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FUNGAL AND BACTERIAL PHYSIOLOGY - REVIEW

A general overview of the multifactorial adaptation to cold: biochemical mechanisms and strategies

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Abstract

Cold environments are more frequent than people think. They include deep oceans, cold lakes, snow, permafrost, sea ice, glaciers, cold soils, cold deserts, caves, areas at elevations greater than 3000 m, and also artificial refrigeration systems. These environments are inhabited by a diversity of eukaryotic and prokaryotic organisms that must adapt to the hard conditions imposed by cold. This adaptation is multifactorial and includes (i) sensing the cold, mainly through the modification of the liquid-crystalline membrane state, leading to the activation of a two-component system that transduce the signal; (ii) adapting the composition of membranes for proper functions mainly due to the production of double bonds in lipids, changes in hopanoid composition, and the inclusion of pigments; (iii) producing cold-adapted proteins, some of which show modifications in the composition of amino acids involved in stabilizing interactions and structural adaptations, e.g., enzymes with high catalytic efficiency; and (iv) producing ice-binding proteins and anti-freeze proteins, extracellular polysaccharides and compatible solutes that protect cells from intracellular and extracellular ice. However, organisms also respond by reprogramming their metabolism and specifically inducing cold-shock and cold-adaptation genes through strategies such as DNA supercoiling, distinctive signatures in promoter regions and/or the action of CSPs on mRNAs, among others. In this review, we describe the main findings about how organisms adapt to cold, with a focus in prokaryotes and linking the information with findings in eukaryotes.

Keywords Cold adaptation · Psychrophiles · Cold-shock proteins · Metabolism · Compatible solutes

Introduction

The current view of the phylogenetic tree of life is based on the comparison between the so-called 16S and 18S ribosomal RNA sequences of living beings on earth. We must thank this molecular taxonomy to Carl Richard Woese (USA, 1928–2012) who defined three domains of life (bacteria, archaea, and eukarya), all of them coming from a last common ancestor (LUCA) [1]. Regardless of the domain of life, extremophilic organisms are found in all of them. Extremophiles are organisms that can thrive in extreme environments such as acid or basic conditions (acidophiles or alkaliphiles, respectively), high pressure (barophiles or piezophiles, as found in the deep-sea), hypersalinity (halophiles, as found in the Dead Sea), shortage of available nutrients to sustain life (oligotrophiles), high osmotic pressure (osmophiles), and cold environments (psychrophiles and psychrotolerant). As an extreme case, we can mention tardigrades (water bears) that are ubiquitous invertebrates resistant to extreme conditions. They survive in the vacuum of space and withstand very high pressures of almost 6000 atm, as well as in extreme temperatures (such as − 200 °C and up to 150 °C), under prolonged dehydration (they survive up to 10 years without water) or under ionizing radiation [2].

Microbes can be classified according to their optimal growth temperature. For a practical purpose, we use a classification based on the report of Morita (1975) [3] as follows: mesophiles grow between room temperature (around 20 °C) and about 45 °C (e.g., the normal human microbiota

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and pathogens, *Bacillus* spp., *Escherichia* spp., *Clostridium* spp., *Staphylococcus* spp., and *Lactobacillus* spp.), and hardly grow at low temperatures; thermophiles do not grow at room temperature and have an optimum temperature of growth that ranges between 50 and 80 °C (e.g., *Thermus* spp. and *Geobacillus* spp.; hyperthermophiles, with growth ranges between 80 and 110 °C) and cold-adapted microbes which include (i) psychrophiles (which can grow at 0 °C, and have an optimum growth temperature near 15 °C and do not grow at 20 °C; e.g., *Marinobacter* spp., *Flavobacterium* spp., *Arthrobacter* spp.), and (ii) psychrotolerant or psychrotrophs (that can grow at 4 °C and have an optimal growth temperature above 20 °C; e.g., *Pseudomonas* spp., *Hymenobacter* spp., and the pathogens *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*).

Mesophiles are the most studied microbes, probably because all human pathogens belong to this group, followed by microbes with biotechnological potential uses such as those involved in fermented foods. However, most of the terrestrial biosphere (85%) has low temperatures, remaining below 5 °C throughout the year. Some terrestrial ecosystems are permanently frozen, or thaw for only a few weeks in summer, while 90% of the oceans are at temperatures below 5 °C. Cold ecosystems include deep oceans, cold lakes, snow, permafrost, sea ice, glaciers, cold soils (mainly subsoils), cold deserts, caves, and areas at elevations greater than 3000 m [4]. What types of microorganisms predominate in cold marine and terrestrial environments? Based on several reports, we can state that psychrophilic microorganisms prevail in permanently cold environments (< 5 °C); whereas, in terrestrial environments, with great temperature fluctuations, psychrotolerant microorganisms predominate [5]. Thus, cold-adapted microbes are present in most ecosystems.

Why is it important to study the mechanisms of cold adaptation in microorganisms?

We will mention a few reasons. Although most microorganisms do not multiply below 4 °C or do it at a very slow rate below 10 °C, the so-called emerging pathogens multiply at refrigeration temperatures or close to 0 °C. The most prominent cold-adapted pathogens are *L. monocytogenes*, *Y. enterocolitica*, and *A. hydrophila*, and they are involved in foodborne diseases (mainly of gastrointestinal etiology) such as Yersiniosis and Listeriosis [6]. Understanding how these microbes deal with low temperatures may be useful to control these diseases and for cold chain logistics.

Let's remember that global warming leads to a loss of cold environments. Understanding the effects of this disturbance on organisms can give clues for predicting the influence of climate change on their abundance, distribution, and ecology. In this regard, global warming implies a loss of microbial diversity in cold environments, including the poles and snowy peaks. Their biological activities sustain the nutrient flux in these cold environments and contribute to the global biogeochemical cycles. Deciphering the mechanisms involved in cold adaptation will help us to face the loss of diversity.

In addition, cold-adapted microbes and/or their enzymes (adapted to function at low temperatures, with high catalytic constants) are relevant to developing bioremediation strategies and industrial processes, including the use of microorganisms that promote plant growth [7, 8]. Thus, cold-adapted biological material (microbes or their molecules) has important biotechnological potential.

In this review, we focus on cold-adapted microorganisms and the mechanisms they use to deal with the cold. We will see that some of these mechanisms are also found in eukaryotes; we also included a brief description of cold adaptation in animals.

What modifications are found in membranes at low temperatures? The response triggered by a decrease in temperature

Biological membranes are critical life structures that set the boundary between the interior and exterior of the cell. They are dynamic structures and regulate the exchange of information with the environment. Membranes have selective permeability to molecules and are flexible and self-repairing. These properties are determined by the chemical nature of their components. Besides maintaining cell permeability, the prokaryotic cell membrane plays a key role in cellular respiration, since enzymes associated with this process reside in the membrane. Its destruction leads to cell death, so it must have mechanisms that allow its adaptation to different environmental conditions.

Microbial membranes are organized according to the Mosaic Fluid Model proposed by Singer and Nicolson [9] (1972), where the phospholipid molecules are highly mobile, generating a fluid structure. The lipids of the membranes can be found, depending on the temperature, in two different states or phases: a gel phase and a liquid–crystal or fluid one. When the temperature drops, the liquid-crystalline phase changes to the gel phase in which molecules are tightly packed and show greatly reduced motions. This packed condition also modifies membrane thickness, so fluidity and thickness are linked [10]. The temperature at which this transition occurs (Tm) depends on the lipid composition of the membrane, and changes should take place in order to adapt to a new condition. So, to survive at low temperatures and avoid freezing and death, organisms adapt the composition of their membranes to maintain the liquid crystalline phase. These changes are often referred to as "homeoviscous

adaptation" [11]. At Tm, 50% of membrane acyl chains melt and the liquid-like state and gel phase coincide. When the temperature is below Tm, the acyl chains align perpendicular to the bilayer [12]. To avoid freezing and restore the liquid crystalline state, a series of strategies are embraced: increase in unsaturation and hydroxylation of acyl chains, decrease in acyl chains length (shorter chains), alteration of the proportion of ω-alicyclic fatty acids, increase in the number of branched-chain lipids, the unsaturation of hopanoids, and incorporation of glycolipids, uncommon polar lipids, carotenoids, and other pigments [13–16] (Fig. 1).

The most common adaptation is the incorporation of monounsaturated fatty acids (MUFA), preferably *cis*-unsaturated ones [17]. The incorporation of unsaturations (double bonds) to fatty acids (FAs) is achieved either anaerobically during FA synthesis, or aerobically by modification of existing FAs by desaturation [18–21]. The bacterium *Escherichia coli* is a well-studied example of the former mechanism, while *Bacillus subtillis* exemplifies the latter [18–23]. According to the environmental temperature, the mesophile *E. coli* regulates the fluidity of the membrane by modifying the rate of saturated/unsaturated FA membrane composition. The mechanism depends on a transcriptional repressor (FabR) that balances the production of unsaturated (UFA) and saturated fatty acids (SFA). FabR binds to exogenous FA (UFA or SFA-CoA; CoA is coenzyme A) or endogenous FA (UFA or SFA-ACP; ACP is acyl carrier protein) and to the promoters of *fabA* and *fabB* genes with different affinities. FabA and FabB are enzymes that catalyze particular steps of the biosynthetic pathway of FAs. FabA introduces *cis* double-bonds into the 10-carbon chain of the FAs, and FabB catalyzes the elongation of the intermediate of 10-carbon unsaturated FAs produced by FabA (Fig. 2a). When a certain amount of UFAs is reached, FabR binds to UFAs, increasing the affinity of the transcriptional repressor for the *fabB* and *fabA* promoters, whereas the binding of SFA to FabR does not trigger DNA binding (Fig. 2b) [24].

The phospholipids from the *E. coli* membrane have three different FAs: palmitic acid (16:0), *cis*-palmitoleic acid (16:1 Δ9), and *cis*-vaccenic acid (18:1 Δ11). When the

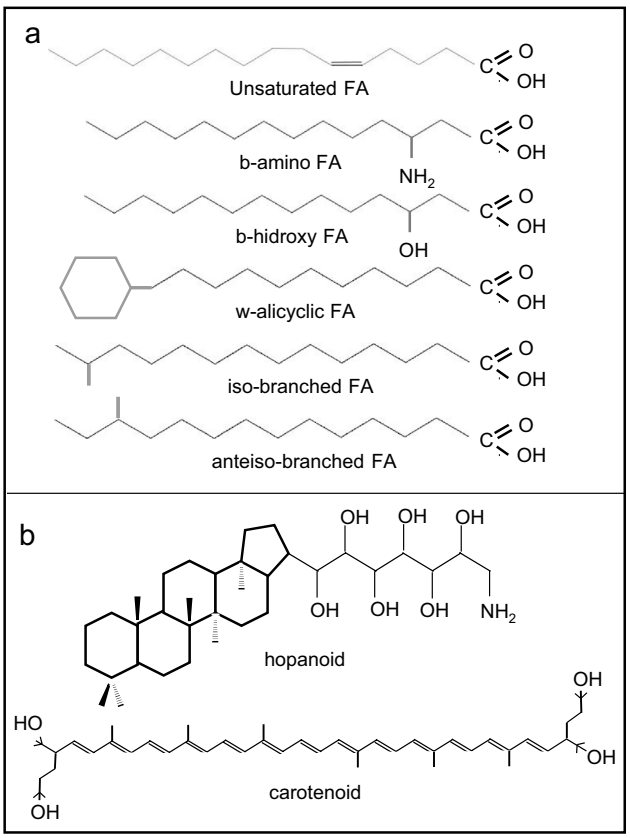

Fig. 1 Chemical structure of some molecules involved in avoiding freezing and restoring the liquid crystalline state of cellular membranes. **a** The structure of some acyl chains and; **b** the structure of carotenoids and hopanoids (a subgroup of triterpenoids)

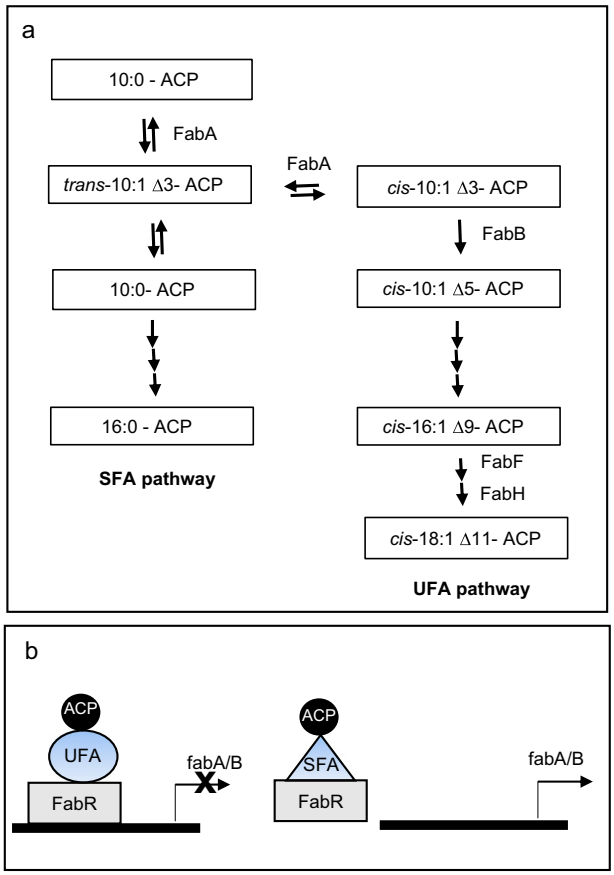

Fig. 2 Production of unsaturated (UFA) and saturated fatty acids (SFA). **a** The pathways for UFA and SFA synthesis in *E. coli* and, some relevant enzymes. FabA introduces *trans* double bonds and also isomerized *trans* to *cis*; FabB elongates the *cis*-FA produced by FabA; FabF and FabH are also elongation enzymes; and ACP means Acyl carrier protein. **b** A simplified diagram of *fabA* and *fabB* transcriptional regulation by FabR

temperature drops, only *cis*-vaccenic acid content increases [25], and it does it quickly. Thus, de novo synthesis is discarded and this quick effect has been attributed to an enzyme (or enzymes) already present at all temperatures of growth. What is more, the overproduction of this FA is independent of translation or transcription activity [26]. The key enzyme in the increase of *cis*-vaccenic acid is FabF (β-ketoacyl-acyl carrier protein synthase II), the enzyme that catalyzes the last elongation step of palmitoleoyl-ACP (16:1 Δ9-ACP) to the keto precursors of *cis*-vaccenoyl-ACP (18:1 Δ11-ACP) [27, 28].

Membrane cold adaptation in *Bacillus subtilis* takes place by introducing unsaturations to existing saturated membrane FAs [29, 30]. The *des* gene is responsible for coding the only FA desaturase found in this microorganism. However, this system represents a backup machinery, since the predominant long-term adaptation to low temperatures occurs via fluidization of the membrane by the introduction of chain branching, the switch from *iso* to *anteiso* branched membrane SFA [13, 23], particularly in the *anteiso* position of methyl groups. This anteiso FA produces a great disturbance in the ordered acyl chains of membrane lipids [31], is species-specific, and depends on the environment. Methyl branching and the introduction of shorter FA chains are dependent on de novo synthesis [16]. *B. subtilis* cells increase and decrease the proportion of lower melting *anteiso*-branched fatty acids and higher melting *iso*-branched fatty acids, respectively. *Iso*- and *anteiso*-branched FAs are derived from valine, leucine, and isoleucine precursors [32]; thus, the occurrence of the branching of FAs in *B. subtilis* depends on the presence of isoleucine in the culture medium [13, 23]. In addition to the results found in the mesophilic microbe *B. subtilis*, studies performed on cold-adapted Antarctic bacteria indicate that the presence of membrane *anteiso*-C15:0 FAs may play a beneficial role in cold adaptation [33].

Also, the introduction of pigments into the cellular membrane is involved in membrane fluidity [15, 33, 34]. The dependence between low temperature of growth and the synthesis of carotenoids and other pigments was observed in several cold-adapted strains such as *Micrococcus roseus* (MTCC 678, IMTECH, Chandigar, India), *Sphingobacterium antarcticus* (MTCC 675, Chandigar, India), *Arthrobacter* species, and *Janthinobacterium* sp. UV13 [35–37].

What pigments are involved in cold adaptation?

Seel et al. (2020) [38] and Alem et al. (2020) [36] found that *Staphylococcus xylosus* strains and *Janthinobacterium* sp. UV13 increased the content of carotenoids and violacein, respectively, after growth at low temperatures, but these pigments were not detectable at 30 °C. An increase in polar holoxantin or zeaxanthin content was found when *Sphingobacterium* strains were cultured at 5 °C compared with 25 °C [35–39]. Seel et al. (2020) [38] also suggested that the introduction of pigments and UFA in membranes has a cooperative effect, but that both strategies are independent cold adaptation mechanisms of the cell membrane.

Are there other molecules involved in membrane adaptation to cold?

Many bacteria synthesize hopanoids (pentacyclic molecules such as bacteriohopanepolyols, among others) to modify membrane properties in a similar way as sterols do it in eukaryotes [40]. Hopanols (the central core of hopanoids) enhance the ordering of lipid chains and modulate phase transitions in membranes [41]. Recent studies on membrane temperature adaptation of the aerobic methanotrophic bacterium *Methylovulum psychrotolerans* showed, for the first time, the importance of the modifications on hopanoid composition [42]. As the growth temperature decreased from 20 to 4 °C, the total percent of unsaturated hopanoids increased from 27 to 49%. Genes that potentially code for hopanoid desaturases were identified in the genome of *M. psychrotolerans* [14] and could be an interesting target to study the relevance of hopanoid production in cold adaptation.

Eukaryotes also adapt their membrane to low temperatures to maintain optimal membrane fluidity. Unfortunately, the molecular mechanisms involved in the adaptation of mammal membranes are still not understood. Brown bears are an interesting example for studying changes in membrane composition during hibernation. Giroud et al. (2019) [43] found that like small heterothermic mammals, bears tend to enrich their membrane phospholipids with unsaturated fatty acids prior to hibernation. They found changes in the major MUFAs and PUFAs (polyunsaturated fatty acids) involved in the functioning of phospholipid membranes and the regulation of membrane fluidity. Poikilothermic organisms (which do not control their body temperature) also incorporate more lipids with poorly packed unsaturated acyl chains as an adaptation strategy at low temperatures. For an excellent review of the membrane adaptation from bacteria to humans see de Mendoza and Pilon (2019) [44].

What about plants?

Oleic (18:1), linoleic (18:2), and α-linolenic (18:3) acids are the most common UFAs in higher plants and act as modulators of cellular membranes [45]. Glycerolipids make up the majority of the membranes and the desaturation of their FAs is involved in the adaptation of some plants to low temperature. Other reported modifications are the decrease in some lipids, such as monogalactosyldiacylglycerol (MGDG), that destabilize membranes [46].

How is a decrease in the temperature detected? The signaling systems in action

Signaling systems help cells to detect extracellular and intracellular variations and to activate adaptive responses. Among the different biological consequences of a decrease in temperature, bacterial membranes become rigid and increase their thickness. This is because, if the hydrophobic regions of proteins and lipids in the membranes do not match, the lipid bilayer stretches or compresses, and/or the proteins change their structure by moving or rotating their helices to compensate for the mismatch between the hydrophobic regions [47]. Indeed, this change in the thickness of the membrane is the signal that sets in motion a series of events that end up in the fluidification of the membrane (by changing the chemical structure of lipids through the introduction of double bonds). Most studies have been conducted using the mesophilic microbes *Escherichia coli* and *Bacillus subtilis* as models, and some considerations can be generalized. Bacteria use a two-component (sensor-regulator) system for the detection of a change in the membrane thickness and to trigger the further response that leads to the activation of the *des* gene (the desaturase that introduces the double-bonds in pre-existing fatty acids).

A two-component regulation system consists of (i) a membrane-bound sensor that senses a specific environmental situation and (ii) a soluble response-regulator that facilitates a cellular response (or the expression of target genes). The sensor has histidine kinase/phosphatase activity (activated/deactivated by phosphoryl-transfer reactions), and activates the corresponding soluble response-regulator, through phosphorylation. When the temperature drops, DesK/DesR is the two-component system involved in membrane adaptation [48, 49]. In response to a decrease in temperature (an environmental fluctuation), the change in the thickness of the membrane acts as a signal that activates DesK, a membrane-bound sensor histidine kinase. All histidine kinases have a highly conserved structure, including an extracellular domain that detects the environmental situation. Unlike most sensor histidine kinases, DesK does not have an extracellular domain [50] (Fig. 3). The DesK sensor is a homodimer. Each protomer contains five transmembrane segments, which compose the sensor domain, and a cytoplasmatic catalytic core (Fig. 3). The last one presents two domains, one in charge of the dimerization between protomers and the histidine-phosphotransferase activity (DHp; also known as 4-HB or four-helix bound), and a second one responsible for the ATP-binding activity [50]. The transmembrane domain is connected to the DHp domain through a linker known as the 2-HCC (2-helix coiled-coil), responsible for transmitting the information from the sensor domain to the catalytic core. DesK dimers are stabilized through hydrophobic interhelix interactions between the DHp domain, and alternate between phosphorylation and phosphatase activity, depending on membrane composition [47].

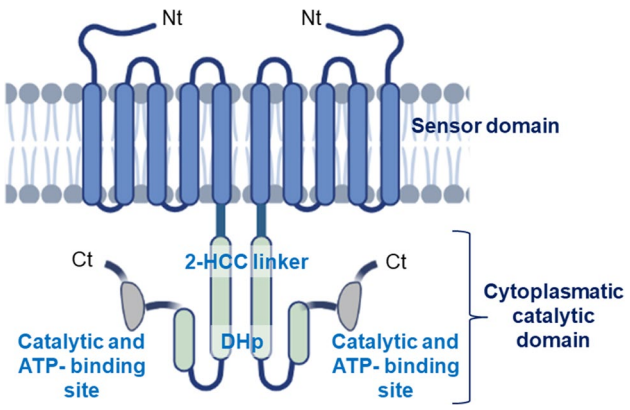

Fig. 3 Representation of the membrane-bound sensor with histidine kinase/phosphatase activity, DesK. The figure shows the two protamers with the five transmembrane segments (sensor domain) and the regions (2-HCC and DHp) that link the sensor domain and the catalytic and ATP-binding domain. Nt and Ct means N-terminal and C-terminal domain

How does the thermo-sensing domain work?

At high temperatures (Fig. 4a), the membrane is thin and, DesK is in the phosphatase state because the N-terminal portion of DHp forms a coiled-coil structure and the ATP binding site is hidden and close to DHp. As the temperature drops, the membrane becomes thicker and DesK switches to its kinase state disrupting of the structure of the DHp domain; the rotation of the helices and the release of the ATP-binding domain occur, promoting the kinase activity [47] (Fig. 4(b1)). In this structural transformation, a few hydrophilic amino acids from the N-terminal transmembrane domain (Lys10-Leu11-Asn12; KLN) are important; this motif of amino acids is called "the buoy". At low temperatures, when the membrane is thicker, the motif keeps buried in the hydrophobic region of the lipid bilayer [50, 51]. Inda et al. (2014) [50] found that, as a consequence of burying the buoy in the membrane, the linker region acts as a transmission switch, changing its conformation. Thus, in response to the change in the position of the buoy, the linker region (2-HCC) adopts two conformations: (i) randomly coiled, interacting with the phospholipid head groups at the water-membrane interface, promoting the phosphatase state, or (ii) forming a continuous helix that spans the membrane into the cytoplasm, promoting the kinase state. There would be a 90° rotation and separation of the signaling helices that form the 2-HCC (linker) that connect to DHp, which drives the

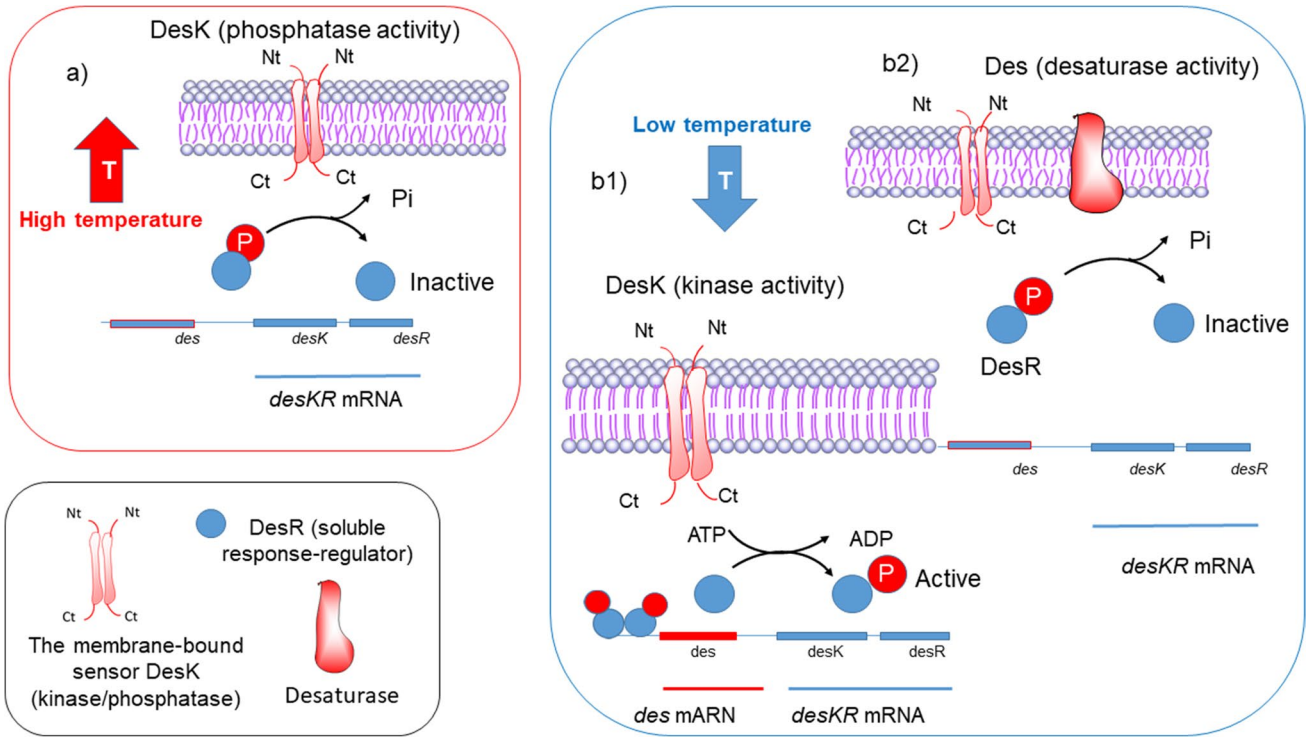

Fig. 4 Sensing temperature; the DesKR two component system. At high temperature, **a** the *desKR* mRNA is expressed; the membrane is in liquid-crystalline phase and DesK shows phosphatase activity; thus, DesR is inactive (non-phosphorylated). At low temperature, (**b1**) *des* mARN and *desKR* mRNA are expressed; the membrane is in gel phase and DesK shows kinase activity; DesR is active (phosphorylated) and acts as transcriptional activator of *des* mARN; Des is active and introduce insaturations in membrane acyl chains. After the introduction of insaturations by Des (**b2**), the membrane goes back to the liquid-crystalline phase; DesK goes back to the phosphate activity and inactivates Des

destabilization of the 2-HCC. But, *what drives the helical rotations?* The disruption of the alpha-helix structure produced by a Pro148 (located in segment 5 of the transmembrane domain) could guide the rearrangements of helices 1 and 5 to drive the 2-HCC conformational change [52].

How is the transcriptional regulator DesR thus activated?

DesK, in its kinase state, transfers a phosphoryl group to a conserved aspartate in DesR (the cognate response-regulator), whose phosphorylated form acts as a transcriptional regulator of *des*, and thereby induces the expression of the desaturase that adds double bonds in the membrane fatty acids, fluidizing the membrane (Fig. 4(b2)). Once the membrane turns to its fluid state, the effect is reversed (DesK in phosphatase conformation). Phosphates bind covalently and reversibly to DesK and DesR, functioning as a chemical mark that changes the functional properties of these proteins by changing their 3D conformation.

Some multicellular organisms can also sense a decrease in temperature through their membranes. In plants, low temperatures likewise produce membrane rigidification and cytoskeleton reorganization, and it is considered the primary event that triggers a downstream cold-stress response. Currently, there is not any available support for the existence of a protein with both kinase and phosphatase activity involved in plant response to cold stress, but some consideration can be done. The decrease in temperature activates a Ca^{2+} uptake system that increases the concentration of Ca^{2+} in the cytosol and as a consequence, several Ca^{2+}-related protein kinases activate. These kinases switch on transcription factors that in turn lead to the activation of CORs (cold-regulated genes) [53]. In addition to Ca^{2+}, reactive oxygen species (ROS) and abscisic acid (ABA), among others, work as second signal messengers that induce a downstream signal transduction cascade (kinases, transcription factors, etc.) to regulate cold-responsive genes and cold tolerance [54].

Even if there is no evidence of the existence of a thermal receptor in the membrane of plants, Cano-Ramirez et al. (2021) [55] recently showed that the *Arabidopsis* plasma membrane fluidity is sensitive to small changes in temperature. *Arabidopsis* has a cytoplasmic cold-responsive receptor kinase1 (CRPK1) that is activated by phosphorylation. Then, it phosphorylates 14–3-3 proteins, which causes their translocation from the cytosol to the nucleus, where they interact

with the c-repeat binding factor (CBFs), which promotes their degradation via the 26S proteasome pathway [53].

A putative sensor was identified in rice. It is a transmembrane protein located in the plasma membrane and the endoplasmic reticulum, known as COLD1 [56]. It was speculated that COLD1, in association with RGA1 (rice G-protein a subunit 1), leads to a phosphorylation cascade that finally activates the cold-induced influx of intracellular Ca^{2+} and also different transcription factors. Zhang et al. (2019) [57] showed that OsCIPK7, a CBL-interacting protein kinase (CBL means calcineurin B-like protein), has a kinase domain that after a conformational change increases its activity and participates in a cascade conferring cold tolerance.

What features are involved in protein adaptation to cold? How do low temperatures affect the kinetic of psychrophilic enzymes?

Now we will face how enzymes deal with the cold, adjusting their structure and thermodynamic activation parameters for activity at low temperatures. All enzymes have a range of temperatures where they are active, but there are certain temperatures where they work optimally. We will present information for answering the following question: *What adaptation mechanisms do they have, compared with homologous enzymes that work better at higher temperatures?*

The native 3D structure of a protein is essential to its function. In this regard denaturation (unfolding: disruption of the quaternary, tertiary, and secondary structures, keeping the primary structure) is a factor to keep in mind, mainly when thinking in the denaturation-stability/activity relationship its relationship with stability/activity.

Thermodynamic activation parameters adaptation

Enzymes are essential catalysts of life processes, controlling the rate of metabolic reactions to the organisms' requirements. Temperature is a critical variable since an exponential decrease in reaction rate is produced as the temperature is lowered. To maintain an appropriate rate of enzymatic reactions at low temperatures, psychrophilic organisms produce cold adapted enzymes with high activity at low temperatures. According to the Arrhenius equation, reaction rates (the number of substrate molecules converted to product per active site, per unit of time, measured by the catalytic constant k_{cat}) increase with temperature and exponentially decrease with activation energy (the energy that must be supplied to the reacting molecules for an effective collision to take place).

Activation free energy ($\Delta G^{\ddagger}$), the energetic barrier between the ground enzyme–substrate complex (ES) and the transition state of a reaction, is composed by two components, as follows:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (1)$$

In this equation $\Delta H^{\ddagger}$ is the activation enthalpy; $\Delta S^{\ddagger}$ is the activation entropy, and T is the absolute temperature. As much lower the barrier of energy ($\Delta G^{\ddagger}$) is, the faster the reaction rate will be (higher k_{cat} value).

What is the relationship between k_{cat} and the activation parameters? According to the transition state theory, this relationship is defined by the following equation:

$$k_{cat} = \kappa\left(\frac{k_B T}{h}\right) e^{\frac{-\Delta G^{\ddagger}}{RT}} = \kappa\left(\frac{k_B T}{h}\right) e^{\frac{\Delta S^{\ddagger}}{R}} e^{-\frac{\Delta H^{\ddagger}}{RT}} \quad (2)$$

In this equation, κ is the transmission coefficient (usually assumed to be ~ 1), k_B is Boltzmann's constant, and h is Planck´s constant. Moreover, by decomposing $\Delta G^{\ddagger}$ in activation enthalpy $\Delta H^{\ddagger}$ and activation entropy (-T $\Delta S^{\ddagger}$), it is evident that as the temperature decreases there is an exponential decrease in the reaction rate caused by the $\Delta H^{\ddagger}$ component. A decrease in $\Delta H^{\ddagger}$ implies a reduction in the number or type of interactions that must be broken for going from the ES (ground state) to the transition state (activated state). All psychrophilic enzymes reported to date present a reduced activation enthalpy and more negative activation entropy than their mesophilic orthologues [58]. So, activity of psychrophilic enzymes is less temperature-dependent than that of mesophilic enzymes, thus maintaining a high reaction rate (k_{cat}) at low temperature.

For enzyme catalysts, the reaction rate exponentially increases with the temperature reaching a maximum value or optimum temperature of activity (T_{opt}) above which the activity decreases. Psychrophilic enzymes have their T_{opt} shifted to lower temperatures and are less stable than mesophilic orthologs [58]. For mesophilic and thermophilic enzymes, the T_{opt} coincides with T_m (melting temperature; when the amount of native and denatured enzyme is equal), and the decrease in activity at temperatures above T_{opt} is generally explained by a thermal inactivation effect, due to protein unfolding. But for most psychrophilic enzymes, this decrease in the enzymatic activity by the increase of temperature occurs before the protein unfolds [59] and this behavior has led to different interpretations.

As an example, let us remind the experiments of thermodependency of the psychrophilic and mesophilic α-amylase on their activities, which showed a maximal catalytic efficiency at 20 °C and 60 °C, respectively [60]. In addition, D'Amico et al. (2006) [61] showed that psychrophilic enzymes are inactivated before losing their 3D structure, probably because their active sites are more labile to

temperature, compared to the entire protein structure, introducing the "localized increase in flexibility" concept. This locally increased flexibility at the active site would allow an enthalpy-entropy redistribution, such as a decrease in $\Delta H^{\ddagger}$ at the expense of $\Delta S^{\ddagger}$ [62]. However, the amino acids in the catalytic cleft remain conserved between the psychrophilic enzymes and the mesophilic counterparts, and there is little evidence supporting that the active site flexibility is linked to the enzyme stability [63].

Reduction of $\Delta H^{\ddagger}$ values in psychrophilic enzymes, to achieve efficient activity at low temperature compared with the values found in mesophilic enzymes, is supported by experimental evidence. It was proposed that a decrease in activity at temperatures above T_{opt} (without thermal inactivation) is due to a rapid equilibrium between two alternative conformations of the active site (an active state, and an inactive state; being the last one native-like but enzymatically inactive). This hypothesis is known as the "equilibrium model" [64]. In this model, the inactive state undergoes subsequent irreversible thermal inactivation.

Another model proposed by Isaksen and co-workers [65] accounts for the redistribution of the enthalpy-entropy of activation values in psychrophilic enzymes. This model was supported by computational experiments and proposes that the structural origin of the enthalpy-entropy trade-off of psychrophilic enzymes is due to an increased flexibility localized at protein surface regions, while the active site residues show identical mobility. The flexible surface of psychrophilic enzymes produces a broader reactant-free energy landscape (greater conformational sampling) that causes a more negative $\Delta S^{\ddagger}$, compared with mesophilic ortholog enzymes. Initial studies were carried out on citrate synthase [66] and similar results were obtained for trypsin [65, 67], triosephosphate isomerase [68], and elastase [69].

An alternative model was proposed by Arcus and co-workers (macromolecular rate theory or MMRT) [70]. It proposes that, for enzymes catalyzed reactions, $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ are temperature dependent. Since enzymes are macromolecular catalysts, with high molecular weight and dynamical structure, they have high heat capacity (C_{p}). C_{p} is the thermodynamic parameter that quantifies the temperature-dependence of the enthalpy and the entropy. Formally, it is the change in internal energy resulting of a change in temperature, and for proteins in a water milieu, the greatest contribution to high values of C_{p} is the number of vibrational modes. Moreover, it was proposed that if the C_p value for the ES complex is larger than the C_{p} for the enzyme-transition state complex ($ES^{\ddagger}$), then the change in heat capacity of activation ($\Delta C_p^{\ddagger}$) is negative (in enzymes for which the chemical reaction is not rate limiting a small positive value of $\Delta C_p^{\ddagger}$ is possible). For a macromolecular system, negative values of $\Delta C_p^{\ddagger}$ reflect that the enzyme weakly binds the substrate but shows tight binding at the transition state.

For psychrophilic enzymes, reported $\Delta C_p^{\ddagger}$ values are more negative than those reported for mesophilic and thermophilic enzymes, suggesting that the magnitude of $\Delta C_p^{\ddagger}$ reflects the evolutionary adaptation of enzymes. The correlation between T_{opt} and $\Delta C_p^{\ddagger}$ was proved for MalL (alpha-glucosidase); MalL mutants with smaller negative $\Delta C_p^{\ddagger}$ showed higher T_{opt} as compared with the wild type MalL [71]. Applying MMRT, the $\Delta C_p^{\ddagger}$ values for ketosteroid isomerase (dimeric enzyme) and MalL (large monomeric enzyme) were experimentally determined and by molecular dynamics simulations in good agreement [72]. Simulations also evidenced dynamic changes between transition state and ES complex, arising through the entire enzyme, small domains surrounding the active site and distant auxiliary domains (MalL) and dimeric unit (ketosteroid isomerase).

Structural adaptations

The psychrophilic enzymes dogma suggests that they show a very unstable and flexible active site and a larger cleft compared to mesophilic enzymes. Crystal structures and homology modeling of psychrophilic enzymes, compared with mesophilic and/or thermophilic homologous enzymes, helped to establish some general structural statements. Although each protein has its own strategy some generalizations can be done. Psychrophilic enzymes showed more accessible catalytic clefts (as a consequence of the replacement of bulky side-chains with smaller groups), a distinct conformation of the loops bordering the active site or small deletions (explaining the heat-labile and cold-active catalytic centers' properties) and, an increase in the internal solvation that contributes to the increase in flexibility [73–76]. On the other hand, all side chains involved in the catalytic mechanism usually remain strictly conserved. Some general characteristics of psychrophilic enzymes are shown in Fig. 5.

The crystal structures of psychrophilic phosphoglycerate kinase (PGK) showed that multiple (global and local) specific adaptations increase the flexibility of the enzyme allowing a better accessibility to the active site, accompanied by the destabilization of some catalytic residues [77]. Therefore, a larger catalytic cleft can accommodate substrates at a lower energy cost. Hence, psychrophilic enzymes are less substrate-specific than mesophilic ones. Comparing the crystal structure of some homologous mesophilic enzymes and their psychrophilic counterparts, Paredes et al. (2011) [74] observed an increase in the amount of carboxylic amino acids and water molecules of the enzyme cavities (embedded with water molecules and present in the interior of the protein) and concluded

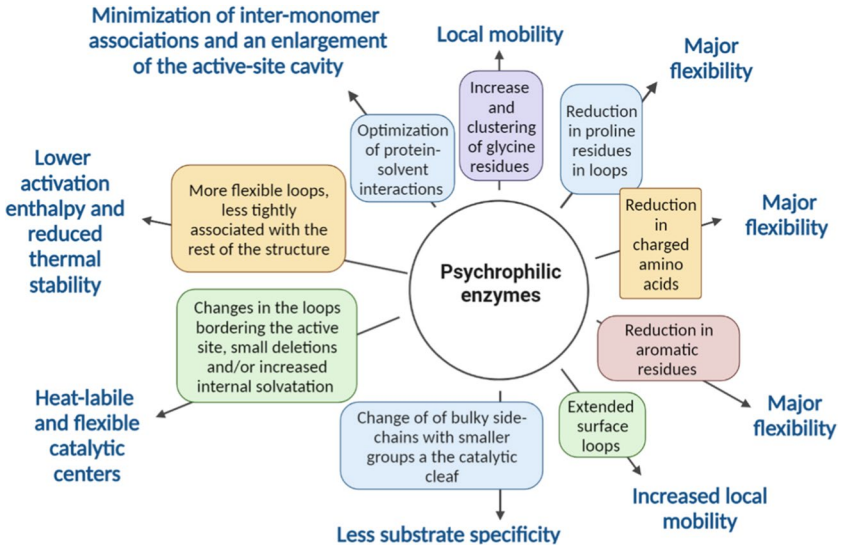

Fig. 5 General features involved in cold adaptation of psychrophilic enzymes

that psychrophilic enzymes tend to be more solvated in the core as compared to mesophilic enzymes. The authors suggested that water molecules may play a significant role in cavity flexibility, and therefore, overall protein flexibility.

We wonder what forces stabilize the tertiary structures of proteins, which may explain the properties described above. Different interactions between the side chains of amino acids build the tertiary structure of a protein; these interactions have different bond energy (ionic or saline, hydrogen bonding, van der Waals and hydrophobic effect, with -1 to -80, -3 to -6, -0.5 to -1, and -0.5 to -3 kcal/moL, respectively). At low temperatures, the molecular motion of proteins is strongly slowed down, thus the improved mobility of the psychrophilic protein structure should be due to an increase in weak interactions (or decrease in strong chemical interactions). Effectively, the activity of a psychrophilic enzyme arises from the disappearance of various stabilizing interactions, resulting in improved flexibility of the enzyme conformation.

Psychrophilic enzymes are also characterized by their thermal-instability. Some amino acid modifications may explain the decrease in thermal-stability, increase in flexibility and increase in k_{cat} of psychrophilic enzymes [78] and can be related to the interactions that stabilize the tertiary structures of proteins. The structure of cold-adapted enzymes could be explained by one or more changes such as the higher content of glycine (which promotes the formation of loops) and neutral amino acids, the lower number of prolines (that disrupts protein secondary structures), arginine/glutamate/aspartate (involved in salt bridges and hydrogen bonds interactions), and aromatic residues (which participate in hydrophobic interactions), as compared with mesophilic or thermophilic enzymes [78]. Glycine and proline are important in protein conformation. Glycine contains a hydrogen atom as its side-chain which imposes an increased conformational flexibility to the protein, while proline, the most rigid residue, can be introduced to rigidify flexible regions of proteins [79]. Glutamate and aspartate are negatively charged at physiological pH and are frequently involved in salt bridge formations, where they pair with a positively charged amino acid such as arginine. Arginine frequently establishes salt bridges and hydrogen-bond interactions. Another general feature of psychrophilic proteins is that they present an increase in the lysine/arginine ratio; although both amino acids function as basic residues, arginine endows the protein structure with more stability than lysine, because it shows a better potential to form hydrogen bonds and saline interactions.

Proteins undergo vibrations and structural rearrangements, the latter being called "conformational transitions." Recently, research employing computer simulation (molecular dynamics; MD) for analyzing the physical movements of atoms has provided information on the differences between psychrophilic and mesophilic enzymes. *What can we learn from these studies?* Visual MD allowed Parvizpour et al. (2021) [80] to perform an analysis of the abundance of salt bridges in the structure of psychrophilic and mesophilic mannanases, performing a simulation from 0 to 100 ns. They found that the psychrophilic enzyme contains fewer salt bridge interactions and longer and abundant cones (graphical summary of the motions in a position), reflecting its highly flexible structure as compared with mesophilic mannanases. Åqvist (2017) [68] examined the average side-chain positional *root mean square fluctuations* (RMSF) of the triosephosphate isomerase from a psychrophilic bacterium identified as *Vibrio marinus* and its yeast mesophilic ortholog. The author determined the RMSF of amino acids that interact with the substrate dihydroxyacetone phosphate (DHAP) at the active site (Asn10, Lys12, His95, Cys126, Glu 165, Ile170, and Leu230), of the DHAP substrate and of the backbone atoms of each amino acid of the

enzyme. For the psychrophilic enzyme, results showed that the overall RMSF for the active site residues is a little smaller than that of the mesophilic yeast enzymes. More importantly, the overall backbone mobility of the two enzymes differs in regions corresponding to some surface-exposed loops; these loops connect secondary structure elements, being higher in the psychrophilic enzyme. These higher flexibility regions show differences in their sequences, particularly amino acid insertions in the psychrophilic enzyme. In general, the works confirm that psychrophilic proteins have more flexible loops which are less tightly associated with the rest of the structure, and again explain why these proteins have lower activation enthalpies and reduced thermal stabilities. In addition, it predicts that evolution utilizes random surface mutations to confer high catalytic activity at low temperatures.

What about proteins with quaternary structure?

Zhang et al. (2021) [81] reported interesting results obtained by comparative MD simulations of mesophilic and psychrophilic serine hydroxymethyltransferases. This enzyme is a dimer and a tetramer in prokaryotic and eukaryotic cells, respectively. The authors found a higher global-flexibility in the psychrophilic enzyme, mainly due to the optimization of protein-solvent interactions which causes the minimization of inter-monomer associations and an enlargement of the active-site cavity, which thus explains the reduced substrate specificity of this enzyme (the active-site cavity is located at the inter-monomer interfaces).

In a few words, some amino acid substitutions may explain the adaptation of psychrophilic enzymes to optimally work at low temperatures: the increase and clustering of glycine residues (providing local mobility), the reduction in proline residues in loops (enhancing chain flexibility between secondary structures), the reduction in arginine, glutamate, and aspartate residues (which are capable of forming multiple salt bridges and H-bonds), and a lower number of aromatic interactions or H-bonds, among others. This is accompanied by the presence of extended surface loops with increased mobility. These structural modifications explain the changes in the kinetic parameters at low temperatures. Psychrophilic enzymes show a larger catalytic cleft that can accommodate substrates at lower energy (less specificity), higher catalytic efficiency (k_{cat}/K_M), and finally a much higher activity at low temperatures, compared with mesophilic homologous enzymes.

What proteins are involved in cellular cold adaptation?

We will next review the identity and function of the main proteins produced during a decrease in temperature, including those involved in the adaptation to water freezing. Again, we will make an emphasis on what is known in prokaryotes, but we will mention examples in eukaryotes.

After a cold shock, microbes enter an acclimation phase. In this phase, which can take up to several hours, a sharp reduction in the rate or speed of growth (or cell multiplication) is observed, as well as a marked reprogramming of protein synthesis. Then, a recovery phase occurs, when the microbial cells change again their protein profile and grow significantly faster. Cells are then considered to be cold-adapted. Finally, cells enter the stationary growth phase, where their gene expression is altered again, but in response to other factors rather than to a change in temperature [82]. The adaptation to a decrease in temperature involves the over- or down-production of proteins (including enzymes) that assist during this stress endurance (a stress endurance for mesophilic/thermophilic organisms). In this regard, some proteins are specifically overproduced during a cold shock: (i) CIPs (cold-induced proteins that are produced transiently) and/or (ii) CAPs (cold acclimatization proteins that are quickly produced and whose levels remain high after the acclimation of cells. CSPs (cold-shock proteins) are a third group of the proteins involved in cold adaptation and can be CIPs or CAPs. They are characterized by the presence of a conserved nucleic acid-binding domain and, as will be shown immediately, are usually "in the eye of the storm" [82, 83]. Interestingly, CSPs from mesophilic organisms act as CAPs, while in cold-adapted bacteria they are constitutively expressed or differentially expressed with changes in temperature [61].

Cold-shock proteins

CSPs belong to a family of proteins which include small polypeptides (65 to 75 amino acids; c.a. 7 kDa) that bind to single-stranded nucleic acids through a cold-shock domain (CSD). CSPs have been found in psychrophilic, mesophilic, thermophilic, and even hyperthermophilic bacteria, as well as in plants and animals (where they present a larger molecular size as compared with microbial ones) [84]. They have been mostly studied in *E. coli* and *B. subtilis* (both mesophilic microbes). *E. coli* possess nine CSPs, although only four are cold-induced: CspA (the main over-produced cold shock protein), CspB, CspG, and CspI. These proteins have overlapping or alternative functions [85], just like the three CSPs identified in *B. subtilis* (CspB, CspC, and CspD) [86].

The presence of the CSD (the five-strand β-barrel) is the common characteristic of this family of proteins. This CSD was found also in the ribosomal protein S1, NusA, PNPase, and IF1 [87, 88], the initiation factor-1a and aIF1 from the archaea *Methanococcus jannaschii*, the eIF1a from *Homo sapiens*, and the Rho terminator factor from *E. coli*, among others [89]. The CSDs have two canonical nucleic acid

binding motifs called RNP1 and RNP2, characterized by the consensus sequences (K/S)G(F/K/Y)G(F/L)IXX and (L/I/V)(F/Q)(V/A/L)HX(STR), respectively [90]. These motifs are located in β-sheets 2 and 3 and contain conserved basic and aromatic residues, which are arranged on one side of the protein and would form the nucleic acid binding surface [89].

What are the different functions of CSPs? Low temperatures induce the formation of RNA secondary structures that can affect cell viability both in prokaryotes and eukaryotes. CSPs function as chaperones that destabilize those secondary structures and maintain a single-stranded molecule. This can facilitate efficient transcription, anti-termination and translation [91], as well as stimulate the activity of RNA nucleases (RNases) that degrade misfolded RNA, and that together with helicases [92] participate in fine tuning the metabolism of RNA. The activities of some CSPs involved in cold adaptation are summarized in Table 1 and are also described more in depth below.

CSPs are also transcriptional activators. In *E. coli* CspE and CspA strongly activate the transcription of *gyrA* and the genes encoding histone-like H-NS proteins, probably due to their effect on stabilizing the open complex formation by RNA polymerase (in addition to a role as transcription anti-terminators) [93–95].

CSPs were first found as over-produced proteins during a cold-shock, but they also have other functions, not related to the adaptation to low temperatures. As an example, CspE is over-produced during nutrient starvation [96] and it is essential for motility and biofilm formation in *Salmonella typhimurium* [97] and *Acinetobacter oleivorans* DR1 [98], and in the attachment, biofilm formation and virulence of the bacterial plant pathogen of grapevine *Xylella fastidiosa* [99]. Interestingly, Zhou et al. (2021) [100] reported that a cold-shock protein (CspL; an RNA chaperone) from a lactate-producing thermophile *Bacillus coagulans* strain, confers high-temperature resistance to *Escherichia coli* and *Saccharomyces cerevisiae*.

In psychrophilic/cold-tolerant microbes, CSPs are constitutively expressed at low temperature, as shown in the psychrophilic yeast *Glaciozyma antarctica* PI12 [101], in cold-tolerant *Janthinobacterium* bacterial strain [102] and *Psychrobacter arcticus* 273–4 [103]. It is accepted that a set of CSPs is constitutively produced, while a different set of CSPs is over-produced under cold stress, as shown in *Pseudomonas* [104, 105].

As we mentioned, CSPs are conserved from bacteria to higher plants and animals. In higher organisms, CSPs exhibit broad functions including a role in growth and development, as shown by Nakaminami et al. [106], who demonstrated that a CSP from *Arabidopsis thaliana* is involved in flowering and silique development. Also animals have CSD-containing proteins, such as the human Unr, an 85 kDa protein involved in the development, as shown in mice. The human *unr* transcript codes for five CSDs. The protein was shown to interact in vitro with RNA, binding to purine-rich consensus sequences with a conserved core motif (AAGUA/G or AACG) downstream of a purine stretch, and is probably involved in cytoplasmic mRNA metabolism [107].

Although a role in cold adaptation has not been described for CSD-containing proteins from plants and animals, there are two examples of animal proteins that respond to cold stress, the cold-inducible RNA binding protein (CIRP) and the RNA binding motif protein 3 (Rbm3). They have one conserved RNA-recognition motif which contains the two RNP1 and RNP2 ribonucleoprotein domains, characterized by the (K/R)G(F/Y)(G/A)FVX(FY) and (L/I)(F/Y)(V/I)(G/K)(G/N)L consensus sequences, respectively. The expression of both proteins increases by cold stress (in

Table 1 CSPs activities in cold adaptation

	Activity*	Organism	Consequence	Reference
Involved in bacterial cold adaptation	Chaperones that destabilize RNA secondary structures	*E. coli*	Facilitate efficient transcription and translation	[91]
			Stimulate the activity of RNA nucleases, for fine tuning the RNA metabolism	[92]
	Transcriptional anti-terminator	*E. coli*, *B. subtilis*	Regulation of the efficiency of RNA production, preventing the transcription termination in functional terminator regions	[86, 93–95]
	Transcriptional regulators	*E. coli*	Activate *gyrA* and the genes encoding histone-like H-NS proteins	[93–95]
		B. subtilis	Activation or repression of genes involved in different cellular functions	[86]
Involved in animal cold adaptation	Regulator of gene expression at the translation level	Hibernating mammals	Activate CIRP that regulates mRNA stability and Rbm3 that enhances global protein synthesis	[108, 109]

*CSPs with activities other than cold adaptation are only described in the body text

hibernating and non-hibernating animals), and they act in regulating gene expression at the translation level, exerting protective effects on cells exposed to low temperature. At least CIRP expression is regulated at the level of pre-mRNA splicing [108]. Interestingly, both proteins are also upregulated in cancer tissues [109]. The most studied human protein containing a CSD is probably YBX1 (Y-box binding protein 1), a basic protein that specifically binds to the Y-box (a *cis*-acting element for expression from some promoters, containing an inverted CCAAT box -ATTGG). YBX1 stabilizes mRNA molecules by an association of its CSD with the cap-structure and destabilizes the interaction with the cap-binding complex eIF4F. For more information regarding this and other CSD-containing proteins from eukaryotes, see Heinemann and Roske [84].

What other proteins are involved in cellular cold adaptation? Ice-binding and anti-freeze proteins

Water is essential for life, but it can also cause cell death. As the temperature decreases, the water turns into ice (formation of crystals inside and outside the cell) affecting its ability to solvate salts and other solutes, thus creating zones of high salinity (osmotic stress, meaning dehydration). As a consequence, it causes damage to cell membranes and cell lysis (cell disruption by mechanical rupture of membranes and macromolecules). Crystal formation has two stages: (i) nucleation from an initial structure or preexisting small crystal, or foreign particle, and (ii) crystal growth (addition of new constituents to the initial nucleus, leading to the formation of crystals). Both stages (nucleation and growth) are driven by supersaturation [110].

How do organisms adapt to water freezing?

They have developed from behavioral (hibernation, migration to warmer areas) to physiological strategies, such as avoiding freezing (fluids are kept in a liquid state) and developing freezing tolerance (controlling the formation of ice). These strategies are used by organisms that live permanently in extreme environments and organisms living in regions where temperatures fluctuate (cycles of freezing and thawing), respectively.

What molecules are involved in the protection of cells against crystal formation?

Ice-binding proteins (IBPs) can be divided in ice-nucleating proteins (INPs) and anti-freeze proteins (AFPs).

AFPs lower the water freezing point, avoiding frostbite due to their thermal hysteresis (TH) and/or ice recrystallization inhibition (IRI) activity. The term thermal hysteresis refers to the temperature difference between the melting and freezing point in a system. IBPs with AFP activities increase thermal hysteresis, and/or inhibit the recrystallization process [111]. AFPs present different degrees of TH and IRI activities, giving rise to different biological roles. Microorganisms use proteins with both mechanisms (TH and IRI). Plants basically avoid freezing through a strategy based on the inhibition of ice crystal growth by producing IBPs with IRI activity. On the contrary, fishes and insects mainly produce IBPs with TH activity; let us remember that the TH is dependent on the concentration and specific characteristics of the IBPs [112].

Several IBPs structures were obtained by nuclear magnetic resonance (NMR) imaging or X-ray crystallography, which showed that they have a great diversity of structures. This information allowed Bar Dolev et al. [113] to perform a phylogenetic tree of organisms that produce IBPs and to assign the structure of the proteins to each branch. The authors clearly showed that IBPs have an independent origin in the tree of life. They have diverse structures (Fig. 6) that go from amphiphilic α-helix proteins [114] to β-solenoid proteins [115], including globular proteins [116]. Let us discuss two examples.

The fungus *Typhula ishikariensis* produces a 223-amino acid AFP that presents a solenoid structure formed by six loops of 18 (or more) amino acids each, that lie alongside an α-helix (22 amino acids). The loops are formed by an irregular rearrangement of β-helix structures that show regularly placed threonine residues. These residues are involved in the binding of the ice via hydrogen bonding of the hydroxyl groups [117]. Most information about IBPs was obtained from microbes, but they are present in all domains of life and are involved in cold adaptation.

The art or the artful of freezing

INPs initiate the formation of ice crystals, catalyzing the arrangement of water molecules to fit into a lattice pattern as they do it in ice. They are glycolipoproteins anchored by phosphatidyl inositol to outer membranes in Gram-negative bacteria [118] or can be released into the milieu [119]. INPs are produced by pathogenic bacteria, being InaZ from *Pseudomonas syringae* the most studied one. It adopts a β-helical structure that interacts with water molecules and imposes a structural ordering; this order increases the melting point of water molecules [120]. *P. syringae* is a plant pathogen that invades the plants through wounds. It produces toxins to suppress host defenses and causes frost damage through the production of InaZ. The ice damages the cell walls of the plant allowing bacteria to gain access to the interior and to use the plant material for nutrients [121].

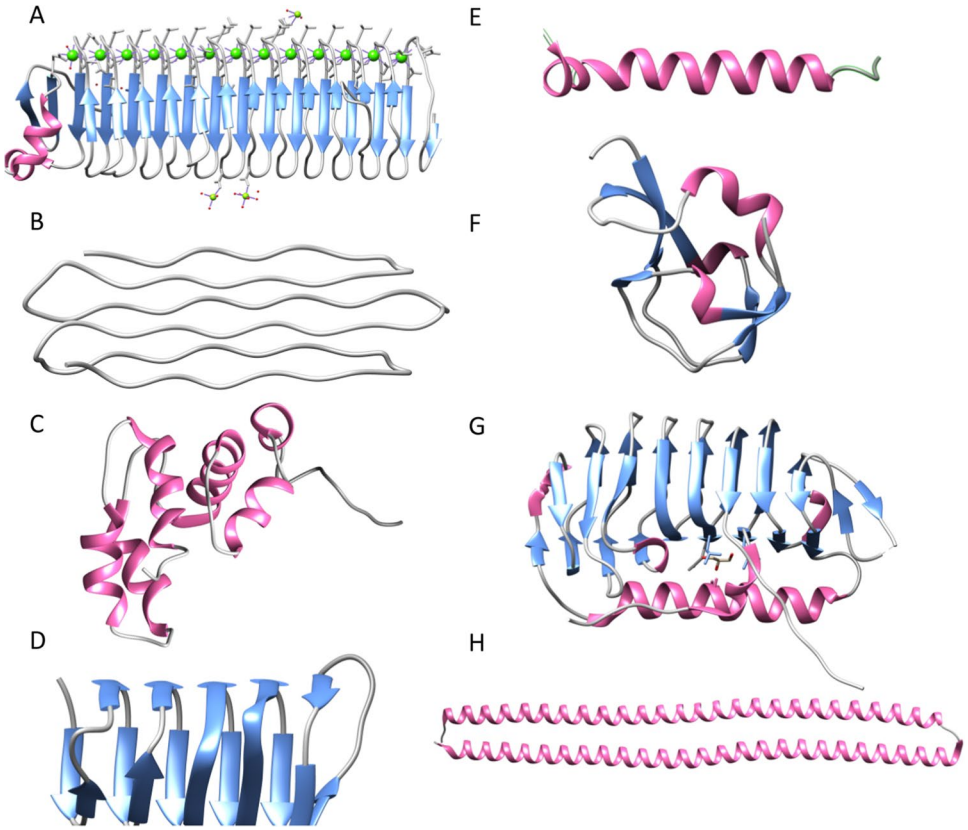

Fig. 6 The diversity of antifreeze protein structures. The figure shows examples of structures of antifreeze proteins produced by **a** *Marinomonas primoryensis* (3p4g.pdb), **b** *Hypogastrura harveyi* (3boi.pdb); **c** *Tenebrio molitor* (1c3y.pdb); **d** *Choristoneura fumiferana* (1l0s.pdb); **e** *Myoxocephalus scorpius* (1y03.pdb); **f)** *Zoarces americanus* (2msi.pdb); **g** *Leucosporidium* sp. AY30 (3uyu.pdb); **h** *Pseudopleuronectes americanus* (4ke2.pdb). This figure was generated using Chimera 1.16 (Goddard et al., 2004)

The structure of InaZ from *P. syringae* (> 120 kDa) was described by Han et al. [122]. The authors analyzed a 240-residue fragment of InaZ and they found it is formed by five 48-residue repeat units mostly composed of β-sheet and random coil, with conserved threonine residues probably involved in water binding. InaZ from *Pseudomonas borealis* is a dimer that increases the active surface area for water binding. It is formed by repetitive units of planar anti-parallel β-sheets (or β-helix) with tyrosine and serine residues in the interface, and the ice-binding motifs SLTA and TQTA on the surface of each side of the dimer [115].

Which strategies lead to the specific induction of cold-shock and cold adaptation genes?

In response to cold-shock, cells use different strategies to specifically induce those genes that will allow the cell to cope with this adverse situation. Some of these strategies are shown in Fig. 7 and will be discussed below.

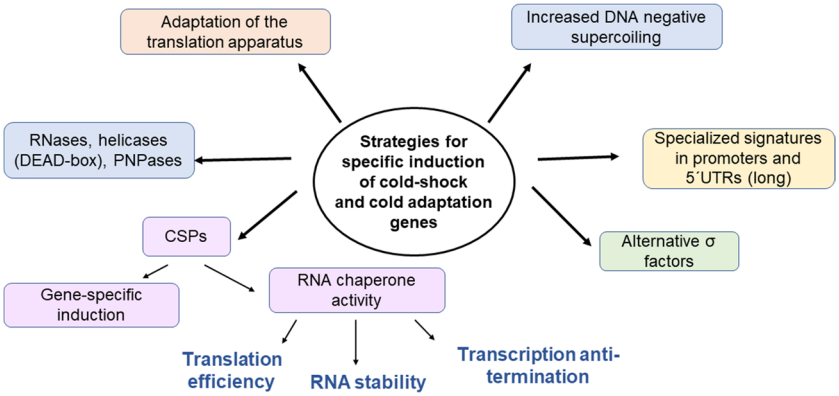

Fig. 7 Mechanisms involved in the induction of cold-shock and cold adaptation genes. See main text for details

Increased DNA negative supercoiling

Temperature fluctuations influence nucleic acid conformation, thus affecting gene expression. Both DNA supercoiling and RNA secondary structure and stability can be altered in response to a temperature drop, and hence cellular mechanisms will be triggered to allow the expression of those genes necessary to maintain cellular homeostasis in these conditions.

DNA supercoiling variations are elicited by almost all kinds of environmental stress [123, 124]. As a consequence, the expression of a number of genes is induced (or repressed). Stress by low temperatures is not an exception. Changes in DNA-negative supertwist at low temperatures occur in *Bacillus subtilis*, *Escherichia coli*, and *Synechocystis* sp. [125–127], among others. As in other types of stress, the involvement of the histone-like protein HU and of DNA gyrase, which introduces negative supercoils in DNA, was suggested for both, *E. coli* and *Synechocystis* sp. [126, 127].

Gene expression variations associated with changes in helical supercoiling could take place through different mechanisms: (i) by modulating the tertiary structure of DNA, which could bring in proximity DNA regions otherwise distant in the genome; (ii) by inducing transitions to alternative secondary structures (local denaturation, or transitions to Z or H form, for example) which could alter particular binding sites; or iii) by affecting the spacer between the − 35 and − 10 elements in promoters, thus altering the ability of σ [70] to bind to certain promoters which are especially sensitive to changes in the relative orientation of these two regions [124] As a particular example, the cold-induced gene *recA* from *E. coli* has a spacer of 16 bp. It was proposed that the increase in the negative DNA supertwist that occurs in response to a temperature drop would place the − 35 and − 10 elements in a more favorable position to induce transcription [128].

Very recently, Forquet et al. [129] signaled that the discriminator element, a G/C-rich sequence located between the − 10 element and the transcription start site, which is denatured during promoter open complex formation, is able to operate as a sensor of supercoiling variations. Hence, it could be predicted that DNA supercoiling could modulate the expression rate of a promoter, depending on the G/C content of its discriminator sequence, and independently of promoter-specific regulatory proteins. The authors observed that a cold-shock increases the negative supercoiling of G/C-rich promoters in *E. coli*, and that these promoters are preferably activated.

Distinctive signatures in identified cold-induced promoters

Relatively few reports have shown the specific induction of transcription by low temperatures from promoters and 5′ untranslated regions (5'UTRs) of cold-responsive genes [130–136]. To our knowledge, the only work that fully discloses the transcriptional activation elements and mechanisms of a cold-induced gene is that of Saita and De Mendoza [47], related to the transcriptional activation of the *des* gene, which encodes for the membrane phospholipid desaturase, involved in membrane fluidity optimization in response to cold (discussed above).

The compilation and analysis of regulatory sequences of cold-induced genes have allowed identifying some sequence signatures important for gene regulation [137, 138]. The role of long 5′UTRs (> 100 pb) in the expression of a number of cold-inducible genes was proposed [137]. Some conserved elements were identified in these regions, which include − 10 and − 35 elements, the UP element, the cold-box, and the DEAD-box (for the characteristics, role and conservation of these elements, see Singh et al. [137] and Faßhauer et al. [86]). Notwithstanding, to our knowledge, the *trans* factors responsible for the recognition of these sequences have not been identified so far.

Alternative sigma factors

Apart from a housekeeping sigma factor, all bacteria have alternative forms, which have been found to respond to environmental signals and redirect RNA polymerase (RNAP) to the promoters of genes necessary for adaptive responses. Österberg et al. [139], Helmann [140], and Rodriguez-Ayala [141] are excellent reviews on the subject. Some sigma factors were implicated in cold adaptation, even though none has been found to be exclusive to this function. Ten sigma factors were identified in *B. subtilis* which regulate the expression of different genes involved, for example, in spore formation or stress adaptation [141]. σ^B (the stress-responsive alternative σ [37]), which regulates the general expression of genes in response to heat-shock, high salinity, ethanol, or nutrient limitation [141–143] was essential for the adaptation of cells continuously growing at low temperatures (15 °C). In these conditions, σ^B-regulated general stress proteins were shown to present a slow and long-lasting induction. This response would be triggered by a signal transduction pathway different from those operating in response to other stress signals [144]. Wiegeshoff et al. [145] reported on the role of σ^L (the sigma factor RpoN or σ [54]) in the cold-shock response. Strains bearing deletions of the gene encoding for this alternative σ, as well as for associated transcriptional regulators BkdR and YplP (previously shown to be linked to the cold-shock response), become cold sensitive. The authors suggest that the association of σ^L with each of these transcriptional enhancers would control a specific adaptation pathway. σ^L/BkdR induce the expression of the *bkd* operon, encoding genes necessary for

membrane adaptation to low temperature. The role of YplP was not yet elucidated.

In the food-borne pathogen *L. monocytogenes*, the expression of the *sigL* gene was induced in cold (but also in the presence of organic acid and by salt stress) and accordingly, the growth of *sigL*-deleted *(DsigL)* strains was impaired under low temperatures. In such a genetic context, the expression of the *oppA* gene significantly decreased. This gene encodes a protein that mediates the transport of oligopeptides and that is required for bacterial growth at low temperatures [146], which suggests a role of σ^L as an activator of *oppA*. More recently, Mattila et al. [147] conducted, in *L. monocytogenes*, a transcriptional comparative analysis to assess the effect of DsigB, DsigL, and DsigBL mutations, which caused growth defects in cold. In those mutants, the number of genes repressed in the cold (3 °C) was significantly augmented with respect to the wild-type strain, when compared to growth at 37 °C. Among them, the authors found genes involved in transcription regulation, energy metabolism, nutrient transport, and viral-associated processes. Some of the genes were co-regulated by σ^B and σ^L, through mechanisms that remain to be studied. The participation of alternative σ factors in the adaptation to cold also was reported for *E. coli* [148, 149] and *Yersinia pseudotuberculosis* [150].

In summary, sigma factors seem to be important actors in the transcriptional regulation of genes induced in response to cold in bacteria, but how general those mechanisms are, still needs to be determined. The transduction pathways which sense the cold signal and activate σ factors are also far from being identified.

CSPs acting on mRNAs

As we mentioned before, low temperatures induce the formation of stable secondary structures in nascent transcripts. These structures, as well as the stability of mRNAs, are of paramount relevance in the regulation of the expression of cold-adaptation genes, both at the level of transcription and translation. In accordance, it is not surprising that many of the cold-induced proteins in *E. coli* have RNA chaperone activity. As aforesaid, this activity stands out among those attributed to CspA. Besides, RNA helicases and exoribonucleases are involved in the degradation of misfolded RNAs, as part of base recycling. Next, we will review in more detail the role of these players at the level of RNA metabolism.

How does the chaperone activity take place? How is the CSP-DNA/RNA interaction?

The interaction involves stacking interplays as follows. Single-stranded nucleic acids bind or interact with a positively charged surface with exposed aromatic residues of CSP; the nucleic bases are oriented toward the aromatic residues of CSP, forming π-π interaction (stacking forces) between the bases and the residues. This stacking weakens the RNA base interactions, thus leading to the "zippering" that unfolds the double-stranded RNA structures [151, 152]. CspA's surface basic amino acids also contribute to its interaction with RNA and to chaperone activity, by compensating for the negative charges on the RNA backbone, thus favoring and stabilizing the formation of CspA/RNA complexes in a sequence-specific way [61, 151].

Due to this chaperone activity, CspA and its homologs can act as anti-termination and anti-pausing factors. Let us remember that the anti-termination activity in prokaryotes occurs when the RNA polymerase ignores the signal of transcription termination, avoiding premature RNA polymerase dissociation. This anti-termination activity can be dependent or independent of the *rho* protein. Bae et al. [93] demonstrated the role of CspA, CspE, and CspC as anti-terminators both in vivo and in vitro, inducing at cold-shock the expression of the *nusA*, *infB*, *rbfA*, and *pnp* genes of the *metY-rpsO* operon, which lay downstream of multiple transcription terminators. The products of those genes are well-known cold-induced proteins. The correlation between the nucleic acid melting ability of CspE and its antitermination activity was demonstrated by Phadtare et al. [94], by using a mutant unable to destabilize secondary structures, which was also unable to induce CspE-induced transcription.

The regulation of CspA itself illustrates different mechanisms which involve mRNA structure and stability. In response to a temperature shift from 37 to 15 °C, the mRNA and protein levels increase, so CspA constitutes almost 13% of the total cell proteins [153]. At 37 °C, even if the gene is well transcribed the mRNA is very unstable and almost undetectable [135, 136]. The 5'UTR of the mRNA of CspA acts as a thermosensor, adopting in the cold a highly stable structure [135, 154, 155] which would be less susceptible to degradation. This structural change also leads to a more efficient translation, since in the mRNA conformation at low temperature the Shine-Dalgarno sequence and the start codon remain exposed [154]. When CspA levels are high, it is also able to down-regulate its own expression by binding to a cold-box, an RNA conserved hairpin motif frequently found among the 5'UTR of the mRNA of CSP genes [86, 137, 156, 157].

The destabilization of RNA secondary structures also has an effect at the level of RNA degradation, by stimulating the activity of RNA nucleases, probably by preventing the formation of refractory secondary structures [91]. However, in other cases, CSPs can stabilize RNAs. This is well exemplified by the action of CspE on poly(A) tails, that by binding to these regions in RNA, inhibits the 3' to 5' exonucleolytic cleavage by polynucleotide phosphorylase (PNPase), and also inhibits internal cleavage by RNaseE [158].

These ribonucleases, and also RNA helicases, are other RNA-acting proteins that have a fundamental role in stimulating RNA degradation at low temperatures. In eukaryotes and eubacteria, these enzymes associate with RNase E in the degradosome, a multi-enzyme complex in charge of RNA turnover and quality control, and in the processing of structured RNA precursors during their maturation [159].

DEAD-box proteins are ATP-dependent RNA helicases found in the three domains of life [160]. In bacteria, the five DEAD-box proteins found in *E. coli* (CsdA, DbpA, RhlB, RhlE, and SrmB) are the best studied [161]. Among these, CsdA and SrmB are involved in cold-shock acclimation, especially the former one. Both have functions in ribosome biogenesis, while functions at the level of translation initiation and degradation of mRNA have also been attributed to CsdA [92, 162, 163]. These proteins would accelerate rearrangements of the highly stable secondary and tertiary RNA structures that arise in the cold when conformational changes can become very slow [164]. CsdA is part of the RNA degradosome of cold-adapted bacteria, taking the place of the RhlB helicase in these conditions [165]. It has been suggested that since CsdA is a "stronger" helicase, it would replace or reinforce the action of RhlB in the cold, when RNA structures are more stable [160]. The regulation of the expression of the CsdA gene (*deaD*) was recently analyzed [166]. The authors report that DeaD is autoregulated through a mechanism presumably involving its mRNA 5' UTR, whose conformation is regulated by its own helicase activity and would then control the access of ribonuclease and Rho.

We have previously mentioned PNPases, which are conserved enzymes in bacteria, involved in the response to different kinds of stress, and that catalyze the 3' to 5' phosphorolytic degradation of RNA and also function as a secondary poly(A) polymerase [167]. The authors showed that PNPases are essential, not only for growth at low temperatures of *E. coli*, *B. subtilis*, and *Y. enterocolitica* [168–170], but also for the degradation of the cold-shock protein mRNAs at the end of the acclimatization phase, and the resumption of growth [92, 170–172]. The expression of the *pnp* gene is subjected to a complex autoregulation process both at post-transcriptional and translation levels, which results in the stabilization and high mRNA-levels during cold acclimation, which involves even the participation of small RNAs [173]. The activity of PNPase is modulated through the interaction with different factors, and by being recruited to different complexes, like the bacterial degradosome [174]. For a comprehensive review of the catalytic activity, regulation, and cellular functions of PNPase, sees Briani et al. [175]. RNase R is another 3' to 5' exoribonuclease induced by cold-shock [176, 177], which has the unusual ability to digest RNAs rich in secondary structure without the aid of a helicase. As demonstrated by Awano et al. [178], RNase R has different domains with separate and independent RNase and helicase activities. In fact, RNase R complements the absence of a functional CsdA. At low temperatures, a high level of RNAse R occurs which is also the result of the stabilization of the levels of the *rnr*-transcript that takes place in these conditions, which is counteracted by PNPase at the end of the acclimation phase [176]. It has also been shown that proteolytic degradation may have a relevant contribution to the control of the turnover of RNase R [177].

Finally, it is also essential to address the general role of RNA secondary structures and its aforementioned protein partners at the level of translation, in response to cold shock and further acclimation. To this respect, Zhang et al. [179] have proposed a mechanism through which CSPs and RNase R carry out the surveillance of bulk mRNA structure and lead to the translation recovery characteristic of the acclimation phase that follows cold-shock. Briefly, together with the genome-wide increase of RNA secondary structure, in response to a descent in temperature CspA and RNaseR expression is induced. As a result, they act together to destabilize highly structured mRNAs, leading to a global increase in translation. The circuitry is finally turned off by the CspA regulatory mechanisms previously described.

Other mechanisms regulating gene expression in response to cold at the translation level

In growth temperatures below 20 °C, the *E. coli* translational apparatus adapts, in order to selectively translate cold shock-induced genes. The mechanisms that contribute to this translational bias are: differential *cis-elements* present in the 5´UTR of up-expressed genes (see above); *trans*-acting factors that bind those *cis-elements* (*e.g.* CspA, which is also up-regulated in the same conditions); and an induced imbalance of the IF1, IF2, or IF3/70S ribosomal subunit ratio [180, 181] for a review see Gualerzi et al. [182]. Besides, the maturation of the rRNA and the assembly of ribosomes suffer a considerable slow-down [183]. This imbalance is proposed to be important to counteract the increased affinity between ribosomal subunits which occurs because of the reduced number of translating ribosomes, and hence ensure that the available dissociated subunits are enough to initiate the translation of specific cold-induced proteins [182].

What about ribosome assembly?

It is worth mentioning that cold adaptation also operates at the level of ribosome assembly, and we will briefly cite a couple of examples. In *E. coli*, the pY protein, which at physiological temperature associates to ribosomes in cells at the stationary phase of growth, is present in ribosomes as a result of a temperature drop. This protein inhibits the elongation of translation by blocking the ribosomal A site,

thus hampering the binding of aminoacyl-tRNA [184] and also by hampering the dissociation of 70S ribosomes [185]. The proposed role for pY is that of a hibernation factor, that "silences" the ribosome, but renders them ready for rapid activation when translation resumes after cold-shock.

Another example is that of RbfA, a cold-shock protein essential for the cells to adapt to low temperature and to render functional ribosomes, due to its role in the processing of the 16S rRNA 5' end during the assembly of the 30S subunit and other stages of the 30S subunit maturation process [186, 187]. It was proposed that the increase in RbfA after a cold-shock is necessary to overcome the translation block that takes place in these conditions, probably because it promotes the production of mature 30S subunits [188]. After 30S maturation, RbfA is displaced by initiation factor 3 (IF3), which promotes translation initiation [189]. Notwithstanding, there is no evidence of the regulation of specific genes as a consequence of differential ribosome assembly in response to low temperature.

What other molecules are involved in cellular cold adaptation?

Not only proteins have a role in adaptation to low temperatures. Two groups of chemical compounds such as compatible solutes and extracellular polysaccharides also have important roles.

Compatible solutes

Some molecules replace water molecules on the surface of biomolecules (water replacement theory) alleviating the effect of ice formation. Among them, compatible solutes are low molecular weight and neutral organic compounds that maintain osmotic balance without interfering with the cellular metabolism. They accumulate after their transport into the cell from the external environment and/or by synthesis. The advantage of the accumulation of compatible solutes is the rehydration of the cell (reduction of water activity, restoration of cell volume, and turgor pressure) without interfering with the cellular functions; they act by stabilizing cellular proteins and membranes at low temperatures [190].

Dawson et al. [191] found that a downshift in temperature affects the Antarctic sea-ice diatom *Nitzschia lecointei*, which responds by increasing the concentration of some compatible solutes, such as glycine betaine (GBT; the most common compatible solutes), dimethylsulfoniopropionate, and proline. Other diatoms such as *Navicula cf. perminuta* and *Fragilariopsis cylindrus*, bacteria, and higher organisms transport and accumulate GBT as an adaptation strategy to freeze-drying [192–195]. The accumulation of GBT is also a common strategy for osmotic stress adaptation [196, 197] but it must be kept in mind that freezing and osmotic stress goes hand in hand.

Free amino acids also act as compatible solutes. Proline accumulates both in prokaryotes and eukaryotes in response to different stresses, including cold stress [198], even though cold stress only produces proline accumulation in plants [199–201] and insects, such as *Drosophila melanogaster* [202]. On the contrary, in bacteria, it is involved in cell protection against thermal stress. In *E. coli* it was demonstrated to suppress the thermo-sensitive phenotype (at 42 °C) of a mutant in the *dnaK* gene, which encodes a critical heat shock chaperone [203]. The authors also showed that proline reduces the denaturation and aggregation of proteins by heat, acting as a chemical chaperone.

Trehalose has a role as compatible solute in cold adaptation both in prokaryotes and eukaryotes [204, 205], too. Due to its unusual ability to alter the water environment surrounding proteins, it has a stabilizing role, by maintaining the protein in its native conformation.

Extracellular polysaccharides

Extracellular polysaccharides (EPS) have functions of protection or adaptation to conditions such as desiccation, salinity, presence of heavy metals, UV irradiation, oxidative stress, low pH, and temperature [206–209]. They are composed of monosaccharides and some of their decorations. These latter are non-carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate), or carbohydrate derivatives such as uronic acids (D-glucuronic acid is the most common one, although D-galacturonic and D-mannuronic acids are also found), and/or phosphate which confers a polyanionic state to the molecule.

EPS from cold-adapted microbes have diverse chemical structures but similar functions such as inhibition of recrystallization. Among other examples, *Colwellia psychrerythraea* and *Psychrobacter arcticus* cells produce an EPS composed of a trisaccharide repeating unit (N-acetyl quinovosamine unit and two residues of galacturonic acid both decorated with alanine) and a mannan EPS, respectively, both with significant inhibitory effect on ice recrystallization [207, 210]. Recently, Kumar et al. [211] reported that the glacier bacterium *Mucilaginibacter* sp. ERMR7:07 produce a linear EPS with a glucose backbone and methylations that provides viability to *E. coli* under freezing conditions. Thus, the EPS produced by a cold-adapted microbe can assist a mesophilic microbe in the cold tolerance. In addition, these compounds have potential biotechnological applications, as discussed by Lo Giudice et al. [212].

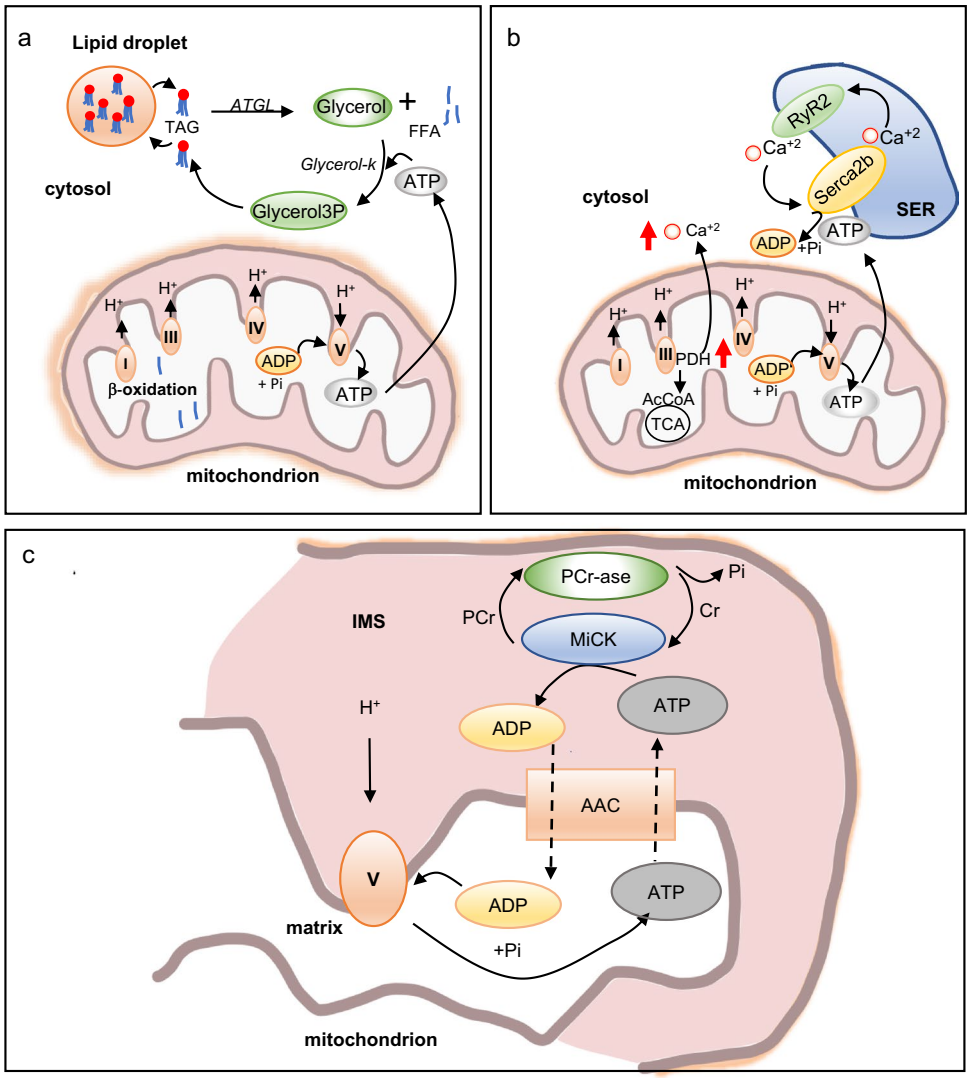

Fig. 8 Futile cycles in brown adipose tissue (BAT) cells. The cells can increase energy expenditure by activating futile cycles; these cycles need ATP derived from mitochondria that remain coupled. This figure shows a schematic vision of the cycles and how they work. **a** Lipid cycling, ATGL: adipose triglyceride lipase; FFA: free fatty acid; glycerol k: glycerol kinase; TGA: triacylglycerides. **b** Calcium cycling, RyR2: ryanodine Ca^{2+} channel; SERCA 2b: sarcoendoplasmic reticulum Ca^{2+} ATPase; PDH: pyruvate dehydrogenase; AcCoA: acetyl-CoA. **c** Creatine cycling, ACC, ATP/ADP carrier; MiCK, mitochondrial creatine kinase; PCr-ase, phosphocreatine phosphatase; IMS, mitochondrial intermembrane compartment

What are the adaptations in eukaryotes?

The biochemical reactions occurring in mammals are optimized to take place in the physiological range of temperature. For most mammals, this temperature ranges from 36.1 to 37.2 °C, with the average human body temperature being 37 °C. Exposition to temperatures lower than the physiological one may affect the rate of reactions, causing a slowdown in the metabolism. To achieve stability, mammals developed mechanisms to regulate body temperature, such as those that involve the neuroendocrine system [213, 214]. However, when the environmental conditions change and the habitat temperature drops, the organisms promote adaptive adjustments to maintain the body temperature [215]. This cold response involves a combination of behavioral and physiological constituents. Animals' curling up into a tight ball is a behavioral response where the surface area exposed to cold is reduced; animals also huddle together to keep warm and to reduce a loss of heat [216, 217]. Likewise, cold weather is rarely a restrictive factor for humans because we have developed a behavioral response of building shelters, using insulation and warm clothing, and increasing our activity [218].

Mammals show two types of physiological responses to cold exposure that can occur alone or in combination: insulative response, and metabolic response or adaptive thermogenesis (namely metabolic energy transformation). The insulating response involves a decrease in the skin temperature, mainly at the extremities; it is caused by vasoconstriction, which restricts heat transfer from the internal organs to the skin and from the skin to the environment. The sympathetic nervous system release norepinephrine from nerve endings, inducing vasoconstriction via α-receptors [219] and thus reducing the loss of convective heat [218, 220].

Table 2 Mechanisms/strategies for cold adaptation (see main text for details)

Adaptation mechanism/strategy	Microorganism or proteins	Reference
Changes in membrane fluidity	*E. coli*, *B. subtilis*	[17–32]
Increased content of pigments in membranes	*Staphylococcus xylosus, Janthinobacterium* sp., *Sphingobacterium antartcticus, Micrococcus roseus*	[13–16, 33–39]
Changes in hopanoid composition	*Methylovulum psychrotolerans*	[14, 42]
Transduction signaling; DesKR	*E. coli*, *B. subtilis*	[48–52]
Structural and thermodynamic adaptation of proteins	Amylase, citrate synthase, trypsin, thriosephosphate isomerase, elastase, alpha-glucosidase, phosphoglycerate kinase, mannase, serine hydroxymethyltransferases	[58, 66, 6, 68–78, 80, 81]
Synthesis of specialized cold adaptation proteins (CIPs, CAPs, CSPs, IBPs)	*E. coli, B. subtilis*, *Pseudomonas syringae Glaciozyma antárctica*, *Psychrobacter arcticus, Typhula ishikariensis, P. syringae*	[61, 82, 85, 86, 91, 92, 101–105, 111–121]
Changes in DNA supercoiling	*E. coli*, *B. subtilis*, *Synechocystis* sp.	[126–130]
Distinctive signatures in promoters	*E. coli*, *B. subtilis*, *Pseudomonas syringae*	[86, 137, 138]
Alternative σ factors	*B. subtilis*, *L. monocytogenes,Y. pseudotuberculosis*	[141–150]
Regulation of mRNA structure and stability	*E. coli*, *B. subtilis, Y. enterocolitica*	[137, 156–158]
Adaptation of ribosomes and translation apparatus	*E. coli*	[180–186, 185, 188]
Accumulation of compatible solutes and extracellular polysaccharides	*L. monocytogenes*, *Vibrio anguillarum*, *Colwellia psychrerythraea*, and *Psychrobacter arcticus, Mucilaginibacter* sp.*	[192, 193, 204, 205, 207, 210, 211]

*Extracted EPS allows increased viability to mesophilic *Escherichia coli* under freezing conditions (− 80 ℃)

What about the metabolic response?

When the temperature drops, mammals increase resting energy expenditure which serves as a source of heat production to counterbalance the loss of it. This response is named adaptive thermogenesis and it is different from obligatory thermogenesis; this last one is related to normal cell functions and organ activity, and it does not attempt to produce heat. Adaptive thermogenesis increases energy expenditure by the so-called shivering thermogenesis, or by non-shivering thermogenesis [218, 221]. When exposed to a cold environment, the body reacts by producing a shivering reflex. How is this shivering generated? The skin detects the cold and sends messages to the thermoregulatory center of the preoptic area, located at the hypothalamus. This preoptic area responds by producing a descending excitatory signal through the hypothalamus and spinal cord, causing shivering as a result of the contraction of the skeletal muscles. Thus, shivering is the involuntary contraction of skeletal muscles induced by exposure to cold and leads to heat production due to ATP-dependent movements [222, 223]. In humans, lipids are the oxidative fuel used during low-intensity shivering; while carbohydrates are the preferred substrate used under severe cold conditions, playing the muscle glycogen an important role during intense shivering [224, 225]. The mammalian body also reacts by inducing piloerection when the arrector pili muscles raise the body hair follicles, making it easier to trap the generated heat [226, 227].

Likewise, non-shivering thermogenesis produces heat from stored energy. This occurs in several systems, including skeletal muscle and, mainly in the brown adipose tissue (BAT). Animals have a thermogenic organ known as BAT that is activated by cold. BAT increases (up to 4–5 times) the resting energy expenditure at a temperature near 4 °C. For a long time, it was thought that BAT disappeared in human adults. However, recent studies (using positron emission tomography and computed tomography scans, along with the tracer 18F-fluorodeoxyglucose) showed that adult humans have BAT and, that this becomes active upon cold exposure [228, 229]. In adult humans, BAT is found in the neck, supraclavicular and thoracic tissue; it is also found in the abdomen at the paraspinal level. This tissue is characterized by marked innervation of the sympathetic nervous system and extensive vasculature [230, 231].

BAT consumes calories to generate heat. Many studies showed that intracellular triglycerides are likely the primary energy source in this tissue and that cold promotes the uptake of triglyceride-rich lipoproteins into BAT [232]. Brown adipocytes regulate energy expenditure through their

numerous and large mitochondria. The inner membrane of these mitochondria has a BAT-specific uncoupling protein 1 (UCP-1), an anion/H + symporter, unique in BAT and beige adipocytes. The beige adipocytes are "brown-like" cells, thermogenically active, that appear in the white adipose tissue (WAT) in response to cold, catecholamines, and other β-adrenergic agonists. UCP-1 allows for the re-entry of protons into the mitochondrial matrix bypassing ATP-synthase. When it is turned on, the uncoupling protein dissipates the intermembrane proton gradient. Thereby, mitochondrial uncoupling is a process that burns up energy by oxidizing nutrients to generate heat, instead of ATP synthesis [233, 234]. Thyroid-releasing hormone, secreted from the hypothalamus in response to cold exposure, stimulates the release of the thyroid-stimulating hormone (TSH), which raises the production of L-triiodothyronine (T3). T3 binds to the nuclear T3 receptor in BAT and stimulates lipolysis and heat production in an UCP1-dependent manner. Furthermore, norepinephrine (NE) secreted in response to cold binds to the β3-BAT receptor (β3-AR) and activates the cAMP-PKA signaling pathway, stimulating lipolysis and thermogenic gene programs in a way also dependent on UCP1 [234, 235].

Brown and beige adipocytes have a very active cellular metabolism as the result of their protein machinery that allows them thermogenic activities. Their oxidative metabolism is regulated by transcriptional factors and co-factors, such as the PR domain containing 16 (PRDM16) and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α). PRDM16 has a critical role in maintaining proper tissue structure and function, and with PGC-1α plus DNA-binding transcription factors cooperate to coordinately induce mitochondrial biogenesis and fatty acid oxidation required by thermogenesis [232, 236]. Therefore, BAT consumes its own lipid reserve when activated in response to cold in a process called non-shivering thermogenesis. Cold also induces the uptake of glucose into BAT; however, most of these molecules are metabolized to lactate or serve for glyceroneogenesis, or eventually de novo lipogenesis (see later) and do not contribute to increased BAT oxidative metabolism [237].

In addition to the UCP1-dependent thermogenesis that involves mitochondrial uncoupling, energy is also dissipated as heat through futile cycles. Recent studies showed that BAT and beige adipocytes can increase energy expenditure by activating futile cycles that need ATP derived from mitochondria which electron transport chain and oxidative phosphorylation processes remain coupled [238–240]. In these cycles, two opposite metabolic pathways work just consuming ATP and releasing the energy as heat; examples are the lipolysis/fatty acid re-esterification cycle, creatine/phosphocreatine cycle, and the SERCA-mediated calcium import and export cycle [241]. These useless cycles are present not only in BAT and beige adipocytes but also in the skeletal muscle [242, 243].

How do these cycles work? Recently, Oeckl et al. [244] showed that the *futile ATP-consuming triglyceride/fatty acid cycle* contributes to non-shivering thermogenesis (NST) in brown adipocytes of UCP1 knockdown mice, allowing survival in the cold (Fig. 8a). The hydrolysis of intracellular triglycerides (TAGs) is critical for cold-induced thermogenesis in human BAT, being the adipose triglyceride lipase (ATGL), the hormone-sensitive lipase (HSL), and the monoacylglycerol lipase (MAGL) the main enzymes involved in lipolysis [241, 245]. TAG hydrolysis releases fatty acids and free glycerol molecules. These glycerol molecules are reused by glycerol kinase in the anabolic pathway, consuming ATP to regenerate glycerol-3-phosphate that can be used to rebuild TAG and resulting in net heat production [246].

Brown and beige adipocytes also respond to cold inducing a *futile calcium cycle* (Fig. 8b). In this cycle, the endoplasmic reticulum (ER) releases Ca^{2+} through the ryanodine Ca^{2+} channel (RyR2), and then it is pumped back by the Ca^{2+}-ATPase (SERCA2b) for maintaining a low cytosolic Ca^{2+} concentration. For the uptake of calcium back to the ER, SERCA2b consumes ATP, so this calcium cycle causes a useless consumption of ATP that leads to energy dissipation and heat production [247]. Additionally, the increased cytosolic Ca^{2+} is also delivered to the mitochondria, triggering the activation of pyruvate dehydrogenase and ATP synthesis which is necessary to sustain this futile cycle [239].

In skeletal muscle, the regulatory protein sarcolipin (SLN) promotes ATP hydrolysis by SERCA1a activity resulting in non-shivering muscle thermogenesis. When SLN is unphosphorylated, it induces conformational changes in SERCA structure which decreases its affinity by calcium; this means that skeletal muscle will consume more ATP to achieve similar levels of calcium transport [248]. Therefore, SLN enhances energy dissipation and heat production in skeletal muscle [242].

The *creatine-dependent ADP/ATP substrate cycle* provides ATP in muscles, and also acts as a futile cycle involved in the thermogenic pathway in the mitochondria of brown and beige adipocytes, dissipating chemical energy as heat (Fig. 8c). This mechanism depends on creatine levels and ATP synthesis; thus, it is linked to mitochondrial respiration and ATP synthase activity [241]. The mitochondrial creatine kinase B (CKB) is a key effector of the futile creatine cycle. CKB catalyzes the phosphoryl transfer from ATP (produced by oxidative phosphorylation) to creatine, yielding phosphocreatine (PCr) and ADP [249]. Otherwise, a phosphatase is required for the hydrolysis of PCr and the regeneration of creatine, allowing thus a new round of the futile cycle. Recently, a phosphocreatine phosphatase activity, which was attributed to tissue-nonspecific alkaline phosphatase

(TNAP), was localized in the mitochondria, where futile creatine cycling occurs [250].

In response to chronic cold exposure, BAT produces heat relying on metabolic adaptations such as lipogenesis, mitochondrial biogenesis, mitophagy, UCP-1 induction. In addition, the involvement of autophagy was also seen more recently [251]. This agrees with previous reports that showed an increase in autophagy during long-term cold exposure [252]. After 72 h of cold exposure, the authors observed the transcriptional induction of autophagy genes that would play an essential role in sustaining mitochondrial integrity and function, which is a requisite for thermogenesis during chronic cold exposure in vivo [251].

In summary, mammals use many mechanisms to face the adversity imposed by cold weather and its adaptive plasticity enables them to live even in extremely cold environments. Furthermore, in the course of evolution, they have acquired a tissue, the BAT, specialized in generating heat and specifically activated by cold. Given the ability of BAT to consume calories for heat production, this tissue, and in general the response of mammals to cold, has become the center of the study of biomedical problems. Thereby, basic research on mammals to cold adaptation could bring clues in the resolution of medical problems such as obesity, diabetes, and cancer.

Concluding remarks

The first step in cold adaptation is the detection of a decrease in temperature. Cellular membranes have an important role in this detection. They become rigid, affecting their normal function. To restore membrane activity the lipid composition changes, increasing the number of unsaturated and eventually short-chain FAs. In microbes, a two-component system is involved in the detection and transmission of the information, followed by the transcriptional activation of a desaturase that introduces double bonds in membrane FAs.

As a strategy for cold adaptation, organisms produce proteins known as CIPs and CAPs, including the well-studied cold-shock proteins, and also may produce antifreeze proteins (to protect themselves from intracellular and extracellular ice), compatible solutes such as trehalose and glycine-betaine (which protect the cell interior from the osmotic environment), pigments (that fluidize the cell membrane), and extracellular polysaccharides (that bind ice and lower the melting point of water, protecting extracellular proteins from the hostile environment). But they also overproduce compatible nutrient and solute transporters, enzymes involved in energy metabolism, as well as enzymes adapted to low temperatures, chaperones that affect the secondary structure of nucleic acids, nucleases, etc.

Adaptation to cold is multifactorial (Table 2 summarizes main mechanisms and strategies found in prokaryotes). The mechanisms activated when adapting to low temperatures are varied, and each one of them fulfills a function. The failure of just one of these mechanisms can lead to a lack of adaptation, even cell death.

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