid charged tRNAs over uncharged tRNA pools. With respect to this idea and the fact that the dramatically decreased growth rates observed in the cold are tightly connected to correspondingly reduced requirements for metabolite turnover and hence substrate uptake, we would expect a global downregulation of metabolically relevant enzymes that is accompanied by selective upregulation of those individual proteins which constitute regulatory and functionally critical checkpoints. Indeed, two recently published DNA array studies presenting global gene expression profiles of two different cold-shocked *B. subtilis* strains[64,229] in combination with several earlier proteomic approaches[29–31] appear to support this concept. While mRNA levels of many enzymes involved in metabolic processes such as glycolysis, citric acid cycle, and the biosynthesis of amino acids, nucleotides, and cofactors are reduced, mRNAs encoding proteins required to restore membrane fluidity, transport, lipid metabolism, and the function of the translation machinery are present at elevated amounts[64,229].

Interestingly, our comparison of the results reported for *B. subtilis* strains Marburg 168 and JH642 revealed two significant differences concerning the cold adaptation of the cytoplasmic membrane. While *B. subtilis* Marburg 168 contains an increased mRNA level of the *bkd* operon[229] which encodes enzymes involved in funneling isoleucine into the lipid biosynthesis pathway, in strain JH642 abundance of this mRNA is reduced after cold shock[64]. This matches previous data according to which cold-adaptation of the *B. subtilis* JH642 membrane by increasing synthesis of anteiso-branched fatty acids is impaired in the absence of exogenous isoleucine sources[127,129] and suggests that the suspected defect in JH642 isoleucine metabolism at low temperature could be attributed to downregulation of the *bkd* operon that can be cured by simply increasing the substrate concentration for the enzymes encoded by this locus. Moreover, in contrast to *B. subtilis* Marburg 168[229], cold-exposed strain JH642 contains increased levels of the mRNA encoded by the *desKR* operon[64] whose products constitute the two-component system essential for expression of the FA-D Des[58,132]. Hence, upregulation of the membrane-associated cold perception system in order to amplify and/or accelerate the incoming signal required for desaturase activation might be interpreted as an attempt to compensate for the reduced capacity to incorporate isoleucine into anteiso-branched FAs. Regarding the fact that DNA

array analyses do not generally allow to distinguish between transcriptional induction or post-transcriptional stabilization of mRNAs, detailed comparative analyses of the differences in primary structure and regulation of the *bkd* and *desKR* operons in both strains might offer a unique opportunity to gain new insights into mechanisms for control of mRNA abundance after cold shock.

Cryoprotection

Apart from the production of antifreeze proteins (AFPs)[5] or ice-nucleation agents (INAs)[6], after cold shock several bacterial species accumulate low molecular weight compounds known as compatible solutes. These substances comprise polyols, polyamines, sugars, amino acid derivatives, *etc*. and have been shown to protect cells of many different types from damage caused by exposure to salt, cold, heat, and free radicals. Although their precise mode of action is still unclear, it is thought that they function as "chemical chaperones" by stabilizing the native state of proteins, preventing their denaturing/aggregation or by acting as radical scavengers. It seems reasonable to assume that increased concentrations of compatible solutes lead to increased viscosity, reduced diffusion rates, and a lowered freezing point of the cytoplasm and might therefore prove beneficial to the cell at low temperatures. In *E. coli*, a temperature downshift from 37 to 16°C leads to the accumulation of the disaccharide trehalose which is synthesized by the two protein products of the *otsAB* operon whose mRNA is present at elevated levels following cold shock[241]. Interestingly, *otsA* null mutants show no apparent growth defects at 16°C, however, upon exposure to 4°C, viability of these cells is significantly reduced compared to the wild type and can be restored upon *otsAB* expression *in trans*[241]. Thus, the cold-induced synthesis of trehalose appears to be a protective response that occurs in anticipation of a further fall in temperature which constitutes a more severe stress to the cell. In addition to *de novo* biosynthesis of cryoprotectants, some bacteria such as the human pathogen *L. monocytogenes* accumulate glycine betaine, carnitine, and other substances by transporting them across the cell membrane(s) into the cytoplasm[242–244]. Since similar transport systems exist in many other bacteria it seems likely that this strategy represents a common mechanism in bacterial cold shock responses.

In addition to the natural cryoprotection systems bacteria have evolved over time, the fundamental research results summarized in this work point to a number of applications that might prove useful to enhance or reduce survival rates of fermentative or pathogenic bacteria in the cold, respectively. As an example, the efficiency of

yoghurt production might be significantly improved if *L. lactis* strains could be used that have been genetically engineered to contain inducible components for cold-adaptation of the cell membrane and/or the translation machinery prior to freezing of the starter cultures required for this industrial process. On the other hand, avoiding the presence of known cryoprotective substances in combination with appropriate chilling procedures during food preparation can reduce the probability of pathogen survival.

Protein folding and protein chaperones

Correct *de novo* folding and a well defined final conformation is essential for proper protein function *in vivo*. After temperature upshift, increased molecular motion may allow peptide chains to misfold by crossing energy barriers that, under optimal temperature conditions, would guide the protein folding process as well as the final protein conformation by restricting spacial arrangement of amino acid residues to a limited number of possibilities. That this physical principle imposes a major problem on cellular physiology manifests in the fact that the HSR is mainly designed to cope with heat-induced protein damage. After cold shock, the situation is similar with respect to possible temperature-dependent alterations of protein folding pathways and final conformations but completely different as far as the direction of the temperature effect is concerned. Under cold shock conditions even standard energy barriers that have to be overcome during protein folding may not be crossed anymore such that affected proteins require assistance of chaperones. Moreover, some cellular functions might be impaired due to a reduced conformational flexibility of the enzymes involved. As a consequence, one would expect an increased requirement for protein folding catalysts at low temperatures.

Indeed, in *B. subtilis* it has been shown that peptidyl-prolyl isomerases such as PPiB as well as the presumably ribosome-associated trigger factor are cold shock-induced or cold-accumulated proteins, respectively[30,245]. This is in line with observations reported for *E. coli* according to which a mutant in cold-induced trigger factor shows a cold sensitive phenotyp[246]. These ubiquitous enzymes catalyze the *cis/trans* isomerization of peptide bonds N-terminal to proline residues[247], a reaction that often represents a rate-limiting step in protein folding, especially at low temperatures[248]. Moreover, trigger factor is involved in protein secretion[249] which appears to play an important role during cold shock adaptation (see section below).

Another example in *E. coli* is Hsc66 that has been identified as a cold-inducible HSP70 homolog sharing 62% similarity and 42%

identity with endogenous DnaK[250]. The corresponding gene *hscA* is co-transcribed with the *dnaJ*-like upstream gene *hscB* that encodes the HSP40 homolog Hsc20[250]. Both genes are cold-inducible[250] and locate to a cluster of genes (*iscSUA-hscBA-fd*x) whose products either contain iron sulfur clusters such as ferredoxin (*fdx*; presumably cold-induced since co-transcribed with *hscA*) or are involved in their synthesis as has been shown for the NifS and NifU homologs IscS and IscU, respectively[251]. Only recently it has been discovered that the Hsc66/Hsc20 system specifically interacts with the highly conserved IscU protein and appears to represent a specialized chaperone machinery that participates in the biogenesis of iron-sulfur proteins[252].

It is interesting to note that in most bacteria expression of the molecular chaperones induced after heat shock such as *e.g*. the GroEL/GroES machinery is not only generally suppressed immediately after cold shock[30] but, at least in *E. coli*, reduces cell viability at low temperatures when artificially enhanced[246]. One exception are cyanobacteria in which some of the typical HSPs such as GroEL, HtpG (a HSP90 homolog), and members of the Clp protein family are cold-induced and seem to play an important role during cold adaptation[253–255]. Interestingly, in *Synechococcus* sp. PCC7942 the presence of the *htpG* gene is required for low temperature accumulation of a high molecular weight protein complex that comprises GroEL and DnaK[255]. Inactivation of *htpG* resulted in inhibition of both cell growth and photosynthetic activity when the mutant was shifted to low temperature. This latter observation is similar to what has been reported for *Synechococcus* sp. PCC7942 strains carrying a mutation in either *clpB* or *clpP1*[253,254]. Therefore, it is possible that the exceptional requirement for HSPs during cold adaptation of cyanobacteria might primarily be related to the maintenance of their photosynthesis machinery. However, taking into account the time scale after which cold accumulation of HSPs in cyanobacteria has been reported and the fact that most other CSR studies have so far focused on short term processes, it remains to be elucidated whether some of the HSP chaperones might play a cold-relevant role at a later stage during cold adaptation in other bacterial species as well. In this context it is interesting to note that increased mRNA levels of *clpB*, *clpP*, and *groEL* have been detected during a recent analysis of the long term cold adapation in *L. monocytogenes*[256].

Protein transport

One important aspect often overlooked in studies related to bacterial cold adaptation is the fact that presumably all bacterial species transport a considerable number of functionally important proteins from

the cytosol across the cytoplasmic membrane to specific outer locations which depend on the structural features of the cellular envelope present in the bacterium examined (Gram-negatives: cytoplasmic membrane, periplasm-embedded thin cell wall, outer membrane, LPS; Gram-positives: cytoplasmic membrane, thick cell wall, no periplasm). In growing cells these polypeptides comprise: (i) lipoproteins that remain attached to either the cytoplasmic or the outer membrane by means of a lipid anchor; (ii) freely diffusing periplasmic proteins; (iii) cell wall-associated proteins; (iv) outer membrane proteins; and (v) proteins secreted into the extracellular medium whereas during cell differentiation such as spore formation in *B. subtilis*, additional destinations may be addressed. On the basis of known bacterial genomes, the lipoprotein fraction alone has been predicted to represent about 1–3.5% of eubacterial proteomes[257] and it is evident that at least some of these proteins constitute components essential for cell viability[258]. Remarkably, detailed proteomic investigations of *B. subtilis* revealed that the number of actually exported proteins is significantly higher than initially anticipated which further underlines the importance of this protein fraction[259–261] [Weber, M.H.W., Diplomarbeit, 1997]. Upon cold shock, wild type *E. coli* and *B. subtilis* display reduced efficiencies in preprotein processing and translocation compared to optimal growth temperatures and it has been concluded that protein export is an intrinsically cold sensitive process since this effect persists even in cold adapted cells[262,263].

Of the many protein transport systems recognized so far[264], the majority of exported proteins utilizes the general secretory (Sec) pathway[259] that requires a specialized membrane-embedded protein translocation machinery termed translocase to which nascent polypeptides are either cotranslationally or posttranslationally directed via the SRP-SRP receptor route (SRP: Ffh & 4.5S RNA; SRP receptor: FtsY) or via interaction with the SecB molecular chaperone, respectively[260]. In *E. coli*, translocase consists of at least seven proteins and its core components are the integral membrane proteins SecY and SecE and the peripheral membrane protein, SecA. While SecY and SecE interact to form a protein-conducting channel that translocates secretory proteins across the membrane, SecA is an ATPase that binds to the SecYE complex and undergoes cycles of membrane insertion and deinsertion coupled to ATP binding and hydrolysis that are believed to drive the segmental translocation of the preprotein. It has been demonstrated that SecY, SecE, and SecA are necessary and sufficient for translocation, however, optimal activity of this process is achieved only if SecYE associates with either SecG or a heterotrimeric complex formed by another set of integral membrane proteins,

SecD, SecF, and YajC[260]. Interestingly, mutations in almost any of the translocase components result in a cold-sensitive phenotype[262,263] that, in some cases, could be attributed to reduced expression of the respective proteins or manifested merely in conjunction with a defect in lipid synthesis[265]. Moreover, it has been shown that the *B. subtilis lspA* gene encoding the signal peptidase II which is required for the processing of lipoproteins is essential for growth at 15°C[257]. These data indicate that protein transport and preprotein processing plays an important role during cold adaptation. In this context it is interesting to note that a recent study suggests that induction of the ribosome-associated trigger factor appears to retard protein export in *E. coli*[249]. Since the amount of trigger factor is increased in the cold[30,246,245] and an *E. coli* trigger factor mutant shows a cold-sensitive phenotype[246], it seems likely that in addition to its function in protein folding (see section above), trigger factor provides a mechanism to reduce the possibly deleterious insertion of excessive secretory proteins into the membrane during the process of lipid adaptation which is required to restore membrane fluidity at low temperatures. This dual impact of trigger factor on both general protein folding and protein export is representative for the complex functional as well as regulatory interaction of distinct elements within the individual bacterial cold shock response networks for which a number of additional examples exist. For instance, the histone-like protein HBsu in *B. subtilis* has not only been shown to play a role in nucleoid structuring, but is also a component of the SRP[266] that recognizes the ribosome-emerging preprotein signal peptide and targets the respective polypeptide to the Sec translocase[260]. Furthermore, the Sec pathway is not only utilized by the majority of secreted proteins but is also required to recruit a significant fraction of integral membrane proteins into the cytoplasmic membrane[267] which reflects a tight coupling of another two processes, protein export and membrane protein targeting. Hence, it remains to be elucidated whether the cold sensitive phenotypes of mutated Sec components have to be attributed to an impairment of protein secretion alone or simultaneously affect recruitment of proteins into the membrane at low temperatures.

Cell differentiation

Some bacteria such as *Bacillus subtilis* and *Myxococcus xanthus* have the capability to form highly resistant endo- or myxospores, respectively. This complex cellular differentiation process is initiated in response to certain stress conditions such as *e.g.* nutrient deprivation or slow dehydration and requires significant reprogramming of gene expression[268]. On first sight, it appears conceivable that

in some bacterial species this pathway might as well be utilized as a mean to escape from the stress situation imposed on the cell by a sudden drop in ambient temperature. However, the cellular adjustments necessary to successfully complete the differentiation process might be too demanding to be accomplished under conditions where the translation machinery, the membrane integrity, and many other vital aspects of cell function are negatively affected. In fact, only few studies are available that focus on bacterial cell differentiation at low temperatures. A recent investigation revealed that *B. subtilis* cells that have already initiated sporulation at 30°C induce a similar set of proteins when cold shocked as has been described for growing cells[31]. For *B. thuringiensis*, endospore formation is evident in cells that had grown for a prolonged time at 14°C[269] and must therefore be regarded as pre-adapted at the time of sporulation. This result, however, provides important evidence that the general ability to differentiate is not necessarily lost in the cold. Although in *M. xanthus* low temperature alone causes the expression of many developmentally regulated genes, wild-type strains do not sporulate upon cold shock[270]. To our knowledge, there is not a single experimental report available that demonstrates that bacterial cell differentiation is utilized as a response in order to escape from cold stress. Rather it appears that the cold shock response is required and sufficient to cope with a sudden temperature drop. In this context, it is interesting to note that KinA, one of the membrane-bound kinases involved in initiation of sporulation in *B. subtilis*, is inhibited by unsaturated fatty acids *in vitro*[271] that appear to be present in cold-shocked cells of this bacterium[129]. Under optimal temperature conditions, however, combined deletion of the CSP-encoding genes *cspB* and *cspC* or *cspB* and *cspD* results in complete loss of the ability to sporulate which indicates an important role of CSPs in *B. subtilis* cell differentiation[99,197,31]. These data demonstrate that although sporulation does not seem to represent a feasible pathway providing cellular protection during exposure to low temperature, vice-versa cold-induced proteins play significant roles during bacterial development.

Regulation of the cold shock response – first insights

While many functional properties of bacterial CSRs have been analysed down to the protein structure level, much less is known about how the regulatory network controlling cold-dependent gene expression is organized. Only recently, first insights emerged from genome-wide transcriptional profile analyses of cold shocked *B. subtilis*[64,229] and *Synechocystis* sp. PCC6803 cells[57]. Importantly,

apart from providing valuable clues for the identification of additional genes potentially playing a role during cold shock adaptation, these studies further clarified the regulatory significance of the membrane-bound sensor systems DesK and Hik33 in *B. subtilis* and *Synechocystis* sp. PCC6803, respectively, by globally identifying the target genes controlled by these proteins upon cold shock. While Hik33 controls a broader spectrum of genes in *Synechocystis* sp. PCC6803, *B. subtilis* DesK appears to exclusively regulate the Des-dependent membrane adaptation process. Consequently, in both bacteria a majority of cold-regulated genes seems to be controlled by yet uncharacterized mechanisms and since the mRNA abundances measured in the mentioned DNA array experiments cannot reliably be differentiated into cold-induced/repressed transcriptional activity and/or cold-dependent mRNA stabilization/destabilization events, the regulatory information provided is only limited. However, from what has been reported so far, it appears that bacterial cold shock stimulons are organized into several distinct regulatory subsystems which comprise: (i) two-component sensor systems that scan the fluidity of the membrane (Hik33/Hik19/Rer1, DesKR, MCPs)[56,58,65]; (ii) the stringent factors RelA/SpoT which determine the cellular (p)ppGpp level and are controlled by the status of the translation apparatus[25,88]; (iii) DNA gyrase that, probably in concert with histone-like proteins, alters the nucleoid structure thereby modulating promotor recognition by RNA polymerase[106]; (iv) CSPs which act as transcriptional antiterminators[192,209,210] and RNA chaperones[178] and have been shown to globally affect gene expression[13]; (v) PNPase which seems to selectively control the stability of several mRNAs such as those of CSPs[23,272]; (vi) non-coding RNAs like *micF* and *dsrA* that modulate the translation of at least *ompF* and *rpoS* mRNAs encoding *E. coli* outer membrane protein F and stationary phase sigma factor, respectively[144,273]; and finally (vii) the alternative sigma factors RpoS[241] and SigB[274,244] which represent global master regulators serving a number of specialized roles for example during cryoprotection. In order to control the genetic flow of information, these regulatory elements interact/overlap in a complex manner that might be best described using a "multiple filter model" in which RNA polymerase, the cellular RNase pool, and the ribosome each constitute a separate "filter" whose individual output is modulated by the corresponding regulatory subsystems as illustrated in Figure 7. The following section outlines a generalized scenario that, in a strongly simplified manner, might represent a typical cascade of events taking place when bacterial cells are exposed to a sudden drop in temperature.

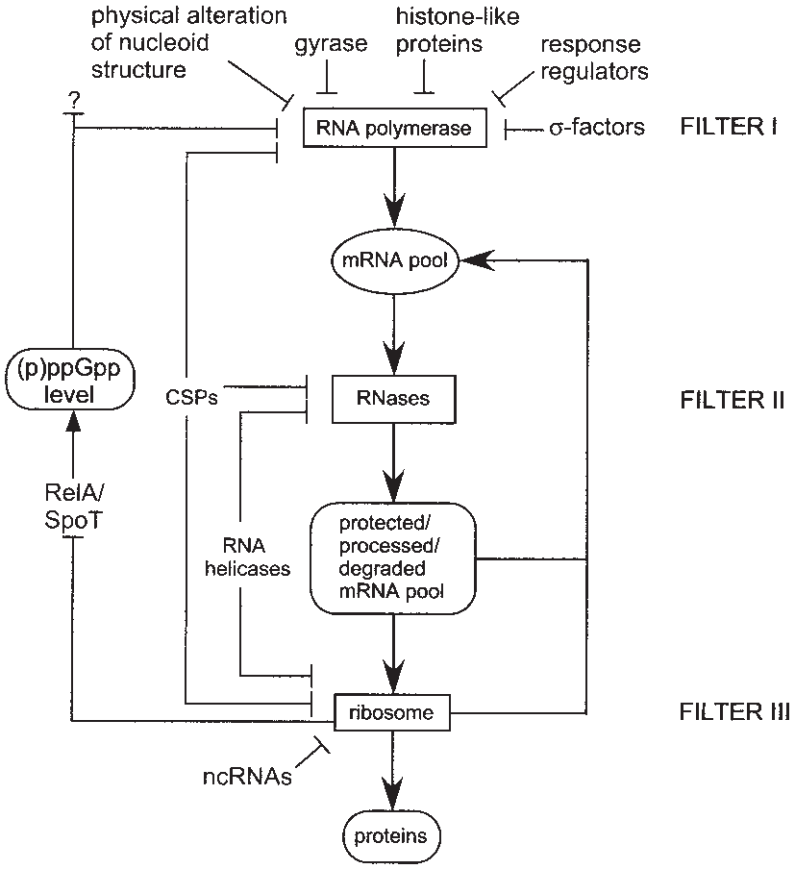

Fig. 7 *Regulation of bacterial cold shock responses – a multiple filter model. Black and grey arrows indicate the flow of genetic information from DNA to protein or the production of effector molecules, respectively, whereas T-arrows have to be read as "modulates activity of". The filter systems are boxed while circled items represent their substrates or products. For further details see text.*

Immediately after cold shock, perturbation of the physical state of bacterial cell membrane(s) triggers a signal cascade that is initiated by membrane-embedded sensor proteins like Hik33 or DesK and finally leads to the transcriptional activation (and/or repression) of specific genes such as *B. subtilis des* whose product is designed to restore the appropriate fluidity required for optimal membrane function. Only shortly delayed, a block of translation initiation due to cold-induced conformational alterations in both mRNAs and ribosomal particles and/or specific induction of ribosome-inactivating pro-

teins as for example *E. coli* pY appears to result in a reduced (p)ppGpp level that, in addition to the signals coming from the membrane, further affects global gene transcription with predominant repression of many metabolically relevant genes and activation of those encoding ribosomal components. Simultaneously, the lowered temperature perturbs the DNA polymer conformation which is likely to modulate transcriptional activity at certain promotors and may locally attract gyrase, possibly in concert with histone-like proteins, to more profoundly modify DNA topology even without an immediate requirement for gyrase *de novo* synthesis. At this stage, as a consequence of the significantly diminished translational activity, the structure of the nucleoid appears to undergo additional compaction probably due to reduced expansion forces resulting from uncoupling transcription and translation of membrane-inserted proteins. Hence, the combined physical effects cold exerts on the status of the membrane, the ribosome, and the nucleoid is effectively converted into specific signals altering the transcriptional output of RNA polymerase. In this context, it is interesting to note that in spite of the presence of a functional *desKR* two-component system, cold-induced transcription of *des* in *B. subtilis* is abolished upon addition of the gyrase inhibitor novobiocin[106]. This observation suggests that, at least in case of *des*, the status of the membrane is compared to the status of the nucleoid before a productive transcriptional activation of the target gene is initiated which further underlines the tight interconnection of individual elements involved in the regulation of bacterial CSRs.

Importantly, as demonstrated for *E. coli* CspA, bacterial CSPs are among the first proteins induced upon cold shock[22] and, together with other CIPs, seem to positively respond to a decrease in the cellular (p)ppGpp level[25] and probable other yet uncharacterized signals resulting from a functional block of the translation machinery[13,88]. Apart from a quantitatively modest transcriptional cold-activation[275–278] and the fact that *cspA* promotor activity is under general growth phase-dependent antagonistic control of the DNA-binding proteins FIS (stimulation) and H-NS (repression) at optimal growth temperature[278], similar to *B. subtilis cspB* and *cspC* mRNAs[279] the *E. coli cspA* transcript is subject to dramatic stabilization at low temperature[280,281]. Comparable stabilizing effects after exposure to cold have been reported for a number of other mRNAs encoding CIPs such as the cyanobacterial DEAD-box RNA helicase CrhC from *Anabaena sp.* PCC7120[217] and *E. coli* PNPase[282]. Although the precise regulatory mechanisms of cold-specific mRNA stabilization and decay processes remain largely unclear, involvement of RNases[23,272], CSPs[178], and RNA helicases[214] has been demonstrated. For example, the DEAD-

box RNA helicase CsdA appears to protect the *cspA* transcript in *E. coli*[278] which is consistent with earlier studies where similar effects have been observed in context with other mRNAs[219]. Such stabilization might be explained by RNA helicase-mediated removal of potential endonuclease recognition elements formed within the *cspA* transcript[278]. Additionally, the evident preferential translation of CIP-encoding mRNAs might generally shield these molecules from rapid RNase attack in the cold. Consequently, in addition to alteration of the RNA polymerase transcriptional output by action of response regulators, adjustment of the (p)ppGpp level, and modification of DNA topology (Figure 7), bacterial CSRs include important post-transcriptional regulatory components involving a combination of protection and degradation of selected mRNAs by CSPs, helicases, and RNases which results in an adaptation phase-dependent modulation of the mRNA pool available for translation. During the initial acclimatization phase of cold adaptation, from this pool the ribosome selects a set of transcripts specialized to bypass the cold-induced block of the translation machinery. It is conceivable that these mRNAs encode mainly proteins designed to restore ribosomal function and regulators designed to increase the transcript pool encoding such proteins. Since the *csp* transcripts *cspA*, *cspB*, and *cspG* are virtually the only *E. coli* mRNAs capable of bypassing a complete block of the translation machinery[200] it seems reasonable to assume that they play a central role during cold adaptation. Indeed, in addition to their proposed activities as RNA chaperones involved in re-establishing the coupling of transcription to translation[13,178], recent investigations revealed that increased amounts of *E. coli* CSPs are capable of upregulating expression of the *metY-rpsO* operon genes *nusA*, *infB*, *rbfA*, and to a lower extent[282] *pnp* by means of transcriptional antitermination[192,209,210]. Strikingly, these genes are similarly organized in *B. subtilis* and encode the cold-induced transcriptional antiterminator protein NusA[22], translation initiation factor IF2[22], ribosome binding factor RbfA[213,115], and the phosphorolytic exonuclease PNPase[283–285], respectively, whose importance during bacterial cold adaptation has been outlined above as far as known. After sufficient enrichment of the mRNA pool with these transcripts and the completion of a number of probably other functions as suggested, CSP expression is downregulated by a rather complex interplay of transcriptional and posttranscriptional regulatory events that is generally subject to controversial discussion[145]. However, it is important to note that the reduction of the cellular *csp* mRNA amount appears to be correlated to the expression of PNPase[23,272] and resumption of growth at the end of the cold acclimatization phase. The relevance of this

finding is conceivable in light of the fact that PNPase null mutants display cold sensitive growth phenotypes in *Y. enterocolitica*[286] and *B. subtilis*[287,288] and suggests that PNPase as part of the degradosome represents one of the master regulators for cold adaptation which further highlights the importance of post-transcriptional regulatory events during bacterial CSRs. Moreover, two non-coding *E. coli* RNAs, *micF*[144] and *dsrA*[273], have been identified to play a role in cold-dependent post-transcriptional regulation of the outer membrane composition and the expression of the two global transcription regulators σ^S and H-NS. Small, stable RNA molecules provide an economical cellular means to achieve global regulation. While cold-dependent downregulation of *micF* leads to enhanced expression of the large outer membrane pore constituted by OmpF due to diminished interference of *micF* with *ompF* translation[144], cold-induction of *dsrA*[289] appears to stimulate translation of *rpoS* and interferes with that of *hns*[273]. Indeed, during the initial hour of the cold shock acclimatization phase the cellular amount of H-NS progressively decreases although its mRNA level rises[101], but after 1.5 hrs of cold exposure H-NS synthesis re-reaches pre-shock levels and is further induced[101]. This is consistent with a CspA-mediated upregulation of *hns* transcription and an initial increase of *dsrA* which would simultaneously repress *hns* translation. However, the observed time-course of H-NS production suggests that at a later stage of cold adaptation, the *dsrA*-dependent translational repression of *hns* is either lost or superimposed by other events that upregulate H-NS production. The most convenient explanation for such an effect would be an excess of *hns* mRNA over *dsrA* due to progressing transcriptional activation of the *hns* promotor as for example mediated by CspA. In parallel to translational repression of *hns*, *dsrA* stimulates translation of RpoS. Although the influence of RpoS expression upon cold shock in *E. coli* appears largely unexplored, it has been shown that this alternative sigma factor is involved in cold-dependent upregulation of the *otsAB* mRNA whose products are required for cryoprotective synthesis of trehalose[241]. In a similar manner, in *L. monocytogenes* SigB participates in the accumulation of cryoprotectants[244,274].

In summary, bacterial cold shock responses involve the typical regulatory elements found in other bacterial stress responses as well, but are differently organized. In contrast to the latter where alternative sigma factors play a central regulatory role especially during initial adaptation stages, cold shock-induced synthesis of RpoS in *E. coli* and increased regulatory activity of SigB in *L. monocytogenes*, although important, rather appear to represent a long-term necessity that is only part of a complex regulatory interplay dominated by the requirement

for adaptation of the translation machinery. Importantly, even though individual bacterial species display subtle differences in their adaptational needs as is conceivably the case for photosynthetic cyanobacteria, large parts of function but also regulation appear to be conserved as for example indicated by the fact that heterologous expression of CspB from *B. subtilis* in *E. coli* results in activation of *hns*[290] and overproduction of the universal stress protein UspA [Weber, M.H.W., Schmid, R. & Marahiel, M.A., unpublished results] which have both been implicated in endogenous CSP-dependent regulation. Most strikingly, however, is the possibility that one of the main *E. coli* gene batteries involved in adaptation of the ribosome, namely the *metY-rpsO* operon appears to be under central control of the CSPs[192,209,210].

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Glossary of terms

ACP:	acyl carrier protein
AFP:	antifreeze protein
CAP:	cold acclimation protein (a protein that is permanently present at a higher cellular amount after shift to low temperature)
CIP:	cold-induced protein (a protein that is transiently induced after cold shock)
Clp:	caseinolytic proteases, a family of bacterial molecular chaperones
CSD:	cold shock domain (a conserved nucleic acid-binding protein fold representing a beta-barrel that consists of five anti-parallel beta-strands which harbor surface-exposed aromatic and basic amino acid residues located in the conserved RNP-1 and RNP-2 motifs on beta strands two and three, respectively)
CSP:	cold shock protein (proteins that belong to the CSD-containing family of *E. coli* CspA-like proteins)

CSR:	cold shock response
EPR:	electron paramagnetic resonance spectroscopy
FA:	fatty acid
FA-D:	fatty acid desaturase
FT-IR:	Fourier transform infrared spectroscopy
GRP:	glycine-rich protein
HBsu:	bacterial histone-like protein encoded by the *hbs* gene in *B. subtilis*
H-NS:	bacterial histone-like protein (encoded by the *hns* gene in *E. coli*)
HSP:	heat shock protein
HSR:	heat shock response
HU:	a heterodimeric bacterial histone-like protein encoded by the *hupA*, *hupB* genes in *E. coli*
IHF:	integration host factor (a heterodimeric bacterial histone-like protein encoded by the *ihfA*, *ihfB* genes in *E. coli*)
INA:	ice-nucleation agent (includes INPs)
INP:	ice-nucleation protein
Lk:	linking number
LPS:	lipopolysaccharide
MCP:	methyl-accepting chemotaxis protein
MW:	molecular weight
NMR:	nuclear magnetic resonance
OB-fold:	oligonucleotide/oligosaccharide binding-fold
ORF:	open reading frame
pI:	isoelectric point
PNPase:	polynucleotide phosphorylase (polyribonucleotide nucleotidyltransferase, EC 2.7.7.8)
(p)ppGpp:	collective term for either guanosine 5′-triphosphate-3’diphosphate or guanosine 5′-diphosphate-3’diphosphate, or both
PUFA:	polyunsaturated fatty acid
rbs:	ribosomal binding site
RelA:	(p)ppGpp synthetase
RNAP:	RNA polymerase
RNase:	ribonuclease
SASP:	small acid-soluble spore protein (encoded by the six *sspA-F* genes in *B. subtilis*)
SD:	Shine-Dalgarno sequence
SFA:	saturated fatty acid
SpoT:	(p)ppGpp hydrolase

SRP:	signal recognition particle (consists of Ffh, scRNA, and HBsu in *B. subtilis* and of Ffh and 4.5S RNA in *E. coli*)
SRP receptor:	signal recognition particle receptor (encoded by the *ftsY* gene in both *E. coli* and *B. subtilis*)
ssDNA:	single-stranded DNA
ΔT:	temperature difference
Tw:	twist
UFA:	unsaturated fatty acid
UTR:	untranslated region
Wr:	writhe

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