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Cold Shock Response and Low Temperature Adaptation in Psychrotrophic Bacteria

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Abstract

Psychrotrophic bacteria are capable of developing over a wide temperature range and they can grow at temperatures close to or below freezing. This ability requires specific adaptative strategies in order to maintain membrane fluidity, the continuance of their metabolic activities, and protein synthesis at low temperature. A cold-shock response has been described in several psychrotrophic bacteria, which is somewhat different from that in mesophilic microorganisms: (i) the synthesis of housekeeping proteins is not repressed following temperature downshift and they are similarly expressed at optimal and low temperatures (ii) cold-shock proteins or Csps are synthesized, the number of which increases with the severity of the shock (iii) a second group of cold-induced proteins, *i.e.* the cold acclimation proteins or Caps, comparable with Csps are continuously synthesized during prolonged growth at low temperature. Homologues to CspA, the major cold-shock protein in *E. coli*, have been described in various psychrotrophs, but unlike their mesophilic counterparts, they belong to the group of Caps. Although they have been poorly studied, Caps are of particular importance since they differentiate psychrotrophs from mesophiles, and they are probably one of the key determinant that allow life at very low temperature.

Introduction

In nature, many bacteria can grow harmoniously in very hostile environments such as polar regions and cold water, acidic hot springs, salterns, dry rock surfaces, deserts, or at depth in the sea. Such organisms are able to withstand harsh conditions, and they are often submitted to rapid variations of the environment. Among the various environmental factors that condition the viability of microorganisms, their growth and physiology, temperature is of particular interest since it affects immediately the interior of the cells. According to their ability to grow at high, intermediate or low temperature, microorganisms have been divided into three broad categories: thermophiles, mesophiles and psychrophiles, respectively. The last category has been further subdivided into psychrophiles *sensu stricto*, which have optimal growth temperatures below 15°C and an upper limit of 20°C, and psychrotrophs (psychrotolerants) which are able to divide at 0°C or below and grow optimally at temperatures around 20-25°C (Morita, 1975). Psychrophilic and psychrotrophic microorganisms are of particular importance in global ecology since the majority of terrestrial and aquatic ecosystems of our planet is permanently or seasonally submitted to cold temperatures: the world's oceans occupy 71% of the earth surface and 90% of their volume is below 5°C; the polar regions represent 14% of the earth surface and if one includes alpine soils and lakes, snow and icefields, fresh waters and caves, more than 80% of the earth biosphere is below 5°C. Microorganisms capable of coping with low temperatures are widespread in these natural environments where they often represent the dominant flora and they should therefore be regarded as the most successful colonizers of our planet (Russell, 1990). Furthermore, the development of the industrialised production of foods and the increased use of refrigeration for their long conservation, have greatly enhanced the importance of psychrotrophic bacteria. Their presence in foodstuffs is a frequent cause of spoilage and food poisoning.

Recently, the effects of hypothermic stress on the protein content of various microorganisms has been investigated: bacteria as well as eucaryotic cells respond to an abrupt decrease in temperature by overexpressing a specific set of proteins, the cold-shock proteins (Csps). It has been suggested that a universal cold shock regulon exists, and that the Csps expressed after an hypothermic stress are probably required for optimal adaptation to the lower temperature. Paradoxically, there is still a paucity of information concerning the cold-shock response in cold-adapted bacteria and its role in subsequent adaptation of cells to low temperatures, whereas it has been extensively studied in *Escherichia coli*, the paradigm of mesophilic bacteria. After a brief review of the physiology of cold-adapted microorganisms, we shall concentrate mainly on the cold-shock response in psychrotrophic bacteria which is different from that of mesophilic bacteria in several respects.

Physiology of Cold-Adapted Microorganisms

Psychrophiles and psychrotrophs belong to extremely diverse genera and, in addition to mechanisms developed during the course of evolution, adaptation to identical thermal constraints may imply common molecular mechanisms that allow the maintenance of vital cellular functions at low temperatures. In this respect, psychrotrophs are interesting since, while being able to grow at temperatures close to or below freezing, they kept their ability to withstand mild temperatures. Psychrotrophic

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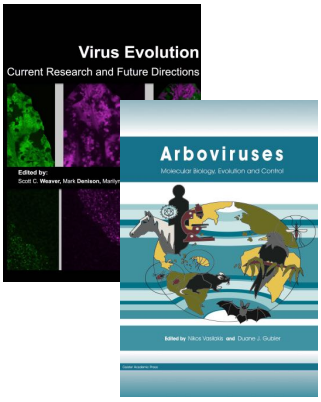

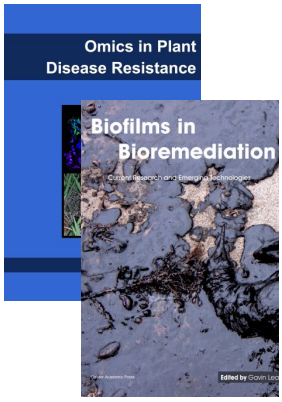

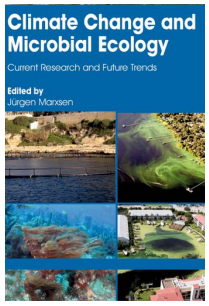

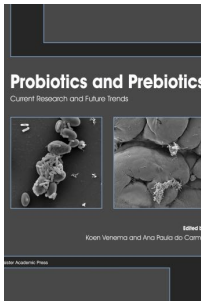

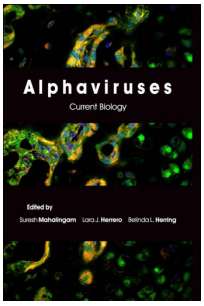

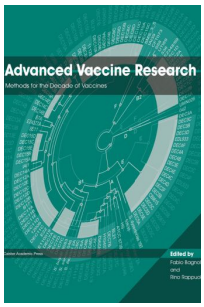

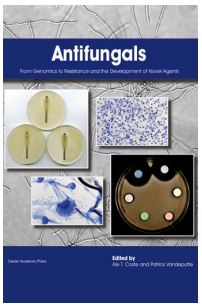

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microorganisms are more ubiquitous than psychrophilic bacteria and they are numerous (both quantitatively and in term of number of species) even in permanently cold environments. In their natural habitats, they are frequently submitted to large and rapid temperature changes and they can develop over a wide temperature range (up to 40°C, whereas the temperature range that permits growth of most other bacteria does not exceed 30°C). This ability to cope with such temperature shifts must be accompanied by adaptative changes in response to alterations of numerous physical and biochemical parameters, including solubility, reaction kinetics, membrane fluidity, protein conformation and stability and changes in gene expression. Therefore, the biochemical effects allowing bacterial cells to adapt to wide temperature changes are likely to be complex, involving a number of interacting phenomena.

Kinetics of Growth at Low Temperature

Microbial growth is the result of a sequence of interrelated chemical reactions on which the effects of temperature can be expressed by the Arrhenius equation by plotting the log of the specific growth rate constant against the reciprocal of absolute temperature in degrees Kelvin. The linear descending portion of the curve corresponds to the physiologically normal temperature range for growth, and indicates the obedience of the growth rate to temperature on the Arrhenius principle regarding a chemical reaction. At temperatures above or below this range, a deviation from linearity occurs, and rather than just a continual decrease in growth rate, the curve finally becomes vertical and growth ceases. The general form of the Arrhenius curve is the same for all microorganisms, but cardinal temperatures (*i.e.* maximum, optimum and minimum) are lower for cold-adapted bacteria. For all microorganisms, temperatures outside the linear range of the Arrhenius plots are stress-inducing temperatures, and what remains to be explained is why, whereas deviation from linearity occurs at about 20°C for mesophilic bacteria, it occurs between 5 and 10°C for psychrotrophs and below 0°C for psychrophiles. Therefore, genotypic adaptation to very low temperatures may represent the sum of many end-points of phenotypic adaptations of cell structure/metabolism, which can be achieved to various extents by different microorganisms (Russell, 1990; Gounot, 1991; Gounot and Russell, 1999).

Lipid Composition and Membrane Fluidity at Low Temperature

One of the best studied effects of low temperatures on bacterial physiology concerns the mechanisms by which microorganisms maintain an optimal degree of fluidity of their membrane, and there is abundant information concerning the lipid composition in psychrotrophs and psychrophiles (for reviews: Herbert R., 1986; Russell, 1990; Russell and Fukunaga, 1990). Depending upon the strain, membrane fluidity at low temperature can be achieved in several ways, such as increasing the ratio of unsaturated fatty-acyl residues and/or cis double bonds, chain shortening, and in some rare cases, methyl branching (Shaw and Ingraham, 1967; McElhaney, 1982; Russell, 1989). This diversity of response reflects the fact that different fatty acid compositions can provide similar thermal properties, and no specific mechanism appears to be linked to psychrophily. Furthermore, the temperature-dependent changes in membrane composition of cold-adapted microorganisms are essentially the same as those observed in mesophiles. Russell (1990) suggested that the relevant factor that differentiates the adjustment of membrane fluidity in mesophiles and cold-adapted bacteria is the timescale of the adaptive changes after a sudden temperature decrease, which would be particularly important for growth in thermally unstable cold habitats where large and rapid thermal fluctuations exist. These temperature-triggered lipid changes can be mediated by (i) enzyme activation responsible for the modification of pre-existing lipids, and (ii) by *de novo* synthesis of specific enzymes following a temperature downshift. In this respect, the product of the *des* gene which is induced following cold-shock in *Bacillus subtilis* was recently identified as a membrane phospholipid desaturase (Aguilar *et al.,* 1998). It is therefore likely that similar cold-shock induced enzymes exist in cold-adapted microorganisms, which would be responsible for the rapid homeoviscous adaptation of their membrane.

Cold-Adapted Enzymes

In contrast to the situation in mesophiles, all structural and metabolic proteins of cold-adapted bacteria have to be functional at low temperature, sometimes near or below 0°C. Catalysis at low temperature is a thermodynamic challenge which raises a number of questions including, whether growth at low temperature involves the synthesis of new proteins, or whether the cellular proteins are sufficiently «heat and cold stable» to function normally at all temperatures at which growth is possible. Both situations seem to coexist within cold-adapted bacteria. Synthesizing more enzymes or synthesizing enzymes characterized by temperature-independent reaction rates (perfectly evolved enzymes) are two other strategies to maintain sustainable growth at low temperature (Feller and Gerday, 1997). The first possibility appears energetically expensive and cannot be extended to a whole organism, whereas perfectly evolved enzymes are quite rare.

Compared with their mesophilic counterparts, enzymes from cold-adapted bacteria are more thermolabile but they are much more active at low temperatures (Rentier-Delrue *et al.,* 1993; Gerike *et al.,* 1997; Choo *et al.,* 1998; Kulakova *et al.,* 1999). It is generally thought that cold-adapted enzymes have evolved toward a high conformational flexibility, which would be responsible for their increased catalytic efficiency at low temperature and their low thermal stability (Feller *et al.,* 1997). The correlation between conformational flexibility and enzyme activity has been studied by comparing a few cold-adapted enzymes with their mesophilic homologues: the β-lactamase from the Antarctic psychrophile *Psychrobacter immobilis* A5 (Feller *et al.,* 1997), the α-amylase from *Alteromonas haloplanctis* (Feller *et al.,* 1992), the subtilisin from the Antarctic *Bacillus* TA41 (Davail *et al.,* 1994). It appears that protein flexibility can be achieved by several means, including the reduction of electrostatic noncovalent weak interactions (salt bridges, polar interactions between aromatic side chains, hydrogen bonds) and the decrease of hydrophobicity. However, no general rule governs this adaptative strategy and each enzyme can gain flexibility by one or a combination of the above modifications (Feller and Gerday, 1997). The need for more flexible molecules in order to gain functionality at low temperature is probably not limited to proteins. For

example, high levels of dihydrouridine have been found in the tRNA of three psychrotrophilic bacteria (ANT-300 and *Vibrio* sp. 5710 and 29-6), which are responsible for the maintenance of conformational flexibility and dynamic motion in RNA at low temperature (Dalluge *et al.,* 1997). This, together with protein modifications, may provide an advantage in organisms growing under conditions where thermal motion, enzymatic reaction rates and intermolecular interactions of biomolecules are compromised.

Protein Content at Low Temperature

Another aspect of adaptation to cold concerns the synthesis at low temperature of a specific set of proteins that are not (or poorly) present at milder temperatures. This particular class of proteins, referred to as cold acclimation proteins (Caps), is permanently synthesized during continuous growth at low temperature. Such Caps have been described in a variety of phylogenetically unrelated cold-adapted bacteria (Potier *et al.,* 1990; Araki, 1992; Roberts and Inniss, 1992; Whyte and Inniss, 1992; Hébraud *et al.,* 1994; Berger *et al.,* 1996) and they are likely to play a fundamental role for life in the cold. Therefore, the presence of Caps appears to be a general feature of cold-adapted microorganisms that differentiates them from mesophiles. These proteins may be involved in important metabolic function(s) at low temperature by maintaining membrane fluidity and/or by replacing cold-denaturated peptides. A low-temperature-specific proteolytic system has been described for the psychrotrophic *Arthrobacter globiformis* SI 55 (Potier *et al.,* 1987a, b), and some Caps could act as cold-specific proteases that eliminate denatured proteins whose accumulation would be deleterious for the cells.

Protein Synthesis at Low Temperature

The important points that remain to be explained is how protein synthesis proceeds in cold-adapted bacteria at temperatures not suitable for mesophiles and what are the factors that preclude protein synthesis in mesophiles at temperatures below 8-10°C. Szer (1970) isolated a protein (factor P) by washing ribosomes of the psychrotrophic *Pseudomonas* sp. 412. The washed ribosomes, while retaining activity at 25-37°C, largely lost their capacity to function at low temperature but this could be restored by addition of the protein washings. It has been established that protein synthesis is inhibited at 0°C in *E. coli* while all macromolecules necessary for translation are present and energy is still available (Das and Goldstein, 1968; Friedman *et al*., 1969; Okuyama and Yamada, 1979). The ability to synthesize more protein is almost instantly restored after only a brief exposure to 37°C. The primary effect of cold results in a polysomal run-off and accumulation of 70S ribosomal particles (Das and Goldstein, 1968; Broeze *et al*., 1978). These particles are then dissociated and subunits are accumulated at the expense of polysomes (Friedman *et al*., 1969). Das and Goldstein (1968) proposed that the cold-labile step in protein synthesis was the reattachment of ribosome to mRNA after they had run-off the 3' end at the completion of the cycle of transcription. Broeze *et al.* (1978) found that the initiation of protein synthesis was much more resistant to a sudden decrease in temperature in the psychrotroph *Pseudomonas fluorescens* compared with *E. coli* showing that this early step in translation might be the one that is adapted in cold-loving bacteria.

Cold-Shock Response in Psychrotrophic Bacteria

Cold-shock is characterized by a sudden transfer of cells to lower temperatures. Although such rapid temperature downshifts are unlikely to occur in natural environments, they provide interesting laboratory situations that largely contribute to the elucidation of the molecular mechanisms by which cells respond to cold. Most of our knowledge on how cells respond to rapid temperature downshifts originates from studies on mesophilic microorganisms such as *E. coli* and *B. subtilis* (for review, see Jones and Inouye, 1994; Wolffe, 1995; Graumann and Marahiel, 1996; Thieringer *et al*., 1998 and articles of Graumann and Marahiel and Yamanaka in this written symposium). For these bacteria, a sudden temperature decrease to 10-15°C creates a stress situation to which cells respond by specific adaptative mechanisms which, to a certain extent, allow their survival and subsequent growth at the lower temperature. A similar cold stress also exists, but at lower temperatures, when psychrotrophic bacteria are shifted outside the linear range of their Arrhenius plot. The question which arises is what are the cold-shock specific adaptative mechanisms that allow psychrotrophs to cope with lower temperatures than mesophiles. Although the cold-shock response in psychrotrophic bacteria is still poorly documented, recent data indicate that it presents similarities but also differences which may provide a partial answer to this question.

Effect of Cold-Shock on Growth

Psychrotrophic bacteria respond to temperature downshifts by a lag period before growth resumes at a rate characteristic of the new temperature (Phan-Thanh and Gormon, 1995; Bayles *et al*., 1996; Berger *et al*., 1996; Chasseignaux and Hébraud, 1997; Michel *et al*., 1997). The duration of the lag period increases with the magnitude of the downshift and/or with the lowering of the final shock temperature (Table 1). The same phenomena are also observed for *E. coli* (Ng *et al*., 1962; Jones *et al*., 1987) whereas for other mesophilic bacteria (Panoff *et al*., 1994; McGovern and Oliver, 1995; Lottering and Streips, 1995; Kim and Dunn, 1997) and for the psychrophilic *Vibrio* sp. (Araki, 1991a), no lag phase is observed and growth continues at an intermediate rate followed by a growth rate characteristic of the final low temperature. Obviously, different mechanisms exist for bacteria to respond to temperature downshifts, and the time to re-adapt to the low temperature is not directly dependent on the range of growth of the bacterium, *i.e*., of its psychrophilic, psychrotrophic, or mesophilic character.

Effect of Cold-Shock on Protein Synthesis

In mesophilic bacteria, cold-shock results in the transient inhibition of the synthesis of the bulk of proteins, the so-called housekeeping proteins. In *B. subtilis*, this inhibition is only partial and at least 75 proteins are permanently synthesized upon cold-shock (Graumann and Marahiel, 1996). The growth of *B. subtilis* continues at a reduced doubling time without apparent growth lag. The response of *E. coli* is somewhat different since the transient inhibition of housekeeping proteins is total and results in a growth lag period (Jones *et al*., 1987; Thieringer *et al*., 1998). One of the most significant difference between mesophiles and cold-adapted bacteria is that the relative rate of synthesis

Table 1. Duration of Lag-Phase According to the Magnitude of Temperature Downshifts

Micro-organisms	Cold-shocks [a]	Lag phase [b]	References
Pseudomonas fragi	20 ➔ 5	3	Michel *et al.*, 1997
	30 ➔ 5	5	
Listeria monocytogenes LO28	**15 ➔ 5**	1*	Chasseignaux and Hébraud, 1997
	20 ➔ 5	3*	
	25 ➔ 5	5*	
	30 ➔ 5	> 11*	
	37 ➔ 5	> 11*	
Listeria monocytogenes 10403S	37 ➔ 10	2	Bayles *et al.,* 1996
	37 ➔ 5	>2	
Arthrobacter globiformis	25 ➔ 15	1 - 2	Berger *et al.,* 1996
	25 ➔ 10	4 - 6	
	25 ➔ 8	6 - 7	
	25 ➔ 6	7 - 8	
	25 ➔ 4	10 - 12	
Escherichia coli	37 ➔ 10	4	Jones *et al.*, 1992
	24 ➔ 10	2	

[a] initial and final temperatures (°C).
[b] lag phase in hours except * (days).

of most cytosolic proteins is maintained after cold-shock. This has been demonstrated in *Vibrio* sp. ANT-300, (Araki, 1991a, b) *B. psychrophilus* (Whyte and Inniss, 1992), *A. globiformis* (Berger *et al*., 1996) and *P. fragi* (Michel *et al.*, 1997). Obviously, it can be assumed that, as opposed to mesophiles, regulatory factor(s) exists in cold-adapted bacteria prior to cold-shock that allows the maintenance of a functional translational machinery at low temperature. However, in spite of this continuous protein synthesis, growth of psychrotrophic bacteria ceases transiently after a cold-shock and additional regulatory mechanisms may exist which allow growth resumption at temperatures close to freezing.

An intermediate adaptative mechanism seems to exist in those pathogenic bacteria that are not psychrotrophic *per se*, but are capable of residual growth at low temperature (*i.e.* around 4°C). For example, Phan-Thanh and Gormon (1995) reported that the synthesis of half the proteins synthesized at 25°C is turned off while many others are depressed more than twofold in the first hours following a cold-shock to 4°C in *Listeria monocytogenes* and *Listeria innocua*. However, a lag phase is systematically observed which increases with the severity of the cold shock (Table 1). It is therefore likely that several strategies exist for adaptation to cold that may account for the continuum of lower growth temperatures within the microbial world.

Cold-Induced Proteins in Psychrotrophic Bacteria

The outstanding common feature of the microbial response to cold-shock is that all bacteria studied to date, including thermophiles (Schröder *et al*., 1993), mesophiles (Jones *et al*., 1987; Schröder *et al*., 1993; Jones and Inouye, 1994; McGovern and Oliver, 1995; Graumann *et al*., 1996), psychrotrophs (Cloutier *et al*., 1992; Whyte and Inniss, 1992; Schröder *et al*., 1993; Berger *et al*., 1996; Gumley and Inniss, 1996; Michel *et al*., 1997), and psychrophiles (Araki, 1991a, b; Roberts and Inniss, 1992), overexpress a specific subset of proteins: the cold-shock proteins (Csps). In psychrotrophic bacteria, a basal set of Csps exists which is overexpressed even after mild shocks, and additional Csps appear with more severe cold-shocks (Table 2). In *A. globiformis* SI 55, a mathematical analysis of protein appearance revealed that the sequence of cold-induced proteins expression following cold-shocks of increasing magnitude is not random, and the Csps fall into different groups: one is common to all shocks, one is specific of mild shocks, and one is specific of large shocks that occur outside the range of linearity of the Arrhenius plot (Berger *et al*., 1996). Hence, the number of Csps together with the magnitude of their induction depend upon the range of the temperature shift: the larger this range, the more pronounced the response (Table 2). It appears therefore that the synthesis of Csps in psychrotrophic bacteria results from active regulatory mechanisms and it is likely that some if not all these proteins are essential for helping cells to recover from the temperature downshift.

A general feature of cold-adapted bacteria is that the relative levels of cold-induced proteins are much lower than those reported in mesophiles (Araki, 1991a, b; Cloutier *et al*., 1992; Roberts and Inniss, 1992; Whyte and Inniss, 1992; Schröder *et al*., 1993; Berger *et al*., 1996; Gumley and Inniss, 1996; Michel *et al*., 1997). This is probably due to the fact that, as opposed to mesophiles in which Csps are the prevailing proteins that are synthesized following cold-shock, the overall synthesis of cellular proteins is not inhibited in cold-adapted bacteria. This diversion of the translational machinery may therefore account for the lower rates of cold-induced protein synthesis in psychrotrophs.

Although there are very few data on the overall cold-shock response in cold-adapted microorganisms, more Csps seem to be overexpressed than in mesophilic bacteria, such as *E. coli* (Jones *et al*., 1987; Goldstein *et al*., 1990; La Teana *et al*., 1991; Jones *et al*., 1992; Jones and Inouye, 1994; Lelivelt and Kawula, 1995; Jones *et al*., 1996) and *Lactococcus lactis* (Panoff *et al*., 1994). Julseth and Inniss (1990) reported the induction of 26 Csps after a 24 to 4°C cold-shock in the psychrotrophic yeast *Trichosporon pullulans*. Cloutier *et al*. (1992) showed that Arctic *rhizobium* strains respond to very low temperature (-10°C) by synthesizing more proteins than temperate strains do at higher temperatures. When the psychrotrophic bacteria *P. fragi* and *A. globiformis* are subjected to cold-shocks from their optimal growth temperature to 4-5°C, 25 and 26 Csps can be detected, respectively (Berger *et al*.,

Table 2. Cold-Induced Proteins, Including Caps (in parentheses), and Common Csps between Cold-Shocks of increasing Magnitude, in *P. fragi* (Michel *et al.*, 1997) and *A. globiformis* (Berger *et al.*, 1996)

Micro-organisms	Cold-shocks [a]	Number of Csps	Number of common Csps [b]	Total number of Caps [c]
Pseudomonas fragi	20 ➔ 5	15		
	30 ➔ 5	25 (11 Caps)	12 (20 ➔ 5)	20
Arthrobacter globiformis	25 ➔ 15	13		
	25 ➔ 10	14	12 (25 ➔ 15)	
	25 ➔ 8	17	12 (25 ➔ 15) 13 (25 ➔ 10)	
	25 ➔ 6	19	12 (25 ➔ 15) 13 (25 ➔ 10) 17 (25 ➔ 8)	
	25 ➔ 4	26 (9 Caps)	11 (25 ➔ 15) 12 (25 ➔ 10) 16 (25 ➔ 8) 18 (25 ➔ 6)	18

[a] initial and final temperatures (°C).
[b] in parentheses, the cold-shock considered.
[c] determined during prolonged growth at 4°C.

1996; Michel *et al.*, 1997). It is important to underline that among the total number of proteins overexpressed upon cold-shock in these two bacteria, some behave like Caps and are still synthesized during prolonged growth at the shift temperature (Table 2). Therefore, the number of Csps *per se* in *P. fragi* and *A. globiformis* is similar to that reported for mesophilic bacteria. However, this simple numeric deduction is certainly not sufficient to say that a totally common pattern exists for Csps synthesis between these two thermal bacterial groups, and some of Csps in mesophiles are Caps in psychrotrophs (see below). Synthesizing large numbers of Csps is probably not a pre-requisite for adaptation to very low temperatures since *B. subtilis*, which is unable to grow at temperatures near or below freezing, was shown to synthesize 37 cold-shock proteins after temperature downshift from 37 to 15°C (Graumann *et al.*, 1996).

Kinetics of Expression of Cold-Induced Proteins

Studies with *A. globiformis* (Berger *et al.*, 1996) or *P. fragi* (Michel *et al.*, 1997) indicated that some cold-induced proteins appear very rapidly following temperature downshift. This number increases during post-shift to reach maximal induction before or at mid-lag phase. A similar situation exists in *E. coli* in which Csps are very quickly induced after a downshift from 37 to 10°C, with an optimal level of induction towards 2 hours (Goldstein *et al.*, 1990).

In *P. fragi,* the kinetic pattern of protein expression following cold-shocks from 30 or 20 to 5°C allows to differentiate the Csps and the Caps according to their transient or permanent overexpression, respectively (Michel *et al.*, 1997). Each of these two classes can be further divided in two subclasses according to their immediate or delayed overexpression. Induction of these late cold-induced proteins usually takes place one hour after the shift or later during the lag period. A similar pattern of expression exists in *A. globiformis*, in which the appearance of cold-induced proteins is sequencial: early Csps and Caps are synthesized immediately following cold-shock, followed by late Csps and Caps, and finally growth resumes (Berger *et al.*, 1996). It can be speculated therefore that early cold-induced proteins are necessary for the expression of the late cold-induced proteins, both of them being essential for growth resumption at the low

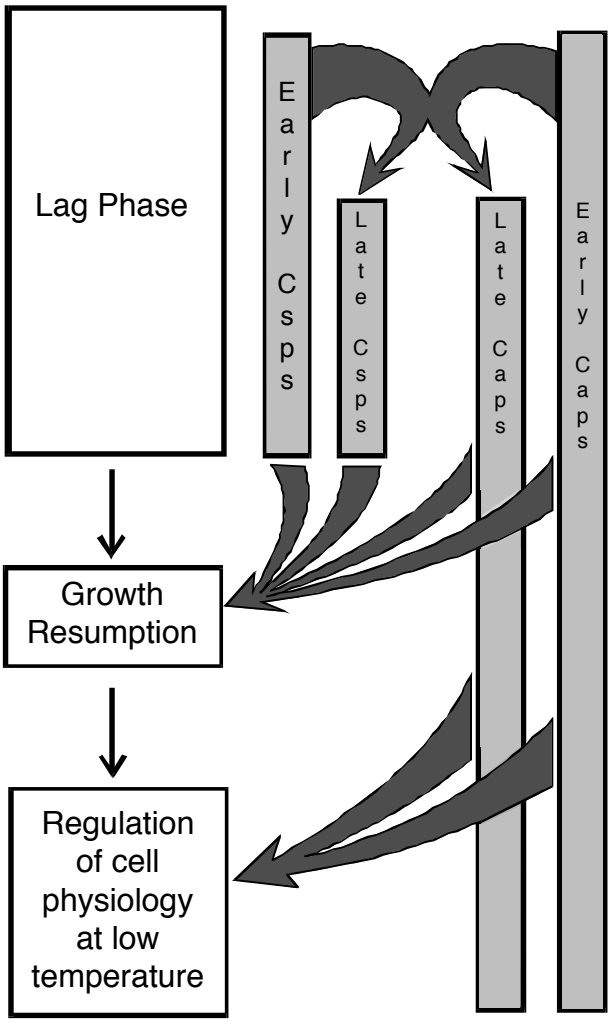

Figure 1. Schematic representation of the hypothetical interactions between cold-induced proteins and acclimation to low temperature in psychrotrophic bacteria.

temperature (Figure 1). The persistence of some of these proteins (early and late Caps) during prolonged growth in the cold may be important to maintain a balanced physiology at low temperature and may constitute one of the key determinant of psychrotolerance.

The CspA-Like Proteins of Psychrotrophic Bacteria

All the recent studies concerning the cold-shock response in psychrotrophic bacteria are generally descriptive and most Csps and Caps have not been identified. This situation originates from the fact that these microorganisms are poorly studied, their genetics is totally unknown and, due to their relative low levels of synthesis, insufficient amounts of Csps and Caps are generally present on two-dimensional gel electrophoresis to allow micro-sequencing. Therefore, most of what we know so far about cold-inducible proteins in these bacteria comes from analogies with *E. coli* and *B. subtilis*, for which the nature of the majority of Csps has been identified. In particular, most studies concerning cold-induced proteins in psychrotrophs have focused on proteins that resemble CspA, the major cold-shock protein in *E. coli*. CspA is believed to play a central role in the cold-shock response in this bacterium. It is specifically produced after cold-shock and its production reaches 13% of total cellular protein synthesis following temperature downshift. It belongs to a family of nine homologous proteins, CspA to CspI, of which only CspA, CspB, CspG and CspI are cold-shock inducible, but are differentially regulated (Etchegaray *et al*., 1996; Etchegaray and Inouye, 1999; Wang *et al*., 1999). Similar to *E. coli*, *B. subtilis* also contains a family of CspA-like proteins, and three homologous proteins (CspB, CspC and CspD) have been found to be essential for low temperature adaptation (Graumann *et al*., 1996). CspA homologues have been identified in a wide number of Gram negative and Gram positive eubacteria whatever their growth range of temperature (Av-Gay *et al*., 1992; Willimsky *et al*., 1992; Schröder *et al*., 1993; Hébraud *et al*., 1994; Ray *et al*., 1994; Berger *et al*., 1996; Graumann *et al*., 1996; Mayr *et al*., 1996; Chapot-Chartier *et al*., 1997; Francis and Stewart, 1997; Mayo *et al*., 1997; Michel *et al*., 1997; Kim *et al*., 1998; Perl *et al*., 1998; Database Accession nos. U70990 and L23115). However, most of these studies do not detail the low-temperature inducible or noninducible character of these genes.

Genes homologous to *cspA* have been found in several cold-tolerant bacteria including *Listeria* (Francis and Stewart, 1997) and *Bacillus cereus* (Mayr *et al*., 1996), as well as in psychrotrophic bacteria such as *Arthrobacter* (Ray *et al*., 1994; Berger *et al*., 1996), *Pseudomonas* (Hébraud *et al*., 1994; Ray *et al*., 1994; Michel *et al*., 1997), *Bacillus globisporus* (Schröder *et al*., 1993), and *Micrococcus roseus* (Ray *et al*., 1994). All these genes encode highly conserved proteins that share a high level of identity with all members of the CspA family in *E. coli* and with the homologous proteins in other mesophilic bacteria. No particular amino-acid sequence nor domain seems to exist in the CspA-like proteins of cold-adapted bacteria that could differentiate them from their mesophilic counterparts. They also contain the highly conserved RNP-1 and RNP-2 motifs, both domains being involved in the binding to RNA and single-stranded DNA, thereby suggesting related functions of these proteins.

The number of *cspA*-like genes varies in the cold-adapted bacteria. Only one gene is present in *A. globiformis* SI 55 (Berger *et al*., 1997; Berchet and Potier, unpublished) whereas at least 4 and 6 genes exist in *P. fragi* (Hébraud *et al*., 1994) and *B. cereus* WSBC 10201 (Mayr *et al*., 1996), respectively. The *cspA*-like gene in *A. globiformis* SI 55 and the four ones in *P. fragi* are cold-inducible. On the contrary, only one of the six *cspA* homologues in *B. cereus* WSBC 10201 is overexpressed at low temperature and following cold-shock from 30 to 7°C. In a collection of 100 strains of *B. cereus* belonging to different thermal groups, the presence of a cold-inducible *cspA* gene correlates with the ability of some strains to grow at or below 7°C (Francis *et al*., 1998). It is therefore likely that, as their mesophilic counterparts, the products of cold-inducible *cspA*-like genes play a major role in cell adaptation to low temperatures.

Similar to *B. subtilis* in which a *cspB/cspC/cspD* triple deletion mutation is lethal (Graumann *et al*., 1997), allelic exchange of the unique copy of the *cspA*-like gene with a deleted copy is lethal in *A. globiformis* SI 55 (Berchet and Potier, unpublished). This suggests a more general role of these genes than their sole implication in cold acclimation processes, and the presence of at least one copy is essential for survival.

Expression of CspA-Like Proteins in Psychrotrophic Bacteria

Expression of CspA-like proteins is an immediate response to temperature downshift in all the psychrotrophic bacteria studied to date. For example, they appear within the first hour postshift in *P. fragi* (Michel *et al*., 1997) and as soon as 20 minutes in *A. globiformis* SI 55 (Berger *et al*., 1997). In this latter bacterium, the transcripts of this gene pre-exist prior to cold-shock and post-transcriptional regulation is likely to account for its induction.

In *A. globiformis* SI 55 submitted to various cold shocks at temperatures inside the range of linearity of the Arrhenius plot, *i.e.* down to 6°C, synthesis of the CspA-like protein is transient and ceases between 2-4 h postshift (Berger *et al*., 1996). However, when cells are transferred to a colder temperature, this protein is continuously synthesized at a similar level, not only during the lag phase, but also during prolonged growth at the shift temperature. Hence, this CspA-like protein in *A. globiformis* SI 55 was named CapA for «cold acclimation protein A». The particular continuous expression of CspA homologues at low temperature has also been described in *B. cereus* (Mayr *et al*., 1996), and in *P. fragi* (Hébraud *et al*., 1994). Therefore, the CspA-like protein in all these microorganisms can be referred to as an early Cap, and this may represent a common feature of cold-adapted bacteria that distinguishes them from mesophiles.

The four CspA homologues in *P. fragi* are the products of four independent genes, and their expression varies at different growth temperatures as well as following temperature shifts. All four proteins are overexpressed following a 30 or 20°C to 5°C shift: two of them (CapA and CapB) are Caps since they are still synthesized during prolonged growth at 5°C, whereas the two others cannot be considered as Caps *per se* since their level decreases slowly after growth resumption at 5°C (Michel *et al*., 1997). Surprisingly, these two last CspA homologues are also heat-shock inducible (Michel *et al*., 1996) and were therefore referenced as TapA and TapB for «temperature adaptation proteins A and B». In cultures at steady-state

temperatures, CapA and CapB are optimally expressed between 4 to 10°C, whereas TapA and TapB are optimally expressed at 25 to 30°C, *i.e.* at temperatures around their optimal for growth (Hébraud *et al.*, 1994). The fact that either of these protein pairs is overexpressed according to the growth temperature means there is a subtle way of regulation with the probable involvement of temperature-sensitive molecules. Such a pattern of expression has not been described in other bacteria and further studies are necessary to gain insights on the precise role of these four small proteins in *P. fragi* at extremes of temperature.

Finally, an attempt to correlate CapA overexpression to cold adaptation was made in *A. globiformis* SI 55 (Berger *et al.*, 1997). A temporary block in protein synthesis during cold-shock treatment results in an additional lag that increases with the duration of the inhibition. CapA expression is delayed for similar periods of time and whatever the length of protein synthesis inhibition, growth resumption systematically occurs 12 to 14 h after CapA synthesis. Therefore, a positive correlation exists in *A. globiformis* SI 55 between CapA appearance and growth resumption at low temperature, but whether or not CapA synthesis is causal remains to be determined.

Coming in from the Cold?

For several decades, it was commonly thought that life on our planet appeared at high temperature, and the primitive character of hyperthermophiles has become a paradigm. The recent discovery of psychrophilic archaea (DeLong *et al.*, 1994; DeLong, 1998), which branch close to the base of the universal phylogenetic tree, revived the idea of a cold origin (Forterre, 1999). Therefore, the presence of Csps in thermophiles, mesophiles and psychrotrophs raises interesting questions about the warm or cold origin of their relevant genes. If the «warm hypothesis» is correct, it can be speculated that these genes, being involved in some vital processes, have been conserved throughout evolution from thermophiles to psychrotrophs. Caps would therefore be the manifestation of a recent evolutionary process by which some bacteria gained the ability to colonize cold habitats. According to the «cold hypothesis», a complete set of genes necessary for life at low temperature would have existed in MRCA, the so-called Most Recent Common Ancestor (Galtier *et al.*, 1999), which would have been conserved in psychrotrophic and psychrophilic bacteria. Part of these genes, such as those encoding Caps, may have been lost during the course of evolution that led to the appearance of mesophilic microorganisms. If such is the case, perturbations of the translational machinery, the dramatic overexpression of a few Csps and the absence of Caps following cold-shock would be the manifestation of an incomplete and deregulated mechanism that prevents these cells from growing in the cold. Therefore, Csps and Caps could provide interesting models to gain an understanding on the origins of life on our planet.

Conclusions

Psychrotrophic bacteria can grow harmoniously at temperatures not suitable for mesophilic bacteria. In spite of their importance in natural environments and in refrigerated food, cold-adapted bacteria have been largely ignored and their biotechnological exploitation has remained untapped. This situation is changing and recent interest has focused on the biotechnological potential of psychrophilic and psychrotrophic microorganisms and their cold-active enzymes: expression of eucaryotic recombinant proteins of medical interest that are naturally thermosensitive (*e.g.* insulin, interferon, enkephalins, growth hormones, etc), production of cold-active enzymes (cellulases, amylases, lipases, pectinases, etc) that could be used in low-energy processes, soil or water bioremediation in cold environments. A better knowledge of the physiology and genetics of these microorganisms is necessary to control their degradative abilities in natural cold environments, to optimize the production of their enzymes for biotechnological purposes, or to prevent their growth in foods. The fundamental question of how they function at low temperature remains to be elucidated. There is probably no general and unique explanation for adaptation of psychrophilic and psychrotrophic bacteria to temperatures near or below freezing. Growth and cell multiplication at such temperatures underlie that these bacteria developed adaptative strategies in order to maintain enzyme activity and membrane functionality. Cold-shock inducible proteins are certainly involved in the mechanisms by which these cells overcome temperature downshift. Among them, cold acclimation proteins (Caps) are probably the specific determinants that differenciate psychrotrophs from mesophiles and allow continuous growth at very low temperature. Determination of the nature and of the role of these Caps is certainly the major task for further studies in order to gain an understanding in psychrophily.

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