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# Adaptation to low temperature and regulation of gene expression in antarctic psychrotrophic bacteria

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Exposure to extremes of temperatures cause stresses which are sometimes lethal to living cells. Microorganisms in nature, however, are extremely diverse and some of them can live happily in the freezing cold of Antarctica. Among the cold adapted psychrotrophs and psychrophiles, the psychrotrophic bacteria are the predominant forms in the continental Antarctica. In spite of living in permanently cold area, the antarctic bacteria exhibit, similar to mesophiles, 'cold-shock' response albeit at a much lower temperatures, e.g., at 0–5°C. However, because of permanently cold condition and the long isolation of the continent, the microorganisms have acquired new adaptive features in the membranes, enzymes and macromolecular synthesis. Only recently these adaptive modifications are coming into light due to the efforts of various laboratories around the world. However, a lot more is known about adaptive response to low temperature in mesophilic bacteria than in antarctic bacteria. Combined knowledge from the two systems is providing useful clues to the understanding of basic biology of low temperature growing organisms. This article will provide an overview of this area of research with a special reference to sensing of temperature and regulation of gene expression at lower temperature.

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## 1. Introduction

Low temperature induced stress and subsequent effects on synthesis of proteins and induction of genes have been studied in some detail in various mesophilic microorganisms (Jones and Inouye 1994; Murata and Wada 1995; Lottering and Streips 1995; Graumann *et al* 1996). For example, *Escherichia coli* which grows optimally at 37°C produces more than a dozen of new proteins when transferred to 10–15°C. These proteins, called cold shock proteins are perhaps required for low temperature adaptation of the organism (Jones *et al* 1987; Jones and Inouye 1994). The temperature range of 10–15°C is quite a 'cold shock' for the mesophilic organisms. However, the antarctic microorganisms which live and grow permanently in a cold environment of freezing temperature are little studied, especially as regards to their adaptive response to low temperature, such as 0–4°C where mesophiles do not grow. In fact, no clear experi-

ment has been conducted to find out whether 0–4°C is at all a cold shock for them. Therefore, the biochemical and genetic studies of these organisms are of importance to understand the basic biology of the low temperature growing organisms (Gounot 1991; Shivaji and Ray 1995; Feller *et al* 1996).

Broadly speaking, all antarctic microorganisms could be classified into two groups: the psychrophiles which grow at zero or sub-zero to 18–20°C with an optimum growth at 10–12°C, and the psychrotrophs which grow at 0°C or below but are capable of growing at temperature as high as 30–32°C (Morita 1975). This latter group of microorganisms which are the dominant forms in the continental Antarctica grow optimally at 20–24°C in laboratory (Wynn-Williams 1990; Shivaji and Ray 1995). Most of the antarctic microenvironments, however, may not rise above 10°C even during austral summer. Therefore, the antarctic psychrophiles and psychrotrophs may or may not exhibit a typical 'cold shock' response which

**Keywords.** Antarctic bacteria; cold adaptation; cold-shock proteins; temperature sensing; cold induced gene expression

is observed in mesophiles. Indeed very little is known about the adaptive response and adaptive modifications of these unique bacteria. In order to understand as to how the antarctic microorganisms respond to cold temperatures, such as 0–4°C, and how the genes are regulated at low temperature, we have chosen an antarctic psychrotroph *Pseudomonas syringae* (Shivaji *et al* 1989) as a model system. In this review we will discuss the present state of knowledge of the adaptive modifications and adaptive response of these organisms in the light of data from both psychrophiles, psychrotrophs and mesophiles when they are subjected to cold shock. We will also discuss how the cold inducible gene expression has been used to study the temperature sensing mechanism at cellular level in these organisms. Some of these aspects have recently been reviewed (Murata and Wada 1995; Shivaji and Ray 1995; Feller *et al* 1996; Graumann and Marahiel 1996).

## 2. Adaptive modifications of antarctic bacteria

### 2.1 Changes in enzymes

The major adaptive modifications so far encountered in the antarctic psychrotrophs and psychrophiles are documented in the structure and activity of the enzymes. The enzymes are generally cold-active which means they are capable of functioning at 0°C. Most mesophilic enzymes however do not function at 0°C. The low temperature activity of the antarctic enzymes is found to be due to both increased  $K_{cat}$  and decreased  $K_m$  (Jaenicke 1990; Feller *et al* 1996; Marshall 1997). The gain of function at lower temperature has probably been at the cost of thermal stability. As a result the enzymes are comparatively heat labile. Interestingly, however, the enzymes have retained the temperature optima of activity at or around 37°C, similar to mesophiles. The examples include acid protease, subtilisin, lipase,  $\alpha$ -amylase, citrate synthase, RNase, alkaline phosphatases, etc. (Kobori *et al* 1984; Ray *et al* 1992; Feller *et al* 1994a,b; Reddy *et al* 1994; Adler and Knowles 1995; Chattopadhyay *et al* 1995; Davail *et al* 1995; Gerike *et al* 1997). Some of the genes for these enzymes have been cloned and amino acid sequences were deduced. When the primary and predicted secondary structure of these enzymes were compared with their mesophilic counterparts, a few interesting observations were made (Feller *et al* 1996): (i) an increased occurrence of charged residues in the protein; (ii) decrease in isoleucine content and reduction in aromatic-aromatic interactions in the hydrophobic core-cluster; (iii) decrease in arg/(arg + lys) content; (iv) the occurrence of extended surface loops; (v) decrease in number of proline residues. These observed changes suggest that the psychrophilic enzymes might have adapted structurally to be more flexible which is important

for functioning at low temperature. Recently, a suitable crystal for X-ray diffractions study has been obtained for an  $\alpha$ -amylase of the psychrotrophic *Alteromonas haloplanctis* but a detailed structural analysis is awaited (Aghajari *et al* 1996).

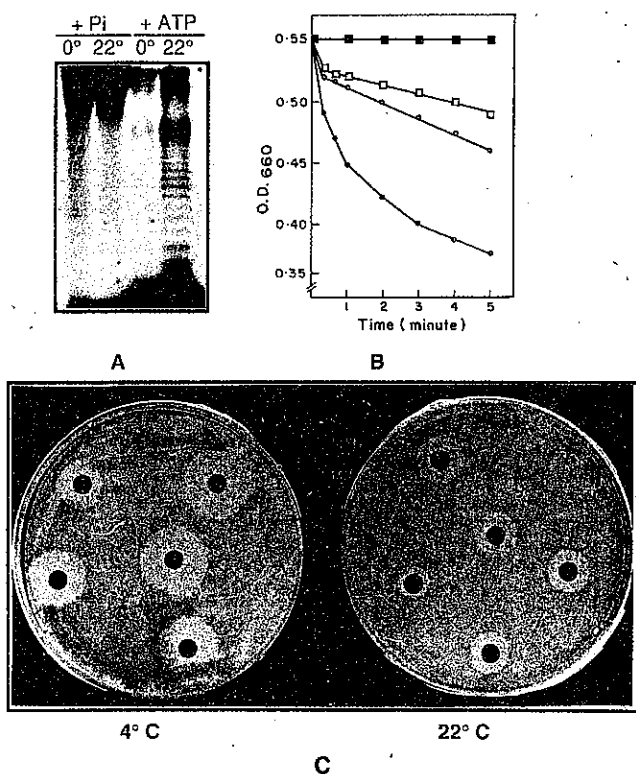
### 2.2 Changes in membrane

It is known for a long time that the microorganisms, when shifted from a high to low temperature, respond by increasing the level of unsaturated fatty acids in the membrane phospholipids (Russel 1984a,b; Herbert 1986; Murata and Wada 1995). Presumably this helps to maintain the homeoviscosity of membrane so that membrane function (e.g., permeability) is not affected (Cossins 1994). Concurrent with this view, antarctic bacteria have been found to have increased level of unsaturated fatty acids in their membrane phospholipids (Russel 1990). Recent data also show that some of the antarctic marine psychrophiles possess polyunsaturated fatty acids (PUFAs), such as eicosaentaenoic acid (20 : 5) and docosahexaenoic acid (22 : 6), in their membrane to adapt to extreme cold temperatures (Nichols *et al* 1993; Bowman *et al* 1997). Interestingly, it has also been found that some bacteria (e.g., *Vibrio* sp. and *Micrococcus cryophilus*) could also adapt to low temperature by decreasing the chain length of the fatty acids (Russell 1984a,b).

The outer membrane of Gram-negative bacteria has a different structure where the outer leaflet of the membrane bilayer is made up of lipopolysaccharides (LPS) and inner leaflet is made up of phospholipids. It has been observed that the LPS molecules of antarctic *P. syringae* undergo changes by phosphorylation and dephosphorylation (figure 1) in the 'core' region of the molecules (Ray *et al* 1994a). An LPS kinase activity which was shown to be associated with a specific membrane fraction *in vitro* probably brings about this change. At high temperature (22°C) the phosphate content in the LPS is high. But at low temperature (4°C), there is a decrease in phosphate content of the LPS molecules which, as a result, bind less divalent cations, such as  $Ca^{2+}$  and  $Mg^{2+}$  that can be easily titrated out with a small amount of EDTA. Therefore the cells become more sensitive to EDTA at low temperature (figure 1). Concomitantly, it has been found that the psychrotrophic *P. syringae* becomes more susceptible to some of the cationic antibiotics, such as polymyxin B, gentamycin, etc. The molecular basis of alteration of outer membrane permeability at low temperature in the psychrotrophic *P. syringae* is currently under investigation. Preliminary evidence suggests that the LPS from low and high temperature grown cells of *P. syringae* differ in their acyl chain composition which may have a bearing in low temperature adaptation (G Seshukumar and M K Ray, unpublished observation).

### 2.3 Other modulators of membrane fluidity

Although increased unsaturation and decreased chain length of fatty acids constitute the major modifications of cell membrane, it has been suggested that other membrane associated molecules may also play important role in low temperature adaptation of antarctic psychrotrophic bacteria (Jagannadham *et al* 1991; Chauhan and Shivaji 1994). One such molecule is carotenoid pigment which remains associated with cell membrane. Studies *in vitro* have indicated that the pigments interact with cell membrane and increase its 'rigidity' (more ordered) (Jagan-



**Figure 1.** Phosphorylation of lipopolysaccharides (LPS) and changes in sensitivity to EDTA and polymyxin B in *P. syringae*. (A) *In vitro* phosphorylation of membrane fraction containing LPS in the presence of  $^{32}\text{P}$ i and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  at  $0^\circ\text{C}$  and  $22^\circ\text{C}$ , respectively. The LPS kinase activity associated with the membrane fraction phosphorylates the LPS molecules predominantly at higher temperature ( $22^\circ\text{C}$ ). (B) EDTA sensitivity of the *P. syringae* cells grown at low ( $4^\circ\text{C}$ ) and high ( $22^\circ\text{C}$ ) temperatures. The sensitivity to EDTA was monitored by assaying cell-lysis at  $\text{OD}_{660}$  in the presence of 2.5 mM ( $\square$ ,  $22^\circ\text{C}$ ;  $\bullet$ ,  $4^\circ\text{C}$ ) and 5 mM ( $\circ$ ,  $22^\circ\text{C}$ ) EDTA, or in the absence of EDTA ( $\blacksquare$ ). (C) Polymyxin B sensitivity of the cells grown at  $4^\circ\text{C}$  and  $22^\circ\text{C}$ . The sensitivity was assayed by putting filter discs containing various amounts (1–5  $\mu\text{l}$ ) of polymyxin B (2 mg/ml) on a growing lawn of *P. syringae*. The discs contained 1, 2, 3, 4 and 5  $\mu\text{l}$  of the antibiotic solution, anticlockwise, starting at 10 O'clock and 8 O'clock positions for the plates at  $4^\circ$  and  $22^\circ\text{C}$ , respectively. For details, see Ray *et al* (1994a).

nadham *et al* 1991). Since a large number of antarctic bacteria have been found to contain carotenoid type of pigments in their membrane, it has been speculated that these pigments may have a role in buffering membrane fluidity when there is an upshift of environmental temperature. The antarctic bacteria which normally contain a high proportion of unsaturated fatty acids in their membrane may face adverse effect immediately following an upshift of temperature, and in such a situation carotenoid type of pigments may maintain the homeoviscosity of membrane till there is a *de novo* synthesis of saturated fatty acids. A direct experimental evidence for such a hypothesis, however, is lacking. In pigmentless psychrotrophic bacteria, such as marine *Vibrio* sp ABE-1 and *Pseudomonas* sp strain E-3, *trans*-monounsaturated fatty acids [e.g., 16:1(9t)] which also increase membrane 'rigidity' may play a similar role in maintaining membrane structure and/or fluidity (Okuyama *et al* 1991).

### 2.4 Transcription and translation machinery

When mesophilic organisms such as *E. coli* are subjected to cold shock (for example, a shift of culture from  $37^\circ\text{C}$  to  $15^\circ\text{C}$ ), both the transcription and translation machinery of the cells are affected. The translation machinery is most severely affected (Das and Goldstein 1968; Broez *et al* 1978). However, the antarctic psychrotrophic organisms which have been studied did not exhibit such an effect (Shivaji and Ray 1995). *In vitro* studies indicated that an S-30 fraction containing ribosomes from mesophilic *E. coli* could not translate the exogenously added poly (U) message at  $0^\circ\text{C}$  whereas a similar S-30 fraction from psychrotrophic strain of Antarctica such as *P. syringae* could do so efficiently (Shivaji and Ray 1995). Another psychrotrophic *Pseudomonas* sp. was shown earlier to synthesize polyphenylalanine from poly (U) message *in vitro* at  $0^\circ\text{C}$  (Szer 1970). The same study also demonstrated that a salt-eluted fraction of the ribosomes from the psychrotrophic *Pseudomonas* could induce *E. coli* ribosomes to translate poly (U) at  $0^\circ\text{C}$ . The nature of the protein or any other molecules in the salt eluted fraction which could induce translation, is, however, still not known.

The studies *in vitro* from our laboratory demonstrated that the transcription efficiency of a cell-free extract with enriched RNA polymerase correlates grossly with the generation time of the antarctic *P. syringae* at various temperatures, except at  $15^\circ\text{C}$  (figure 2). The cell extract exhibited maximum transcription activity at 15– $25^\circ\text{C}$ , and about 65% activity at  $0^\circ\text{C}$ . The reason for a lack of correlation between the transcription activity and generation time at  $15^\circ\text{C}$  could not be ascertained in this study due to the crude nature of the cell extracts. The purified RNA polymerase which exhibited a typical eubacterial subunit composition with  $\beta$ ,  $\beta'$ ,  $\alpha$  and sigma

factor(s), however transcribed only at about 10–15% of its maximum activity which was observed at 37°C (S Uma and M K Ray, unpublished observation). In contrast, *E. coli* RNA polymerase could not transcribe at all at 0–4°C. The RNA polymerase of *P. syringae* was also found to be relatively thermolabile when compared with *E. coli* RNA polymerase. An earlier study indicated that the RNA polymerase of the marine psychrophilic bacterium *Pseudomonas* BAL-31 also had  $\beta\beta'\alpha_2$  subunit structure but exhibited optimum activity at temperature of 28–30°C. The enzyme was extremely thermolabile and lost 50% activity following 10 min preincubation at 40°C (Zimmer and Millette 1975).

### 3. Adaptive response to cold shock

When organisms are shifted to sub-optimal temperature of growth (i.e., cold shocked) the organisms immediately exhibit transient response by synthesising a new set of cold shock proteins (CSPs) which presumably help them to acclimatize to newer growth temperature. Mesophilic organisms such as *E. coli* and *Bacillus subtilis* have been studied to some detail in this respect (Jones et al 1987; Williamsky et al 1992; Graumann et al 1996). Similar studies with psychrotrophs and psychrophiles of antarctic or other cold-environment origin have been initiated in a few laboratories but the results are preliminary in nature (Araki 1991; Whyte and Innis 1992; Ray et al 1994d).

#### 3.1 Cold shock proteins of mesophiles

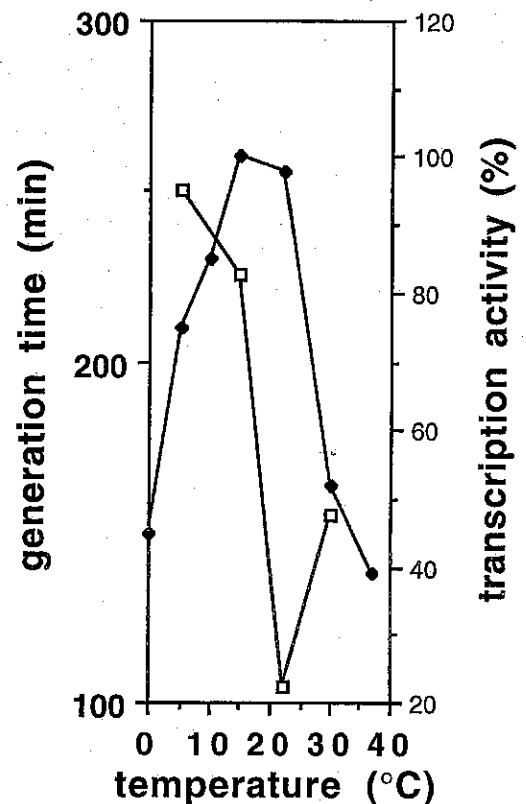
In *E. coli* about 13–15 cold shock proteins have been identified (Jones and Inouye 1994). The genes for these proteins are induced immediately after the downshift of temperature (e.g., 37°C to 15°C) followed by the repression. Although most of these cold shock proteins are involved in transcription and translation they can be classified into several groups (table 1) as described below.

**3.1a Proteins associated with DNA:** RecA, subunit A of DNA gyrase, and histone like protein HNS belong to this groups. While HNS has been demonstrated to be important for low temperature adaptation (Dersch et al 1994), similar experimental evidence regarding other proteins is lacking. Only circumstantial evidences suggest that the increased supercoiling of DNA is important for transcription at low temperature and the gyrase might be involved in bringing about this change (Goldstein and Drilca 1984; Rhode et al 1994).

**3.1b Proteins associated with transcription and post transcriptional events:** Major cold shock protein CspA, NusA, polynucleotide phosphorylase (PNPase), cold

shock induced DEAD-box protein (CsdA) belong to this group.

The prominent among above proteins is the CspA which is induced about 200-fold and constitutes about 13% of the total proteins which are newly synthesized in *E. coli* upon cold shock (Goldstein et al 1990). The homologue of this protein, CspB has been identified in mesophilic *Bacillus subtilis* which exhibited freezing-sensitive phenotype upon disruption of the encoding gene, *cspB* (Williamsky et al 1992). Recently, nine members of the family of *cspA* has been identified in *E. coli*, three of them (*cspA*, *cspB* and *cspG*) are cold inducible (Lee et al 1994; Mitta et al 1997). It is to be noted that *cspB* of *E. coli* and *B. subtilis* are two different genes; the same nomenclature is coincidental. The proteins of the CspA family have distinct RNA binding motifs, RNP1 and RNP2. Recent evidence (Jiang et al 1997) favours a 'RNA-chaperone' function for CspA and its family members. The crystal and solution structures of CspA of *E. coli* and CspB of *B. subtilis* are known (Schindelin et al 1993, 1994; Schnuchel et al 1993; Newkirk et al 1994). The eight surface aromatic



**Figure 2.** *In vitro* transcription efficiency of a RNA polymerase-enriched cell-free extract as a function of generation time of *P. syringae*; (■) transcription activity and (□), generation time. The antarctic *P. syringae* does not grow above 30°C.

amino acids (W11, F12, F18, F20, F31, H33, F34, and Y42) of the five stranded  $\beta$ -barrel structure which probably binds to the single stranded DNA (ss DNA) were identified. The RNA binding motifs, RNP1 and RNP2, are located on  $\beta$ 2 and  $\beta$ 3 strands, respectively (Jiang *et al* 1997). CspA cooperatively binds to single stranded RNAs, with low sequence specificity. Presumably it binds to mRNA to prevent the formation of secondary structure

Table 1. Different classes of proteins that are induced upon down-shift of temperature.

Classes of proteins	Functions	Organisms
Proteins associated with DNA		
Histones like protein (HNS)	Bacterial chromatin structure; regulation of gene expression	<i>E. coli</i>
Subunit A of DNA gyrase	Supercoiling of DNA	<i>E. coli</i>
RecA	Recombination; repair of DNA	<i>E. coli</i>
Proteins associated with the transcriptional and post transcriptional events		
CspA family of low molecular weight proteins (~70 kDa)	Transcriptional activation (?); RNA chaperone	<i>E. coli</i> , <i>B. subtilis</i> , Psychrotrophic <i>Pseudomonas</i> and <i>Arthrobacter</i> sp.
RNA binding proteins (RbpA1, A2, B, C and D)	RNA Chaperone (?)	<i>Anabaena variabilis</i> , <i>Synechocystis</i> sp.
RNA helicase (CsdA)	RNA unwinding activity	<i>E. coli</i>
Transcription factor (NusA)	Transcriptional termination and antitermination	<i>E. coli</i>
Polynucleotide phosphorylase (PNPase)	Degradation of RNA	<i>E. coli</i>
Proteins associated with ribosomes and translation		
Translation initiation factor (IF2, IF2 $\alpha$ and IF2 $\beta$ )	Translation of protein	<i>E. coli</i>
30S ribosome binding factor (RbfA)	Translation of proteins at low temperature	<i>E. coli</i>
Proteins with chaperone function		
Hsc66 (Hsp70 homologue)	Binding to cold-damaged proteins (?)	<i>E. coli</i>
Trigger factor (TF)	Peptidyl-prolyl isomerase activity; enhances binding of unfolded proteins to GroEL; promotion of degradation of certain polypeptides	<i>E. coli</i>
Proteins associated with desaturation of phospholipids		
$\Delta$ 6, $\Delta$ 12, $\Delta$ 15 acyl-lipid desaturases	Unsaturation of acyl chains in phospholipids	Cyanobacteria, e.g., <i>Synechocystis</i> 6803, <i>Anacystis nidulans</i>
Enzymes		
Urocanase, histidase of <i>hut</i> operon	C and N source utilization	Psychrotrophic <i>P. syringae</i>
Pyruvate dehydrogenase (lipoamide), dihydrolipoamide acetyl transferase	Oxidative decarboxylation of pyruvate	<i>E. coli</i>
$\gamma$ -glutamyltranspeptidase	Glutathione metabolism; assimilation of exogenous $\gamma$ -glutamyl peptides as amino acid source	<i>E. coli</i>

for their efficient translation. Interestingly, the cyanobacteria including *Anabaena variabilis* and *Synechocystis* PCC 6803 do not seem to have any homologue for CspA-family of proteins. These organisms have evolved a class of analogous RNA binding proteins (Rbp), such as RbpA1, A2, B, C and D (Sato 1995). Recent genome sequence analysis also suggests that the archaeobacteria, cyanobacteria and parasitic eubacteria, such as *Mycoplasma genitalium* do not have any homologue of *cspA* in their genomes.

Recently, the cold shock protein (CsdA) of *E. coli* has attracted much attention of the workers in the area (Jones *et al* 1996). The 70 kDa CsdA contains an acidic region ('DEAD' in single letter amino acid code) and exhibits RNA helicase activity which may help in unwinding of RNA secondary structure for efficient translation at low temperature. The CsdA is also responsible for derepression of heat shock proteins at low temperature. In contrast, the specific roles of PNPase and NusA during cold shock are not known. The PNPase is known to degrade RNA in cells *in vivo* (Donovan and Kusher 1986). The transcription factor NusA is known to be involved in both termination and antitermination of transcripts in *E. coli* (Friedman *et al* 1984).

**3.1c Proteins associated with ribosomes and translation:** Translation initiation factors 2 $\alpha$  (IF 2 $\alpha$ ) and 2 $\beta$  (IF2 $\beta$ ) and the 30S ribosome binding factor (RbfA) are also induced at low temperature in *E. coli* (Jones *et al* 1987; Jones and Inouye 1996). Because of their known role during initiation of translation of mRNA, IF 2 $\alpha$  and IF 2 $\beta$  are thought to play important role during low temperature acclimation of cells. But the exact significance of these two factors are yet to be determined. The 30S ribosome binding factor, RbfA, on the other hand was originally identified as a multicopy suppressor of a dominant cold-sensitive mutation of 16S rRNA. The absence of RbfA causes severe growth inhibition at low temperature. Interestingly *rbfA* is in the same operon that contains *nusA*, a known transcription factor (Jones and Inouye 1996).

**3.1d Proteins with chaperone function:** Although the roles of molecular chaperones in protein folding during heat shock response have been illustrated for various living cells (Hendrick and Hartl 1993) similar proteins during cold shock are not known. However, recent experimental evidence suggests that there might be low temperature specific chaperones for overcoming cold shock induced anomalies of proteins. A Hsp70 homologue, HSc66 has been shown to be induced by low temperature in *E. coli* (Lelivelt and Kawula 1995). Whether this protein has a role in protein folding similar to the classical heat shock protein, Hsp70 (DnaK) remains unknown. Recently, a cold inducible trigger factor (TF)

has been shown to be important for adaptation of *E. coli* to low temperature (Kandror and Goldberg 1997). The cells with reduced or no TF die exponentially, while over-expression causes enhanced viability at 4°C. The protein has been found to have multiple properties. It exhibits peptidyl-prolyl isomerase activity, binds to GroEL, enhances binding of unfolded proteins to GroEL and promotes degradation of certain polypeptides. However, whether this protein promotes the refolding of cold damaged proteins is not clear as yet.

**3.1e Proteins associated with desaturation of phospholipids:** In the mesophilic cyanobacterium *Synechocystis* PCC 6803, the desaturases which introduce double bonds into specific place of the acyl chains of phospholipids are also induced upon a temperature downshift (e.g., 35° to 22°C) (Murata and Wada 1995). Of the four desaturase enzymes, namely  $\Delta 6$ ,  $\Delta 9$ ,  $\Delta 12$  and  $\Delta 15$  or  $\omega 3$  desaturases, only  $\Delta 6$  (encoded by *desD*),  $\Delta 12$  (encoded by *desA*) and  $\Delta 15$  (encoded by *desB*) desaturases are inducible at low temperature (Los *et al* 1997). The  $\Delta 12$  desaturase is only essential for chill-tolerance of *Synechocystis* PCC 6803 (Wada *et al* 1990).

**3.1f Other cold inducible proteins:** A few other proteins which were shown to be induced during cold shock in *E. coli*, include enzymes such as pyruvate dehydrogenase (Lipoamide), dihydrolipoamide acetyl transferase,  $\gamma$ -glutamyl transpeptidase (Goldstein *et al* 1990; Hashimoto *et al* 1997). What specific role do these enzymes play during cold shock adaptation is not clear as yet.

**3.1g Other cold-shock related changes:** Apart from the induction of cold-shock proteins, several other important changes were found to be associated with the downshift of temperature in *E. coli*. These are temporary inhibition of DNA and RNA synthesis, repression of heat shock proteins, continued synthesis of ribosomal proteins despite a lag period of growth, and decrease in (p)ppGpp level (Jones and Inouye 1996). Among these changes (p)ppGpp was shown to have inverse correlation with the adaptation to low temperature (Jones *et al* 1992a).

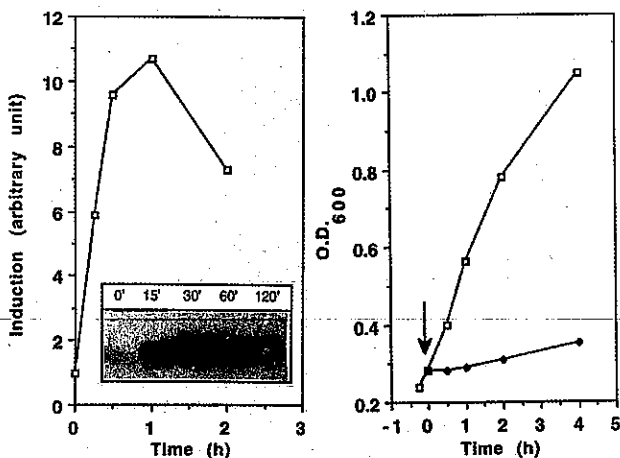
### 3.2 Cold shock proteins of psychrotrophs and psychrophiles

Studies of cold shock proteins from psychrotrophs and psychrophiles are still in infancy. 2-D gel analyses of cold inducible proteins following [<sup>35</sup>S]-labelling have been carried out in a few psychrotrophs, such as *Arthrobacter globiformis* S55, *Bacillus psychrophilus*, *Pseudomonas fragi* and in psychrophiles *Aquaspirillum arcticum*, *Vibrio* sp strain ANT-300 (Bobier *et al* 1972; Araki 1991; Whyte and Innis 1992; Hebraud *et al* 1994). It was found that 5–9% of the proteins are newly synthesized

in the psychrotrophic bacteria when the temperature is shifted from 24°C to 4°C. In *Aquaspirillum arcticum*, 39 new proteins were synthesized upon a temperature downshift of 13° to 0°C (Araki 1991). None of these proteins, however, was characterized. Immunoblot analyses suggested that the homologous of low molecular weight proteins, CspA are induced in the psychrotrophic *P. fragi* and *B. cereus* (Hebraud *et al* 1994; Mayr *et al* 1996). Our own study indicated that most of the antarctic bacteria of Gram-positive and Gram-negative genera contain homologous of *cspA* genes (Ray *et al* 1994d). In one of them, *P. fluorescens* 1W, it was found that the transcription of the homologue was inducible after a shift of the cells from 22°C to 4°C (figure 3). Interestingly, the resumption of growth at low temperature in *P. fluorescens* started only after 30-min to 1 h, coinciding with the maximum induction of the *cspA*. In contrast, in *E. coli*, although the maximum induction was observed within 1 h of the down-shift (37° to 15°C), the resumption of growth took place only after 4–5 h.

Since not much information is available as regards to the nature of cold inducible proteins of psychrotrophs and psychrophiles from the 2D-gel analyses, an alternative genetic approach was undertaken in our laboratory to find out the genes that are upregulated at low temperature (Kannan *et al* 1998). A Tn-5 based promoter probe vector (*pOT182*) containing the promoterless *lacZ* gene (Merriman and Lamont 1993) was used to generate a number of random promoter fusions in the genome of *P. syringae* to identify the frequency of genes which are expressed differentially at low (4°C) and high temperature (22°C). About 70–80% of the promoter fusions

produced  $\beta$ -galactosidase ( $\beta$ -gal) at both the temperatures. Only about 5% of the fusions produced more  $\beta$ -galactosidase at low temperature. Northern hybridization analyses indicated that the latter fusions indeed produced more *lacZ* mRNA at 4°C. One such fusion (F43) which exhibited 10-fold increase in  $\beta$ -gal activity at 4°C was studied in detail. DNA sequence analysis of the flanking region of the fusion revealed that the fused gene has homology (more than 80% identical) with the *hutU* gene of *P. putida* (Fessenmaier *et al* 1991). The *hutU* encodes the enzyme urocanase which is involved in the metabolic pathway of histidine utilization for carbon and nitrogen sources in bacteria (Hu and Phillips 1988). A direct assay of urocanase and histidase (the product of the second gene, *hutH*) in the wild type *P. syringae* indicated that both the enzymes are produced at elevated level at 4°C (figure 4). The urocanase activity was 14-fold higher in the low temperature grown cells of *P. syringae*. Two other antarctic species of *Pseudomonas* strains, *P. putida* and *P. fluorescens* were also found to produce more urocanase and histidase at low temperature (4°C). This was in contrast to the mesophilic *P. putida* which produced very little urocanase at low temperature (10°C) (figure 4). There is no apparent advantage of the *hut* operon for low temperature growth of *P. syringae*. However, based on our earlier observation (Ray *et al* 1992) that a protease is produced more at low temperature in the antarctic yeast *Candida humicola* (figure 5) and the experimental results of other workers (Feller *et al* 1994a) on the production of protease, lipase and  $\alpha$ -amylase in antarctic bacteria, we speculate that the biodegradative operons are in general upregulated at low temperature. This probably enables the antarctic microorganisms to utilize the scarce nutrient sources of antarctic soil.

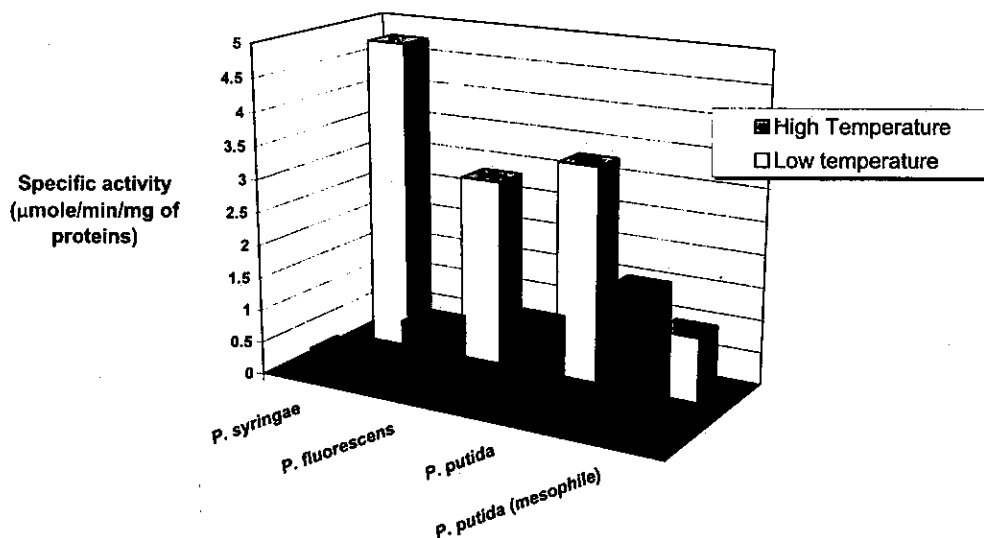


**Figure 3.** Induction of *cspA* and reinitiation of growth of the antarctic *P. fluorescens* 1W following a downshift of temperature (22° to 4°C). The left panel shows the fold of induction of *cspA* mRNA, with a representative Northern blot result as inset. The right panel shows the growth of cells (measured as increase in OD<sub>600</sub>) following a shift from 22°C (□) to 4°C (◆). The arrow indicates the time of temperature shift. For details, see Ref. Ray *et al* (1994d).

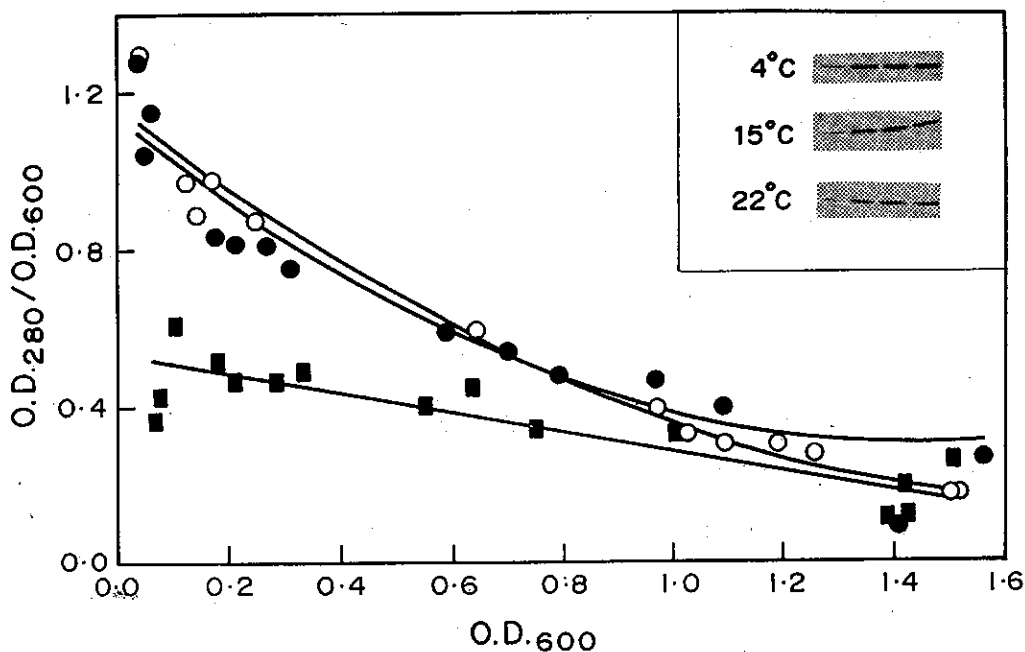
#### 4. Mechanism of up-regulation of cold inducible genes

##### 4.1 Regulation in mesophilic bacteria

Initial Northern blot analyses and RNase protection data suggested that most of the induction of genes at low temperature takes place at the level of transcription in mesophilic bacteria, such as *E. coli* and *B. subtilis* (Goldstein *et al* 1990; Williamsky *et al* 1992). However, promoter fusion studies (e.g., *cspA* promoter fused to *lacZ*) indicated that, while the CspA is found to be induced about 200-fold,  $\beta$ -gal activity is induced only 5–10 folds. Later studies however suggested that the induction of the cold shock genes is regulated not only at the level of transcription but also at the level of post transcriptional stability of the mRNAs (Brandi *et al* 1996; Goldenberg *et al* 1996; Fang *et al* 1997). This was not only true for *cspA* of *E. coli* but also for *desA*, *desB* and *desD* genes of the cyanobacterium *Synechocystis*



**Figure 4.** Urocanase activities of psychrotrophic and mesophilic bacteria grown at low and high temperatures. The antarctic *P. syringae*, *P. fluorescens*, and *P. putida* were grown at 4°C (low temperature) and 22°C (high temperature) while the mesophilic *P. putida* was grown at 10°C (low) and 37°C (high) temperatures.



**Figure 5.** Production of extracellular protease by the antarctic psychrotrophic yeast *C. humicola* at various temperatures (4°C, ○; 15°C, ●; and 22°C, ■). The ratio,  $\text{OD}_{280}/\text{OD}_{600}$  is a measure of protease activity per a constant number of cells which has been plotted against  $\text{OD}_{600}$  to indicate the stage of growth of cells. The inset shows silver nitrate stained protein bands corresponding to the protease which was secreted into the growth medium. For further details, see Ray et al (1992).



PCC6803 (Los *et al* 1997). It is now believed that the abundance of cold inducible proteins at low temperature is perhaps due to a combined effect of increased transcription, increased stability of the transcripts, preferential translation and post translational stability of the proteins. The preferential translation of the low-temperature specific mRNAs is probably assisted by RNA chaperones (e.g., CspA) and other ribosome associated proteins (e.g., CsdA and RbfA) which destabilize the secondary structures of mRNA.

Any positive activator which may induce transcription from the promoters of cold shock genes remains elusive. Early observations in *E. coli* suggested that CspA might work as a cold specific transcriptional factor due to its binding at the upstream regulatory region containing Y-box or "CCAAT" element of cold inducible genes, such as *hns* and *gyrA* (Le Teana *et al* 1991; Jones *et al* 1992b; Lee *et al* 1994). It was also observed that CspA could stimulate transcription from *hns:cat* fusion at low temperature, and the over-production of CspA results in a higher rate of synthesis of other cold-shock proteins, e.g., G55.0, G 41.2, and gyrase A following a downshift of temperature (Jones and Inouye 1994). However, whether CspA functions as a transcription factor, or as a RNA chaperone, or both remains to be determined. It is also to be determined whether there is a division of functions among the three of the cold inducible homologous of CspA, CspB, and CspG in *E. coli* (Mitta *et al* 1997).

#### 4.2 Regulation in psychrotrophs and psychrophiles

Although psychrotrophs and psychrophiles induce cold-temperature specific genes at relatively low temperature (e.g., 0°–4°C) after a downshift from a high temperature of about 20°–24°C, the mechanism of induction remains unknown. A low temperature specific protein in the cell extract of antarctic *P. syringae* was found to cross react with polyclonal antibodies raised against sigma subunit ( $\sigma^{70}$ ) of *E. coli* (S Uma and M K Ray, unpublished results). Whether this cross-reacting protein is a genuine low temperature specific sigma factor, or a degraded product of the major vegetative sigma subunit is yet to be determined. Interestingly, a deep sea *Photobacterium* sp. which is both barophilic and psychrophilic was found to become sensitive to growth at low temperature following a transposon mediated disruption of a  $\sigma^E$  homologue (Chi and Bartlett 1995). Therefore, a role for homologue of  $\sigma^E$  in regulation of cold specific gene expression cannot be ruled out. The *hutU* gene which was found to be upregulated at low temperature (4°C) in antarctic psychrotrophic *P. syringae* is probably controlled by a derepression mechanism (K Janiyani and M K Ray, unpublished observation). A repressor protein which binds to the intragenic *hutU* region and possibly regulates the locus is yet to be characterized.

#### 5. Sensing of temperature and transduction of signal

The temperature dependent expression of genes suggests that there might be a way by which bacteria perceive the signal for a change of temperature and transmit it to the transcriptional apparatus for expression of temperature-specific genes. The temperature signalling mechanism and temperature perception is a fascinating new area of research. Although very little is known, the temperature signalling at cellular level has generally been addressed by asking three broad questions: what is the nature of the sensor(s) and where are they located? What and how many of the molecules are involved in the signal transduction pathway? How do they communicate with each other for the ultimate cellular response?

A few recent studies have attempted to answer the first and a part of the second question. These studies have come up with some novel ideas and interesting models in various systems in which the studies were carried out. The working models which have been proposed in the above studies can be divided into four basic types as follows:

(i) *Ribosomes as cellular sensor for temperature*: This model (van Bogelen and Neidhardt 1990) was proposed on the basis of works on *E. coli*. The model proposes that an alteration in the environmental temperature is perceived at the level of ribosomes which act as the primary sensor. A shift-up or shift-down of environmental temperature alters the translational capabilities of the ribosomes which probably then produces a secondary signal (e.g., synthesis or break down of ppGpp) which is transmitted to the regulator for eliciting a suitable response at the transcription level. Evidence for this model was mainly based upon the production of heat shock and cold-shock proteins in *E. coli* at the normal or optimal temperature of growth, namely 37°C, by treating the cells with sub-inhibitory concentrations of antibiotics which inhibit translation of proteins at the level of ribosomes. It was shown that a group of antibiotics (e.g., chloramphenicol, erythromycin, fusidic acid, spiramycin, and tetracycline), which either block the "A" site of ribosomes directly or by binding of an aminoacyl tRNA, could induce the production of cold shock proteins while another group of antibiotics (e.g., kanamycin, puromycin, and streptomycin) which block protein synthesis by keeping the "A" site empty could induce the heat shock proteins in *E. coli*. The recent finding that the disruption of *rbfA* which encodes a 30S ribosomal associated factor derepresses the heat shock proteins (Jones and Inouye 1996) provides a partial support to the model. A similar suggestion comes from the study of conditional suppression of the *rpsL31* mediated streptomycin resistance at low temperature in certain mutant (*gicB1*) strains of *E. coli* which suggests

that ribosome function (and structure) may be altered at cold (Mangoli *et al* 1997).

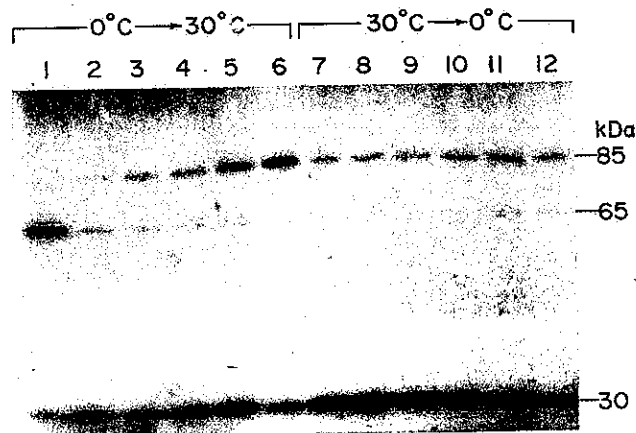
(ii) *Change of membrane fluidity as a primary sensor for temperature*: This model was proposed on the basis of work on expression of the genes for desaturases in the cyanobacterium *Synechocystis* PCC6803 (Vigh *et al* 1993). The cyanobacterium *Synechocystis* PCC6803 could normally induce transcription of *desA* (encoding  $\Delta 12$  desaturase) upon a down-shift of temperature, such as  $36^\circ$  to  $22^\circ\text{C}$ . However, if the saturation level of the membrane lipids is increased to alter the membrane fluidity by catalytic hydrogenation *in vivo*, the cells could induce the transcription of *desA* at  $36^\circ$  to the same level as seen for the temperature down-shift. Therefore it was proposed that an alteration of cell membrane fluidity due to a change in environmental temperature might act as a primary signal for temperature and the cellular sensor is the membrane.

(iii) *Change in phosphorylation states of membrane proteins in response to temperature*: This model was proposed on the basis of work on antarctic bacteria, specifically on the phosphorylation and dephosphorylation of membrane proteins (figure 6) in the antarctic *P. syringae* (Ray *et al* 1994b). In this study a correlation was observed between phosphorylation and dephosphorylation of a set of membrane proteins in response to up-shift and down-shift of temperature both *in vitro* and *in vivo*. This study, however, did not demonstrate the effect of this phosphorylation/dephosphorylation of proteins on the expression of any heat or cold inducible genes. Interestingly, it was observed in *P. syringae* that the phosphorylated membrane protein could induce phosphorylation of a cytosolic 66 kDa protein at tyrosine residue, probably by a tyrosine kinase (Ray *et al* 1994c). Although the tyrosine kinases are known to play important roles in signal transduction of eukaryotes, a similar role for bacterial tyrosine kinase is yet to be established.

(iv) *Calcium channels and  $\text{Ca}^{2+}$  influx/efflux in response to temperature*: That calcium channel could work as a sensor for environmental temperature came from the studies mainly with plants (Monroy and Dhindsa 1995). It was observed that upon a down-shift of temperature there is an increased calcium influx into the cells of alfalfa (*Medicago sativa*) and the subsequent induction of cold acclimation-specific genes or *cas* genes (such as *cas15*, *cas18*). The induction of the *cas* genes at  $4^\circ\text{C}$  could be blocked by using calcium chelator, such as BAPTA (1,2-bis(o-aminophenoxy)ethane) and by specific calcium channel blockers, such as lanthanum ( $\text{La}^{3+}$ ) which blocks the channels from outside. The *cas* genes could also be induced at normal temperature ( $25^\circ\text{C}$ ) by using a calcium ionophore, such as A23187 or a calcium

channel agonist Bay K8644 which allows the calcium ions to get into the cells directly. Therefore, it was proposed that the calcium channel proteins directly, or due to alteration of the membrane fluidity, are activated by the down-shift of temperature and allow the calcium ions to enter into the cells from outside. The cytoplasmic calcium in turn probably activates a cascade of calcium dependent kinases which would lead to the expression of *cas* genes. Therefore, calcium channel proteins and calcium ions could act as a sensor and second messenger, respectively, in the temperature signal transduction pathway.

Among the four models, the last three have something in common in that they presume the primary sensor for temperature perception is located on the surface of the cells, namely plasma membrane. The last three models also could be linked by a hypothesis that the modulation of calcium pump activity is brought about by the change in its phosphorylation state by specific kinase(s) or phosphatase which are located in the cell membrane and are activated by alteration in the membrane fluidity following changes in environmental temperature. In essence, therefore, the basic mechanism for temperature perception may lie either on surface of the cells (Vigh



**Figure 6.** Temperature dependent phosphorylation of membrane proteins of the antarctic *P. syringae*. The triton X-100 solubilized proteins of the membrane fraction of *P. syringae* were phosphorylated *in vitro* in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  at  $0^\circ$  (lane 1) and at  $30^\circ\text{C}$  (lane 7), and then shifted to the experimental temperatures for various time periods. The phosphorylated proteins were detected by autoradiography. Low temperature specific 65 kDa and high temperature specific 30 kDa and 85 kDa protein bands has been marked. Time kinetics of change of phosphorylation of the proteins, following the shift of temperatures have been shown in lanes 1–6 (for  $0^\circ$  to  $30^\circ\text{C}$  shift) and in lanes 7–12 (for  $30^\circ$  to  $0^\circ\text{C}$ ). The time points are 0 (lanes 1 and 7), 5 (lanes 2 and 8), 10 (lanes 3 and 9), 15 (lanes 4 and 10), 30 (lanes 5 and 11), and 60 min (lanes 6 and 12). For further details, see Ray *et al* (1994b).

et al 1993; Ray et al 1994b; Monroy and Dhindsha 1995), or in the intracellular ribosomes (van Bogelen and Neidhardt 1990), or could be located in both places. The possibility of existence of multiple sensors for temperature perception also could not be ruled out, neither could we rule out the variation in the temperature perception mechanism in different living systems. A genetic screening for mutants which exhibit deregulated expression of luciferase from the chromosomal *PdesB:lux* fusion has been initiated in *Synechocystis* PCC6803 (Los et al 1997; Ray and Murata, unpublished results) which might shed new light in identifying various molecular candidates of temperature signalling pathway.

## 6. Concluding remarks

Understanding the molecular basis of cold adaptation is still in its infancy. Relatively more progress has been made in mesophiles than in psychrophiles and psychrotrophs. Among the psychrophiles and psychrotrophs antarctic microorganisms are unique in that they have evolved in extremely cold, and relatively unfluctuating temperature for a long period of time. However, the biota inhabiting the region today is probably the result of the population which already existed in the tropics before the drift of the continent from the super continent of Gondwana about 38 million years ago, and relatively recent long-range colonization by new population of species. According to one estimate, the ice probably started accumulating on the antarctic land mass about 30 million years ago (Wynn-Williams 1990). Since then the continent has remained permanently covered with ice sheet along with the massive floating ice shelves over the surrounding ocean. Therefore, the original population of microorganisms (mesophiles?) might have evolved very slowly due to low metabolic activities and reduced cell division rate in the prevalent low temperature of the last 38 million years. It would therefore be doubly rewarding to study the biology of antarctic bacteria, as it would help not only to understand the molecular basis of cold adaptation but also the basic evolutionary process and adaptive radiation of microorganisms. The antarctic microbial organisms may also provide a new source of novel enzymes and cellular metabolites for future biotechnological applications.

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