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Cold Shock Response in Bacteria

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Abstract

Bacteria often encounter temperature fluctuations in their natural habitats and must adapt to survive. The molecular response of bacteria to sudden temperature upshift or downshift is termed the heat shock response (HSR) or the cold shock response (CSR), respectively. Unlike the HSR, which activates a dedicated transcription factor that predominantly copes with heat-induced protein folding stress, the CSR is mediated by a diverse set of inputs. This review provides a picture of our current understanding of the CSR across bacteria. The fundamental aspects of CSR involved in sensing and adapting to temperature drop, including regulation of membrane fluidity, protein folding, DNA topology, RNA metabolism, and protein translation, are discussed. Special emphasis is placed on recent findings of a CSR circuitry in *Escherichia coli* mediated by cold shock family proteins and RNase R that monitors and modulates messenger RNA structure to facilitate global translation recovery during acclimation.

1. INTRODUCTION

The hallmark of bacteria is their ability to adapt promptly and efficiently to diverse, challenging environments. Accounting for a significant portion of the world's biomass, bacteria are among the most widely distributed organisms on earth. Temperature is a pervasive challenge, requiring bacteria to adapt to both temperature extremes and frequent fluctuations to ensure their survival and growth. For host-associated bacteria such as pathogens and microbiome constituents, temperature changes upon entering and exiting a warm-blooded host are also important signals that often influence virulence. In addition, bacteria growing at refrigerated or even freezing temperatures have become an increasing threat to food safety and human health. Thus, bacterial adaptation to temperature shifts has been the focus of much research, and how bacteria sense and transduce the temperature signal and regulate gene expression upon temperature stresses have been studied extensively.

This review focuses on the bacterial cold shock response (CSR). We first define this response and then use recent findings to discuss two important questions: What structural and functional properties of bacterial cells are affected by cold shock (i.e., the inputs to CSR)? How do different bacteria overcome these challenges to adapt to cold shock (i.e., the outputs of CSR)? For the latter, we focus on several important cellular constituents and discuss the key players involved that promote their adaptation to low temperature, as well as the regulation and functionalities of these players.

CSR: cold shock response; sum of cellular responses required for efficient adaptation to a sudden temperature downshift outside the normal temperature range

Psychrophile: microbe that has a maximum growth temperature at 20°C or below and is usually restricted to permanently cold habitats

Psychrotroph: microbe that can grow at 0°C but prefers higher temperatures, with a maximum growth temperature above 20°C

Mesophile: microbe adapted to moderate temperatures, with optimal growth temperatures ranging from about 20 to 45°C

Thermophile: microbe that thrives at relatively high temperatures, ranging between 41 and 122°C

Hyperthermophile: microbe with optimal growth temperatures above 80°C

2. THE EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

Temperature effects are governed by the Arrhenius equation, a relationship indicating that the logarithm of the velocity of a chemical reaction is linearly correlated with the reciprocal of the absolute temperature (K). A similar correlation between growth rate and temperature is observed within the normal temperature range of a bacterium (61). This law applies to all bacterial species, but their cardinal temperatures (maximum, optimum, and minimum) are species specific, leading to bacteria being classified as psychrophiles, psychrotrophs, mesophiles, thermophiles, and hyperthermophiles. Bacteria tolerate shifts within their normal temperature range with little change in cellular composition, but shifts outside this range induce stress (heat shock and cold shock), with growth first slowing and then ceasing as this range is progressively exceeded. The CSR is defined as the sum of all cellular reactions required for efficient adaptation to a sudden temperature downshift outside the normal temperature range of that bacterium. As bacteria have colonized different natural environments, some necessitating evolutionary adjustments to macromolecules and biological processes, including cold temperature, it is not surprising that the CSR strategies of different categories of bacteria can differ.

3. WHAT ARE THE INPUTS TO THE CSR?

Water is the major constituent of life, and its chemical and physical properties are strongly affected by temperature. Understanding how water properties change with temperature downshift is a starting point for understanding how organisms cope with cold shock. One direct consequence is the general inhibition of biochemical reactions and cellular processes. This is partially due to decreased water ionization, which lowers the concentration of H^+ and OH^- ions. As these ions participate in most biochemical reactions, the change directly affects equilibrium and kinetics of cellular processes. Additionally, cold temperature reduces substrate diffusion rates and increases water viscosity both inside and outside cells, which slows cellular processes

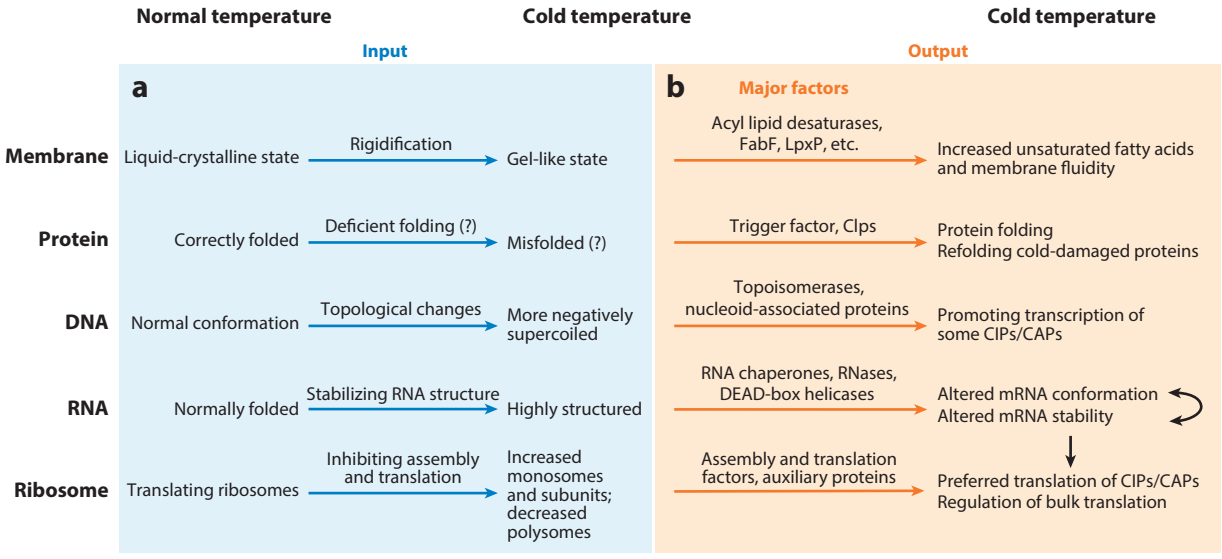

Figure 1

Overview of bacterial CSR. Two important aspects of CSR are summarized here: (*a*) the inputs to CSR (*blue*), including the changes of cellular constituents directly impacted by cold shock, and (*b*) the outputs of CSR (*orange*), adaptation of bacteria to mitigate changes in panel *a*, with major factors involved in different pathways highlighted. Black arrows show the cross-interaction between regulation of mRNA metabolism (folding and stability) and regulation of translation. Abbreviations: CAP, cold acclimation protein; CIP, cold-induced protein; CSR, cold shock response.

mediated by macromolecules and also makes it more difficult to acquire limited resources from the extracellular environment. All of these factors render cell growth sensitive to cold shock.

Low temperature slows enzymatic reactions, both because decreased kinetic energy results in less frequent collisions and slower reaction rates and because of temperature-dependent changes in the enzymatic activity of proteins. Some psychrophilic bacteria have evolved to produce cold-adapted enzymes, which often optimize activity at the expense of substrate affinity, via reducing the activation energy barriers. This optimization is reached by increasing protein flexibility using a menu of changes, including changing the electrostatics and hydrophobicity of or near the core site and altering the amino acid composition of the protein (123). For mesophilic bacteria without cold-adapted enzymes, adaptation to cold shock can be challenging.

Low temperature directly impacts the structure and function of various cellular constituents with thermodynamically sensitive properties. These changes may serve as inputs to trigger the CSR and are also mitigated by the response (**Figure 1**): (*a*) Membrane bilayers undergo a reversible phase transition from a liquid crystalline state to a more rigid, gel-like state when the temperature drops. Rigidification affects membrane permeability and influences the topology, interactions, and functions of the membrane-associated proteins. (*b*) Protein folding is intuitively expected to be hindered by low temperature, although its effect on CSR is poorly studied. (*c*) Nucleic acid conformation is altered by cold shock, likely due to the temperature-dependent changes in hydrogen bond strength—DNA is compacted and RNA secondary structure is stabilized, which can affect transcription, translation, and mRNA decay. (*d*) Ribosomes are directly impacted, with low temperature inhibiting assembly and activity, and likely promoting CSR remodeling of gene expression.

4. NEW OMIC APPROACHES TO STUDY CSR

The CSR is complex as cold temperature sensors are distributed in various processes and structures, which may be connected and coordinated via unknown mechanisms. Therefore, identification of all critical players is a prerequisite to disentangling these pathways and establishing their connections. Cold-inducible genes seem to be a good starting point for dissecting the response, but not all are essential for cold adaptation, and some key players are not strongly induced. Therefore, unbiased global phenotypic analyses are a useful starting point for molecular characterization of cold adaptation. Furthermore, regulation of cold-induced genes is multilayered. There is no cold-specific sigma factor or transcription regulator, with much regulation being post-transcriptional. Thus, quantification of different regulatory levels of gene expression, including transcription, RNA stability, translation, and protein stability, would both enable accurate measurement of gene expression output and help elucidate the mechanism of induction. Together, these unique features and challenges motivate high-throughput omic approaches to study the CSR.

4.1. Proteome

Protein expression during cold shock was classically studied using 2D gel electrophoresis (2D gels) (13, 50, 74). First described by O'Farrell in 1975 (106), 2D gels separate a complex mixture of proteins first by their pI values (dimension 1) and then by their relative molecular weights (dimension 2). With radioactive or fluorescent labeling, 2D gels allow quantitative comparison of protein production between different samples. Although 2D gel technology identified many cold shock–induced proteins, it is also limited because some proteins are not well resolved; determining protein identity is difficult, and low abundance proteins are beyond the detection limit. Mass spectrometry–based quantitative proteomics partially circumvents these difficulties (21, 105), has led to the discovery of previously unannotated proteins (such as small proteins) (34), and provided novel insights into biological function during the CSR.

4.2. Transcriptome

Microarrays and, more recently, RNA-seq have been used to quantify the change of RNA levels during CSR (6, 15, 111, 136). The high coverage of RNA-seq allows quantification not only of low-abundant transcripts but also of small RNAs (153), which exhibit important roles in gene regulation after cold shock. Changes in RNA level reflect altered transcription and/or stability. It is currently challenging to disambiguate these two pathways on a global scale because we lack global technologies to measure each separately. A combination of transcriptome and proteome analysis can separate effects on RNA and protein levels, potentially revealing multilayered gene regulatory mechanisms.

4.3. Translatome

Ribosome profiling (or Ribo-seq, deep sequencing of ribosome-protected mRNA fragments) measures translation globally by quantifying ribosome occupancy on mRNAs at genome scale (64). Translation efficiency, the rate of protein production per mRNA, is determined by normalizing the population-averaged ribosome density of an mRNA (from Ribo-seq) to its level (from RNA-seq). Thus, changes in translation efficiency are independent of whether the altered mRNA level results from changes in transcription or degradation. Recent studies used Ribo-seq and RNA-seq to study both heat shock and cold shock in bacteria (102, 153), providing a deeper understanding of translational control and ribosome trafficking during stress adaptation.

4.4. RNA Structurome

mRNA becomes more structured after cold shock, altering its translatability and/or stability and impacting gene regulation. Recent advances in next-generation sequencing and in vivo chemical probing have provided unprecedented insights into genome-wide RNA folding. DMS (dimethyl sulfate)-seq and SHAPE (selective 2′-hydroxyl acylation analyzed by primer extension) are commonly used in bacteria. DMS modifies the Watson-Crick face of adenines and cytosines such that reactivity directly estimates the likelihood that the nucleotide is unpaired (124). SHAPE reagents (e.g., 2-methylnicotinic acid imidazolide or NAI) react with the ribose sugar, modifying all four nucleotides, so the reactivity will be diminished by base-pairing (98). DMS-seq monitoring of the genome-wide changes of mRNA folding during the CSR revealed an mRNA structure surveillance system in *Escherichia coli* that is critical for restoration of protein synthesis after temperature shift (153; see Section 5.8.3). New improvements of the technologies enable single-molecule RNA structure probing in vivo by identifying correlated multiple modifications in a single RNA, i.e., DMS-MaPseq (154) and SHAPE-MaP (132), offering the potential for future mechanistic characterization of structural polymorphism.

4.5. Metabolome

Global metabolite profiling can simultaneously monitor precursors, intermediates, and products of metabolic pathways. It can enable discovery of unidentified metabolites and quantify changes in known metabolites during stress responses. Previous work incorporated the metabolite composition with gene expression data to describe a time-resolved *E. coli* response to different perturbations, including cold, which provides a more comprehensive insight on system-level stress adjustments (75). Interestingly, the metabolic response displays a higher level of specificity than the transcriptomics response especially during early time points after perturbation. This highlights the importance of metabolic analysis in addition to gene expression analysis when studying stress responses, as the combination will reveal potential mechanisms other than changing the protein level for altering protein function and activity.

4.6. Phenotypome

Above we discussed methodologies monitoring different steps of cellular processes, but to determine whether these effects are physiologically important during the stress adaptation, we need a measurement of the final output—i.e., cell survival and growth. Previous studies used large-scale transposon mutagenesis to screen for cold-sensitive mutations in diverse bacteria (91, 96). Recently, genome-wide screening of single-gene deletion libraries identified genes that may contribute to long-term growth at low temperatures in *E. coli* and *Bacillus subtilis* (83, 131). Furthermore, the novel CRISPR interference–based gene knockdown libraries allow for tunable downregulation of essential genes (58), some of which encode critical translational regulators and ribosome components that are hypothetically important for cold adaptation. These libraries will enable us to monitor the change of fitness of individual mutants during the entire course of adaptation.

5. WHAT ARE THE OUTPUTS OF THE CSR?

Here we discuss how bacteria respond to the inputs of cold shock, focusing on the following features as major outputs of the CSR: (*a*) a general description of the outputs of CSR; (*b*) compatible solute accumulation; (*c*) cell membrane adaptation; (*d*) protein folding regulation; and (*e*) global

regulation of gene expression, including alterations in DNA topology and transcription, RNA metabolism, and protein translation (**Figure 1**).

Acclimation: growth lag period that some bacterial species experience after cold shock before resuming growth at the low temperature

CIP: cold-induced protein; protein transiently induced after cold shock and tuned down before cells reach steady state at the low temperature

CAP: cold acclimation protein; protein permanently synthesized at an elevated level during the continuous growth at low temperature

5.1. A General Description of the Outputs of CSR

Upon cold shock, bacteria dramatically slow growth. Most cellular processes are inhibited by the low temperature. However, a subset of genes are induced by temperature downshift, and many of these play critical roles in CSR and low temperature adaptation. Here, we first summarize growth rate changes and then discuss the general features of proteins induced upon cold shock in different bacteria.

5.1.1. Bacterial growth after cold shock. Bacterial growth slows after exposure to a sudden cold shock, but the pattern of growth rate change varies with bacterial species and cold shock conditions. Some bacteria exhibit a lag period, termed the acclimation phase, immediately after the temperature downshift, before resuming growth at a rate characteristic of the new temperature. These species include the mesophilic bacteria *E. coli* (74, 103), *Mycobacterium smegmatis* (130), and *Vibrio cholerae* (31) and the psychrotrophic bacteria *Listeria monocytogenes* (13), *Arthrobacter globiformis* (16), and *Pseudomonas fragi* (99), as well as the thermophilic bacteria *Streptococcus thermophilus* (147) and *Thermoanaerobacter tengcongensis* (90). The duration of the acclimation phase is affected by the magnitude of the temperature downshift and/or the final cold temperature (59). However, some bacteria do not exhibit acclimation. Instead, growth continues at an intermediate rate until reaching the steady state at the final cold temperature. These species include the mesophilic bacteria *B. subtilis* (50), *Lactococcus lactis* (109), and *Enterococcus faecalis* (108) and the psychrophilic *Vibrio* sp. (8). These observations suggest that species-specific mechanisms may exist for different bacteria to respond to cold shock, and the time required to readapt cannot be fully explained by the range of optimal growth temperatures or the phylogeny of the bacteria species.

5.1.2. Cold-induced proteins and cold acclimation proteins. Time-resolved proteomic profiling has identified many proteins whose expression levels are modulated during different stages of the CSR. The upregulated proteins have different induction times and dynamic changes after cold shock. In the face of differences in nomenclature, we follow the definitions proposed by Weber & Marahiel (145) that assign upregulated proteins as either cold-induced proteins (CIPs) or cold acclimation proteins (CAPs). CIPs are transiently induced early and then decreased later before reaching their steady state at the low temperature. CAPs are permanently synthesized at an elevated level during the continuous growth at low temperature, with their induction generally, but not always, occurring at the later phase of CSR. It is a straightforward speculation that the time-dependent up- and downregulation of individual proteins reflects a temporally organized regulatory network where different factors with specialized functions are important at specific stages of CSR.

The expression profiles of CIPs and CAPs of bacteria with different temperature preferences also show species-dependent differences. In cold-adapted bacteria, the relative levels of CIPs are generally lower than those in mesophiles, whereas CAPs can be particularly important in allowing their prolonged growth at low temperature (16, 99, 122). This is in line with the fact that the global protein synthesis is largely inhibited in mesophiles with CIPs being the prevailing proteins synthesized after cold shock, whereas in psychrotrophic bacteria, the overall synthesis of cellular proteins is less inhibited (reviewed in 59). These different translational signatures are likely due to diversion of the translational machinery and/or the species-specific modification of the structure and function of ribosomes upon temperature downshift.

5.1.3. Cold shock proteins. The cold shock family proteins (Csps) are among the most upregulated proteins by cold shock and are usually CIPs or CAPs. Csps are small (~7.4 kD), nucleic acid–binding proteins with a highly conserved cold shock domain (CSD) that harbors the nucleic acid–binding motifs, RNP-1 and RNP-2 (126). With the general exception of cyanobacteria, which possess a functional analog of Csps called Rbps (RNA-binding proteins) (92), Csps are present in almost all bacteria studied thus far, regardless of normal growth temperature range (13, 16, 31, 50, 74, 90, 99, 147), as well as in the earliest diverging bacterial branches, *Thermotoga* and *Aquifex*, indicative of an ancient origin (51).

Csp: cold shock family proteins; the protein family most strongly induced by cold shock, harboring a highly conserved CSD

CSD: cold shock domain; a nucleic acid–binding domain that is widely conserved from bacteria to higher plants and animals

Csps were initially identified in *E. coli*, with the finding that CspA comprised ~15% of total protein synthesis after cold shock (47, 74). Initially, CspA was thought to function only during cold adaptation; subsequently, it was observed that CspA was also abundant during the early exponential phase at 37°C (18), where it was present at ~40,000 copies/cell (87), with synthesis decreasing when transiting to late-exponential phase (18). There are nine Csp family proteins in *E. coli* (CspA–I), which have semiredundant functions. Four of the nine Csps, CspA, CspB, CspG, and CspI, are CIPs, and CspE can be induced by cold shock under certain conditions (53). A quadruple knockout of CspA, CspB, CspG, and CspE is unable to divide at low temperature (148). This cold sensitivity can be suppressed by all members of the Csp family except for CspD, whose overexpression leads to lethality by inhibiting DNA replication (151).

As a family of nucleic acid–binding proteins, Csps bind to both DNA and RNA in vitro with fairly relaxed sequence specificity (70, 110). With the exception of binding to the promoter regions of two cold shock genes, *hns* (85) and *gyrA* (72), to regulate their transcription, the remainder of Csp functions derive from their ability to bind to RNA and promote the formation of single-stranded RNA either by unwinding or by capturing transiently unwound bases (70, 113). Csps modulate RNA stability, as supported by the observation that CspE binds to mRNA and impedes polyA-mediated 3′ to 5′ exonucleolytic decay by PNPase (polynucleotide phosphorylase) and inhibits internal cleavage by RNase E (37). Csps also increase *nusA* expression after cold shock via an antitermination mechanism by binding to and disrupting the rho-independent terminator RNA stem-loop located upstream of *nusA* in the *metY-rpsO* operon (12). Csps facilitate protein translation, as CspA stimulates translation of cold shock mRNAs, and were proposed to contribute to translational selectivity at low temperature, although further evidence is required (53). Although interacting with promoter DNA, binding RNA, and melting RNA are all important, the critical role of Csps is to melt RNA, as a Csp mutant proficient in RNA/DNA binding but unable to destabilize RNA secondary structure cannot complement the cold sensitivity of quadruple *csp* deletion strains (112). These observations highlight the importance of Csps as RNA chaperones during cold adaptation.

The multiple Csp homologs are differentiated by letters following the Csp (e.g., CspA and CspB). Csp nomenclature does not denote function (e.g., CspBs in different organisms may have different functions). In *E. coli*, although Csps are required for expression of genes with high translation efficiency at 37°C, their deletion does not cause a strong growth defect (153). In contrast, in *B. subtilis*, of the three cold-inducible Csps (CspB–D) identified, at least one copy is required for growth at 37°C and during cold shock, with CspB the most important, followed by CspD or CspC during optimal or low-temperature growth, respectively (49). *L. monocytogenes* also possesses three Csps, CspA, CspB, and CspD, all of which are dispensable for growth at optimal temperature (37°C). However, they are required for efficient cold and osmotic stress tolerance (127) and are involved in virulence, cell aggregation, and flagella-based extracellular motility of *L. monocytogenes* (35). Interestingly, Csps may regulate the virulence gene *hly*, which encodes the pore-forming cytolysin listeriolysin O needed in intracellular survival, by modulating its transcript stability (125). Thus, the post-transcriptional regulatory role of Csps is likely to be broadly conserved.

5.2. Compatible Solute Accumulation

Compatible solutes are a group of low molecular weight compounds that can be amassed to high intracellular levels without interfering with biological function. They are important during osmotic stress, heat shock, and cold shock, and they act to reduce the stress by various mechanisms, including balancing osmotic pressure, altering freezing properties, and protecting cellular constituents. Known cold-protective compatible solutes include trehalose (76), glycine betaine (62, 82), and carnitine (14), which were observed to accumulate by increased synthesis and/or acquisition during CSR. In *E. coli*, *otsA* and *otsB* encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, which are important for biosynthesis of trehalose from glucose (41). The *otsBA* operon can be induced during cold shock, osmotic stress, and entering stationary phase, in an RpoS-dependent manner (41, 60, 146). *otsBA* mRNA is also stabilized at low temperature (76). Deletion of *otsA* strongly reduces cell viability at 4°C, indicating a defense mechanism of trehalose to overcome stress at low temperature (76). Moreover, several osmosensing transporters in *E. coli* are induced after cold shock (153), including the osmolyte:H^+ symporter ProP and the ABC transporter ProVWX, which mediate the uptake of proline, glycine betaine, and other osmoprotectants (52, 54), and BetT, which transports choline, a substrate for synthesizing glycine betaine (86). The overlap between osmo-inducible and cold-inducible genes involved in protection suggests a general crosstalk between the two stress response systems.

5.3. Cell Membrane Adaptation

A functional membrane is crucial for survival of all organisms. In general, cells seek to maintain normal fluidity of the membrane by adjusting the melting point of its lipid constituents to a particular set point regardless of temperature, a process called homeoviscous adaptation (133). After cold shock, membrane fluidity rapidly decreases, resulting in a local to global phase transition from its normal liquid-crystalline state to a more rigid gel-like state. Cells respond by incorporating lower melting point fatty acids into the lipid membrane, such as fatty acids with shorter chains, branched fatty acids, or ones with *cis* carbon double bonds to restore the optimal fluidity to the extent possible.

In *B. subtilis*, membrane adaption is initially carried out by the fatty acid desaturase Des, which makes a *cis* double bond at the Δ5 position of existing saturated fatty acids attached to membrane phospholipids (3). Des is transiently induced upon temperature downshift and then repressed about 30′ later. Its regulation is under control of the two-component system DesKR (4), in which the histidine kinase DesK directly senses the membrane state [presumably changes of membrane thickness due to altered fluidity (29)] and triggers conformational changes to activate or deactivate DesR, which regulates the transcription of *des* (4). For long-term adaptation, saturated anteiso-branched C-15 and C-17 fatty acids are substituted for iso-branched chain fatty acids, which increases membrane fluidity (81), but the mechanism is still unclear. Likewise, in *E. coli*, composition of inner membrane lipids gradually shifts toward unsaturated fatty acids as a result of increased β-ketoacyl-ACP synthase II enzymatic activity (39, 40). In cyanobacteria, the low temperature membrane adaptation also relies on synthesis of fatty acyl lipid desaturases and polyunsaturated fatty acids (reviewed in 104).

Interestingly, the fatty acid composition of the outer membrane (OM) of *E. coli* is also modified. Immediately after cold shock, the fatty acyl chains in lipid A that are predominantly laureate at 37°C shift to palmitoleate, an unsaturated acyl chain, at 12°C. This shift is due to induction of *lpxP*, one of the most highly cold-induced genes, which encodes a palmitoleoyl acyltransferase (22, 141). An *lpxP* mutant does not exhibit a significant growth defect at 12°C; however, it shows a cold-dependent hypersensitivity to certain antibiotics, indicating that palmitoleate is required

for an effective OM barrier (141). Interestingly, many bacteria that survive outside of their host exhibit cold-induced lipid A acyl transferases, indicating that OM remodeling at cold temperatures is generally important (141).

Carotenoids have also been discussed as regulators of membrane fluidity at low temperature. Studies of two psychrotolerant bacteria, *Sphingobacterium antarcticus* (67) and *Micrococcus roseus* (25), showed that the relative amount of polar carotenoids increased at low temperature compared to nonpolar carotenoids. Polar carotenoids were reported in vitro to decrease the membrane fluidity when above the phase transition temperature and increase it below that temperature (137). Hence, it was suggested that the increase of membrane fluidizing fatty acids might be counterbalanced by the synthesis of membrane-stabilizing polar carotenoids in Antarctic bacteria (24). A recent study of two *Staphylococcus xylosus* strains showed that carotenoid levels increase at 10°C but are not detectable at 30°C. Increased carotenoid levels correlate with an increased resistance of the cells against freeze-thaw stress (128). All of these suggest that increased carotenoid synthesis might be a constitutive component in the cold adaptation strategy broadly applied by diverse bacteria.

5.4. Regulation of Protein Folding

Dysregulation of protein folding homeostasis is the primary signal for the heat shock response (HSR) and led to the discovery of the key role of chaperones, both in proteostasis and in sensing temperature upshift. However, its role during cold shock is limited and specific, consistent with the fact that low temperature generally leads to more stable proteins. There are two well-documented effects of chaperones on low temperature growth—trigger factor (TF) and some Clp (caseinolytic protease) proteins—although their precise roles are unclear.

In *E. coli*, TF is induced upon cold shock at a modest level after a lag period of 2–3 h and viability at 4°C is increased by TF overexpression and decreased by TF reduction (77). TF binds to ribosomal protein L23 near the polypeptide exit channel and assists in the cotranslational folding of nascent proteins (84). Recent research using selective ribosome profiling enriching for TF-associated ribosomes revealed a role of TF in the biogenesis of β-barrel OM proteins (OMPs) (107), consistent with the discovery that TF chaperoned OMPs in vitro (28) and with the finding that deleting *tig* (encoding TF) leads to decreased OMP levels and increased sensitivity to OM stresses (107). As remodeling the OM to improve its functionality is a major cold-induced regulation in *E. coli* (22, 141), it seems likely that supporting the folding and/or transport of OMPs is the major role of TF in CSR. In addition, TF may also play an auxiliary maintenance and repair role by refolding the cold-damaged proteins (94).

Among the Clp proteases, there is some evidence that ClpB, distinguished by its ability to resolve heat-induced protein aggregates cooperatively with the Hsp70 (DnaK) chaperone system (101), also has a minor role in cold adaptation. In the cyanobacterium *Synechococcus* sp. strain PCC 7942, ClpB is strongly induced during moderate cold stress (37 to 25°C) but not when shifting to more extreme cold (to 15°C) (115). However, as deletion of ClpB or ClpP1, the proteolytic subunit of Clp protease, leads to severe defect in cell survival and growth at cold temperature (115, 116), the Clps are potentially important for cold adaptation.

5.5. Changes in DNA Conformation

DNA structure exhibits both global and local changes in response to cold shock. Globally, DNA tends to become more negatively supercoiled at low temperature in both *E. coli* and *B. subtilis* (48, 100). In *E. coli*, this may in part rely on induction of several DNA topology-relevant factors (GyrA, Hup-B, H-NS) (43, 72, 85, 100), but *gyrA* and *gyrB* (encoding DNA gyrase) are not strongly

induced by cold shock in *B. subtilis* (15). It is not yet clear what is responsible for increased negative supercoiling. Regardless, DNA supercoiling status impacts bacterial transcription globally (57), making it an important parameter in temperature-dependent gene regulation, and the global DNA topology modulators, including DNA topoisomerases and nucleoid-associated proteins, are important transcription regulators during temperature fluctuations. Indeed, the general ability of *E. coli* to cope efficiently with cold environments (12 and 25°C) is strongly impaired by mutations in *hns* (encoding nucleoid-associated protein H-NS) (33). Inhibiting DNA gyrase activity in *B. subtilis* prevents the induction of unsaturated fatty acid synthesis at low temperature (48). In the cyanobacterium *Synechocystis* sp., a large number of the cold-induced genes require an excess of negative supercoiling of DNA for their induction (117), although the mechanism is still unknown.

Additionally, local DNA conformation changes can also alter expression in response to cold temperatures. This might result from the spreading of globally increased negative supercoiling and/or sequence-/structure-specific features that affect the affinity of DNA-binding proteins. In fact, changes in DNA curvature at the promoter region significantly modulate the location of the −35 and −10 regions relative to each other. Moreover, intrinsically curved DNA regions characterized by AT-tracts upstream of −35 region influence binding of RNA polymerase. Therefore, temperature-induced changes in the topology within these regions directly affect transcription. Indeed, the *plc* gene of *Clostridium perfringens* coding for phospholipase C is induced by cold temperature via bending of the phased A-tracts upstream of its promoter (78). However, this effect might be sequence or context dependent, as the upstream element of *E. coli rrnB* P1 promoter stimulates the *plc* promoter less prominently at low temperature compared to the endogenous phased A-tracts (78).

5.6. Changes in RNA Structure

The secondary and tertiary structure of RNA, as well as its ability to form intermolecular RNA-RNA hybrids, can alter gene expression by affecting transcription, translation, and/or RNA turnover. Following temperature downshift, RNA molecules generally become more folded, as is expected because lower temperature increases the stability of RNA structure. Increased structure generally contributes to the global decrease of translation (153). However, in specific cases, cold-induced changes of RNA structure can also lead to increased expression. In *E. coli*, expression of the major cold shock protein, CspA, is regulated by a change in the secondary structure in the 5′ untranslated region (UTR) of its mRNA, which is a *cis*-acting thermosensor. At 37°C, 5′ UTR conformation destabilizes the mRNA, so it is rapidly degraded, whereas its low temperature conformation stabilizes mRNA and facilitates translation (17, 36, 45, 46). The small RNA, DsrA, is an example of *trans*-acting RNA transducer. It controls expression of the general stress sigma factor RpoS in *E. coli* (134). DsrA level increases at low temperature as a consequence of increased transcription and RNA stability (120). DsrA binds to and changes the folding of the 5′ UTR of *rpoS* mRNA to enhance both the stability and translatability of *rpoS* mRNA at low temperature (97). In addition, RprA, another small RNA, which regulates RpoS production upon osmotic shock (93), is also cold induced and therefore likely to regulate *rpoS* expression after cold shock as well (153). Thus, the temperature-dependent regulation of *rpoS* mRNA is mediated by multiple small RNAs simultaneously and cooperatively.

5.7. Regulation of RNA Degradation

There are massive changes in mRNA stability during the CSR because of alterations in either the RNA degradation machinery itself (degradosome) or auxiliary proteins. In fact, many

cold-inducible proteins are involved in RNA metabolism, including components of RNA degradosome (e.g., PNPase), the DEAD-box helicases, and other ribonucleases such as RNase R (20, 73, 152).

5.7.1. The degradosome. The RNA degradosome is a large, multiprotein complex central to mRNA degradation in bacteria. In *E. coli* and other gram-negative bacteria, the long multidomain protein RNase E forms the core of this machine and is essential for viability. Its N-terminal catalytic domain carries out single-strand-specific endonuclease activity, and its long C-terminal unstructured region is a scaffold to recruit both its RNA substrate and the other constituents of the degradosome, including PNPase (a 3′ to 5′ exoribonuclease), RhlB (an ATP-dependent DEAD-box helicase that unwinds RNA structures), and enolase (a glycolytic enzyme whose role is still unclear). The degradosome is modified during adaptation to cold shock, likely to make it more efficient at cold temperatures. PNPase is induced and essential for growth at low temperature (152). Because PNPase can associate with RhlB to form a small RNA degradation machine independently of RNase E (89), increased PNPase may modify its distribution between the degradosome and the PNPase-RhlB complex. Although it is unclear whether the increased PNPase level leads to change in RNA degradation efficiency or substrate selectivity, PNPase was shown to degrade a number of cold-inducible mRNAs, including those of *cspA*, *cspB*, *cspG*, *cspI*, *rnr*, and putative prophage genes *ydfK* and *ynaE* (20, 114, 150). In addition, the S1 RNA-binding domain of PNPase, which folds into a β-barrel structure similar to CspA, can also suppress the cold sensitivity of the quadruple *csp* mutant (148), suggesting common functional properties.

The RNA degradation machinery is different in gram-positive bacteria, whose constituents and functions are less understood. Lacking RNase E, the *B. subtilis* degradosome is suggested to use RNase Y as the scaffold endoribonuclease and contains RNase J1 and RNase J2. Similar to *E. coli*, deletion of *pnpA* (encoding PNPase) in *B. subtilis* leads to cold sensitivity (143). CshA, the functional analog of RhlB of *E. coli*, and another RNA helicase, CshB, have increased mRNA levels after cold shock (15) and are important for cold adaptation in *B. subtilis* (63). However, how the degradosome and RNA degradation are regulated in gram-positive bacteria upon cold shock is largely unknown.

5.7.2. DEAD-box RNA helicases. DEAD-box proteins are the largest family of superfamily 2 helicases, which are named for the D-E-A-D sequence in motif II of these proteins (88). DEAD-box proteins are ubiquitous enzymes that use ATP to modulate RNA or RNA-protein structures. In bacteria, DEAD-box helicases are generally dispensable for normal growth, but their absence often results in cold-sensitive phenotypes (65). *E. coli* has five DEAD-box helicases: RhlB, CsdA, DbpA, RhlE, and SrmB. RhlB is a constitutive member of the degradosome, and CsdA may be degradosome associated at low temperature (118). RhlB, the best-characterized DEAD-box helicase, facilitates degradation of highly structured RNA intermediates by PNPase (79). CsdA (DeaD) is induced by cold shock, and its deletion causes a cold-sensitive growth defect (73). CsdA is involved in RNA degradation at low temperature (10) and copurifies with RNase E and other degradosome components after a shift of culture to 15°C but not at 37°C (118). It can functionally replace RhlB in a "minimal degradosome" reconstituted in vitro, suggesting that CsdA that accumulates during early stages of cold acclimation may subsequently assemble into cold-specific degradosomes (118). However, because RhlB is still present in cold-shocked cells, it remains questionable whether degradosomes contain both helicases or different subpopulations contain either of them, and whether/how this affects RNA degradation. Interestingly, in *Caulobacter crescentus*, the cold shock inducible DEAD-box helicase RhlE was also found to associate with degradosome at 15°C. Both RhlE and the canonical degradosome component RhlB are necessary for optimal

growth at low temperature (5). These observations suggest that the cold-induced remodeling of degradosome mediated by DEAD-box helicases might occur in diverse organisms.

5.7.3. RNase R. RNase R is the only *E. coli* exonuclease with two distinct activities: It is both an RNase and a helicase. Its ability to unwind secondary structures of RNA appears to be dependent on its CSDs (9). RNase R is cold shock inducible, increasing its level seven- to eight fold after temperature shift (20). Overexpression of RNase R complements the cold-sensitive phenotype of a *csdA* mutant (10), where its helicase activity was shown to be essential (9). As RNAs are generally more structured at low temperature, the ability of RNase R to degrade highly structured RNA is likely the main reason for its importance in the CSR. RNase R was named for its ability to carry out ribosomal quality control by degrading damaged ribosomal RNA (rRNA) (26). It is required for maximal growth, maturation of transfer mRNA (tmRNA) and presumably tmRNA-mediated degradation of nonstop mRNAs at low temperature (20, 121). Recent transcriptome and RNA decay studies in *E. coli* showed that deletion of *rnr* (encoding RNase R) almost completely abolished mRNA degradation during acclimation (153), suggesting that RNase R may act alone or as an essential accessory protein for the canonical degradosome pathway to promote mRNA degradation following cold shock. Indeed, RNase R is associated with the degradosome in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W (119).

5.7.4. RNase III. RNase III is an endonuclease that cleaves double-stranded RNA (dsRNA). It is required for processing rRNA, phage RNA, and some mRNAs, including that of PNPase and its own mRNA, usually by cleaving dsRNA created by the formation of stem-loop structure (reviewed in 27). In *E. coli*, YmdB, an evolutionarily conserved stress-responsive protein, inhibits RNase III activity by binding in its catalytic region to prevent its dimerization/activation (80). YmdB, controlled by RpoS, is transcriptionally activated by cold shock (80), suggesting that YmdB inhibits RNase III activity during the CSR. In support of this idea, an *rnc-lacZ* fusion transcript, which is sensitive to RNase III cleavage, showed lower β-galactosidase activity in the *ymdB* deletion mutant than in wild-type cells after cold shock (80). Moreover, as the PNPase transcript is an RNase III substrate, inhibition of RNase III might be important for PNPase accumulation in cold-shocked cells.

5.8. Regulation of Translation

Early work of VanBogelen & Neidhardt (139) showed that ribosome-directed antibiotics administered to *E. coli* cells growing at 37°C resulted in induction of either heat shock proteins or CIPs/CAPs, depending on the antibiotic, leading them to suggest that ribosomes are temperature sensors via an unknown mechanism. We now know that at low temperature, ribosome assembly is hindered, and translation initiation and elongation are significantly slowed due to decreased enzymatic activity of translation factors, increased mRNA secondary structure, and possibly direct changes in ribosome structure and functionality. However, whether the ribosome itself is a sensor has not been resolved.

5.8.1. Ribosome assembly. Ribosome assembly is sensitive to low temperature, as indicated by the fact that in general, ribosome assembly mutants are cold sensitive (55). Indeed, two cold-induced ribosome-binding proteins, RbfA and CsdA, participate in ribosome assembly. RbfA, a 30S ribosome-binding factor, was isolated as a multicopy suppressor of a cold-sensitive mutant in the 5′ helix of 16S rRNA in *E. coli* (30). It is required for efficient 16S rRNA processing and 30S subunit assembly (19, 32), where RbfA release appears to be a checkpoint for 30S maturity (129).

Following cold shock, RbfA is induced and a larger fraction of 30S ribosomal subunits contain RbfA (149). Cells lacking RbfA show a cold-sensitive growth phenotype (30) and constitutive induction of the CSR after shift to low temperature (71). Although the molecular mechanism of RbfA is not clear, these findings are compelling evidence that ribosome assembly is hindered during cold shock and that the production of modified/reassembled ribosomes is critical for CSR.

CsdA, the DEAD-box helicase discussed above for its role in RNA degradation at low temperature, was initially isolated as a suppressor of a ribosomal protein S2 (*rpsB*^{ts}) mutant (138) and participates in the assembly of large ribosomal subunit (23). A *csdA* mutant accumulates unprocessed 23S rRNA and shows an altered ribosome profile compared to wild-type cells (68). Together, CsdA, RbfA, and likely other not-yet-identified cold-specific ribosome components might be required not only for assembly but also to adjust the cold-induced conformational change of the ribosomes, to generally promote translation initiation during the CSR.

Ribosome silencing/hibernation is another feature of cold adaptation. Protein synthesis is dramatically reduced at cold temperatures but needs to reactivate rapidly after a shift back to normal temperature, indicating the necessity for creating quiescent ribosomes. pY (RaiA), a cold-induced ribosome-associated protein, is one such silencing factor. It stabilizes 70S ribosomes against dissociation (1) and inhibits translation at low temperature by blocking the binding of aminoacyl-tRNA to the ribosome (2, 140). Sra, another ribosome-associated protein induced by cold shock (153), is likely a second factor. Little is known about the function of this small, basic protein that binds tightly to the 30S subunit (142), but recent studies indicate that *sra* is downregulated in fast-growing *E. coli* isolates from cystic fibrosis patients (95). Interestingly, both pY and Sra are among the ribosome-associated factors induced upon transition to stationary phase (2, 66). This is not surprising, as stationary phase, like cold shock, demands that cells inactivate but not destroy ribosomes.

5.8.2. Translation initiation. Translation initiation starts with binding of initiation factors (IFs) IF1 (*infA*) and IF3 (*infC*) to the 30S subunit, followed by binding of the mRNA, IF2, and fMet tRNA. Upon binding the 50S subunit, the IFs dissociate and protein synthesis begins. In *E. coli*, the de novo transcription and translation of *infA* and *infC* after cold shock contribute to the transient increase of the IF1/ribosome and IF3/ribosome ratio, which is partially responsible for preferentially translating cold shock mRNAs over bulk mRNAs in cold-stressed cells (42, 44). The increased level of IFs during cold adaptation can be important for overcoming the higher stability of the 70S ribosomes at low temperature and for providing a sufficient pool of free 30S subunits for translation initiation.

B. subtilis lacks an acclimation phase, instead continuing to grow at a significantly reduced rate after shift to cold. Likewise, overall protein translation is maintained at the low level without significant recovery at 15°C (Y. Zhang & C.A. Gross, unpublished data), suggesting a different translation regulation mechanism. Although *B. subtilis* has CsdA and RbfA homologs, whose mRNA levels are about twofold induced by cold shock (15), their function is unclear. *B. subtilis* appears to induce/accumulate a number of canonical ribosomal or ribosome-binding proteins after cold shock, including L7/L12, S6, IF1, and IF2 (15, 50), and so far there are no reports of the cold-specific auxiliary factors. Although there are speculations that *B. subtilis* might adjust the ribosome pool by assembling a subpopulation with different rRNAs to facilitate translation (144), the cold shock regulation of translation in *B. subtilis* is still a mystery.

5.8.3. A case study: the circuitry controlling translation recovery during acclimation in *E. coli*. By combining omic approaches monitoring global changes in RNA level (RNA-seq), translation (ribosome profiling), and RNA secondary structure (DMS-seq) with molecular genetic

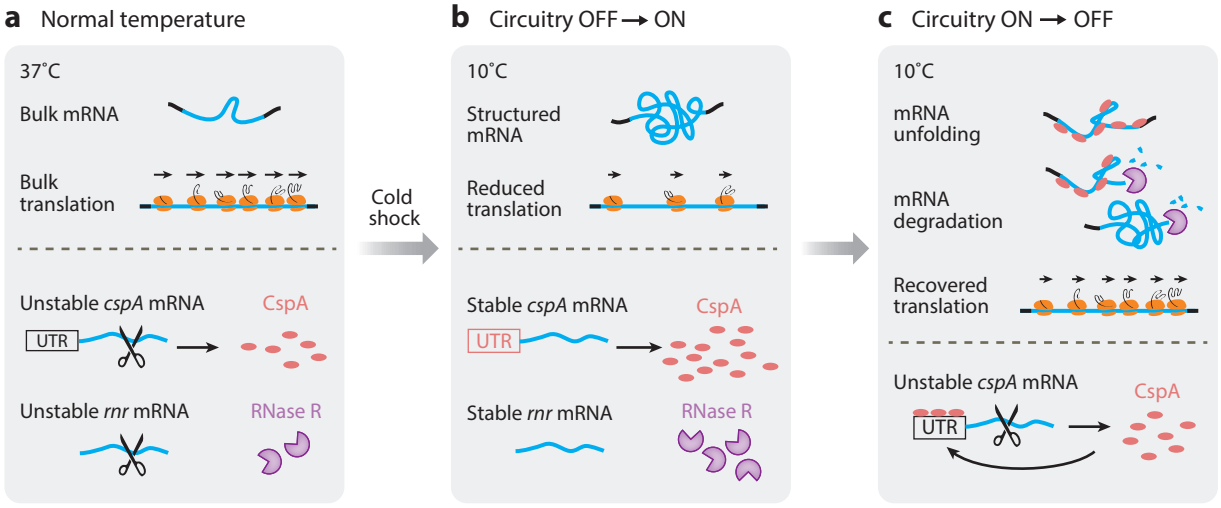

Figure 2

The circuitry controlling translational recovery during acclimation in *Escherichia coli*. The three panels depict the changes in the bulk of mRNA metabolism and translation (above the *dashed line*) and the regulation of two major players of the circuitry (below the *dashed line*) after cold shock. (*a*,*b*) As a consequence of cold shock, translation is massively inhibited, and mRNA becomes more structured. The circuitry is turned on by induction of CspA and RNase R. The structural change in the 5′ untranslated region (UTR) of *cspA* mRNA (shown by the *red box* in *b*) leads to increased mRNA stability and translatability. Induction of RNase R mainly results from stabilizing its mRNA at low temperature. (*c*) Increased CspA destabilizes mRNA structure globally to facilitate translation. RNase R degrades highly structured mRNA via its helicase activity, which promotes CspA coverage on mRNAs and is likely to contribute to translation reprogramming during acclimation. The circuitry is turned off by CspA binding to its own 5′ UTR and reversing the RNA structure back to its 37°C conformation, which decreases CspA expression. Figure adapted with permission from Reference 153.

analysis and building on prior knowledge, a recent study on *E. coli* identified the minimal circuitry required for increasing translation during acclimation (153) (**Figure 2**). Zhang et al. (153) found that immediately after cold shock, the deep reduction in protein synthesis was accompanied by a sharp increase in mRNA structure. As acclimation proceeds, there is a 3.5-fold increase in protein synthesis accompanied by mRNA unfolding. The profile of proteins synthesized confirmed most previously identified CIPs, identified new CIPs, and revealed the kinetics of the response. Csps and RNase R are among the most highly induced early responders, whose expression first dramatically increases following cold shock, together reaching up to 40% of total early protein synthesis, and then decreases later during acclimation. By analyzing the effects of individual CIP deletions on protein synthesis, Zhang and colleagues showed that only CspA and RNase R are necessary for increased protein synthesis during acclimation.

How is this circuit constructed? Basically, CspA and RNase R function as an RNA structure surveillance system that monitors and modifies RNA folding status to promote translation recovery (153). The role of CspA (and several other Csps) is to destabilize mRNA structure globally to facilitate translation initiation: In the absence of the Csps, there is neither global unfolding of mRNAs nor increased translation. This finding provides the mechanistic basis for previous work showing that the critical molecular function of Csps during CSR was to destabilize RNA secondary structure (112). The role of RNase R is to degrade mRNA, either alone or as part of the degradosome: After cold shock, mRNA degradation is almost completely abolished in the absence of RNase R, possibly because its strong helicase activity is necessary to unwind mRNA structure

at low temperature. This function may help remove the excessive mRNAs synthesized prior to cold shock, which promotes Csps coverage on mRNA substrates and/or facilitates the translation reprogramming to predominantly translate cold shock mRNAs.

The circuit is turned on by massive overexpression of Csps (enough to coat mRNA) and turned down by decreased Csp expression. Induction of CspA expression results from the previously described cold-induced change in the mRNA structure of its 5′ UTR, which increases the mRNA stability and translatability (17, 36, 45, 46). Shutoff of CspA expression results from reversal of this conformational change, as a consequence of CspA binding to its own 5′ UTR (153). This finding is consistent with earlier research showing that *cspA* mRNA is negatively autoregulated in a process dependent on an 11-base sequence called the cold box in its 5′ UTR (11, 69). Indeed, CspA binding to its 5′ UTR most prominently alters the conformation in the cold box region to resemble that at 37°C (153). The fact that this autoregulation is dependent on the relative CspA protein concentration suggests that Csps tie their own regulation to their role of structure surveillance in the cell.

6. CONCLUSION

Studying bacterial responses to temperature changes has been and continues to be a source of new insights into cellular functions and control mechanisms. Central to each response are the sensory pathways that receive and transmit the stress signals, the players that reprogram the molecular profile of the cells, and eventually the factors that maintain the steady state of the adapted cells under the new condition. Notably, most organisms from prokaryotes to eukaryotes share similar features in CSR, including induction of proteins with the conserved CSD. The eukaryotic CSR involves a coordinated response that modulates protein expression, metabolism, cell cycle, and the cell cytoskeleton (reviewed in 7, 38, 56, 135). In bacteria, the CSR also exhibits similarity and crosstalk with other stress responses, including antibiotic treatment, stationary phase transition, osmotic stress, sporulation, and virulence in pathogenic bacteria. Some of these reflect the cross-protection mechanism in bacteria. In fact, the general stress response regulators (e.g., RpoS in *E. coli* and SigB in *B. subtilis*) are involved in responding to different stresses. Another common feature is the global inhibition of protein synthesis. Therefore, to some extent, the cell uses common strategies to cope with stresses resulting in slow growth. Interestingly, both HSR and CSR exhibit RNA folding regulation as a major output of the response. After heat shock, unfolding of *rpoH* mRNA mediates the feedforward response, resulting in enhanced translation of σ^{32}. Likewise, upon cold shock, the RNA chaperone Csps underlie the global regulation of mRNA metabolism and translation. Although a large body of knowledge on bacterial CSR has accumulated, lots of open questions remain to be answered concerning the exact function of different CIPs and CAPs and their regulation during the response. Further research should help us understand the specific temperature adaptation mechanisms in diverse bacteria living in different environments.

SUMMARY POINTS

1. The cold shock response (CSR) is the sum of cellular responses required for efficient adaptation to a sudden shift from a temperature within the normal growth range to a lower one.
2. Low temperature generally inhibits biochemical reactions and cellular processes.

3. Cellular constituents with thermodynamically sensitive properties include membrane state, protein conformation, nucleic acid (DNA and RNA) structure, and ribosome assembly and function; these may serve as inputs to the CSR.
4. Adaptation to cold shock mitigates changes in thermosensitive components by increasing membrane fluidity, facilitating protein folding, and regulating global gene expression through changes in DNA topology, transcription, RNA metabolism (RNA folding and degradation), and translation (ribosome and translation factors).
5. The strategies of cold shock adaptation differ for bacterial species with different temperature preferences (i.e., psychrotrophic/psychrophilic, mesophilic, or thermophilic/hyperthermophilic).
6. Cold shock globally inhibits protein synthesis but stimulates expression of cold-induced proteins (CIPs) and cold acclimation proteins (CAPs), which are important for the short-term response and long-term adaptation to low temperature, respectively.
7. The cold shock family proteins (Csps), which harbor nucleic acid–binding domains that are highly conversed in almost all bacteria, are the most strongly cold shock–induced proteins.
8. A minimal circuitry in *Escherichia coli* controlling translation during cold acclimation consists of two main players, Csps and RNase R, which respectively unfold and degrade highly structured mRNA, and together act as an RNA structure surveillance system that monitors and modulates mRNA folding to globally facilitate translation initiation.

FUTURE ISSUES

1. Further work is required to dissect the multilayered regulation of the entire set of cold shock–induced proteins, including changes in mRNA transcription, stability, and translatability.
2. The functions of CIPs and CAPs during CSR are largely unknown. Even for the factors whose general role has been studied, such as Csps and DEAD-box helicases, their gene-specific effect remains unclear. What transcripts do they target? What determines their specificity during CSR?
3. It remains questionable whether cold-specific ribosomes exist and are responsible for the translation bias during CSR. If so, what structural and functional alterations do they exhibit compared to the canonical ribosomes? Are these changes conserved in different bacteria species?
4. Not all the factors critical for cold shock adaptation have been identified. High-throughput phenotypic analyses are required to identify factors important for optimal cell fitness at different stages during the response.
5. It is unclear why some bacterial species exhibit growth lag (acclimation) after cold shock whereas others do not. Is having acclimation beneficial for faster or more efficient adaptation to temperature downshift?

6. Thus far, the circuitry controlling CSR has been extensively studied only in *E. coli*. Mechanistic studies are needed to characterize how the response is monitored and regulated in nonmodel bacteria, especially for psychrotrophic/psychrophilic and thermophilic bacteria.
7. The molecular mechanism of cold-induced membrane adaptation is largely understudied in bacteria other than several *Bacillus* species. Do gram-negative bacteria have other outer membrane–specific regulations during CSR? Is their inner membrane–specific regulation conserved with that in gram-positive bacteria? It will be particularly interesting to investigate the temperature-dependent membrane regulation in pathogenic bacteria with noncanonical membranes, such as mycobacteria.
8. The response of bacteria when shifting back from cold temperature to an optimal growth temperature has not been studied in detail. How does this response compare to the general heat shock response?

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