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Importance of *trmE* for Growth of the Psychrophile *Pseudomonas syringae* at Low Temperatures▽†

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Transposon mutagenesis of *Pseudomonas syringae* Lz4W, a psychrophilic bacterium capable of growing at temperatures between 2 and 30°C, yielded 30 cold-sensitive mutants, and CSM1, one of these cold-sensitive mutants, was characterized. Growth of CSM1 was retarded when it was cultured at 4°C but not when it was cultured at 22°C and 28°C compared to the growth of wild-type cells, indicating that CSM1 is a cold-sensitive mutant of *P. syringae* Lz4W. The mutated gene in CSM1 was identified as *trmE* (coding for tRNA modification GTPase), and evidence is provided that this gene is induced at low temperatures. Further, the cold-inducible nature of the *trmE* promoter was demonstrated. In addition, the transcription start site and the various regulatory elements of the *trmE* promoter, such as the −10 region, −35 region, UP element, cold box, and DEAD box, were identified, and the importance of these regulatory elements in promoter activity were confirmed. The importance of *trmE* in rapid adaptation to growth at low temperatures was further highlighted by plasmid-mediated complementation that alleviated the cold-sensitive phenotype of CSM1.

Psychrophilic bacteria (57) constitute a sizeable proportion of bacterial diversity because a large proportion of Earth's biosphere (75%) is either transiently or permanently cold (temperature, <5°C) (2). Studies have indicated that psychrophiles adapt to low temperatures by being able to sense changes in temperature (41, 48, 59), by modulating membrane fluidity (11–13, 28, 29), and because they possess enzymes and genes which are active at low temperatures (8, 10, 19, 35, 50, 60, 64). In psychrophilic bacteria *pnp* (encoding polynucleotide phosphorylase) (23), *oppA* (mediation of the transport of oligonucleotides) (5), and *recD* (51) have been identified as genes required for low-temperature growth. In contrast, in mesophilic bacteria many genes are induced following a downshift in temperature; these genes include genes for fatty acid desaturases and other enzymes (26, 32, 62), cold shock genes (33, 47), and genes involved in replication transcription and translation (3, 9, 26, 33, 69). The question is whether such genes are induced in psychrophiles, which, unlike mesophiles, are not cold stressed but are cold adapted. The present study investigated the role of *trmE* in low-temperature growth.

MATERIALS AND METHODS

Generation of cold-sensitive mutants. Psychrophilic *Pseudomonas syringae* Lz4W (referred to as *P.syringae* below) and *Escherichia coli* strains DH-5α and S-17-1 (Table 1) were grown in Antarctic bacterial medium (58) or Luria-Bertani medium (66). *P. syringae* was mutagenized with a Tn*5* transposon-based suicide plasmid vector (pOT182) (35, 42), and cold-sensitive mutants were identified based on their inability to grow or their delayed growth on plates incubated at 4°C for 1 week. Growth characteristics were also analyzed using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) (36, 37).

Identification of the disrupted gene. Southern analysis using genomic DNA of CSM1 (one of the cold-sensitive mutants) and α-^{32}P-labeled pOT182 (35, 36) showed that CSM1 produced two bands, confirming that a single copy of the transposon was integrated (data not shown). The disrupted gene was identified by inverse PCR (46) using 35 cycles and transposon-specific outward primers (primers Ash 1 and Ash 3 for upstream regions and primers Ash 1 and Ash 2 for downstream regions) (Table 2). PCR-amplified products were purified and sequenced with a ABI Prism 3700 DNA sequencer (Applied Biosystems, California) (12, 13, 36, 37) using Ash 1 and Ash 3.

Molecular biology techniques. Genomic DNA of *P. syringae* was isolated (36), fragmented (35 to 40 kb), and cloned in pCC1FOS (Epicentre Technologies, Madison, WI). All other DNA manipulation techniques, including colony hybridization, were performed as described by Sambrook et al. (54).

For reverse transcription PCR RNA was isolated from *P. syringae* (1, 13, 36), and first-strand cDNA was synthesized by RT. The cDNA was then amplified using Ash 71 and Ash 72 for the β-galactosidase gene and Ash 5 and Ash 44 for *trmE* for 21 cycles (Table 2).

Primer extension analysis and cloning of *trmE* promoter. Transcription start site mapping of *trmE* was carried out by performing primer extension analysis (37) using [γ-^{32}P]ATP-end-labeled PextR2 (Table 2) and total RNA of *P. syringae* grown at 4°C and 22°C. The putative *trmE* promoter of *P. syringae* was amplified by PCR using Ash57 and Ash59 (Table 2) and then cloned in pKZ27 with the β-galactosidase gene as the reporter gene (Promega Corporation, Madison, WI). The resulting pKZ27::promoter construct was then electroporated into *P syringae* (37).

Deletions of a specific promoter element were obtained by performing overlap extension PCR (65) (see Fig. 4A) using primers listed in Table 2. PCR products were cloned in pKZ27, and deletions were confirmed by DNA sequencing. The promoter constructs were electroporated into *P. syringae*, and transformants positive for β-galactosidase activity (43) were selected.

Complementation of CSM1 with *trmE*. The open reading frame (ORF) of *trmE*, along with its promoter, was amplified from *P. syringae* using primers Ash 57 and Ash 47 and cloned in pGL10 (3). The resulting pGL10::promoter-*trmE* construct was mobilized into CSM1 (52), and the complemented strain was checked for recovery of the phenotype.

RESULTS AND DISCUSSION

Cold-sensitive mutants of *P. syringae*. A total of 3,500 mutants of *P. syringae* were generated using a Tn*5* transposon-based suicide plasmid vector (pOT182) (47). Thirty of these mutants were cold sensitive and exhibited delayed growth at 4°C but not at 22°C or 28°C (Fig. 1). Growth analysis of CSM1, one of the cold-sensitive mutants, indicated that its growth is not altered at 22°C and 28°C compared to the growth of the *P.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
Escherichia coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*M15) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Laboratory stock
Escherichia coli S-17-1	RP4–2Tc::Mu-Kn::Tn*7 pro hsdR recA*	61
Pseudomonas syringae Lz4W	Wild type, Antarctic isolate	58
Plasmids		
pMOSBlue	Cloning vector, Amp^r	Amersham Life Sciences (Buckinghamshire, United Kingdom)
pGL10	Broad-host-range cloning vector, IncP replicon, *mob*$^+$ Kan^r	3
pKZ27	*lacZ* transcription fusion vector, Kan^r *incQ oriT*	68

syringae wild-type strain (Fig. 1). But at 4°C, CSM1 exhibited a prolonged 60-h lag period, compared to the 20-h lag period for the wild type. Thus, CSM1, like the *pnp* mutants of the psychrophile *Yersinia enterocolitica* (24), is a "psychrotrophy-defective" mutant since it exhibits delayed growth at 4°C but not at 22°C and 28°C compared to the growth of the parental strain.

The gene that was disrupted in CSM1 following amplification yielded a 700-bp fragment which, as determined by sequencing, exhibited high levels of similarity (98 to 99%) with *trmE* (encoding tRNA modification GTPase) of *Halomonas cupida*, *Brevibacterium linens*, and *Bacillus megaterium*.

TABLE 2. Primers used in this study[a]

Primer	Oligonucleotide sequence	Gene
Ash 1	5′CTGCAAGGCGATTAAGTTGGG3′	Transposon
Ash 2	5′CCATGTTAGGAGGTCACATGG3′	Transposon
Ash 3	5′CCTGATGCAGTAATCCTACGG3′	Transposon
Ash 5	5′CAATCGCAGCTTCGACGTAG3′	*trmE*
Ash 44	5′CCGGAATTCATGAGTGTTGCTGCTGAA3′	*trmE*
Ash 47	5′CCGGAATTCCTATTTACCGATACAGA3′	*trmE* promoter
Ash 57	5′GCTGCAGCAGCTACAAGTCGATGGC CC3′	*trmE* promoter
Ash 59	5′GGGGTACCCCACACCACCTCGGCCTTG3′	*trmE*
Ash 60	5′ATCAAGGACCCGTTCTTGCTTTCCATCA CT3′	*trmE* promoter
Ash 61	5′AGTGATGGAAAGCAAGAACGGGTCCTT GAT3′	*trmE* promoter
Ash 62	5′ACTTCTGGAAAGTGTGCGCCAGGCGCC GTT3′	*trmE* promoter
Ash 63	5′AACGGCGCCTGGCGCACACTTTCCAGAA GT3′	*trmE* promoter
Ash 64	5′GCGCCGTTCATGCTCACCGACCTGTCGA TC3′	*trmE* promoter
Ash 65	5′GATCGACAGGTCGGTGAGCATGAACGG CGC3′	*trmE* promoter
Ash 66	5′GCCTGTCCATCGCCCGGTCGAAGCTGC TAC3′	*trmE* promoter
Ash 67	5′GTAGCAGCTTCGACCGGGCGATGGACA GGC3′	*trmE* promoter
Ash 71	5′ACCGACTACACAAATCAGCGA T3′	β-*gal*
Ash 72	5′TTCATTCCCCAGCGACCAGAT3′	β-*gal*
Ash 77	5′GGTCGAAGCTGCTACGCAGCCGAGTAA CTT3′	*trmE* promoter
Ash 78	5′AAGTTACTCGGCTGCGTAGCAGCTTCGA CC3′	*trmE* promoter
PextF	5′ATGCCGGTCTTCCTGTCCTTGTAC3′	*trmE* promoter
PextR2	5′TGCATCGGATCCGGAGGAGTCGG3′	*trmE* promoter

[a] PCR primers were designed using SeWeR (http://iubio.bio.indiana.edu/webapps/SeWeR.xx).

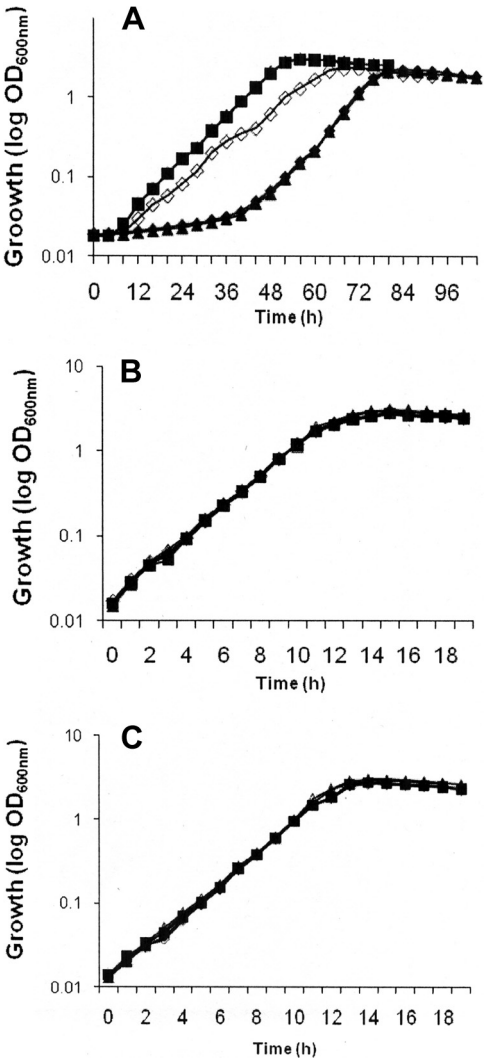

FIG. 1. Growth of the psychrophile *P. syringae* (■), *P. syringae* harboring pGL10 (△), a cold-sensitive mutant of *P. syringae* (CSM1) (▲), complemented *P. syringae* cold-sensitive mutant CSM1 harboring the pGL10::*trmE* promoter plus *trmE* (□), and *P. syringae* cold-sensitive mutant CSM1 harboring only pGL10 (◆) cultured at 4°C (A), 22°C (B), and 28°C (C). OD_{600nm}, optical density at 600 nm.

Characteristics of *trmE* of *P. syringae*. Colony hybridization screening of a genomic library of *P. syringae* using the ^{32}P-labeled 700-bp PCR product of *trmE* identified two positive clones (5pCC1FOS and 11pCC1FOS). Sequencing of the insert of 5pCC1FOS (35 to 40 kb) using Ash 5, Ash 44, and Ash 59 yielded a 2,371-bp sequence corresponding to the complete ORF of *trmE* (1,377 bp) (accession number AM944531), an upstream incomplete ORF of the putative inner membrane protein translocase component *yidC* gene subunit (587 bp), and a downstream incomplete ORF of the *abc* transporter ATP-binding protein gene (407 bp). *abc* was in the same orientation as *trmE*, and transcriptional terminators were not detected downstream of *trmE*. The transposon insertion site was identified between bp 829 and 830 in *trmE*.

trmE codes for a 458-amino-acid protein (accession number CAQ16331). BLAST analysis (www.ncbi.nlm.nih.gov) showed

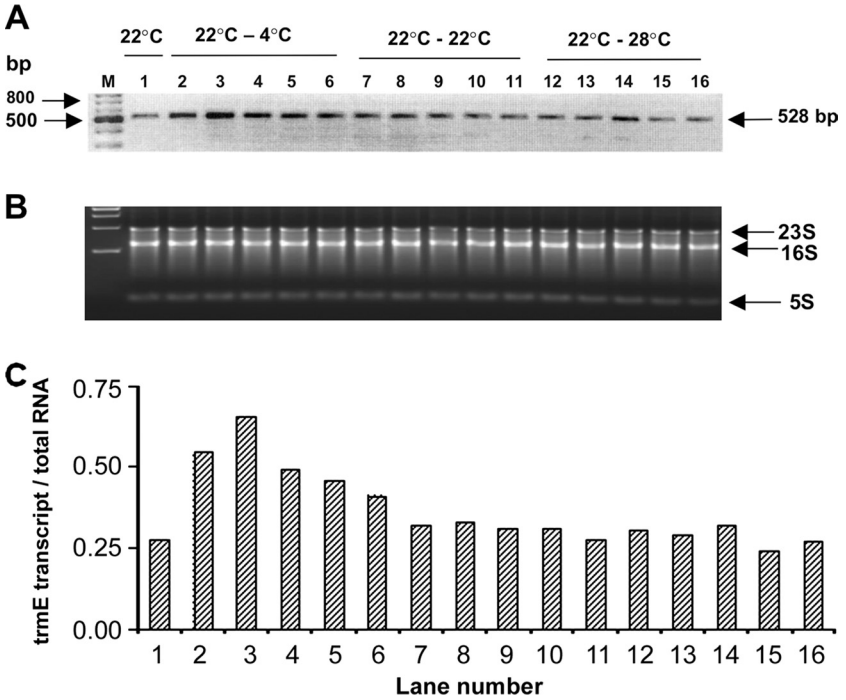

FIG. 2. RT-PCR analysis of *trmE* expression in *P. syringae*. RT-PCR was carried out using total RNA isolated from 3 ml of *P. syringae* grown to an optical density at 600 nm of ~1.1 at 22°C and shifted to 4°C, 22°C, and 28°C for 0, 10, 20, 30, 45, and 60 min. Lane 1 contained the culture grown at 22°C (before the shift); lanes 2 to 6 contained the culture grown at 22°C and shifted to 4°C for 10, 20, 30, 45, and 60 min, respectively; lanes 7 to 11 contained the culture grown at 22°C and shifted to 22°C for 10, 20, 30, 45, and 60 min, respectively; and lanes 12 to 16 contained the culture grown at 22°C and shifted to 28°C for 10, 20, 30, 45, and 60 min, respectively. Lane M contained markers. (B) Amount of total RNA used in each of the RT-PCRs. (C) Ratios of the *trmE* transcript to total RNA (as determined by densitometry) for lanes 1 to 16 in panels A and B. The experiment was done three times, and the data are the results of a representative experiment.

that the amino acid and nucleotide sequences of *trmE* of *P. syringae* were similar to the *trmE* sequences of various bacterial species, and the highest level of similarity was 99% similarity with *H. cupida* (nucleotide sequence accession number AM944535 and amino acid sequence accession number CAQ16333).

CLUSTAL W analysis (www.chembnet.org/software/clustals.html) indicated that in TrmE of *P. syringae* the GTP binding (G-1, G-3, and G-4) and effector molecule binding (G-2) motifs involved in GTPase activity are conserved (8). In addition, four other regions (regions I, II, III, and IV) are conserved (see Fig. S1 in the supplemental material).

Complementation of *trmE* in CSM1. The growth of complemented strain CSM1 (harboring pGL10::promoter-*trmE*) matched the growth of the wild type and the mutant at 22°C and 28°C. But at 4°C the complemented strain grew faster than the mutant, and the phenotype was rescued significantly (Fig. 1). The lag period for the complemented strain was 32 h, compared to 60 h for the mutant. Thus, it appears that the cold-sensitive phenotype was due to inactivation of *trmE*. However, the mutant did not recover totally compared to the wild type (Fig. 1). The slower growth of the complemented mutant was not due to the vector since analysis of wild-type cells transformed either with the vector (Fig. 1) or with the vector with the insert did not show any effect on the growth kinetics (data not shown). The inability of the complemented mutant to recover totally may have been due to the polar effect of another gene that is downstream of *trmE* (22, 53) or due to a gene dosage effect, or *trmE* may not have been expressed properly.

TrmE helps modify the uridine (U34) at the wobble position to 5-methylaminomethyl-uridine (72). This modification influences frameshift during the translation process (4, 6), thus causing a transient block in initiation of translation, as a consequence of which growth at low temperatures is affected, as observed for *E. coli*. Further, many genes involved in translation, such as *fus* and genes for RNA helicases (63) and ribosomal proteins (14, 26, 33, 69), have been implicated as genes that may be required for growth of mesophilic bacteria at low temperatures. *trmE* should be added to this list of genes.

Studies of *E. coli* have led to identification of a number of cold shock proteins and cold-inducible genes (20, 24, 25). CspA, the major cold shock protein in *E. coli*, belongs to a family of nine homologous proteins (27), but not all nine proteins are cold inducible (38, 45, 56). *cspA* has been identified in various psychrophilic bacteria, and this gene is constitutively expressed at 4°C and 22°C (49). Interestingly, none of the cold-shock-inducible genes are singularly responsible for cold adaptation (70).

Expression of *trmE* and mapping of the transcription start site. In vivo expression of *trmE* in *P. syringae* that was shifted from 22°C to 4°C increased transiently more than twofold up to 30 min after the shift (Fig. 2A to C), and the maximum increase, about 2.5-fold, was observed at 20 min after the shift. This temporal increase was consistently observed in three independent experiments using RNA isolated specifically in each experiment. In contrast, cells which were grown only at 22°C (control) or cells which were shifted from 22°C to 28°C did not

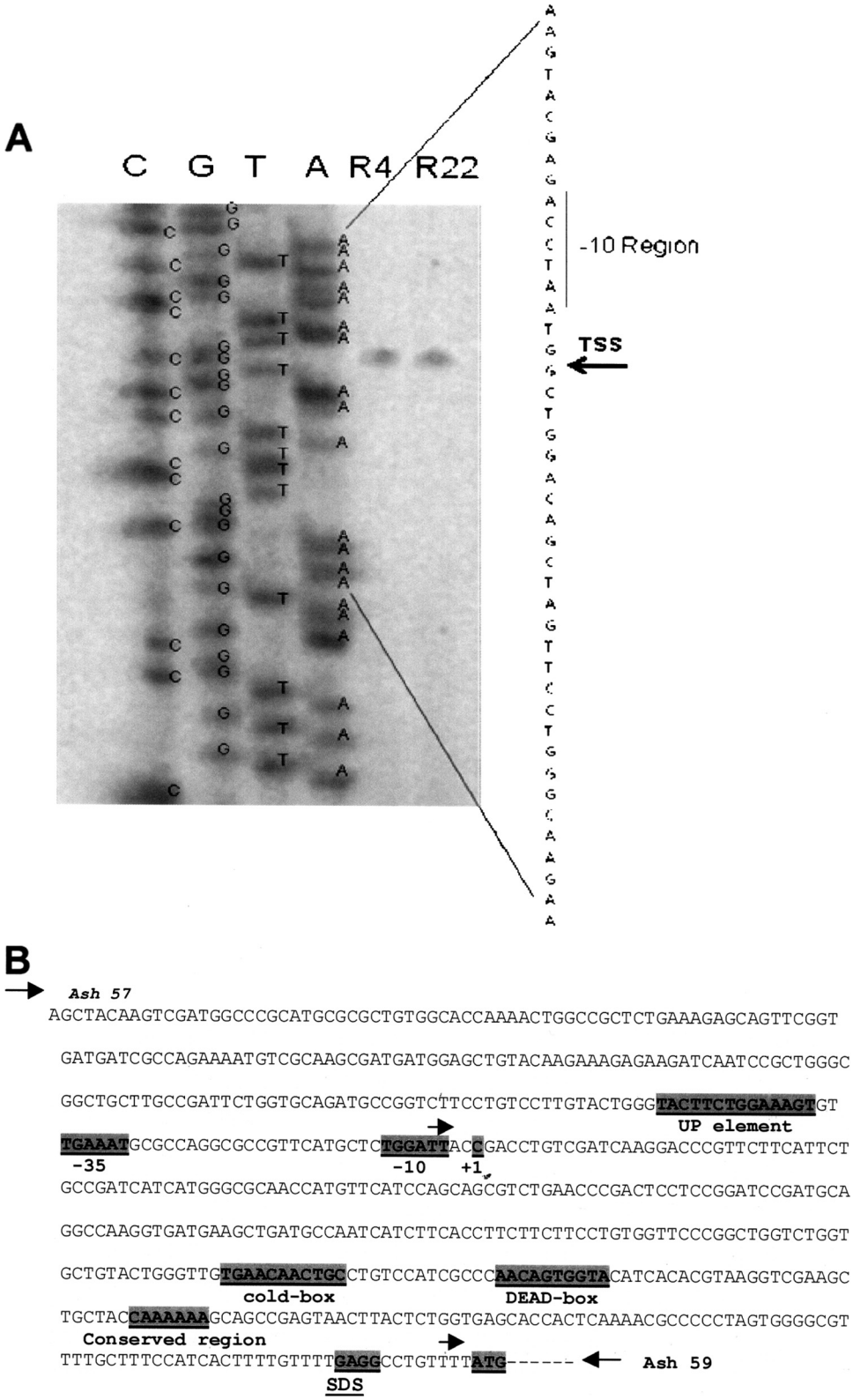

FIG. 3. (A) Primer extension analysis of the *trmE* transcript of *P. syringae* cells grown to mid-log phase (optical density at 600 nm, ~1.1) at 4°C and 22°C. The primer extension reactions were carried out using 5′-end-labeled primer PextR2 and reverse transcriptase. Primer extension products R4 and R22 are from cultures grown at 4°C and 22°C, respectively, and represent the transcription start site (TSS) (arrow). (B) Nucleotide sequence of the *trmE* promoter of *P. syringae* and its regulatory elements. The transcription start site at position +1 (C), −10 region (TGGATT), −35 region (TGAAAT), UP element (TACTTCTGGA AAGT), cold box (TGAACAACTGC), DEAD box (AACAGTGGTA), conserved region (CAAAAA), Shine-Dalgarno sequence (SDS) (GAGG), and translation start site (ATG) are highlighted. The arrows indicate the direction of the primers, the transcription start site, and the translation start site.

TABLE 3. Length of 5′-UTRs in some cold-inducible genes of bacteria

Microorganism	Gene	5′-UTR length (bp)	Reference
Pseudomonas syringae Lz4W	*trmE*	345	This study
Pseudomonas syringae Lz4W	*hutU*	170	30
Pseudomonas putida	*rpoS*	368	34
Escherichia coli	*cspB*	161	38
Escherichia coli	*cspA*	159	20
Escherichia coli	*csdA*	226	63
Escherichia coli	*cspG*	161	45
Escherichia coli	*cspI*	145	6
Anabaena sp.	*chrC*	116	9
Methanococcoides burtonii	*deaD*	113	39
Sinorhizobium meliloti	*cspA*	119	47

show any change in the transcript levels for up to 60 min after the shift. These results imply that *trmE* expression is under the control of a cold-inducible promoter. Expression of *pnp* and *oppA* in the psychrophiles *Y. enterocolitica* and *Listeria monocytogenes*, respectively, was also reported to increase in cells growing at 5°C compared to cells growing at 30°C (5, 23).

Primer extension analysis revealed a primer extension product of an expected size (100 bp) in *P. syringae* grown either at 4°C or at 22°C (Fig. 3A). The transcription start site was identified as a C (Fig. 3B).

Activity and expression of the *trmE* promoter. Primers Ash 57 and Ash 59 amplified a 655-bp product, which corresponded

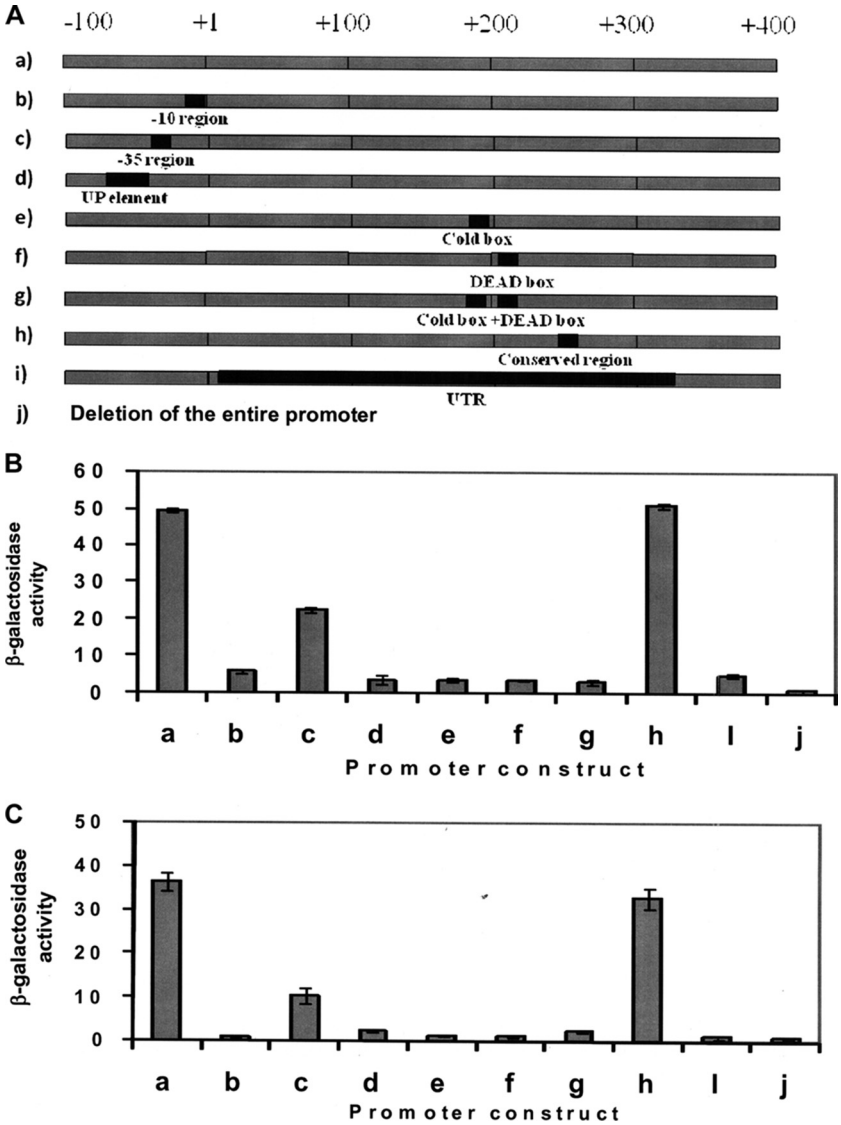

FIG. 4. (A) Schematic diagrams of *trmE* promoter fragment deletion constructs of *P. syringae*. The deleted regions are indicated by dark gray boxes. The intact *trmE* promoter is shown on line a, whereas lines b to j show the regions that were deleted, as follows: line b, −10 region (deletion from position −5 to position −10); line c, −35 region (deletion from position −33 to position −38); line d, UP element region (deletion from position −41 to position −54); line e, cold box region (deletion from position 184 to position 194); line f, DEAD box (deletion from position 208 to position 217); line g, cold box and DEAD box regions (deletion from position 184 to position 194 and from position 208 to position 217); line h, conserved region (deletion from position 246 to position 252); line i, long 5′-UTR (deletion from position 9 to position 322); and line j, the entire promoter. (B and C) *trmE* promoter activities in *P. syringae* harboring the promoter deletions indicated above, assayed using cells grown at 22°C (B) and at 4°C (C), based on the activity of the β-galactosidase gene, the reporter gene.

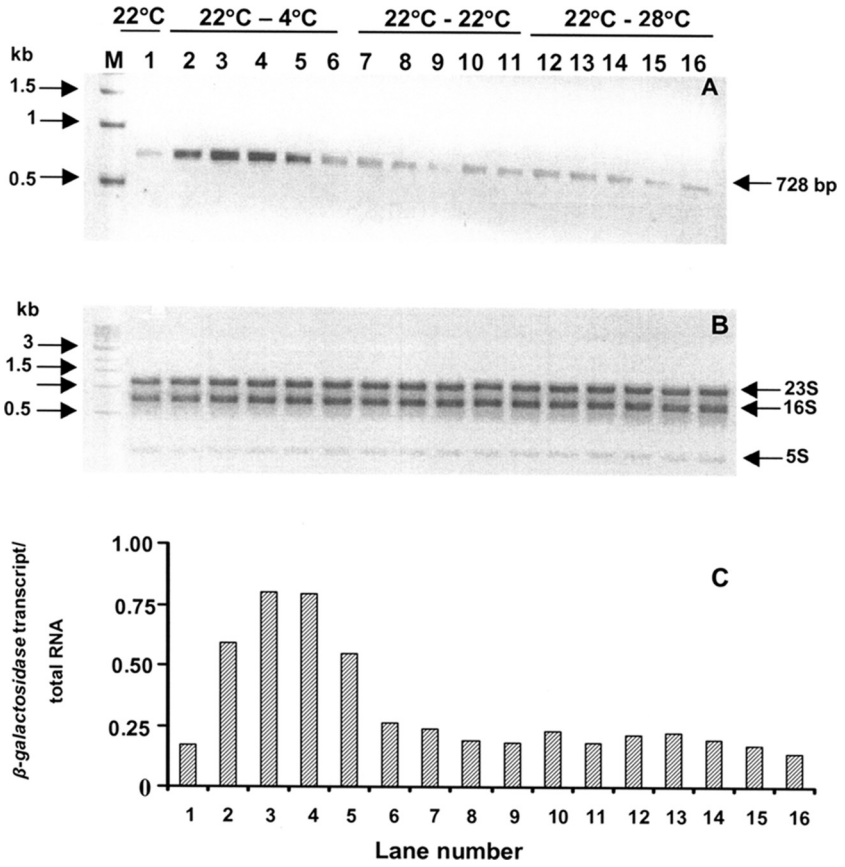

FIG. 5. RT-PCR analysis of the expression of the *trmE* promoter based on expression of the β-galactosidase gene, the reporter gene in *P. syringae* (harboring pKZ27::promoter). (A) Lane 1, β-galactosidase gene transcript level in a culture grown at 22°C (before a shift); lanes 2 to 6, transcript levels in cultures grown at 22°C and shifted to 4°C for 10, 20, 30, 45, and 60 min, respectively; lanes 7 to 11, transcript levels in cultures grown at 22°C and shifted to 22°C for 10, 20, 30, 45, and 60 min; lanes 12 to 16, cultures grown at 22°C and shifted to 28°C for 10, 20, 30, 45, and 60 min, respectively; lane M, markers. (B) Amount of total RNA used in each of the RT-PCRs in panel A. (C) Ratio of the β-galactosidase gene transcript to the total RNA (as determined by densitometry) for lanes 1 to 16 in panels A and B. The experiment was done three times, and the data are the results of a representative experiment.

to 591 bp upstream and 64 bp downstream of the translation start site (ATG). Between the transcription and translation start sites, a long 345-bp 5′ untranslated region (5′-UTR) was identified (Fig. 3B), which is one of the longest 5′-UTRs in cold-inducible genes (Table 3). Further, the −10 region, the −35 region, the UP element, the cold box, the DEAD box, and a conserved region (CAAAAAA) were identified (Fig. 3B; see Fig. S2 in the supplemental material). These promoter elements are highly conserved in the *trmE* promoters of other bacteria (see Fig. S2 in the supplemental material). When the putative promoter (655-bp fragment) upstream of the *trmE* gene was ligated upstream of the β-galactosidase gene and cloned in *P. syringae*, it exhibited β-galactosidase activity (Fig. 4A to C). But when the promoter elements were deleted (except for deletion of the −35 region, which resulted in 50 and 70% reductions in activity at 22 and 4°C, and deletion of CAAAAA, which had no effect), a drastic reduction in β-galactosidase activity was observed irrespective of whether the transformants were cultured at 4°C or 22°C (Fig. 4A to C). The CAAAAAA sequence was reported previously for the *hut* (30) and *cti* (37) promoters of *P. syringae* (see Fig. S2 in the supplemental material), but it does not appear to influence the promoter activity of *trmE*. The present study demonstrates that the 5′-UTR, −10 region, −35 region, UP element, cold box, and DEAD box (Fig. 3B) are essential for the activity of the *trmE* promoter, as they are for other genes (18, 31, 39, 44, 47, 66, 67) (Fig. 4A to C). Further, the *trmE* promoter elements of *P. syringae* had sequences identical to those in *B. linens* and *Marinomonas primoryensis*, which are psychrophiles (see Fig. S2 in the supplemental material).

The sequences of the −10 and −35 regions were similar to the sequences of the −10 (TATA/GAT/C) and −35 (TA/GG A/GTA/T) regions of 11 promoters of the psychrophile *Pseudoalteromonas haloplanktis* but differed from the consensus sequences of the *E. coli* −10 and −35 regions (TATAAT and TTGACA, respectively) based on 300 promoters (40). Whether the observed similarity is a reflection of the cold-inducible nature of the promoter is difficult to predict. The UP element (17) in *P. syringae* is an AT-rich region (64.3%) and shows limited similarity with *E. coli* (21) and *P. haloplanktis* sequences (15) (see Fig. S2 in the supplemental material). The activity of the *trmE* promoter was also analyzed by monitoring the levels of β-galactosidase (reporter) transcripts in *P. syringae* harboring pKZ27::promoter. The β-galactosidase gene

transcript level was unaltered in control cultures, which were cultured continuously at 22°C, and in cultures shifted from 22°C to 28°C (Fig. 5A to C). But when cultures were shifted from 22°C to 4°C, a transient increase in the β-galactosidase gene transcript level was observed for up to 30 min (Fig. 5A to C), confirming that the *trmE* promoter is under transcriptional regulation at low temperatures.

This temperature-dependent regulation is consistent with the conclusion that *trmE* plays a significant role in the adaptation to cold temperatures. Whereas previous studies have shown that TrmE modifies tRNA and has GTPase activity, this report is the first report to identify a low-temperature function (7, 55, 71). When TrmE activity is inhibited in *E. coli*, the loss of U34 modification causes a transient block in the initiation of translation (16). Additional studies are needed to determine the exact relationship between protein translation and the TrmE-mediated mechanism of cold resistance. This report is the first report demonstrating the importance of *trmE* for growth at low temperatures.

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