"At the end of three yards I shall repeat them—
for fear of your forgetting them.
At the end of four, I shall say good-bye.
And at the end of five, I shall go!"

Through the Looking Glass
STANDING IN THE THRESHOLD of the transition from pre- to post-genomic era, microbiology will never be the same again. Looking at the types of the genomes sequenced so far, it is quite obvious that the extremophiles caught the fancies of the modern day biologists. Primary reason for this is that extremophiles, as a group, are extremely important, economically. One more reason for this interest is more fundamental, and that is, the quest for an insight into how, being strictly governed by orthodox biological dogmas, life thrives under apparently unorthodox circumstances.

Temperature, as a physical factor has the most profound influence on biochemical machinery of any organism. Yet we find active microbial community in all ranges of temperatures. If the flexibility of a system determines its adaptability, then psychrotrophs are the most adaptive of all the microbes with the widest and the most flexible growth temperature range.

The work presented in this thesis was an attempt to shed some lights on the adaptability of transcription process of a psychrotrophic bacterium, originally classified as *Pseudomonas syringae* strain Lz4W, from Antarctica.

Transcription, the process of cellular RNA synthesis, is the central point of integration of signals controlling gene expression. A particular subunit of RNA polymerase, σ, plays central role in transcription, conferring sequence specific DNA binding capabilities to otherwise non-specific core RNA polymerase. There are several different types of σ factors in a cell. The bulk of the transcription in Gram-negative bacteria is carried out by RNA polymerase containing σD, the primary σ factor, encoded by *rpoD* gene. In Gram-negative bacteria, one of the σ factors, σS, encoded by *rpoS*, is known to act as the master controller of stress-response. σS was, therefore, expected to play an important role in the adaptation process of Antarctic bacteria.

Earlier studies have shown that RNA polymerase from Antarctic psychrotroph *P. syringae* Lz4W has the ability to carry out transcription at low temper-
ature (Uma et al. 1999). The enzyme has subunit composition similar to its mesophilic counterpart, but differs in its ability to distinguish cold-inducible promoter of *E. coli* in a temperature-dependent manner.

This thesis looked into the role of $\sigma^5$ in the cold-adaptation of this Antarctic psychrotroph, and the associated stress response. The work also examined any change that might have taken place in the primary sequence of $\sigma^5$ and $\sigma^D$ during the evolution of these cold-adapted bacteria. This Chapter is an attempt to summarize the findings of this work with the existing knowledge into a coherent model.

### 7.1 A summary of the results

#### 7.1.1 Cloning and analysis of $\sigma^D$ and $\sigma^5$

Using a variety of techniques, the DNA fragments containing a partial ORF encoding *rpoD* and full-length ORF encoding *rpoS* were cloned from the genomic library of Lz4W (Chapter 3). Comparison of these sequences with the existing sequences in the database revealed close similarity with their homologs from other bacteria (Chapter 4). The comparison also revealed that the DNA fragment containing the *rpoD* has the C-terminal half of the gene containing the major functional elements. The DNA fragment containing the *rpoS* contained the full-length ORF of the gene, including the putative promoter elements. Sequence comparison also revealed that *rpoD* in its functional regions, particularly, region 2.3 which is involved in promoter melting, a rate limiting process in transcription at low temperature, is not different from its mesophilic counterpart (Chapter 4).

Interestingly, the reading frame of *rpoS* was found disrupted with an amber mutation. The mutation was found to be present in all natural isolates of Lz4W. Sequence comparison revealed that the amber is located in a position that unless suppressed, the resulting $\sigma^5$ will lack the C-terminal region 4 of the $\sigma$ factor. The region 4 has been shown to be involved in recognition of $-35$ element of the promoter (Chapters 3 and 4).

#### 7.1.2 Transcriptional activity of the mutated $\sigma^5$

When transformed into *E. coli*, the amber-mutated *rpoS* of Lz4W could activate *bolA* and *csiD* promoters, which were shown to be *rpoS*-controlled in *E. coli* (Chapter 5). Two other *rpoS*-controlled promoters, *katE* and *osmY* in *E. coli* were not activated. Nucleotide sequence comparison of the *rpoS*-controlled promoters in *E. coli* revealed that the two activated promoters (of *bolA* and *csiD*) had G at $-14$ of the promoter, characteristic of extended $-10$ promoters in *E. coli* (Chapter 5).
7.1.3 Expression and requirement of σ^5 during growth

Direct detection of σ^5 protein in cell extract by immunoblot analysis indicated that the amber indeed resulted in a truncated protein in Lz4W. Moreover, the σ^5 protein level was repressed during growth at 4 °C. rpoS gene was disrupted before the amber, thus, generating a rpoS null-mutant. The null-mutant was marginally slow grower at low temperature than the wild-type Lz4W. The null-mutant was also severely retarded in its ability to grow at low pH, both in high and low temperature. This results indicated that in spite of harboring an amber the rpoS of Lz4W was, indeed, functional (Chapter 6).

In a related study of the catalases of Lz4W it was found that Lz4W cells possess only one catalase isoform, which, although growth phase related, is perhaps not rpoS-controlled (Chapter 6).

7.1.4 The significance of the amber mutation in σ^5

To assess the the significance of the amber mutation, a chimeric gene was created by fusing the N-terminal half of rpoS gene of Lz4W with the C-terminal half of rpoS gene of P. aeruginosa. Sequence analysis predicted that the resulting chimeric protein will be in all respect similar to the native rpoS gene of Lz4W. When expressed from a plasmid, the chimeric protein was expressed in high level in Lz4W, and caused severe cold-sensitivity. When expressed from the same plasmid, the native gene (with an amber) also caused growth defect at low temperature but not to the extent as observed for the full-length protein (Chapter 6).

In contrast to its effect on growth at low-temperature, the full-length rpoS did improve the viability of the stationary phase culture at low temperature. Surprisingly, the amber-mutated rpoS also improved the viability, almost to the level of the full-length protein (Chapter 6).

7.2 rpoS of Lz4W—a GASP allele?

As discussed in Section 1.13, rpoS in E. coli and Salmonella, frequently accumulates mutations. It was also discussed in Section 1.14, that prolonged incubation of E. coli culture at stationary phase selects for mutant rpoS allele, which could outcompete the ancestral wild type population at stationary phase. These rpoS alleles are severely deficient in rpoS function. Because rpoS function is required for the maintenance of the viability at stationary phase, GASP mutants create a paradox in the field. It was also discussed in Section 1.14 that the selection advantage of the GASP allele over the wild-type allele, could be explained in terms of prisoner's dilemma (Vulić and Kolter 2001).
A closer look at the prisoner's dilemma theory, however, makes it obvious that the "defection" can only be selected in the presence of the other player in the game. In absence of the "conformers," the defectors lose its selective advantage. The fitness data presented in Vulic and Kolter (2001) also shows that rpoS GASP alleles can dominate the culture only when inoculated as minority and could have a selective advantage only in presence of a large excess of cells, carrying wild-type rpoS allele.

According to Game theory (Axelrod and Hamilton 1981), in a population solely consisting of defectors, the punishment, $P$ (see Section 1.14) is less than $S$ (sucker's payoff) resulting in Chicken run ($T>\cdot R>S>P$), which according to the theory will allow the wild-type cells to invade the defectors and will force the population to harbor both the defectors (GASP allele) and the cooperator (wild type allele) to coexist. A mutant allele, therefore, will be under considerable selection pressure to revert back to the wild type. Discovery of a large number of natural isolates containing solely of GASP mutants, therefore, can not be explained by idea proposed by Vulic and Kolter (2001). The obvious alternative is that rpoS mutant may confer a genuine growth advantage to the cells in hitherto unknown environmental conditions.

The first report of such an advantage came from the work done by Notley-McRobb et al. (2002). It was found that in slow growing chemostat culture of E. coli, rpoS mutants get selected and are fixed in the population. Moreover, under dual stress conditions (carbon starvation and low pH) most of the mutants were carrying amber in the rpoS reading frame. The authors also demonstrated that rpoD-controlled genes were upregulated in these mutants. The authors attributed the selection of these mutants on the increasing outer membrane permeability and increased scavenging of the nutrients. The selection of the amber-mutated alleles under dual stress condition, led the authors to conclude that amber mutation is a mechanism to reduce the activity of $\sigma^5$, simultaneously, maintaining enough $\sigma^5$ level to deal with the stress condition.

The work presented in this thesis is a second observation in the same line, and the first report that the GASP allele do play a very vital role during growth at low temperature, at least in case of psychrotrophic bacteria. The amber-mutated rpoS did produce an attenuated $\sigma^5$. A complete loss of rpoS is detrimental to the cell under stress, as shown by the severe acid-sensitivity and marginal cold-sensitivity of the mutant. Moreover, overexpression of rpoS in trans caused severe retardation of growth at low temperature. The amber in rpoS of Lz4W, is therefore, a gene regulatory mechanism to control an optimum level of $\sigma^5$ in the cell and probably confer a selective advantage during growth at low temperature.
7.3 Role of rpoS in cold-adaptation of Lz4W

In the mesophilic E. coli, rpoS is upregulated during growth at low temperature (Sledjeski et al. 1996). rpoS mutant, however, has no growth defect at low temperature (20 °C). The upregulation is mediated by DsrA RNA (see Section 1.11.3.4 on page 25). In pseudomonads, DsrA is not identified till date. In contrary to the finding in E. coli, the rpoS level in Lz4W was actually downregulated during growth at low temperature. It remains to be seen whether this phenomenon is specific to pseudomonads in general, or a consequence of the cold-adaptation of this species. The work presented here suggests the latter, supported by the fact that the high level of rpoS expression caused severe growth-defect, only at low temperature.

How does lowering of σS help in cold adaptation? A very naive hypothesis would be that in Lz4W, the growth at low temperature is analogous to the situation of E. coli growing at chemostat culture, with bulk of the transcription carried out by RpoD. As discussed in Section 1.15, lowering of the activity of σS will increase the activity of σD, thereby promoting growth. An amber-mutated rpoS helps in this process by reducing the activity of σS but still retaining enough σS activity to cope with the stress conditions. There are several ways this can be achieved. As shown in Chapter 5, the truncated rpoS was capable of promoter discrimination within rpoS regulon at least in E. coli. The mutated rpoS, therefore, could have retained the ability to induce the stress-responsive genes, at the same time reduce its own binding capacity to core RNA polymerase. This may be due to a structural defect in the truncated rpoS reducing its affinity to core enzyme, or may be a direct effect on a gene, modulating the control of the core binding properties of σ factor, such as anti-σ factor, or simply by increasing the Km of EoS to the substrates as demonstrated for Eo70 in E. coli (Campbell et al. 2002). Further studies will require to test these hypotheses.

7.4 Lessons learned from rpoD sequence

Strand separation (melting) of the promoter during open complex formation in transcription, is highly temperature dependent. If the extent of DNA melting is plotted as a function of temperature, the resulting plot resembles a DNA-melting curve with the midpoint of the transition around 20–25 °C (Coulombe and Burton 1999; and references therein). The energy requirement of promoter melting is as high as 1 kcal/mol/bp of the double stranded DNA (deHaseth et al. 1998). No external energy source is required to form this open complex. It is therefore, very easy to grasp that the prime energy barrier for transcription at low temperature, is the promoter melting and subsequent open complex formation.
As described in Section 1.7.2.4 on page 14, hydrophobic and charged residues in the region 2.3 of σ factor play crucial roles in promoter melting. It was therefore expected that comparing region 2.3 of Lz4W RpoD with its mesophilic counterpart might give valuable insight into the mechanism of the ability of the purified RNA polymerase of Lz4W to transcribe at low temperature. Surprisingly, there were no changes in this region or any other region that are unique to RpoD of Lz4W (Chapter 4). The secret of transcription at low temperature therefore, lies either in region 1.1 of RpoD of Lz4W, which was missing from the cloned DNA fragment or in some other subunit(s) of RNA polymerase, or even in some external factors.

7.5 Final comments

The mechanism of cold-adaptation is a very complex phenomenon and encompasses the whole physiology of the cold-adapted organism. The work presented in this thesis shows only a glimpse of highly complex, sometimes sophisticated, sometimes crude, but mostly ingenious ways, how living organism adapts to its environment. Extremophiles are the best examples of what mere chance and trials could achieve, when survival is at stake; a mechanism commonly called as Evolution. A lot more work is required before one will be in a position to truly realize the power of this tinkering. But for the time being, this tinkerer leaves us with, nothing but sheer awe and a feeling of humbleness.