Chapter 4

DISCUSSION AND CONCLUSIONS
DISCUSSION

Adaptation of bacteria to low temperature is a complex process initiated by the ability of the bacterium to sense changes in temperature and transduce the signal to the cytoplasm and activate genes required for the survival of the bacterium (Shivaji et al., 2007b). In a few bacteria the sensor and the response regulator have been identified and the various modes of temperature sensing mainly involving the membrane have also been studied (Shivaji et al., 2007b). A few candidate genes required for growth of psychrophilic bacteria at low temperature have also been identified but the available information are very scanty and there is a need to identify gene/genes essential for low temperature survival and growth. The psychrophilic Pseudomonas syringae (Lz4W) grows in the temperature range of 2°C to 30°C and thus could serve as a model system to understand the molecular basis of cold adaptation and for the identification of gene/genes required for survival and growth of bacteria at low temperature (Ray et al., 1994a, 1994b, 1994c, 1998; Kiran et al., 2004, 2005; Chintalapati et al., 2004, 2005, 2007; Shivaji et al., 2007b). Using approaches of DNA microarray or proteomics, one could identify a number of genes or proteins that are up or down regulated in bacteria growing at low temperature in contrast to bacteria growing at optimum temperature but the wealth of information acquired would not be sufficient to conclude whether a particular gene or protein is absolutely essential for growth at low temperature only. Therefore to identify candidate gene or genes essential for low temperature survival and growth in bacteria, a mutagenesis approach, wherein only a single candidate gene is inactivated would help to identify the gene of interest since the mutant strain would be expected to become cold sensitive in contrast to the cold resistant wild type strain. Very few studies have used this approach on psychrophilic bacteria (Kannan et al., 1998; Regha et al. 2005).
In the present thesis, attempts have been made to identify a gene essential for growth of a psychrophilic bacterium *Pseudomonas syringae* (Lz4W) using the transposon mutagenesis approach. *Pseudomonas syringae* (Lz4W) has been earlier used as a model system to understand the molecular basis of cold adaptation (Shivaji et al., 1994, 2007b; Ray et al., 1998) with respect to its ability to transcribe at low temperature (Uma et al., 1999; Ray et al., 1999), ability to sense temperature (Ray et al., 1994a; 1994b), ability to catalyse reactions at low temperatures (Ray et al., 1992; Reddy et al., 1994; Chattopadhyay et al., 1995) and to understand the role of membrane components such as fatty acids in thermal adaptation (Kiran et al., 2004, 2005; Chintalapati et al., 2004, 2005, 2007). This bacterium has also been used for the identification and characterization of a gene involved in cold adaptation (Regha et al., 2005).

The objectives of the present thesis were:

1. Generation of cold sensitive mutants of psychrophilic *Pseudomonas syringae* (Lz4W) using random transposon mutagenesis.
2. Identification and expression of the gene during growth at low and optimum temperature.
3. Identification and characterization of the promoter of the gene.
4. Over-expression of the gene and purification and characterization of the gene product.
4.1 CSM1, A COLD SENSITIVE MUTANT OF PSYCHROPHILIC

*Pseudomonas syringae* (Lz4W)

CSM1, was generated as a cold sensitive mutant of psychrophilic *Pseudomonas syringae* (Lz4W) following transposon mutagenesis. The mutant is defective in growth at 4°C in that it exhibits a prolonged lag period compared to the wild type *Pseudomonas syringae* (Lz4W) and interestingly the growth of the mutant at 22°C and 28°C was like in the wild type cells. Thus these results clearly indicate that CSM1 is a cold sensitive mutant. As of now two types of cold sensitive mutants have been reported. The first type includes those mutants that have totally lost the ability to grow at low temperature and the second type includes those that grow at low temperature but the growth rate is slow and the lag period is very prolonged and thus could be easily recognized as distinct from the wild type. Mutants of the first type are known as severe cold sensitive and those of the second type are known as slow growing. CSM1, is a mutant of the second type and has a phenotype similar to that of other cold sensitive mutants such as the *pnp* mutant in mesophilic *Escherichia coli* and *Bacillus subtilis* (Luttinger et al., 1996) and in the psychrotrophic *Yersinia enterocolitica* (Goverde et al., 1998), *bipA* mutant in the enteropathogenic *Escherichia coli* strain D10 (Pfenning and Flower, 2001) and *rbfA* mutant in *Escherichia coli* (Bing et al., 2003). In contrast, *oppA* mutant in the psychrophilic *Listeria monocytogenes* (Bozic et al., 2000) and *recD* in psychrophilic *Pseudomonas syringae* (Lz4W) (Regha et al., 2005) resulted in mutants of the first type that totally lost the ability to grow at low temperature. These results indicate that the type of mutant generated did not depend on whether the bacterium was a mesophile or a psychrophile and in all the studied cases so far, the cold sensitive phenotype was attributed to the inactivation of a single gene.
4.1.1 *trmE* is mutated in CSM1, a cold sensitive mutant of psychrophilic *Pseudomonas syringe* (Lz4W)

Results unequivocally establish that CSM1 is also a consequence of a single insertion of transposon in the genomic DNA because the genomic DNA of CSM1 following restriction digestion with *EcoR*I and Southern hybridisation using labeled pOT182 as the probe, yielded only two bands. This is possible only if one copy of pOT182 is inserted because *EcoR*I would cut once in the single *EcoR*I site in pOT182 and cut also outside the transposon thus generating at least two fragments having pOT182, which would hybridise with pOT182. If the number of insertions of pOT182 in the genome were more than one, the number of fragments that would hybridise with pOT182 would be at least more than two, which was not the case with CSM1. Thus confirming that only a single copy of the transposon was integrated in CSM1 genome and the resultant phenotype was a direct result of this single insertion (Fig. 3.2).

The insertion of the transposon in CSM1 could be mapped to t-RNA modification GTPase (*trmE*) between 829 and 830 bp and this insertion is assumed to be responsible for the cold sensitive phenotype. In fact this indeed may be so because when CSM1 was complemented with *trmE* from the wild type strain *Pseudomonas syringae* (Lz4W), the growth of the complemented strain at 4°C was faster than the mutant and the phenotype was rescued significantly (Fig. 3.1), thus indicating that the cold sensitive phenotype was due to the inactivation of *trmE*. The growth rate of the complemented strain improved significantly compared to the mutant but it did not recover totally and exhibited slower growth compared to the wild type. The reason could be due to the fact that the plasmid used for the complementation was causing a delay in growth. But, this is not the case since wild type *Pseudomonas syringae* (Lz4W), transformed with pGL10 alone did not show any effect on the growth kinetics. The slower growth of the complemented mutant may also be due to the polar effect of another gene that is situated down stream.
Goverde et al. (1994) also observed that when the cold sensitive *pnp* mutant of *Yersinia enterocolitica* was trans-complemented with *pnp* gene the mutant did not recover completely and it was suggested that one of the downstream genes might also be responsible. In contrast, trans-complementation of the *recD* mutant of *Pseudomonas syringae* (Lz4W) and *oppA* mutant of *Listeria monocytogenes*, which are cold sensitive mutants, resulted in total recovery and the complemented strains were capable of growth like the wild type at low temperature (Borezee et al., 2000 and Regha et al., 2005). The disruption of *trmE* in psychrophilic *Pseudomonas syringae* (Lz4W) altered the psychrophilic strain to a cold sensitive strain but did not in any way alter the ability of the strain to grow and divide at 22°C and 30°C indicating that it is not a lethal mutation.

In contrast, in certain strains of *Escherichia coli* loss of *trmE* function *E. coli* strains JC7623 (recB21 recC22 sbcB15 sbcC201 sal*) and V5701 (bgl, Sal*) in which *trmE* is non-functional are not viable but MC1000 (F′ araD139 Δ (ara-leu) 7679 galUK Δ (lac) X74 rpsL thi) and DEV16 (F′ thi-l rec22 sbcB15 sbcC201 Sal*) are viable (Yim et al., 2006). Earlier studies have indicated that *trmE* mutants (*trmE and trmF*) of *Escherichia coli* exhibits the phenotype of readthrough at UAG codon and are defective in biosynthesis of the hyper modified nucleosides 5-methylaminomethyl-2-thiouridine (mmn⁵S⁵U) which is found in the wobble position of some t-RNA (Elseviers et al., 1984; Kruger and Sorensen, 1998). The *trmD* gene in *E. coli* encodes for t-RNA (mⁱG37) methyltransferase or TrmD and mutant of *trmD* showed a severalfold reduced growth rate (Persson et al., 1995). The *trmA* and *trmH* have also been characterized from *E. coli* and known to have t-RNA methyltransferase activity and help in t-RNA modification (m⁵U54 and Gm18), respectively (Bystrom et al., 1982; Ny et al., 1988). The *trml* in *Thermus thermophilus* HB27 encodes for t-RNA (m⁵A58) methyltransferase (Trml) and is required for growth at extreme temperatures. The mutants of *trml* in *T. thermophilus* HB27 exhibits thermosensitive phenotype at 80°C (Droogmans et al., 2003). The *gidA* mutant of *E. coli* was known to
affects cell division, but only when cells are grown on glucose (Yim et al., 2006). In the plant pathogen *Pseudomonas syringae*, mutations in *gidA* affects antibiotic production, swarming, presence of fluorescent pigment and virulence (Kinscherf et al., 2002).

4.2 IDENTIFICATION OF *trmE* AS THE MUTATED GENE IN CSM1, THE COLD SENSITIVE MUTANT OF PSYCHROPHILIC *Pseudomonas syringae* (Lz4W)

The transposon mutated gene in CSM1 based on its sequence was identified as having high similarity (98-99%) with *trmE* of *Halomonas cupida* (AM944535), *Brevibacterium linens* (AM944530) and *Bacillus megaterium* (AM944534) at the nucleotide level. Subsequent, sequencing of the entire *trmE* from the psychrophilic bacterium *Pseudomonas syringae* (Lz4W) followed by BLAST N analysis also confirmed that the sequence was very similar to *trmE* of *Halomonas cupida* (AM944535). The *trmE* gene, encodes for TrmE, a GTP binding protein and many homologues of TrmE have been reported from prokaryotes and eukaryotes but not in archaea. This gene is known to be essential in *Escherichia coli* and is involved in t-RNA modification (Cabedo et al. 1999). As of now, 19 t-RNA modifying enzymes have been identified from 45 putative t-RNA modifying genes in *Escherichia coli* genome but TrmE is the only t-RNA modifying enzyme, which also has GTPase activity. In *Saccharomyces cerevisiae* also a TrmE homologue was shown to have GTPase activity (Colby et al., 1998; Decoster et al., 1993). BLAST P analysis of TrmE of *Pseudomonas syringae* (Lz4W) also showed maximum sequence similarity of 99% with *Halomonas cupida* (CAQ16333), and minimum sequence similarity of 36% with *Thermotoga maritima* (AAD35356). Further, CLUSTAL W analysis of the amino acid sequence of TrmE of *Pseudomonas syringae* (Lz4W) confirmed that the sequence is highly conserved and is very similar to the TrmE sequence of other bacterial species. Further, various sequences like the GTP binding motifs (G-1, G-3 and G-4) and the effector molecule binding motif (G-2) are conserved
as in other bacteria (Fig. 3.5) and the consensus sequences for G-1 to G-4 were GRPNAGKS, GTTRD, DTAG and NKAD respectively (Fig. 3.5). Earlier studies had also indicated that G-1 to G-4 are conserved in all TrmE homologues (Yamanaka et al., 2000; Martinez-Vicente et al., 2005). Cabedo et al. (1999) had suggested that only the GTP-binding domains are involved both in GTP-binding and GTPase activity in *Escherichia coli* TrmE. The present results imply that the TrmE of *Pseudomonas syringae* (Lz4W) is a GTP-binding protein and also possesses GTP hydrolysis activity, thus making it a GTPase. Yamanaka et al. (2000) suggested that motif G-2 is not involved in GTP-binding but binds to an effector molecule. It is also observed that the G-2 consensus sequence is limited to TrmE homologues and not present in other GTP-binding proteins, suggesting that TrmE homologues constitute a novel subfamily of the GTPase superfamily (Yamanaka et al., 2000). GTPase activity of TrmE has been characterized from *Thermotoga maritima*, a hyperthermophilic bacterium and it has been established that it has maximum activity at 70°C (Yamanaka et al., 2000). Proteins possessing both GTP-binding and GTPase activities play essential roles in cell proliferation, signal transduction, protein synthesis and protein targeting (Bourne et al., 1990; Kjeldgaard et al., 1996).

In contrast to the Ras-like proteins that cycle between a GDP bound inactive and a GTP bound active state (Vetter and Wittinghofer, 2001; Bourne et al., 1990), TrmE GTPase is directly involved in modification of the wobble position uridine (U34) in some t-RNAs in bacteria, yeast and mammals (tRNA-Lys, tRNA-Glu, tRNA-Leu, tRNA-Arg and tRNA-Gln). It modifies U34 to 5-methylaminomethyl-uridine in bacteria, 5-carboxymethylaminomethyl-uridine in yeast and 5-taurinomethyl-uridine in humans. This modified t-RNA also interacts with G and A, but restricts base-pairing with C and U (Yokoyama et al., 1979; Yokoyama et al., 1985; Yokoyama and Nishimura, 1995) to avoid mis-incorporation of amino acids in mixed codon box families (Glu, Gln, Lys, Leu and Arg).
Furthermore, the modification influences frame-shift during the translation process (Brierley et al., 1997; Hagervall et al., 1998; Bjork et al., 1999; Urbonavicius et al., 2001).

In addition to the above conserved motifs (G1-G4) four other conserved regions (I, II, III and IV) have also been identified in the TrmE sequence of *Pseudomonas syringae* (Lz4W). These four conserved regions were earlier identified in TrmE of a number of bacteria and based on the observation that *trmE* mutants of *Escherichia coli* are deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine of t-RNA (Elseviers et al., 1984) it has been speculated that these regions may be responsible for t-RNA modification activity (Yamanaka et al., 2000). It is also interesting to note that the C-terminal of TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) has a sequence CIGK in region IV, which is similar to the consensus sequence CAAX (where A represents an aliphatic amino acid residue and X represents any amino acid residue), which is a site for isoprenylation in the Ras protein (Willumsen et al., 1984). The presence of such a site in bacteria is very intriguing since neither isoprenylation nor genes involved in isoprenylation have been identified in prokaryotes so far (Willumsen et al., 1984). TrmE of *Pseudomonas syringae* (Lz4W) also possessed a number of putative phosphorylation sites (11 serine phosphorylation sites, 6 threonine phosphorylation sites and 2 tyrosine phosphorylation site) and thus it is tempting to speculate that the phosphorylation status of TrmE may be regulating its dual activity based on the phosphorylation status. Previously, it has been demonstrated that *Pseudomonas syringae* (Lz4W) exhibits differential phosphorylation of proteins in response to temperature (Ray et al., 1994a) and in addition, protein kinases have been implicated in cold adaptation (Ray et al., 1994c; Shivaji et al., 2007b).

The *trmE* locus in psychrophilic *Pseudomonas syringae* (Lz4W) genome has been determined by sequencing the upstream and downstream regions of *trmE*. BLAST
analysis of these sequences indicated that preprotein translocase YidC subunit (MW: 60 kDa) is located upstream to \textit{trmE} and ABC transporter (MW: 60 kDa) is located downstream to \textit{trmE}. It has also been observed that the \textit{trmE} promoter sequence overlaps with the ORF of preprotein translocase YidC subunit that is located upstream to \textit{trmE}. Analysis of the \textit{trmE} locus in other bacteria (\textit{Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas mendocina, Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudoalteromonas haloplanktis and Escherichia coli}) also indicated that preprotein translocase YidC subunit is located upstream to \textit{trmE} except in \textit{Escherichia coli} (where, the inner membrane protein OxaA is situated in place of YidC subunit). The downstream gene to \textit{trmE} in \textit{Pseudomonas fluorescens, Pseudomonas stutzeri, Pseudoalteromonas haloplanktis and Escherichia coli} is highly variable and these genes encode for glucose inhibited division protein A, probable NADH dehydrogenase, FMN binding protein and Tryptophanase leader peptide, respectively. Whereas, in \textit{Pseudomonas putida, Pseudomonas mendocina} and \textit{Pseudomonas aeruginosa}, the downstream gene is conserved and it is \textit{gidA}. Thus, from the above discussion, it is clear that the downstream gene in the \textit{trmE} locus is highly variable in different bacterial strains.

4.3 PHYLOGENETIC ANALYSIS OF TrmE OF \textit{Pseudomonas syringae} (Lz4W)

TrmE of psychrophilic \textit{Pseudomonas syringae} (Lz4W) forms a heterogenous clade with TrmE from other species of \textit{Pseudomonas} and with TrmE of \textit{Halomonas cupida} (CAQ16333), \textit{Bacillus megaterium} (CAQ16332) and \textit{Brevibacterium linens} (CAQ16330). This affiliation is unexpected since TrmE of psychrophilic \textit{Pseudomonas syringae} (Lz4W) should have formed a distinct clade with all the \textit{Pseudomonas} species with which at the rRNA gene level it is more related. TrmE sequences of \textit{Marinomonas sp.} (YP001343314), \textit{Vibrio cholerae} (NP062587), \textit{Pseudoalteromonas haloplanktis
(YP341492), Haemophilus influenzae (AAC22664), Yersinia bercovieri (ZP00822202), Serratia proteamaculans (YP001476265), Klebsiella pneumoniae (YP001337760), Salmonella typhimurium (NP462743), Escherichia coli (AAC76729), Shigella flexneri (NP709494) and Thermotoga maritima (YP001343314) formed at least three separate clades which were distinctly separated from the Pseudomonas clade (Fig. 3.6).

4.4 EXPRESSION OF trmE IN PSYCHROPHILIC Pseudomonas syringae (Lz4W) AND CHARACTERIZATION OF THE trmE PROMOTER

Psychrophilic bacteria have the ability to transcribe at low temperature (0-4°C) both in vivo and in vitro (Ray et al., 1998; Uma et al., 1999). However, the information available on the nature of the promoter, its regulatory elements and the mechanism of transcription at lower temperatures are scanty. Kannan et al. (1998), demonstrated that hutU is upregulated in psychrophilic Pseudomonas syringae (Lz4W) and identified the promoter that regulates the hutU operon and established that there are two transcription start sites one functional at normal as well as at low temperature growth i.e. 22°C and 4°C, and the other specific for low temperature i.e. 4°C (Kamala et al., 2002). A promoter for cti (cis-trans isomerase) was also identified in psychrophilic Pseudomonas syringae (Lz4W) (Kiran et al., 2005) and expression studies indicated that the gene is constitutively expressed during different growth stages and under conditions of high temperature and solvent stress. It has also been established that Pseudomonas syringae (Lz4W) requires trans-monounsaturated fatty acid for growth at higher temperature (Kiran et al., 2004).
In the present study the *in vivo* expression of *trmE* in psychrophilic *Pseudomonas syringae* (Lz4W) was analyzed by RT-PCR in cells grown at 22°C and then shifted either to 4°C, 22°C or 28°C. No change in the transcript level was observed when cultures were shifted from 22°C to either 22°C or 28°C (Fig. 3.7 A-C) but in cells shifted to 4°C *trmE* transcript level increased significantly up to 30 min following the shift but by 60 minutes the level decreased to control levels as in cells shifted to 22°C (Fig. 3.7 A-C). The up regulation of *trmE* might be a cold adaptive response of psychrophilic *Pseudomonas syringae* (Lz4W) to compensate for the reduction in GTPase activity of TrmE. The GTPase activity from the bacterial cell lysate which is prepared from the culture grown at 22°C and shifted to 4°C for time interval 0, 5, 10, 30, 60 and 90 min indicated that there was no change in GTPase activity. This is the first report on the *in vivo* expression of *trmE* in a cell.

4.4.1 Characterization of the *trmE* promoter in psychrophilic *Pseudomonas syringe* (Lz4W)

The transcription start site of *trmE* was mapped by primer extension as nucleotide (A), located 343 bp upstream from the translation start site (ATG) thus revealing that *trmE* mRNA contains a long 5'-untranslated region (5'-UTR) of 343 bp which is a characteristic feature of many cold-inducible genes of mesophilic bacteria including *Escherichia coli*, *Anabaena sp.* and *Methanococcoides burtoni* (Fang et al., 1998; Lim et al., 2000). In the 5'-UTR between the transcription start site (A) and the translation start site (ATG) the Shine-Dalgarno sequence (GAGG), conserved region (CAAAAAA), DEAD-box (AACAGTGGTA) and cold-box, (TGAACAACGTG) have been identified (Fig. 3.9). In addition, the conserved -10 region (TGGATT), -35 region (TGAAAT) and the UP element (TACTTCTGGAAAGT) were also identified (Fig. 3.9).
The promoter activity of the above trmE promoter was confirmed using a promoter less promoter probe vector (pKZ27) (with β-galactosidase gene as the reporter gene) which was transformed in to Escherichia coli and Pseudomonas syringae (Lz4W) separately. The results indicated that the promoter is active in Pseudomonas syringae (Lz4W) (Fig. 3.15B) but not in Escherichia coli cells indicating that trmE promoter of Pseudomonas syringae (Lz4W) is highly specific and it is not recognized (functional) in Escherichia coli (Fig. 3.15A). Further, the activity of the enzyme was observed both at 4°C and 22°C indicating that the promoter is functional at both 4°C and 22°C (Fig. 3.16).

4.4.1.1 Role of 5'-UTR and cold-box

trmE of psychrophilic Pseudomonas syringae (Lz4W) which exhibits up regulation in transcription at low temperature contains a 5'-UTR region with a cold-box. Earlier studies had also indicated that in Escherichia coli, Anabaena sp. and Methanococcoides burtonii, the DEAD-box RNA helicase gene (deaD) and in the four csp genes which are cold-regulated contain a 5'-UTR greater than 100 bp length (with the exception of Anabaena) (Table 4.1). The trmE of psychrophilic Pseudomonas syringae (Lz4W) has one of the longest 5'-UTR of 343 bp (Fig. 3.9) compared to the 5'-UTR in cold inducible genes in other bacteria (Table 4.1). Further, based on promoter deletion experiments it is also demonstrated that the 5'-UTR is essential for the regulation of cold inducible trmE. The actA coding for the ActA the major virulence factor in Listeria monocytogenes also has a long 5'-UTR of 150 bp which when deleted dramatically influenced actA expression levels (Wong et al., 2004) thus implying that secondary structural motifs within the actA mRNA 5'-UTR, determine overall levels of actA expression. The 5'-UTR has also been reported to be important for mRNA stabilization following cold shock (Mitta et al., 1997). In most vertebrates the 5'-UTR are less than 100 nucleotides but in genes that are tightly controlled, the 5'-UTR is longer (Uhlmann-Schiffler et al., 2002) and such structures can modulate translation by
formation of secondary structures and by RNA-protein interaction (Gray and Wickens 1998; Uhlmann-Schiffler et al., 2002). The conservation of the long 5'-UTR in low temperature regulated genes strongly suggests that their occurrence is more than a coincidence and supports their role in gene regulation.

In *Escherichia coli* over-expression of the 5'-UTR of *cspA, cspB* and *csdA* mRNA following cold shock induced prolonged synthesis of CspA, CspB and CsdA or simultaneous derepression of *cspA, cspB* and *csdA* (Jiang et al., 1996; Fang et al., 1998). Further, it was also observed that deletion of the cold-box region in the 5'-UTR abolished the derepression caused by over-expression of 5'-UTR of *cspA* mRNA (Fang et al., 1998). These results indicated that derepression occurs at the level of transcription and it is probably brought about by the binding of a putative repressor to the cold-box of the mRNA of cold-inducible genes. Deletion of the cold-box region from *cspA* on the chromosome also caused derepression of *cspA* confirming that cold-box functions as a binding site for the putative repressor (Fang et al., 1998). Overexpression of the *Escherichia coli* *cspl* 5'-UTR however has been reported to cause a weaker derepression of the cold-shock response compared to the *cspA* 5'-UTR (Wang et al., 1999). These authors also indicated that the optimal temperature ranges for induction of the four *Escherichia coli* cold-shock induced *csp* genes (*cspA, B, G* and *I*) are not the same, thereby indicating that while the 5'-UTR and cold-box elements may be involved in regulation of gene expression, specific sequence differences in the 5'-UTR and cold-box elements may play important roles in regulation. Interestingly, it has also been suggested that CspE (a non-cold-shock induced protein in the *Escherichia coli* Csp family) binds to the cold-box of *cspA* and functions as a negative regulator of expression at 37°C (Bae et al., 1999). Thus, it is indeed very well established that the 5'-UTR plays an important role with respect to regulation of cold inducible genes but recent studies using *cspA::lacZ* fusions which contained a variety of deletions of the 5'-UTR (Mitta et
al., 1997; Yamanka et al., 1999) indicated that deleting the cold-box had little effect on cold-shock induction of β-galactosidase activity, and instead a region 11 bp upstream of the ribosome binding site was important for translational efficiency of gene expression. These data indicate that the precise mechanisms by which regulation occurs is unclear. These observations are also not in accordance with the data in the present thesis where in it is observed that deletion of the cold-box inhibits expression of the gene (Fig. 3.19).

The foregoing studies do indicate that the cold-box element has a role to play in the transcriptional regulation of cold-inducible genes. In fact several cold-inducible genes in bacteria are known to contain a cold-box element within the 5'-UTR (Graumann et al., 1997; Graumann & Marahiel 1998; Panoff et al., 1998; Thieringer et al., 1998; Yamanka et al., 1998; Phadtare et al., 1999) and the cold-box sequences are conserved (Fig. 3.12) Further, alignment of the cold-box sequences from cold inducible genes of various bacteria such as Escherichia coli, Anabaena sp., Methanococcoides burtonii, Bacillus subtilis, Brevibacterium linens, Arthrobacter sulfureus, Marinomonas primoryensis, Pseudomonas syringae (Lz4W) and other different species of Pseudomonas sp. led to the identification of a consensus sequence for cold-box as TGAA/CNAACT/AGC/A (where N represents any one of the nucleotides) (Fig. 3.12). 5'-UTR sequences with conserved elements (different to the cold-box sequence) have also been identified in cold-induced genes in Bacillus (Graumann et al., 1997).

**Table 4.1. Length of 5'-UTR in cold inducible genes of bacteria**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Gene</th>
<th>5'-UTR length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas syringae (Lz4W)</td>
<td>trmE</td>
<td>343</td>
<td>This study</td>
</tr>
<tr>
<td>Pseudomonas syringae (Lz4W)</td>
<td>hutU</td>
<td>170</td>
<td>Kamala et al., 2002</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>rpoS</td>
<td>368</td>
<td>Jovic et al., 2008</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>cspB</td>
<td>161</td>
<td>Lee et al., 1994</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>cspA</td>
<td>159</td>
<td>Goldstein et al., 1990</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>csdA</td>
<td>226</td>
<td>Toone et al., 1991</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>cspG</td>
<td>161</td>
<td>Nakashima et al., 1996</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>cspI</td>
<td>145</td>
<td>Wang et al., 1999</td>
</tr>
<tr>
<td>Anabaena sp.</td>
<td>chrC</td>
<td>116</td>
<td>Chamot et al., 1999</td>
</tr>
<tr>
<td>Methanococcoides burtonii</td>
<td>deaD</td>
<td>113</td>
<td>Lim et al. 2000</td>
</tr>
</tbody>
</table>
4.4.1.2 -10 and -35 regions of trmE

In the trmE promoter of Pseudomonas syringae (Lz4W), the -10 region and -35 regions have been identified as TGGATT and TGAAAT. These two sequences were very similar to the consensus sequences TGGATT and GGAAAT for -10 region and -35 region identified based on the alignment of the trmE promoter sequences from different species of Pseudomonas, Brevibacterium linens, Arthrobacter sulfureus and Marinomonas primoryensis (present study Fig. 3.10). However, the consensus nucleotide sequence of the -10 and -35 region in trmE promoter differed from the sequences reported previously for the hutU (Kamala et al., 2002) and cti (Kiran et al., 2005) in Pseudomonas syringae (Lz4W) and were very dissimilar compared to the consensus sequences for -10 region and -35 region identified for Escherichia coli (TATAAT and TTGACA, respectively) based on 300 genes (Lisser, S.; and Maragalit, H. 1993) and Pseudoalteromonas haloplanktis (TRGRTW and TATRAY where, R: A/G, Y: T/C, W: A/T, X: T/G and S: G/C, respectively) (Duilio et al., 2004). Thus, it would appear that these regions vary from organism to organism and may also depend on the gene that has been analyzed. Deletion analysis of these regions indicated that both the -10 and -35 regions are essential for transcription but probably the -10 region is more important since deletion of this region resulted in almost total loss of promoter activity in trmE of Pseudomonas syringae (Lz4W) (Fig. 3.19).

4.4.1.3 UP element sequences

Based on the analysis of 31 promoters in the region upstream to the -35 region (Estrem et al., 1998) identified an AT-rich region (64–91%) called the UP element with a consensus sequence (nn AAA (A/T)(A/T)T(A/T) TTTT-nnAAAAnn) between -38 to -59, A similar UP element (TACTTCTGGAAAGT) which was AT rich (64.3%) was identified between from -41 to -54, in the trmE promoter of Pseudomonas syringae (Lz4W) but this UP element is unique, in that it did not match with the consensus sequence (nn AAA
In fact, the present study based on analysis of *trmE* promoters from different bacterial species of *Pseudomonas, Brevibacterium linens, Arthrobacter sulfureus* and *Marinomonas primoryensis* identified the consensus UP element sequence as TACTNCTGGAAAGT (Fig. 3.11). Earlier studies have indicated that the UP element of bacterial promoter stimulates transcription by interacting with the α-subunit of RNA polymerase (Ross et al., 1993; Mitta et al., 1997). This may indeed be the case also in the case of psychrophilic *Pseudomonas syringae* (Lz4W) since site-specific deletion of UP element resulted in drastic loss of promoter activity (Fig. 3.19).

### 4.4.1.4 DEAD-box

A sequence "AACAGTGGTA" in the 5'-UTR at position +208 to +217 of *trmE* promoter of *Pseudomonas syringae* (Lz4W) was identified as DEAD-box and the sequence exhibited 70% sequence similarity at the nucleotide level with putative Box A (AACATTATA) of *deaD* gene of *Methanococcoides burtonii* (Lim et al., 2000). In *Pseudomonas entomophila, Pseudomonas fluorescens, Pseudomonas mendocina, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas syringae, Brevibacterium linens, Arthrobacter sulfureus* and *Marinomonas primoryensis*, a consensus sequences for DEAD-box has been defined as "AA/GCAGTGGTA" (Fig. 3.13). The presence of a highly conserved DEAD-box structure in cold-inducible *trmE* of psychrophilic *Pseudomonas syringae* (Lz4W) strongly suggests that it might have a specific regulatory role. In fact, site-specific deletion of the DEAD-box from the *trmE* promoter resulted in drastic loss of promoter activity (Fig. 3.19) confirming that the DEAD-box is essential in the regulation of the *trmE* promoter.
4.4.1.5 Conserved region

In addition to the above promoter regulatory elements a conserved region CAAAAAA at position +246 to +252 in the 5'-UTR region of cold inducible \textit{trmE} of \textit{Pseudomonas syringae} (Lz4W) was identified. This sequence was earlier reported for the \textit{hut} promoter (Kamala et al., 2002) and \textit{cti} promoter (Kiran et al., 2005) of \textit{Pseudomonas syringae} (Lz4W) and is also conserved in the \textit{trmE} promoter of \textit{Brevibacterium linens, Arthrobacter sulfureus, Marinomonas primoryensis} and different species of \textit{Pseudomonas} (Fig. 3.14). Deletion of this region in the promoter construct did not alter the promoter activity of \textit{trmE} of \textit{Pseudomonas syringae} (Lz4W) indicating that the conservation of this sequence is probably by chance and it does not have any functional significance in regulation of \textit{trmE}.

4.4.1.6 Low temperature increases the promoter activity of \textit{trmE}

Temperature shift experiments where in \textit{Pseudomonas syringae} (Lz4W) was first grown at 22°C and then shifted to 4°C, 22°C or 28°C clearly indicated that the expression of \textit{trmE} is upregulated when shifted from 22°C to 4°C but not when the cultures were shifted to 22°C or 28°C (Fig. 3.7). Therefore it is likely that the promoter activity of \textit{trmE} is low temperature regulated. In fact, using transcriptional fusion promoter probe vector (pKZ27) having \textit{trmE} promoter and \textit{β}-galactosidase as the reporter gene in psychrophilic \textit{Pseudomonas syringae} (Lz4W) it is demonstrated that \textit{β}-galactosidase transcripts increased when cultures were shifted from 22°C to 4°C up to 30 minutes of the shift but not when the cultures were shifted to 22°C or 28°C (Fig. 3.20) thus confirming that the promoter is upregulated at low temperature. At the same time, it was also observed that \textit{β}-galactosidase activity from the wild type \textit{Pseudomonas syringae} (Lz4W) (harboring the promoter construct) grown at 22°C and then shifted to 4°C for up to 120 min remained unaltered (Fig. 3.20). These results confirm that the \textit{trmE} of \textit{Pseudomonas syringae} (Lz4W) is under transcriptional regulation at low
temperature (due to the presence of a cold inducible promoter) rather than at translational level.

4.5 BIOCHEMICAL PROPERTIES OF TrmE

TrmE has so far been purified and characterized only from *Escherichia coli*, a mesophilic bacterium (Carbedo et al., 1999) and *Thermotoga maritima*, a thermophilic bacterium (Yamanaka et al., 2000). This is the first report on the purification and characterization of TrmE from *Pseudomonas syringae* (Lz4W), a psychrophilic bacterium. The purified protein shows intrinsic GTPase activity, hydrolyses both the oxy- and deoxy- form of GTP and requires K⁺ and Mg²⁺ for activity, thus resembling the GTPase activity of the TrmE of *Escherichia coli* and *Thermotoga maritima* (Yamanaka et al., 2000; Cabedo et al., 1999). Despite these similarities, TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) is unique in that its requirement of Mg²⁺ for GTPase activity could be substituted by Mn²⁺ and exhibits optimum GTPase activity between 12-18°C and retains 60% of its optimum activity even at 4°C indicating that it is a cold-active enzyme. In contrast to the TrmE of this psychrophilic bacterium, TrmE of *Escherichia coli* and *Thermotoga maritima* exhibit optimum activity at 37°C (Cabedo et al., 1999) and 70°C (Yamanaka et al., 2000) respectively. At 37°C the GTPase activity of TrmE of *Pseudomonas syringae* (Lz4W) was severely inhibited (70% loss in activity compared to the activity at 15°C) and at 70°C it is irreversibly inhibited thus clearly indicating that the enzyme is heat labile. The denaturation of TrmE at 37°C may also be the reason for the protein going into an inclusion body during over expression in *Escherichia coli* strain BL21 at 37°C. It is very well known that once the protein gets denatured its hydrophobic amino acids get exposed and due to hydrophobic interactions the proteins aggregate and accumulate in inclusion bodies. Thus, it is logical to speculate that the high GTPase activity of TrmE could be an adaptive response in psychrophilic *Pseudomonas syringae* (Lz4W). In fact, comparison of the Kₘ and Vₘₐₓ for GTPase activity of TrmE of
psychrophilic *Pseudomonas syringae* (Lz4W) (K<sub>m</sub> = 888.2 μM and V<sub>max</sub> = 176 μM/min), *Escherichia coli* (K<sub>m</sub> = 378 μM and V<sub>max</sub> = 250-500 nmol of GTP/min/mg of protein) (Cabedo et al., 1999) and *Thermotoga maritima* (K<sub>m</sub> = 833 μM and V<sub>max</sub> = 37 μM/min) (Yamanaka et al., 2000) indicate that the K<sub>m</sub> value for GTPase activity of TrmE of *Pseudomonas syringae* (Lz4W) is comparable to the GTPase activity of TrmE of *Thermotoga maritima* where as the V<sub>max</sub> is 4.75 times higher suggesting that the higher GTPase activity of TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) might be playing a role in cold adaptation. Generally, psychrophilic enzymes have low K<sub>m</sub> in comparison to their mesophilic counterparts (Feller et al., 1997; Marshall 1997; Smalas et al., 2000; Siddiqui et al., 2006) but the K<sub>m</sub> for GTPase activity of TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) is higher than the TrmE of *Escherichia coli*, a mesophile. Cross-linking studies with glutaraldehyde indicated that TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) exists both as a monomer (53 kDa) and a dimer (106 kDa), unlike TrmE of *Escherichia coli* which exists as a dimer (100 kDa) and a trimer (150 kDa), (Cabedo et al., 1999) and TrmE of *Thermotoga maritima* which exists as a trimer and a hexamer, with a minor fraction existing also as a monomer (Yamanaka et al., 2000).

**4.6 WHY IS trmE REQUIRED FOR GROWTH AT LOW TEMPERATURE IN Pseudomonas syringae (Lz4W)**

From the foregoing discussion it is clearly established that the mutated gene in CSM1, the cold sensitive mutant of *Pseudomonas syringae* (Lz4W) is *trmE* which is transcriptionally up-regulated at low temperature and possess a promoter which is also up-regulated at low temperature. In addition, the gene product of *trmE* i.e. TrmE exhibits GTPase activity and the enzyme exhibits optimum activity between 12-18°C and is heat labile (irreversibly inactivated at 70°C) and thus possess all the features of a cold active
and heat labile enzyme. Nevertheless there is a need to demonstrate that \textit{trmE} is required for growth at low temperature (4°C) and this aspect is amply demonstrated by the gain of function of growth at 4°C, which was clearly visible in CSM1 which was complemented with \textit{trmE} (Fig. 3.1). One of the major differences in the growth characteristics of \textit{Pseudomonas syringae} (Lz4W) and CSM1 is the prolonged lag period in CSM1 compared to the wild type and this difference was indeed overcome in CSM1 into which \textit{trmE} was cloned. Further the complemented strain exhibited a better growth rate than the mutant but the rate did not match that of the wild type. The reason as to why the phenotype did not totally recover following complementation of the mutated gene in CSM1 is not clear but definitely it is not due to the vector used since cells transformed only with vector did not show any change in the growth kinetics (Fig. 3.1).

Finally it is essential to hypothesize as to why \textit{trmE} is required for growth at low temperature. It may depend on the dual function of \textit{trmE} product i.e. TrmE which is involved in t-RNA modification (Yim et al., 2006; Scrima et al., 2005) and also exhibits GTPase activity (Cabedo et al., 1999). The ability of TrmE to modify a specific uridine residue namely U34 (into 5-methylaminomethyl uridine) in t-RNA may be very crucial since this uridine residue is in the wobble position and is required for codon-anticodon interaction. This interaction if altered due to malfunctioning of TrmE could lead to a frame shift during translation thus affecting protein synthesis. It is well known that during cold adaptation, only a specific set of proteins are synthesized and translation is stringently controlled (Giuliodori et al., 2004) alteration of which could lead to retarded growth or lethality. In fact, the \textit{trmE} mutant of \textit{Escherichia coli} was found to be lacking the modified nucleotide (5-methylaminomethyl-2-thiouridine) and the mutant was found to exhibit reduced readthrough at UAG codon (Elsevier et al., 1984). Since, \textit{trmE} mutant of psychrophilic \textit{Pseudomonas syringae} (Lz4W) is cold sensitive (having a prolonged lag phase) it is logical to assume that during cold adaptation, stringent control of translation.
might be required as an adaptive response in psychrophilic *Pseudomonas syringae* (Lz4W) which in turn depends on t-RNA modification brought about by TrmE. Further, the GTPase activity of TrmE may also be associated with adaptation to temperature. This indeed may be the reason as to why both in thermophilic (*Thermotoga maritima*) and psychrophilic (*Pseudomonas syringae* Lz4W) bacteria GTPase activity of TrmE was observed to be optimum at temperatures coinciding with the optimum growth temperature that is 12-18°C (present study) and 70°C respectively (Yamanaka et al., 2000).

### 4.7 CONCLUSIONS

Taken together, the results of the present study indicate that psychrophilic *Pseudomonas syringae* (Lz4W) requires *trmE* for survival at low temperature. The *trmE* is up regulated during low temperature shift and a cold inducible promoter regulates its expression during the shift. The highlights of the present study are as follows:

1. A cold sensitive mutant (CSM1) of psychrophilic *Pseudomonas syringae* (Lz4W) has been generated using transposon mutagenesis.

2. The disrupted gene in CSM1 of psychrophilic *Pseudomonas syringae* (Lz4W) was identified as *trmE* and it exhibited 99% homology with *Halomonas cupida* at both the nucleotide and protein level.

3. The cold sensitive mutant exhibited slow growth at low temperature. The presence of this gene in *trans* form in cold sensitive mutant helps to significantly restore its ability to grow at low temperature.

4. TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) has intrinsic GTPase activity and K\(^+\), Mg\(^{2+}\) or Mn\(^{2+}\) are essential for its GTPase activity.

5. TrmE of psychrophilic *Pseudomonas syringae* (Lz4W) is a psychrophilic enzyme (cold active) having optimum activity at 12 to 18°C and it is thermolabile.
6. The *in vivo* expression of *trmE* in psychrophilic *Pseudomonas syringae* (Lz4W) indicated the up regulation of *trmE* when cultures grown at 22°C are shifted to 4°C.

7. A cold inducible promoter has been identified upstream to *trmE* in psychrophilic *Pseudomonas syringae* (Lz4W).

8. The location of transcription and translation start site indicated the presence of a long 5'-UTR, a characteristic feature of many cold inducible genes in mesophilic bacteria.

9. Deletion constructs of the promoter confirmed that the long 5'-UTR, the -10 region, -35 region, cold-box, DEAD-box and UP element are required for the efficient expression of *trmE*.

10. Complementation of CSM1 with *trmE* resulted in substantial recovery in the cold resistant phenotype indicating that *trmE* is required for growth at low temperature in psychrophilic *Pseudomonas syringae* (Lz4W).