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Cold-stress response of low temperature adapted bacteria

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Abstract

The psychrotrophic and psychrophilic bacteria that could grow at 0°C or below, populate the cold environments of our planet including the continent of Antarctica. These bacteria are of particular importance in the global ecology since more than 80% of the earth biosphere is permanently or seasonally subjected to temperatures below 5°C. How these bacteria cope with low temperatures induced stress is poorly understood. This review focuses on the cold shock response and adaptive mechanism of the psychrotrophic/psychrophilic bacteria with references from mesophilic bacteria as well as psychrophilic and hyperthermophilic Archeae for which information are now available.

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1. Introduction

Temperature is one of the major determining factors for selection and distribution of microorganisms on our globe. Accordingly, microorganisms that live in extreme temperature niches have gained more scientific attention in the recent past because of their importance in understanding the molecular basis of their adaptation, in addition to their application in biotechnological processes. These microorganisms can be classified according to the range of temperature over which they can grow: hyperthermophiles (temperature range of 80 – 115^{o}C, with optimum growth at >80^{o}C), thermophiles (40 – 110^{o}C, optimum ~60^{o}C), mesophiles (10 – 50^{o}C, optimum ~37^{o}C), psychrotrophs (0 – 30^{o}C, optimum ~22-25^{o}C) and psychrophiles (0 – 20^{o}C, optimum ~<15^{o}C). Among them, thermophiles and hyperthermophiles are capable of growing at very high temperatures, and are found in places like hot springs, volcanic vents under water, and even in industrial chimneys that exhaust out hot airs. Psychrotrohs and psychrophiles, on the other hand, have the ability to grow at very low temperatures, and are found in natural habitats like deep-sea, polar and alpine regions, glaciers, and snowfields. They are even found in our own home refrigerator and super market freezer, where they are notorious for spoiling foods and drinks. Psychrotrophs (some time referred to as facultative psychrophiles) are considered more versatile than psychrophiles (referred to as obligatory psychrophiles) because of their ability to grow not only at freezing temperature, but also at moderate temperature, where mesophiles live. This is the reason why are they so ubiquitous, and more numerous even in permanently cold environments such as Polar Regions [1-5].

Studies on low temperature biology have been dominated mainly by research on multicellular vertebrates and invertebrates, and more recently by works on bacteria and algae from oceans, glaciers, Arctic and Antarctic regions. Among the latter, the Antarctic bacteria have been in the forefront in recent time, due to its usefulness in understanding both cold temperature biology and evolution at molecular level. The organisms also provide us with nonpathogenic model for identifying targets, which could be useful for combating refrigerated food-contaminating pathogenic and food-spoiling bacteria.

2. Experimental models for ‘cold shock’ response study

The basic features, which underlie the cold adaptive physiology of bacteria, are of immense value to human knowledge. However, for a commercial reason, the heat shock response and high temperature biology were well studied [6-7], whereas cellular response to low temperature and cold temperature biology were hardly studied except in the recent past. Most extensive studies on prokaryotic adaptation to low temperature have been conducted on the laboratory workhorse *Escherichia coli*. In this bacterium a downshift of temperature causes a transient inhibition of most protein synthesis resulting in a growth lag called acclimation phase [8-9]. During this phase there is a dramatic induction of a group of proteins called 'cold-shock proteins'. These proteins are perhaps required for low temperature adaptation. A battery of cold-shock proteins, numbering about 22-24 was identified in *E. coli* by two dimensional polyacrylamide gel electrophoresis (2-D PAGE) [8]. Cold-shock responses have also been studied in some details in mesophilic *Bacillus subtilis* [10] and cyanobacteria [11].

The adaptive response of psychrotrophs and psychrophiles to low temperature (0°C - 4°C) at which mesophiles fail to grow is now being addressed in a few laboratories including ours [3-4]. In this pursuit, Antarctic psychrotrophic bacteria serve as good model systems. We have, in our laboratory, established the Antarctic *Pseudomonas syringae* Lz4W as a model organism to understand the molecular basis for cold adaptation, and addressed various aspects of low temperature biology, such as transcription and translation machinery, cold-active enzymes, membrane permeability, temperature sensing and regulation of gene expression [3]. These investigations, along with work done elsewhere, indicate that psychrotrophs adapt to the cold environment by virtue of some unique properties, which were hitherto unknown from other bacteria [3-4, 12-13].

It is important to point out here that psychrotrophs and psychrophiles are represented by bacteria from diverse genera, and therefore likely to have different characteristics, specific to the genera and family they belong to. Only few of them have been studied in detail to decipher the range of features that they might have been used against the common stress factor, i.e., the low temperature. However, the general principle and strategies they have adopted are likely to be similar, as we see the common principle of adaptation in mesophiles when they are subjected to low temperature incubation. Primarily, the problems at low temperature are associated with membrane viscosity and permeability, synthesis of macromolecules (e.g., replication, transcription and translation), and slower metabolic rate due to lower kinetics of enzyme reactions, and the ability to sense and transduce the temperature signals to regulatory net work of cell for effective response (Fig. 1). The details of molecular machinery recruited for solving these problems might however vary, depending on the generic features, and the biochemical pathways and metabolites, which are available to different groups of bacteria. Therefore, in this article, we will first discuss the cold-shock response in bacteria in general to reflect upon the problems of low temperature physiology, especially when they are subjected to low temperature of growth, and then we will discuss the long-term changes that have occurred in bacteria over the course of evolution for growing at, and perhaps 'liking' the low temperature. The former is synonymous with the short-term cold acclimatization process, and the latter is the long-term evolutionary process, or simply adaptation. The terms psychrophiles and psychrotrophs would be used interchangeably, as they have highly similar cold-adapted physiology. Additionally, a brief comparison of ‘cold shock’ response would be made between mesophilic and psychrophilic bacteria and those with psychrophilic and hyperthermophilic Archaea to get an insight of the generality of the problem.

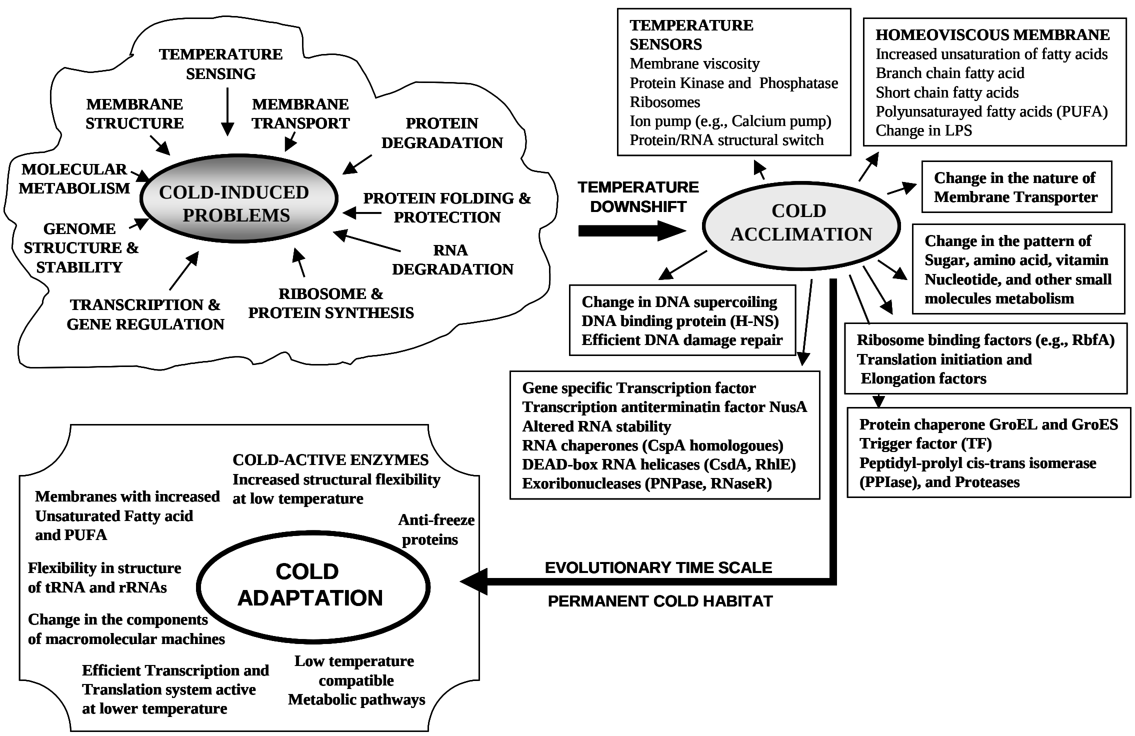

Figure 1. Schematic view of the cold-acclimation and cold adaptive changes of psychrophilic microorganisms.

3. Cold-shock response and acclimatization to low temperature

After a sudden drop of environmental temperature, bacteria face a number of physico-chemical problems that severely affects cellular functions. Immediate cellular response to the problems is to resist the life-endangering influence of low temperature. The sum of all cellular reactions to overcome the problems associated with drop in temperature is called ‘cold shock’ response. The ‘cold shock’ response in microorganisms is a transient phenomenon that affects growth rate of cell, membrane structure and function, and rates of DNA, RNA, and protein synthesis. These have been better documented in mesophiles than in psychrophiles. However, it has been noticed that the responses are similar in both group of organisms, except that the actual temperature which induces them are much lower (0-4^{o}C) in case of psychrophiles than seen in mesophiles (15-20^{o}C).

3.1. Changes in lipids and membrane properties

One of the most well characterized changes that occur in cold-shocked bacterial cell is the change in physical property of membrane, namely membrane fluidity. With the drop in temperature, the cell membrane changes from a liquid crystalline state (disorder or fluid) to gel phase (ordered or rigid). Normally, an optimum fluidity is required for membrane function, which is regained in bacteria by changing the nature of constituting lipids and fatty acids. The process, called 'homeoviscous adaptation' [14], is achieved by one or combination of the following changes in phospholipids: (i) increase in the level of unsaturated fatty acids, (ii) reduction in chain length, (iii) increase in methyl branching fatty acids, and (iv) increase in the ratio of anteiso- to iso-form of branched chain fatty acids.

The biochemical process of introducing these changes may vary between bacteria [15-16]. For example, the unsaturation of membrane phospholipids in *E. coli* occurs by the so-called anaerobic pathway, using β-ketoacyl-ACP synthase II (*fabF* gene product) that converts newly synthesized palmitoleic acid (n-16:1 Δ9-*cis*) to *cis*-vaccenic acid (n-18:1 Δ11-*cis*), which is in turn incorporated into *sn*-2 position of phospholipid backbone, while it is achieved by aerobic pathway, using desaturase enzyme that directly introduces double bond into saturated fatty acids. In mesophilic cyanobacteria (*Synechocystis sp.* PCC6803 and *Synechococcus sp* PCC7942), the membrane bound desaturase introduces double bonds into the fatty acids (mainly at Δ12 and Δ15 (ω3) positions) while these are bound to membrane phospholipids [11]. Low temperature increases activities of the enzyme due to regulation at both transcription and translation level. In mesophilic *Bacillus subtilis* the membrane fluidity following cold-shock is altered both due to increased unsaturation of fatty acids by activity of desaturase (Δ5) and conversion of *iso*-branched to *anteiso*-branched saturated fatty acids. Isoleucine provides the precursors for branched chain fatty acids and regulate the latter activity [17].

3.2. Changes in lipopolysaccharides

Lipopolysaccharides (LPS) are unique component of outer membrane (OM) in the Gram-negative bacteria. These glycolipids are exclusively present in outer leaflet of the asymmetric outer membrane-bilayer, where the inner leaflet is made up of phospholipids, similar to the cytoplasmic membrane. Changes in LPS regulate the properties of OM including permeability, and possibly the transport process by changing the properties of the embedded porin channels. In *E. coli*, the activity of the palmitoleoyltransferase (LpxP), which incorporates a palmitoleoyl moiety into the lipid A is upregulated at low temperature; therefore it has been speculated that incorporation of the unsaturated palmitoeic acid might alter the physical properties of the outer membrane suitable for cellular activity in cold environment [18]. It has been reported that the amount of unsaturated fatty acids in the lipid A moiety of LPS, in general, increases upon downshift of temperature (37^o to 15^oC) in many other mesophilic bacterial species, which might be responsible for maintaining a homeoviscosity of the outer membrane.

Interestingly, in psychrotrophic bacteria such as in the Antarctic *P. syringae*, the amount of hydroxy-fatty acids but not the unsaturated fatty acids increases in lipid A in the outer membrane [19]. Concomitantly, the fluidity of the hydrophobic core of the LPS increases. This suggests a new mechanism by which the outer membrane fluidity can be modulated for functioning at a very low temperature (4^oC) where the mesophiles do not grow. Earlier, it was noted that the amount of phosphates in the LPS changes in Antarctic bacteria, due to a decrease in the phosphate content of 'core' oligosaccharides of LPS during growth at low temperature. It was speculated that the divalent cations (e.g., Mg^{2+} and Ca^{2+}) which bridge the adjacent LPS molecules by binding to phosphate groups on the LPS would also decrease at low temperature [20]. This was found to be consistent with EDTA sensitivity of the cells when Antarctic *P. syringae* was grown at 4^oC. It was suggested that lowering of the divalent metal ion bridges due to lesser amount of phosphate on LPS might affect the packing density of LPS molecules in the outer membrane and OM permeability [20]. The LPS induced changes in the outer membrane is probably responsible for modulating the channel size of OprF in the psychrotrophic *P. fluorescens* [21].

3.3. Change in protein profile

Following cold shock, there is a transient inhibition in the synthesis of most cellular proteins, which is manifested by the lack of cell growth. The extent of protein synthesis inhibition varies in different bacteria. In *E. coli*, there is a total inhibition of house keeping protein synthesis, which is accompanied by transient synthesis of a new set of proteins, which presumably help the cells not only to protect them from cold-induced injury but also help to resume growth by adjusting cellular machinery to slower metabolic rate under low temperature. At least 15 proteins are induced in *E. coli* during the first hour of cold-shock, which are repressed soon to basal level [8-9]. In *B. subtilis* the protein synthesis inhibition is only partial, and at least 75 proteins are synthesized following the cold shock [4, 10]. Two kinds of proteins are mainly synthesized during the growth lag following down shift of temperature: so-called 'cold-shock' proteins (CSPs) or class I protein, whose level increases sharply for short period within the lag phase, and the cold-acclimation proteins (CAPs) or class II protein, the level of which increases gradually to a moderate level and does not fall as fast as CSPs. Examples of the class I protein include cold shock proteins CspA, CspB, CspG, CspI, RNA helicase CsdA, ribosome binding protein RbfA, transcription factor NusA, and exoribonuclease PNPase., while the class II includes histone-like protein H-NS, recombination protein RecA, DNA gyrase subunit A, translation initiation factor IF2α, trigger factor (TF), pyruvate dehydrogenase subunit E1, and dihydrolipoamide dehydrogenase [8-9, 22]. Thus, there is a distinct change in protein profiles of bacteria during acclimatization to lower temperature of growth.

Following a growth lag, the cold-shocked cells resume multiplication, transcription and translation of housekeeping genes, and continue the synthesis of CAPs for some more time before reaching to the steady state level of growth. The growth rate at low temperature is slower, and hence all the metabolic activities are adjusted to the new rate. In fact, global analysis of protein profiles (proteome) in few bacteria suggests a change in large number of proteins in cell at low temperature, and the pattern of change is more complex than the induction of 1-2 dozen of new proteins, the understanding of which, in relation to low temperature biology is only beginning to unfold [23-25].

Table 1 enlists the CSPs and CAPs that have been identified in *E. coli*, and their putative functions in cell. It would appear from the list that the proteins are of diverse type, and involved in diverged cellular functions. Among them, the CspA family of proteins constitute the most common type of ‘cold shock’ proteins in bacterial species. They are small acidic proteins with molecular mass ~7.0 kDa. The first identified member of the family is CspA from *E. coli*, whose homologues were subsequently identified from several bacteria [4, 8, 10]. CspA homologues, in fact, is the most conspicuous group of protein at low temperature in any bacteria, which could constitute more than 10% of total cellular proteins in *E. coli* upon cold-shock. Among the nine homologues, only four (CspA, CspB, CspG and CspI) are cold inducible in *E. coli* [9, 23]. In Gram-positive bacterium *B. subtilis*, CspB of the three homologues (CspB, CspC, and CspD) is cold inducible [10, 25]. This suggests that there is a functional redundancy, as well as probable division of labour among the members of the group. In fact, *E. coli* with only quadruple deletion but not double or triple deletions of *cspA*, *cspB*, *cspG*, and *cspI* genes are cold sensitive [23].

CspA homologues are widely distributed in all psychrotrohic bacteria including those from Antarctica [26]. In the Antarctic *P. fluorescence*, the *cspA* is induced within 30 minutes when the cultures are shifted from 22^{o} to 4^{o}C, suggesting that the psychrotrophic bacteria are also subjected to ‘cold stress’, albeit at a lower temperature [3, 26]. Similar results were obtained with another psychrotrohic strain *P. fragi*, a refrigerated food spoiling bacterium [4].

Functionally, the low molecular-mass Csp group of proteins bind ssDNA and RNA, and have a closed β-barrel structure comprising five antiparallel β-strands [27]. The structure is homologous to the cold shock domain (CSD) of many eukaryotic proteins of diverged function. The conserved aromatic amino residues on the β-strands are involved in binding RNA, with specificity towards 'CCAAT' or 'ATTGG' sequence. Although exact function is yet not clear, most evidences suggest that the proteins work mainly as RNA chaperone by melting RNA secondary structures, which leads either to preferential translation of cold-inducible mRNA at low temperature, or to translation repression in others [27-28].

Cold shock protein (CSP) and Cold acclimation proteins (CAP) include many diverged kind of proteins. Their functions (Table 1) have been shown grouped although broadly under three categories, a close look would suggest that the genes are involved in all major aspects of cellular metabolism that are affected by low temperatures.

3.4. Translation machinery and protein synthetic capacity at low temperature

Immediate effect on bacterial cells upon shift to low temperature is dramatically manifested by inhibition of protein synthesis, presumably due to blockage at the initiation phase of translation, and stabilization of secondary structures of mRNA. Inhibition of translation is reflected by accumulation of 70S monosomes and concomitant decrease in polysomes. RbfA, a 30S ribosomal subunit associated protein is thought to play a crucial role in relieving the translational block at low temperature in *E. coli*. RbfA is a cold inducible protein, and its deficiency leads to cold sensitive phenotype [29]. The stabilization of mRNA secondary structure, a possible cause for hindrance of translation initiation, and pausing of elongating polysome on mRNA are probably overcome by CspA group of RNA chaperone proteins as mentioned earlier. It has been proposed that CspA binds to nascent mRNA during transcription and thereby prevents the formation of intramolecular hydrogen bonds of RNA chain, and thus facilitates coupling of transcription to translation. The 'DEAD box' RNA helicase, like CsdA probably also play a crucial role by actively unwinding duplex structures of RNA at the expense of ATP. Deletion of *csdA* impairs growth of *E. coli* at 15^{o}C [30]. Although a recent study shows the association of CsdA protein to RNA degrading machinery of the bacterium (see below), the possibility remains that CsdA might also play a role in destabilizing RNA helices for facilitating translation at low temperature. That 'cold shock' leads to modification of translation machinery, whereby the ribosomes can preferentially translate cold-inducible

Table 1. Cold shock proteins (CSPs) and cold acclimation proteins (CAPs) of mesophilic *Escherichia coli*. Data have been compiled from both proteomic and micro-arrays data [22-23]. Gene name for proteins has been shown in italic, within the brackets.

Gene/protein functional class	CSPs	CAPs
1. Proteins related to membrane transport and membrane function	~20 in numbers including aerotaxis receptor (*aer*), maltose transport proteins (*malE, malF, malK, malM*), ribose transporter (*rbsA-D*), amino acid ABC transporter (*ybeJ*), acyl synthase I (*fabB*), etc	~21 proteins including chemotactic receotors and regulators (*tap, tar, cheW, cheY*), flagellar proteins (*flg, fli*), fumerate reductase proteins (*frdA, -B, -D*), hydrogenase proteins (*hybA, -B, -C, E*), nucleoside transorter (*nupG*), outer membrane porin (*ompC*), OM protease (*ompT*), etc
2. Proteins involved in cell metabolism	~39 in numbers including alcohol dehydrogenase (*adhE*), aldehyde dehydrogenase (*aldA*), carbamoyl phosphate synthase (*carA*), PTS system enzyme II, pyruvate kinase and pyruvate oxidase, glycerol kinase, glyceraldehydes 3-phosphate dehydrogenase, malate dehydrogenase, fumerate hydratase, thymidine phosphorylase (*deoA*), uridine phosphorylase (*udp*), trehalose synthetic proteins (*otsA, otsB, treC*), etc	~10 proteins including acetate kinase (*ackA*), asparginase (*ansB*) and aspargine synthase (*asnB*), glycerol-3-P-dehydrogenase (glpB, -C), ferritin, formate C-acetyl transferase (*pflB*), etc
3. Proteins involved in gene expression, RNA and DNA metabolism, protein synthesis, protein folding and degradation	~17 proteins including RNA chaperone CspA group (CspA, CspE, CspG, CspI), DNA binding protein (Dps, H-NS), recombination protein (RecA), DNA supercoiling enzyme (GyrA), transcription factor (NusA), exoribonucleases (PNPase, RNaseR), RNA helicase (CsdA), ribosome binding factor (RbfA), translation initiation factor (IF2α, IF2β), Trigger factor (TF), peptidyl-prolyl-*cis-trans*-isomerase (PPIase), chaperone (GroEL, GroES), etc	~5 proteins including glutathione transferase (*gst*), catalase HPI (*katG*), heat shock protein ClpB, spermidine acetyl transferase (*speG*), type I restriction enzyme EcpKI R protein

mRNAs such as those of *cspA* and others is evidenced by the fact that cell-free extracts prepared from cold-shocked cells of *E. coli* could enhance translation of cspA mRNA *in vitro.*

3.5. Transcription and RNA polymerase

Upon cold shock, *E. coli* exhibits transient inhibition of transcription of housekeeping genes, while the cold-shock genes are induced. However, unlike the general heat shock sigma factor (σ^H or σ^{32}), no specific sigma factor for transcription at low temperature has so far been discovered in any bacteria, including low temperature adapted psychrotroph and psychrophiles. Interestingly, the disruption of a sigma homologue of extracytoplasmic group (σ^E) in the *Photobacterium profundum* (a deep sea bacterium) led to a cold sensitive phenotype, in addition to being barosensitive [31]. Whether σ^E plays a role of cold specific sigma factor in other bacteria has not been tested. Similarly, a suspected role of the stationery phase specific sigma (σ^S) in low temperature specific transcription has never been confirmed.

Specific transcription factors for initiation of transcription of the cold-inducible genes, however, could not be ruled out. For example, DesR was found to be responsible for upregulation of *des* gene that encodes desaturase in *B. subtilis* [32]. Similarly, a 'response regulator' protein (Rer1) of temperature sensing two-component regulatory system (Hik33, Hik19/Rer1) was found to be responsible for upregulation of *des* genes in *Synechocystis* PCC6803 [33]. CspA protein was originally reported to bind the promoter region of cold inducible genes *gyrA* and *hns* in *E. coli* [8]. Transcriptional antitermination factor, such as *nusA*, might also play a role in transcription at low temperature. It is generally believed, without any strong evidence, that the derepression, rather than a positive activation, might be responsible for low temperature induced gene expression in bacteria.

3.6. Proteins associated with RNA stability and RNA degradation

It is now believed that RNA stability rather than new initiation of transcription plays a major role in increasing the level of cold-induced mRNAs at low temperature. For example, *cspA* mRNA although transcribed at 37^{o}C is not detectable at this temperature due to quick degradation, where as at 15^{o}C, the level of *cspA* mRNA increases several fold (>10 folds) due to increased half-life (stability) of the mRNA. Similarly, RNA stability was found to play a major role in the induction of des genes (*desA*, *desB*, and *desD*) at low temperature in the cyanobacterium *Synechocystis sp.* PCC6803 [34]. It has been noticed that the low temperature inducible genes generally contain a 100 to 200 base long 5'-untranslated region (5'-UTR), which presumably plays a critical role in the increased stability of mRNA in addition to efficient translation at low temperature [28].

Interestingly, RNA degrading machinery (degradosome) has recently been found to undergo compositional changes at low temperature. In *E. coli* under normal growth conditions, RNA degradosome is comprised of the endoribonuclease RNaseE, exoribonuclease PNPase, RNA helicase RhlB, and the carbohydrate metabolizing enzyme enolase [35-36]. At low temperature, the RNA helicase CsdA probably becomes a component of degradosome, by replacing RhlB [37]. In the psychrotrophic bacterium *P. syringae*, the RNA degradosome has RNaseE complexed with exoribonuclease RNaseR and the RNA helicase RhlE [38]. It is now believed that recruitment of different components to the degradosome under different environmental conditions including low temperature might modulate the RNA degrading machinery leading to altered half-life of different class of mRNAs in cell. In this context, it is to be noted that PNPase is a cold inducible protein, and *pnp* gene inactivation leads to cold sensitive phenotype in mesophilic *E. coli*, and psychrotrophic *Yersinia enterocolitica* [39]. Recently, it has been found that *rnr* gene encoding RNaseR in *E. coli* is also cold inducible like PNPase, but *rnr*-knockout mutants are not cold sensitive. In the psychrotrophic bacterium *P. syringae*, RNaseR is not cold inducible.

3.7. Protein folding and protein degradation

Normally, cytoplasm is protected against the perils of protein misfolding by two mechanisms: molecular chaperones (which facilitate proper folding) and the protein degradation machinery, which degrades misfolded proteins. Low temperature causes denaturation and aggregation of proteins. Studies suggest that proper folding of proteins, and/or maintenance of quarternary structure of proteins at low temperature might be essential for cold adaptation process [40]. Hsc66, a member of the Hsp70 class of molecular chaperone is cold inducible, and was proposed to act as cold shock molecular chaperone in *E. coli* [41]. The importance of chaperone function in proper folding of proteins and promoting growth at low temperature was demonstrated by the ability of the GroEL and GroES homologues (Cpn60 and Cpn10) from the Antarctic bacterium *Oleispira antarctica* strain RB-8T in supporting growth of *E. coli* at 4^{o}C [42]. Trehalose, which has been proposed to function like a chemical chaperone, was also found to protect *E. coli* cells at 4^{o}C. A mutant of *E. coli* that was unable to produce trehalose died much faster than the wild type at low temperature (4^{o}C) [43]. The cold inducible trigger factor (TF), which has multifunctional activities including peptidyl-prolyl-*cis-trans*-isomerase (PPIase), binding to nascent polypeptides on ribosomes, ability to bind GroEL and enhancing GroEL’s affinity to unfolded proteins, and promoting degradation of certain polypeptides was also proposed to have important role for adaptation to low temperature. Inactivation of the gene for TF resulted in decreased viability of *E. coli* at cold [44]. In the psychrotrophic bacterium *Shewanella sp.* S1B1, a homologue of FKBP family of proteins having PPIase activity has been experimentally shown to play a role in the cold adaptation [45]. Two caseinolytic proteases (ClpB and ClpP) of *Synechococcus sp.* strain 7942 were also found essential for low temperature growth [46]. Thus, protein folding and protein degradation also plays a major role in the cold adaptation process.

3.8. Changes in DNA topology and DNA synthesis

Mesophilic *E. coli*, following cold shock, exhibits a transient inhibition of DNA synthesis. Cold-shock also leads to increased supercoiling of plasmid DNA in *E. coli* and *B. subtilis* [47-48]. It has been proposed that low temperature induced negative supercoiling of chromosomal DNA might facilitate DNA unwinding during replication and transcription at low temperature. The enzyme DNA gyrase, and the histone like protein HU probably play a major role in determining DNA supercoiling state. This is supported by the fact that the *gyrA* gene is upregulated at low temperature, and double mutants of *hupA* and *hupB* encoding Hu proteins are cold-sensitive in *E. coli* [49]. Inactivation of another nucleoid associated DNA binding protein H-NS also results in cold-sensitive phenotype in *E. coli* [50].

In the Antarctic *P. syringae*, supercoiled DNA template was found essential for the *in vitro* transcription of *cspA* gene in the presence of cold-active RNA polymerase at low temperature (0-4^{o}C) [51]. Transcriptional activation of some cold inducible genes in *B. subtilis* was found sensitive to supercoiling of chromosomal DNA. For example, inhibition of DNA gyrase by novobiocin prevented production of unsaturated fatty acids in the bacterium on cold shock. It was speculated that transcriptional activation of *des* gene is dependent on topological state of DNA [17].

3.9. Changes in synthesis and uptake of compatible small molecules

Synthesis and/or transport of compatible solutes, such as betaine, choline and trehalose are also important for growth at low temperature. The expression of trehalose synthetic genes (*otsA*, *otsB*, and *treC*) is induced at low temperature in *E. coli* [43]. The betaine transporting BetP is activated at low temperature in the *Corynebacterium glutamicum*, as is the Gbu protein in *Listeria monocytogenes* [52-53]. The mechanism of activation of the betaine transporting BetP and Gbu in the two bacteria however are different, as shown by their *in vitro* reconstitution experiment in proteoliposomes, and functional activity in *E. coli* membrane. For example, Gbu activity can be induced in

E. coli membrane, the BetP can not be activated in the same membrane by low temperature. Thus, the low temperature activation of a protein could be direct or dependent on membrane lipid composition.

3.10. Upregulation of Type II protein secretion system

Upregulation of Type II protein secretion system at low temperature (12^{o}C) has been observed in the pathogenic bacterium *Legionella pneumophilia*. The *lsp* mutant of the bacterium defective for type II secretion system loses the ability to grow at 12^{o}C [54]. The protein secretion machinery also plays a major role in pathogenesis at low temperature in the *L. monocytogenes*. In general, induction of various transporter proteins at low temperature suggests that transport and secretion of molecules through membrane play significant role in the cold adaptive process.

4. Adaptive feature of cold-adapted bacteria

Apart from the structural and physiological changes that are induced by cold shock, which are important for survival and growth at low temperature, there are additional adaptive features that have been gained by the psychrotrophs and psychrophiles over the million of years of evolution (Fig. 1). These adaptive features enable them to successfully colonize the cold environments of our globe. Those features are discussed briefly in the following section.

4.1. Cell membrane

Cell membranes are in direct contact with environment and, therefore, greatly influenced by environmental temperature. Both the physical structure and the biological function of the membrane are affected by prevalent temperature of the environment. The psychrophilic bacteria possess a higher amount of unsaturated fatty acids in their membrane compared to mesophilic bacteria [10]. Further, shortening of chain length and increased branching were also observed to occur in the fatty acids of phospholipids of cold-adapted bacteria. Additionally, polyunsaturated fatty acids (PUFA) that are more preponderant in psychrophilic/psychrotrophic bacteria also play important role in the membrane activity [16]. A modulation of membrane fluidity in these bacteria is brought about by interaction of phospholipids with ‘rigidifying’ molecules like carotenoid pigments, which are known to be associated with the cell membrane [3, 13]. Cis-trans isomerization of double bonds in the fatty acids modulated by *cis-trans* isomerase (encoded by *cti* gene) is also involved in modulation of membrane viscosity in these bacteria [55]. The *trans*-isomers rigidify the membrane, and help the low temperature adapted bacteria to maintain the appropriate membrane fluidity for growth at a higher temperature.

4.2. Structure of enzymes

Enzymes of psychrotrophs and psychrophiles have evolved features to function at low temperature [56]. The features were mainly deduced from the structures of cold-active psychrophilic enzymes in comparison to their mesophilic counterparts. The following facts emerged: (i) an increased occurrence of charged residues, (ii) decrease in isoleucine content, (iii) reduction in aromatic-aromatic interaction in the hydrophobic core cluster (iv) decrease in arg / (arg+lys) content, (v) occurrence of extended surface loops, and (vi) decrease in the number of proline residues. It is now established that the psychrophilic enzymes are cold-active but heat-labile. However, the temperature optima for enzyme activities are similar to those of mesophilic enzymes [3, 38]. It is believed that the gain of function at lower temperature has probably been at the cost of thermal stability [56-57].

From structural comparison of corresponding enzymes from thermophile, mesophile and psychrophile it is now apparent that there is not a single consistent rule for predicting the activity of enzyme at lower temperature. In most of the cases, the structure of the enzymes is highly conserved, except at the substrate binding and/or active site of the protein. Relative distribution of the charged amino acids in and around the substrate binding sites are generally affected for increasing affinity (K_m) of substrates toward enzymes. Although the overall catalytic activity (K_{cat}) of enzymes decreases at lower temperature, the K_{cat}/K_m value is higher for the psychrophilic enzymes compared to mesophilic or thermophilic enzyme.

4.3. Transcription machinery and gene expression

The ability of psychrotrophs and psychrophiles to survive and to grow in the cold temperature (~0°C) demands a functional transcrptional and translational machinery. Studies on a psychrotrophic DNA-dependent RNA polymerase of the Antarctic *P. syringae* revealed that the enzyme has a modest but consistent transcriptional activity even at 0°C [51]. Further, the enzyme could preferentially transcribe the cold-inducible gene *cspA* of *E. coli* only at lower temperature *in vitro*. Thus, RNA polymerase subunits themselves have modified to catalyze transcription at lower temperature (0°C) during evolution.

The mechanism of induction of cold-specific genes in psychrotrophs and psychrophiles at low temperature is largely unknown. As pointed out earlier, the study with the deep sea bacterium, *Photobacterium sp*. suggests that σ^E homologue regulate growth at low temperature, implying that the extra cytoplasmic sigma factor (ECF) might be involved in the regulation of gene expression at the psychrophilic temperature [31]. It is also believed that *cis*-acting elements in the promoter region of the low temperature adapted bacteria play a significant role for initiation of transcription. For example, the ‘cold box’ sequence ‘CCAAT’ was shown to be responsible for cold-induced expression of the thermolabile isocitrate dehydrogenase gene in the psychrophilic *Vibrio sp*. strain ABE-1 [58]. Our study with the cold inducible histidine utilization (*hut*) operon of *P. syringae* Lz4W suggested that occurrence of a ‘CAAAA’ box at the –10 and –15 site of the promoter might be responsible for 4^{o}C specific expression of the operon [59]. However, no systematic studies have been carried out to establish the general nature of the ‘*cis*’-elements that are utilized by the low temperature growing bacteria in nature.

4.4. RNA processing and RNA degrading machinery

Study of the Antarctic *P. syringae* has given us a new insight into the RNA processing and RNA degrading machinery of cold adapted bacteria. In the Antarctic bacterium the degradosomal complex, as mentioned earlier, is made up of endoribonuclease RNase E, exoribonuclease RNaseR, and RNA helicase RhlE [38]. Selection of the highly processive exoribonuclease (RNaseR) and efficient RNA helicase (RhlE) in degradosome of the cold adapted bacterium is significant. Importance of the RNaseR in cold adaptation can be inferred by the fact that *rnr*-inactivation leads to a cold sensitive phenotype in the psychrotrophic bacterium *P. syringae* (Purusharth, R. I. and Ray, M. K., unpublished observation). It was also noticed that degradosomal complex of the Antarctic bacterium undergo dynamic compositional changes, which might have a physiological significance that needs investigation.

The importance of DEAD box containing RNA helicases in low temperature adaptation can easily be inferred by the fact that RNA helicase is induced by different types of bacteria and Archaea when subjected to low temperature shock [60-64]. This signifies that RNA metabolism is highly influenced by the RNA secondary structures at low temperature.

4.5. Translation machinery

Psychrotrophic translational system has acquired the ability to translate mRNAs at low-temperature (0-4^{o}C) but has retained the capacity to function optimally at 37°C *in vitro*. Though the molecular basis of translation at low temperature is not clear yet, one previous study indicated the presence of certain ribosome-associated protein, which facilitates translation at low temperature [65]. Our data from the Antarctic *P. syringae* also demonstrated that the ribosomes of the bacterium is highly efficient in incorporating ^{14}C-phenylalanine into peptide at 0^{o}C when poly(U) is used as a template *in vitro* [3]. The efficiency of translation at 0^{o}C could be as high as 30% of the maximum efficiency seen at 37^{o}C.

4.6. Flexibility in the structure of tRNA and rRNA

The importance of flexibility in the structure of tRNA during translation was advocated by comparing the base composition of the RNAs from bacteria growing under different temperatures [60]. It was observed that there is bias in the composition of bases, especially in the richness of uracil or modified pseudouridine in tRNAs from low temperature growing organisms. The G/C bias of tRNA was earlier established in these RNAs from organisms that grow at higher temperature. On the other hand, in psychrotrophic bacteria, increased post-transcriptional incorporation of dihydrouridine in tRNA is thought to improve the conformational flexibility of RNA [66].

4.7. Chaperone and protein folding machinery

The protein folding machinery in cold adapted bacteria appears to be the key factor for growing at low temperature. The study of cold-adapted chaperones Cpn60 and Cpn10 from the psychrophilic bacterium *O. antarctica* [42] has established that these GroEL and GroES homologues are key to low temperature growth, and sufficient for making a mesophilic *E. coli* to grow at a psychrotrophic temperature (4^{o}C). A recent study indicates that the mechanism of cold-induced systems failure in *E. coli* is due to cold-induced inactivation of the GroELS chaperonins that fails to refold cold-inactivated Dps, ClpB, DnaK and RpsB proteins [40]. Additionally, several members of FKBP family (FK506-binding protein) that exhibits peptidyl-prolyl-cis-trans isomerase (PPIase) activity also play a role in low temperature adaptation of psychrotrophic bacteria and Archeae [45, 60, 62-64].

4.8. Protein degradation machinery

The importance of protein degradation during growth at low temperature has although been speculated, very little is known of the degradation system from cold adapted bacteria. Temperature dependent change in the proteolytic activities has been demonstrated in the psychrophilic *Arthrobacter globiformis* S155 [67]. However, there are now examples of several low temperature inducible genes for proteolytic enzymes and the components of proteosome, which have been identified by microarray analysis, especially in the Archaea at low temperatures [62-64].

4.9. DNA breakage and repair

The importance of DNA breakage and repair during growth of bacteria at low temperature was not known till recently. Studies from plants and bacteria indicated that the cells experience oxidative damage of DNA due to low respiration rate at lower temperature [68-69]. Our group for the first time demonstrated that in the Antarctic *P. syringae* cells accumulate DNA fragments inside the cells when *recD* mutant of the bacterium is transferred to low temperature (4^{o}C) [70]. The cell death also correlated with this accumulation of broken DNA in the mutant. The *recD* mutants were also sensitive to DNA damaging agents, such as UV and mitomycin C. Since RecD functions in a complex with two other proteins (RecB and RecC) to produce RecBCD complex that is involved in DNA double strand breakage (DSB) repair and homologous recombination in bacteria, it is likely that *P. syringae* cells are subjected to greater DNA damage at low temperature, which are repaired by the RecBCD complex. In the absence of RecD (encoded by *recD* gene) the repair pathway becomes defective and leads to cell death. However, it is not clear as to how general this hypothesis is if one looks at the phenotype of *recD* mutants of *E. coli*, which does not show any growth defect.

4.10. Antifreeze protein

Antifreeze proteins (AFPs) are a structurally diverse group of proteins that decrease the freezing point of cellular water (thermal hysteresis or TH activity) and possess ice recrystallization inhibition (RI) activity. These have extensively been studied in polar fish, insects, plants, and fungi but were thought to be absent in bacteria. Subsequently, AFP activity in cell extracts from few bacterial species were demonstrated. Only recently AFPs have been characterized from few Antarctic bacteria, which include *Marinomonas primoryensis* from Vestfold Hills lake water and *P. fluorescens* and *Moraxella sp* from soil samples [71-72]. These bacterial AFPs show week TH activity but modest RI activity.

5. What is ‘cold’ and how much ‘cold’ is cold: Lessons from cold shock response of thermophiles

‘Cold’ is a qualitative description of temperature, mainly in relation to the optimal temperature for growth. For poikilothermous organisms that cannot maintain its cellular temperature constantly but fluctuate with the environmental temperatures, it is assumed that the comfort zone lies within the optimal range of growth temperature. To a homeothermous organism that maintains constant body temperature, sub-optimal temperature might be a zone of comfort, which is reflected by active migration of the organisms to lower temperature. On the other hand, the psychrophilic Antarctic bacteria that grow at or near zero degree Celsius in nature, might or might not, given a choice, preferred the temperature. Most Antarctic organisms grow optimally at ambient temperature (22-24^{o}C) in laboratory. Therefore, it is an open question as to whether low temperature, such as 0-4^{o}C can be called ‘normal’ or a ‘cold shock’ for the psycrophiles and psychrotroph. To recall, 20-22^{o}C is the ‘cold shock' for mesophiles, such as *E. coli*. Similarly, 60°C, which is a lethal temperature for mesophiles, is a 'cold shock' for the hyperthermophiles, such as *Thermotoga maritima* and *Aquifex aeolicus* [60]. These thermophilic bacteria have the homologous genes for CspA family of protein, IF2, and RbfA protein found in mesophilic and psychrophilic bacteria, which are up regulated when the cultures of these are shifted from 90°C to 60°C.

Cold-shock response of hyperthermophilic Archaea, such as *Pyrococcus furiosus* and *Thermococcus sp* strain KS-1 [63-64], which grow optimally at ~ 95-100°C is interesting. When the cultures of these are shifted to 72°C, a typical three phases of cold-shock response seen in mesophiles are observed: an early shock response (1-2 hr), a late shock response (5 hr), and an adapted response (occurring after many generations at 72°C) similar to acclimatized cells of mesophilic *E. coli* growing at lower temperature of growth (15-20°C). These archaea, as well as mesophilic one, do not have homologs of CspA and RbfA as seen from sixteen sequenced genomes (http:://www.tigr.org). These thermophiles display upregulation of about 55 proteins (17 encoded by hypothetical ORFs) in the early response phase at 72°C, of which CipA and CipB (Cip stands for <u>c</u>old <u>i</u>ndicible <u>p</u>roptein) are the prominent one. CipA and CipB appears to be homologue of oligopeptide-binding protein (OppA) involved in transport of di- or tri-peptides in eubacteria. Inactivation of OppA in the psychrotrophic *Liesteria monocytogenes* causes cold sensitive phenotype [73], suggesting that transport of dipeptides into cell might be crucial as a source for amino acids in both hyperthermophilic achaeon and psychrotrophic *Listeria sp*. Cold (72°C) induced proteins in hyperthermophilic *P. furiosus* also include translation initiation factor (e-IF2-β, PF0481), EF-2 like elongation factor (PF1137), his-tRNA synthetase, anaerobic ribonucleotide reductase (for a possible effect on DNA biosynthesis), DNA helicases, tungstoprotein (WOR-5 and AARE), pyrophosphatase, thr/ser and methionine biosynthetic pathway, and protein degradation machinery [64]. Cold shock response of another hyperthermophilic methanoarchaeon *Methanococcus jannaschii* was also recently studied by transcriptional profiling. Upon cold shock to 65°C, a battery of genes including genes for transcription and translation, RNA helicase, proteases, and transport proteins was found upregulated [63]. An 18 kDa protein with homology to FKBP-type PPIase which might have a role in protein folding at low temperature was also observed.

'Cold shock' response of cold-adapted Archaeon, such as Antarctic *Methanococcus burtonii* (growth <4° to 29°C) and *Methanogenium frigidum* (growth <4° to 18°C), however, appears to be different from the hyperthermophilic Archaeae [60]. For example, the cold adapted archaea possess homologues of cold-shock domain (CSD) containing CspA-like protein (*M. frigidum* has one, and *M. burtonii* has two), and *deaD* homologue encoding DEAD-box RNA helicase, which are upregulated at low temperature. Analysis of the predicted proteins from the genome sequence of two low temperature adapted Archaea also shows higher content of noncharged polar amino acids (e.g., Gln and Thr) and lower content of hydrophobic amino acids, particularly leucine. Modeling of quaternary structure indicated that the solvent accessible area of the proteins contain fewer charged residues, and mostly dominated by Gln, Thr, and hydrophobic amino acids [60].

Interestingly, analysis of tRNA sequence of the cold adapted Archaea suggests that GC content largely remained unchanged and were comparable to mesophilic and moderately thermophilic Archaea. In fact, the positive correlation of a high GC content influencing tRNA stability in hyperthermophilic Archaea was found applicable only to those with optimal growth temperature ~60^{o}C. This might indicate that requirement of flexibility of tRNA in psychrophilic Archaea is probably mediated by other mechanism [60]. However, a recent study indicates that uracil content in 16S rRNA has a highly significant inverse correlation with the optimum growth temperature of thermophileas and psychrophiles, that is lower the growth temperature, higher is the uracil (U) content in the rRNA [74]. The A:U base pair in secondary structure of 16S rRNA is more preponderant than G:U mismatches that in theory could have been thermodynamically more flexible, thus suggesting that 16S rRNA sequence in thermophiles and psychrophiles might have been under a strong thermo-adaptive pressure.

6. Sensing of temperature and transduction of the signal

Temperature-dependent gene expression in bacteria suggests that there is possibly a signal transduction pathway for sensing and regulating the temperature-induced response. Ribosomes was first proposed to be the cellular sensor for temperature [75]. Interestingly, Vigh *et al*. had suggested that membrane fluidity might function as a primary sensor in the cyanobacterium *Synechocystis* PCC 6803 [76]. In our laboratory, we demonstrated that the differential phosphorylation of membrane proteins in Antarctic *P. syringae* might have a role in temperature sensing [77]. Monroy and Dhindsa proposed calcium channel as a temperature sensor in higher plant [78]. We had proposed earlier the possible existence of multiple sensors of temperature and suggested a probable links between them [3]. For example, a change of membrane viscosity due to lowering of temperature could be linked to the activation/inactivation of membrane embedded protein kinase/phosphatase activity, which in turn might change the phosphorylation state of ion channels and ion flux, or any other downstream candidate molecules and their activities for an integrated response. Some of these still remains in the realm of conjecture, and some of them now turns out to be correct.

Recently, membrane-located cold-sensor in bacteria has been identified from *Synechocystis* PCC6803 and *B. subtilis*, where histidine sensory kinase has been shown to function as the sensor [32-33]. The downstream signal transduction appears to be carried out by the cognate response regulators (aspartyl phosphorylated protein) in these bacteria. For example, the cold inducible desaturase (*des*) genes are regulated by the two component regulatory system, DesK/DesR in *B. subtilis* and Hik33/Rer1 in *Synechocystis* PCC6803 for production of phospholipid desaturase enzymes leading to adjustment of membrane fluidity in these two bacteria [17]. The upregulation of DesK or Hik33 or a related kinase Hik19 activities by the change of membrane viscosity is a possible clue to the sensing process of temperature in these bacteria. However, the generality of the system in other bacteria as well as the global nature of regulation by this system are yet to be tested.

7. Concluding remarks and Future perspectives

'Cold stress response of low temperature adapted bacteria' may sound like the olympic game March-past announcement of '…now it is the Indians from India'! It reflects the ambiguity of the term 'Indian'. So does the term 'cold-stress'. Complexity of 'cold stress' physiology that one may encounter in nature owing to the fact that microorganisms, in spite of adaptation to grow in a preponderant temperature of the ecosystem, have the ability to survive and grow in a 'range of temperature' where the lowest, highest and the optimal points are different for different organisms. The Antarctic bacteria that live predominantly in a cold environment, for example, are adapted to grow at a very low temperature (zero or sub zero Celsius), but still exhibit cold-shock response when shifted from a relatively high to low temperatures (e.g., 20-22^o to 4^{o}C). On the other hand, mesophilic bacteria with a growth temperature range of 8 to 42^{o}C shows show 'cold-shock' response when shifted from 37^o to 20^{o}C. Thus, the absolute temperature, such as 4^o or 20^{o}C, does not determine the 'cold'; it is the degree of difference (Δ-value) between the growth temperature and the downshifted temperature gives signal to organism for evoking 'cold-shock' response generating molecules that are required for growing at the lower range of growth temperature. These 'cold-shock response' are also critical for survival and growth at lower temperatures. In past, studies have been carried out mainly with mesophilic organisms, and now it is the turn of 'natives' which has been given attention to find out the adaptive mechanism of psychrotrophic bacteria when subjected to 'cooler' temperatures. This would allow one to identify the common mechanism and the molecules that are necessary for growth at 60^{o}C ('cold' for hyperthermophiles), 20^{o}C ('cold' for mesophiles) or in 4^{o}C ('cold' for psychrophiles), which would in turn led to identify the regulatory elements and regulatory mechanisms that organisms have developed for producing common molecules/strategies for the respective cold temperatures in the course of evolution. This would also lead to identification of the unique adaptive features of different groups of organisms for growing at lower temperatures. Additionally it might shed light as to what determines the temperature range of growth of a given organisms, which at present is not known. From biotechnological point of view, the cold temperatures adapted features of different microorganisms would be useful for adopting strategies to control refrigerated food-spoiling as well as food contaminating pathogenic bacteria. The psychrotrophic harmless bacteria might be useful for expression of temperature labile pharmaceutical products, low temperature fermentation process to avoid contamination, and degradation of environmental pollutants in oceanic or other low temperature ecosystems.

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