

Psychrophilic microorganisms: challenges for life

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The ability of psychrophiles to survive and proliferate at low temperatures implies that they have overcome key barriers inherent to permanently cold environments. These challenges include: reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; protein cold-denaturation; inappropriate protein folding; and intracellular ice formation. Cold-adapted organisms have successfully evolved features, genotypic and/or phenotypic, to surmount the negative effects of low temperatures and to enable growth in these extreme environments. In this review, we discuss the current knowledge of these adaptations as gained from extensive biochemical and biophysical studies and also from genomics and proteomics.

Keywords: cold adaptation; enzymes; genomics; membrane fluidity; psychrophiles

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Introduction

Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and polar regions. Some of these organisms, depending on their optimal growth temperature, are also known by the terms psychrotolerant or psychrotroph (Morita, 1975). Nevertheless, we believe that there is a continuum in temperature adaptation for life with wide or narrow growth temperature ranges depending on the microorganism, and we will use the general term psychrophiles in this review to designate all microorganisms growing well at temperatures around the freezing point of water. This unique property implies that psychrophiles have successfully overcome two main challenges: first, low temperature, because any decrease in temperature exponentially affects the rate of biochemical reactions; and second, the viscosity of aqueous environments, which increases by a factor higher than two between 37 °C and 0 °C. Remarkable adaptations have been observed with some organisms such as *Moritella profunda*, which is a psychropiezophilic organism—a microorganism adapted to cold and living in the deep sea—that shows maximal growth rates at 2 °C and a maximum growth temperature of only 12 °C (Xu *et al.*, 2003). This indicates that at temperatures as low as 2 °C, some enzymes or

supramolecular structures already show an altered conformation that negatively affects the metabolic flux.

The aim of this review is to summarize what we know about the cold adaptation of psychrophilic microorganisms. Obvious targets of the deleterious effects of low temperatures are cytoplasmic membranes and enzymes that tend to rigidify when the temperature drops. This affects membrane permeability, and hence the transport of nutrients and waste products, and catalysis, because enzymes require a certain flexibility to function (Goodchild *et al.*, 2004b; Ratkowsky *et al.*, 2005). Impaired protein folding and protein cold-denaturation can also cause problems at low temperatures, in particular for bacterial strains that sustain biological activities at temperatures as low as –20 °C and resist freezing. Cold-shock proteins have also been described. What are their roles in this adaptation? Key biological activities that involve nucleic acids—such as DNA replication, transcription and translation—can also suffer from exposure to low temperatures through the formation of secondary structures or super-coiled structures; how do psychrophiles cope with these phenomena? Finally, several genome sequences of psychrophilic microorganisms have been determined, and partial annotation of these has revealed unpredicted cold adaptations, the number of which will obviously expand after completion of the analysis and genome sequencing of other psychrophiles.

Biodiversity

The lowest temperature limit for life seems to be around –20 °C, which is the value reported for bacteria living in permafrost soil and in sea ice. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or the ice, and to brine channels. These contain high concentrations of salts, exopolymeric substances and/or particulate matter, and fluid flow is maintained by concentration and temperature gradients. Aerobic and anaerobic bacteria are found at these temperatures. Other factors such as osmotic and hydrostatic pressure, solar, earth and cosmic radiation, oxidative stress and nutrient availability also strongly affect living conditions. Consequently, adaptation to cold is often combined with other adaptations. Despite all of these challenges, life thrives in these environments with a remarkable microbial biodiversity of mainly bacteria, fungi (in particular yeasts) and microalgae. Among the bacteria that have been detected, the most commonly reported microorganisms are the Gram-negative α -, β - and γ -proteobacteria (*Pseudomonas* spp. and *Vibrio* spp.) and the *Cytophaga–Flavobacterium–Bacteriodes* phylum. Coryneforms, *Arthrobacter* sp. and *Micrococcus* sp. are the most frequently found Gram-positive bacteria. Bacteria generally dominate in number

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and diversity over Archaea, although in some areas such as deep-sea waters, these are found in equivalent numbers, with *Methanogenium* and *Methanococcus* being the most cited genera. Among identified cyanobacteria, *Oscillatoria*, *Phormidium* and *Nostoc commune* are dominant in most of the Antarctic habitats (Pandey *et al*, 2004). Psychrophilic yeasts, particularly *Cryptococcus* spp., have been isolated repeatedly from soil samples and some researchers have described them as the most important life form in Antarctic desert soils (Vishniac & Klinger, 1986). Refer to Deming (2002) for a detailed description of the types of community associated with specific cold environments.

Membrane fluidity

Decreasing temperatures have an adverse effect on the physical properties and functions of membranes, typically leading to a reduction in membrane fluidity, the onset of a gel-phase transition and, ultimately, a loss of function. The lipid composition governs the physical properties of membranes and hence it is not surprising that this varies with the thermal habitat of the microorganism. In general, lower growth temperatures produce a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length, with studies reporting a high proportion of *cis*-unsaturated double-bonds and anteiso-branched fatty acids (Chintalapati *et al*, 2004; Russell, 1997). This altered composition is thought to have a key role in increasing membrane fluidity by introducing steric constraints that change the packing order or reduce the number of interactions in the membrane. Further adaptations that have been suggested to increase membrane fluidity include an increased content of large lipid head groups, proteins and non-polar carotenoid pigments (Chintalapati *et al*, 2004). However, these adaptive strategies do not seem to be widespread, and studies show more compact lipid head groups (Arthur & Watson, 1976) and decreased non-polar carotenoid pigment synthesis (Fong *et al*, 2001) in some psychrophiles.

Transcription and translation

Some of the main barriers to protein synthesis at low temperatures include: reduced activity of transcriptional and translational enzymes; reduced protein folding, owing primarily to a reduced rate of prolyl isomerization; and a stabilization of DNA and RNA secondary structures. In psychrophiles, enzymes involved in these processes have adapted to be optimally active at low temperatures. For example, a ribosomal extract, RNA polymerase, elongation factor and peptidyl-prolyl *cis-trans* isomerase have all been shown to retain activity near 0 °C in several psychrophilic microorganisms. Indeed, this latter enzyme catalyses *cis-trans* prolyl isomerizations, and its high activity and overexpression at low temperatures might be important for maintaining protein-folding rates at low temperatures. Furthermore, nucleic-acid-binding proteins—for example, *Escherichia coli* CspA-related proteins—and RNA helicases that might be important for the destabilization of DNA and RNA secondary structures are also overexpressed at low temperatures in psychrophiles (Berger *et al*, 1996; Lim *et al*, 2000).

Cold-shock and heat-shock responses

The exposure of mesophilic organisms to sudden temperature changes, both upshifts and downshifts, induces the transient overexpression of several proteins—known respectively as heat-shock proteins (Hsps) or cold-shock proteins (Csps)—that are involved in

various cellular processes such as transcription, translation, protein folding and the regulation of membrane fluidity (Phadtare, 2004). Although studies of these responses in psychrophilic microorganisms are still in their infancy, similarities with the Csps and Hsps that are induced in mesophiles have been observed. In particular, increased levels of nucleic-acid-binding proteins (for example, CspA-related proteins; Inouye & Phadtare, 2004) and chaperones, such as GroEL (Tosco *et al*, 2003) and DnaK (Yoshimune *et al*, 2005), have been frequently reported. However, distinctions do exist between the mesophilic and psychrophilic cold-shock response, including the lack of repression of house-keeping protein synthesis and the presence of cold-acclimation proteins (Caps) in psychrophiles. Many of the Csps observed in mesophiles act as Caps in psychrophiles, being constitutively rather than transiently expressed at low temperatures. Furthermore, this differential regulation of expression indicates that a temperature sensory system exists in psychrophiles, and thermosensors at the cell membrane level—that sense changes in fluidity—have been reported (Ray *et al*, 1994).

Antifreeze proteins and cryoprotectants

Antifreeze proteins (AFPs) have the ability to bind to ice crystals through a large complementary surface and thereby create thermal hysteresis and lower the temperature at which an organism can grow (Jia & Davies, 2002). AFPs have been recently demonstrated in Antarctic lake bacteria (Gilbert *et al*, 2004), one of which, from *Marinomonas primoryensis*, is Ca²⁺-dependent and hyperactive (Gilbert *et al*, 2005). The AFP from the Arctic plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 shows both antifreeze and ice-nucleating activities (Muryoi *et al*, 2004).

Trehalose and exopolysaccharides (EPSs) might also have an important role in cryoprotection in psychrophiles. Trehalose is thought to have a colligative effect, but probably also helps in preventing protein denaturation and aggregation (Phadtare, 2004). Conversely, high concentrations of EPSs have been found in Antarctic marine bacteria (Nichols *et al*, 2005) and in Arctic winter sea ice (Krems *et al*, 2002). These modify the physico-chemical environment of bacterial cells, participate in cell adhesion to surfaces and retention of water, favour the sequestration and concentration of nutrients, retain and protect extracellular enzymes against cold denaturation and also act as cyoprotectants (Mancuso Nichols *et al*, 2005).

Cold-adapted enzymes

The most important selective pressure of low temperatures is exerted towards chemical reaction rates, most of which exponentially drop with decreasing temperature according to:

$$k_{\text{cat}} = \kappa \frac{k_{\text{B}}T}{h} \exp(\Delta G^{\ddagger}/RT)$$

in which k_{cat} is the reaction rate, κ is the transmission coefficient, k_{B} is the Boltzmann constant, T is the absolute temperature in Kelvin, h is the Planck constant, R is the gas constant and ΔG^{\ddagger} is the activation energy. κ is generally considered to be equal or close to one; however, this transmission coefficient significantly varies with viscosity, resulting in a further decrease of k_{cat} (Siddiqui *et al*, 2004). Despite this, psychrophiles produce cold-adapted enzymes that have high specific activities at low temperatures (Fig 1), often up to an order of magnitude higher than those observed for their

mesophilic counterparts (Feller & Gerday, 2003; Georlette *et al*, 2004; Russell, 2000). The commonly accepted hypothesis for this cold adaptation is the activity–stability–flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structure to compensate for the ‘freezing effect’ of cold habitats (Johns & Somero, 2004). This increased flexibility might concern the entire protein or might be restricted to parts of the structure, especially those implicated in catalysis, and is probably also responsible for the generally observed low stability of cold-adapted proteins (Collins *et al*, 2003; D'Amico *et al*, 2003). Conversely, it has been shown that activity and stability are apparently not always inversely linked (Wintrode *et al*, 2001). However, in this study, multisubstrate enzymes and small-size synthetic substrates were mainly used, which might produce different results to those obtained with natural, large substrates; that is, the specificity of the enzyme might simply be shifted towards the substrate used.

Crystallographic structures of psychrophilic proteins indicate that these do not have unusual conformations but instead share a high similarity with their meso- and thermophilic homologues. To enhance flexibility, many structural modifications that lead to attenuation in strength and/or number of stabilizing factors—enthalpic or entropic—have been observed. Common trends include: the reduction of the number of ion pairs, hydrogen bonds and hydrophobic interactions; decreased intersubunit interactions; increased interaction with the solvent; a reduced apolar fraction in the core; higher accessibility to the active site; increased exposure of apolar residues to the solvent; decreased cofactor binding; clustering of glycine residues; and a lower proline and arginine content (see Violot *et al*, 2005).

Comparison of the thermodynamic parameters of activation of psychrophilic enzymes with those of their mesophilic homologues (Lonhienne *et al*, 2000), indicates that the high k_{cat} of these at low temperatures is due to a decrease of the activation enthalpy ΔH^\ddagger —a decrease in the number of enthalpy-driven interactions that have to be broken during catalysis. This decrease is partially compensated by a less favourable activation entropy ΔS^\ddagger . As a result, and as supported by the negative values of $\Delta(\Delta S^\ddagger)_{\text{psychro-meso}}$, the ground-state enzyme–substrate complex shows a broader distribution of conformational states. Consequently, a further effect of the enhanced flexibility should be a looser binding of the substrate, which is observed through high K_M values for many psychrophilic enzymes that interact with large substrates (Collins *et al*, 2002). These catalysts therefore increase k_{cat} at the expense of K_M , whereas in some intracellular enzymes this adaptive drift of K_M is counteracted by the retention of rigid structural domains (Bentahir *et al*, 2000).

Another effect of low temperatures on proteins is cold denaturation, a phenomenon that is thought to occur from destabilizing hydration (Makhatadze & Privalov, 1995).

Genomics and proteomics

Three complete genomes have been sequenced so far: those from *Desulfotalea psychrophila* (Rabus *et al*, 2004), *Colwellia psychrerythraea* 34H (Methe *et al*, 2005) and *Pseudoalteromonas haloplanktis* TAC125 (Medigue *et al*, 2005). Draft genome sequences have been produced from two cold-adapted Archaea: *Methanogenium frigidum* and *Methanococcoides burtonii* (Saunders *et al*, 2003). As expected, several Csp's and proteins involved in unsaturated fatty-acid synthesis have been identified in these genomes. In addition to the classical lipid desaturases,

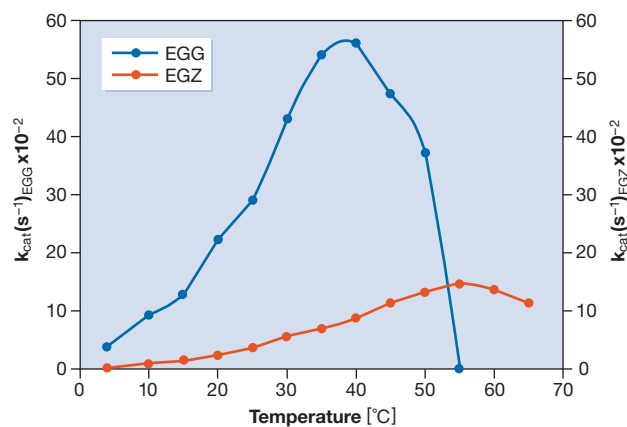


Fig 1 | Thermodependence of activity for the cold-adapted cellulase from *Pseudoalteromonas haloplanktis* (EGG) and its mesophilic homologue from *Erwinia chrysanthemi* (EGZ).

two gene clusters possibly involved in membrane rigidity/fluidity through the degradation of steroids or hopanoids have been found in the *P. haloplanktis* genome. Furthermore, β -keto-acyl carrier proteins, β -keto-acyl-CoA synthetases and a fatty-acid *cis*–*trans* isomerase have been identified in *C. psychrerythraea* and could enhance membrane fluidity depending on either their cold-adapted activity or their upregulated expression.

At low temperatures, the solubility of gasses and the production of toxic reactive oxygen species (ROS) increase significantly. To counteract this, *C. psychrerythraea* and *D. psychrophila* have an enhanced antioxidant capacity owing to the presence of several genes that encode catalases and superoxide dismutases. By contrast, *P. haloplanktis* has evolved by suppressing a series of activities that give rise to ROS—for example, the entire molybdopterin metabolism pathway is absent.

The amino-acid composition of the proteome has also been discussed in terms of temperature adaptation. Saunders and co-workers (2003) showed that working from psychrophilic to thermophilic Archaea, there is a trend in increasing leucine content and decreasing glutamine and threonine content. However, this observation was not corroborated by work on *D. psychrophila*, and an N-driven bias (increase in asparagine content) in the proteins from *P. haloplanktis* has been shown. Therefore, attempts to find specific adaptations to cold by comparing the proteins from various psychrophilic, mesophilic and thermophilic organisms have produced ambiguous results. However, a study published by Khachane and colleagues (2005) found a significant inverse correlation between the uracil content of 16S rRNA and the optimum growth temperature T_{opt} of cultured organisms. They also proposed an algorithm to predict the T_{opt} values of uncultured prokaryotes that might be useful to identify the appropriate cultivation conditions.

The proteomic approach has been used to acquire a global view of cold adaptation at the protein level. Both Csp's and Caps are expressed by the psychrophile *Arthrobacter globiformis* (Berger *et al*, 1996) and the psychrotroph *Aeromonas hydrophila* (Imbert & Gancel, 2004), whereas Seo and colleagues (2004) identified more than 30 overexpressed proteins in *Bacillus psychrosaccharolyticus* under psychrophilic growth conditions. Furthermore, proteins involved in energy metabolism, transcription and translation

processes and in protein quality control were identified at 4 °C in *Methanococcoides burtonii* (Goodchild et al, 2004a) and a prolyl *cis-trans* isomerase was identified in *Shewanella* sp. strain SIB1 (Suzuki et al, 2004).

Conclusion

Psychrophilic microorganisms have successfully confronted the two main physical challenges to which they are exposed: low thermal energy and high viscosity, both of which slow metabolic flux. Proteins are the main targets of these adaptations as they control the equilibrium between substrates and products, influx of nutrients, outflow of waste products, macromolecular assemblies, nucleic-acid dynamics and appropriate folding. Their adaptation seems to rely on a higher flexibility of key parts of the molecular structure or of the whole edifice through a decreased stability that partly compensates the freezing effect of low temperatures on the three-dimensional structure. As shown by genomics and proteomics, cold-shock proteins are also highly expressed and can have crucial roles in protein folding, control of nucleic-acid secondary structure, and transcription and translation. Approaches such as genome sequencing will undoubtedly shed new light on other characteristics of these fascinating organisms.

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