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Cis–trans isomerase gene in psychrophilic *Pseudomonas syringae* is constitutively expressed during growth and under conditions of temperature and solvent stress

Abstract In a recent study, we established that psychrophilic *Pseudomonas syringae* (Lz4W) requires *trans*-monounsaturated fatty acid for growth at higher temperatures (Kiran et al. in Extremophiles, 2004). It was also demonstrated that the *cti* gene was highly conserved and exhibited high sequence identity with *cti* of other *Pseudomonas* spp. (Kiran et al. in Extremophiles, 2004). Therefore it would be interesting to understand the expression of the *cti* gene so as to unravel the molecular basis of adaptation of microorganisms to high temperature. In the present study, the expression of *cti* was monitored by RT-PCR analysis during different growth stages and under conditions of high temperature and solvent stress in *P. syringae*. Results indicated that the *cti* gene is constitutively expressed during different stages of growth and the transcript level is unaltered even under conditions of temperature and solvent stress implying that the observed increase in *trans*-monounsaturated fatty acids (Kiran et al. in Extremophiles, 2004) is not under transcriptional control. A putative promoter present in the intergenic region of the *metH* and *cti* gene has also been characterized. The translation start site ATG, the Shine-Dalgarno sequence AGGA and the transcription start site "C" were also identified. These results provide evidence for the first time that the *cti* gene is constitutively expressed under normal conditions of growth and under conditions of temperature and solvent stress thus implying that the Cti enzyme is post-transcriptionally regulated.

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Introduction

Cis–trans isomerization of fatty acids has been implicated as an adaptive response to changes in ambient temperature and solvent stress in *Pseudomonads*, *Vibrios* and some *Methylotrophs* (Morita et al. 1993; Makula 1978; Okuyama et al. 1990; Kiran et al. 2004) based on the observations that the amount of *trans*-unsaturated fatty acids increased in bacteria subjected to solvent or high temperature (Heipieper and de Bont 1994; Junker and Ramos 1999; Okuyama et al. 1991; Kiran et al. 2004). Increase in *trans*-monounsaturated fatty acid could effectively reduce the fluidity of the membrane (Weber et al. 1994) since the phase transition temperature of membranes containing *trans*-fatty acids lies between membranes containing *cis*-unsaturated and membranes containing saturated fatty acids (Okuyama et al. 1991). Such changes in fluidity of the membrane may be crucial for membrane function and survival of the organism under adverse environmental conditions (Keweloh and Heipieper 1996; Ramos et al. 1997; 2001). Recently, it has been demonstrated using a *cti* (*cis–trans* isomerase gene) null mutant of psychrophilic *Pseudomonas syringae* that mutants which were incapable of synthesizing the *trans*-monounsaturated fatty acid were incapable of growth at higher temperatures since the fluidity of the cell membranes was more fluid compared to that of the wild type cells. This study established for the first time a correlation between *trans*-fatty acid, membrane fluidity and growth at high temperatures (Kiran et al. 2004). The growth of the mutant was not affected when grown at 5°C, indicating that *trans*-fatty acids are not required for growth at low temperatures. The reason for this observation could be because at 5°C

the membrane fluidity was similar both in the mutant and the wild type cells (Kiran et al. 2004). Thus an understanding of the expression of the *cti* gene would be crucial so as to unravel the molecular basis of adaptation of microorganisms to adverse environmental conditions caused due to high temperature, salinity and chemical pollutants.

The fatty acyl *cis–trans* isomerase (*cti*) gene has so far been isolated from four species of *Pseudomonas* viz., *Pseudomonas putida* P8 (Holtwick et al. 1997), *P. putida* (DOT-T1E) (Junker and Ramos 1999), *P. putida* KT2440 (Junker and Ramos 1999), *P. psychrophila* (T. Kusano et al. unpublished), *P. aeruginosa* (Stover et al. 2000) and *P. syringae* (Kiran et al. 2004) and one single species of *Vibrio* viz., *Vibrio cholerae* (Heidelberg et al. 2000). ClustalW analysis of the derived amino acid sequence of the *cti* gene of the above species indicated that the gene is highly conserved and the Cti enzyme exhibits conserved features such as a heme-binding site (CVACHA), a conserved proline after the membrane signal sequence, two putative serine phosphorylation sites (PGSTEAL and SRTPSG), a putative tyrosine phosphorylation site (DMNRYENL) and the sequences RWLYEHLFL, GPVCRGQ and FDSASV, whose functional significance are yet to be elucidated (Kiran et al. 2004). It is still not clear how the activity of fatty acyl *cis–trans* isomerase is regulated (Heipieper et al. 2003; von Wallbrunn et al. 2003). The regulation could be either at the level of transcription (mRNA level) or it could be a post-transcriptional event (at the level of the enzyme).

In the present study, *P. syringae* (Lz4W), a psychrophilic bacterium from Antarctica, (Shivaji et al. 1989) was used to understand the regulation of the *cti* gene by RT-PCR analysis during different growth stages and under conditions of high temperature and solvent stress separately. In addition, promoter fusion studies were done to identify the putative *cti* promoter and primer extension analysis was done to identify the transcription start site "C" and −10- and −35-like sequences, CAAAA and TAGGACT, respectively.

Materials and methods

Bacterial strains, plasmids and growth conditions

All the bacterial strains (Table 1) used in the present study were grown in the appropriate medium at the required temperature (5, 22 and 28°C). Psychrophilic *P. syringae* (Lz4W) isolated from Antarctica was grown in Antarctic bacterial medium (ABM) containing peptone (0.5%, w/v) and yeast extract (0.2%, w/v) (Shivaji et al. 1989). *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani (LB) medium. *E. coli* DH10B served as a cloning host.

The plasmids used in this study (Table 1) were pGEM-T-Easy (PCR cloning vector obtained from Promega Corporation, Madison, USA) and pPR9TT (promoter fusion vector obtained from Santos et al. 2000). Antibi-

otics when required were used at the following concentrations: ampicillin was used at a concentration of 100 μg/ml for *E. coli*, whereas chloramphenicol was used at a concentration of 20 and 100 μg/ml for *E. coli* and *P. syringae* Lz4W, respectively. Cultures in liquid medium were incubated on a rotary water bath shaker or in an environmental incubator at constant temperature with continuous shaking (250 rpm).

RNA isolation

Harvesting the cells

Cultures of *P. syringae* (Lz4W) were mixed with equal volume of 10% (W/V) phenol in ethanol so as to kill the cells instantaneously. The cells were then pelleted by centrifugation (5,000 *g* for 10 min), the supernatant removed, the pellet re-suspended in the residual supernatant, transferred it into a microfuge tube and centrifuged for 1 min to remove the supernatant. The tubes with the cell pellet were then stored at −70°C until further use.

Preparation of RNA

The cell pellet was suspended in 500 μl of TES buffer (50 mM Tris–Cl pH 8.0, 5 mM EDTA and 0.5% SDS), pH 8.0 to which an equal volume of acid phenol was added and the tube incubated at 65°C for 10 min and mixed at 2-min intervals. The mixture was then centrifuged at 10,000 *g* for 10 min and the aqueous phase recovered and extracted with phenol:chloroform:iso-amyl alcohol (25:24:1, v/v/v). Usually the extraction was done three to four times until the white precipitate no longer appeared. The aqueous layer was collected and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol were added to recover the nucleic acids by precipitation at −20°C for 30 min or for 1 h at 5°C. The nucleic acid precipitate was then collected by centrifugation at 10,000 *g* and treated with DNaseI reaction mixture (200 μl) at 37°C for 30 min. The DNaseI reaction mixture contained 5 U of RT-grade DNaseI (RNase free), 20 μl of 10x DNaseI buffer, 20 μl of 10x BSA and 155 μl of DEPC water such that the final volume was 200 μl. The entire mixture was then extracted with 200 μl of phenol:chloroform:iso-amyl alcohol (25:24:1). Subsequently, the supernatant was collected and RNA was precipitated with 2.5 volumes of ethanol and 1/10 volumes of 3 M sodium acetate (pH 5.2). The precipitated RNA was then recovered by centrifugation, the pellet dissolved in 20 μl of DEPC-treated water and stored at −70°C until further use.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (2 μg) was taken in an RNAse free micro-fuge tube and 3 pmoles of primer RTCTR (5′

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
Escherichia coli, DH-10B[a]	F[-], *mcrA*, Δ (*mrr-hsd*RMS-*mcr*BC) φ80d *lacZ* Δ M15 Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ (*ara*, *leu*) 7697, *gal*U, *gal*K, λ[-], *rps*L, *nup*G	Lab collection
E. coli JM109 (pPR9TT)	*E. coli* JM 109, harboring a broad host range promoter probe vector pPR9TT	Santos et al. (2000)
E. coli, (pPR9TT + pro *cti*)	*E. coli* JM 109 containing putative *cti* promoter at *Sma* I site of pPR9TT	Present study
Pseudomonas syringae (Lz4W)	Wild type, Antarctic isolate	Shivaji et al. (1989)
P. syringae, (pPR9TT)	Wild type *P. syringae* harboring promoter probe vector pPR9TT	Present study
P. syringae (pPR9TT + pro *cti*)	Wild type *P. syringae* harboring promoter probe vector pPR9TT containing the putative *cti* promoter	Present study
Plasmids		
PPR9TT	Broad-host-range vector containing RK$_2$ replicon, Chl[r]	Santos et al. (2000)
PPR9TT + pro *cti*	pPR9TT containing putative *cti* promoter	Present study
pGEM-T-Easy	A PCR product cloning vector, Ap[r]	Promega Corporation, Madison, USA

[a]DH-10B is a trademark of Invitrogen

GCTGTCAAAGTGCCGGAAAT 3′) was added and the volume made to 10 μl. The tube was then incubated at 65°C for 15 min and cooled on ice. Subsequently 10 μl of RT-PCR mix (1 μl of 10x RT-buffer, 5 μl of 2.5 mM dNTPs, 2 U of Avian Myeloma Virus Reverse Transcriptase (AMV-RT), 0.2 μl of RNAsin and 3.4 μl of DEPC treated water was added and incubated at 42°C for 75 min. The first strand cDNA thus synthesized was then stored at −20°C until further use.

Ten microlitres of the above RT mix was used as a template to which 40 μl of PCR mix [3 pmoles of each of primers RTCTR (5′ GCTGTCAAAGTGCCGGAAAT 3′) and RTCTF (5′ AAGTTGCGCAACAAGCCTAT 3′), 5 μl of 10x PCR buffer, 5 μl of dNTP mix, 1.5 mM of MgCl$_2$ and 1 U of Taq DNA polymerase] was added and PCR was performed under the following conditions: the entire reaction mix was initially incubated at 95°C for 5 min for denaturation of cDNA prior to carrying out 40 cycles of the polymerase chain reaction. Each cycle consisted of a denaturation step at 95°C for 30 s, primer annealing step at 60°C for 30 s and the primer extension step at 72°C for 30 s. The PCR reactions were performed in MJ Research's DNA Engine thermal cycler (MJ Research Inc, Waltham, USA). All the primers used were synthesized in the in-house facility of our center and the primers were designed based on primer 3 (available at http://iubio.bio.indiana.edu/webapps/SeWeR.xx). The amplified product would correspond to a fragment of 105 bp between +1,287 bp and +1,392 bp position of *cti*.

Quantitative real time PCR

Real time reverse transcription PCR of cti

Real time reverse transcription PCR with SYBR Green was performed using the GeneAmp 5700 system (Perkin-Elmer Biosystems, Foster City, CA, USA) and the SYBR Green Assay kit (Perkin-Elmer Biosystems) according to the instructions of the manufacturer.

Reverse transcription reactions were performed in a 100-μl reaction mixture (1 μg of total RNA, 10 μl of 10x RT-buffer, 10 μl of 5 mM dNTPs, 300 pmoles of random hexamers, 100 units of ribonuclease inhibitor, and 120 units of Adeno virus reverse transcriptase. After incubation at 25°C for 10 min, the reverse transcription reaction was allowed to proceed at 48°C for 30 min and the reaction was stopped by heating at 95°C for 5 min. The cDNA thus synthesized was used for quantitative real time PCR. The 25 μl reaction mixture contained 12.5 μl of 2x SYBR-GREEN PCR Master, 3 pmoles of the primers RTCTR and RTCTF and 7.5 μl of the template. The PCR conditions were as follows: denaturation at 95°C for 10 min for one cycle followed by 40 cycles at 95°C for 15 s and annealing and extension at 1 min at 60°C. Each real time PCR was performed in triplicate and the mean C_T (cycle threshold) values were used for analysis of the data using the untreated control as the calibrator as highlighted by Livak and Schmittgen (2001). In the present study, cells grown at 22°C were used as the control to compare the levels of *cti* transcript in cells grown at 22°C and then shifted to 5 and 28°C.

Promoter analysis

PCR amplification and cloning of a putative cti promoter into a promoter probe vector pPR9TT

The putative *cti* promoter was amplified from *P. syringae* (Lz4W) chromosomal DNA using Apa 2 and *metH* I primers corresponding to 255 bp from intergenic region of *metH* and *cti*, corresponding to −158 bp to +97 bp of *cti* and cloned in a PCR cloning vector pGEM-T-Easy and transformed into *E. coli* DH10B strain. The pGEM-T-Easy vector containing the putative *cti* promoter was then isolated, restricted with *Nco* I and *Sma* I enzymes and separated on 1.2% agarose gel. DNA fragment corresponding to the putative *cti* promoter (142 bp) was purified from the gel and treated with Klenow polymerase to fill-in the ends. This putative *cti*

promoter fragment was ligated into a translational promoter fusion vector pPR9TT at *Sma* I site. The ligation was set up as follows: 142 bp DNA fragments corresponding to putative *cti* promoter was cloned in a suitable vector pPR9TT (50 ng) using T4 DNA ligase (New England Biolabs Inc., Beverly, USA). For this purpose, the DNA fragments and the vector DNA were gel purified using the Qiagen's gel purification kit (QIAGEN, Hilden, Germany) and mixed (1:3 molar ratio) to which 200 units of T4 DNA ligase and 2 μl of 10x DNA ligase buffer were added and the final reaction volume was made up to 20 μl with autoclaved milli-Q water. The reaction mixture was then incubated at 16°C overnight after which the enzyme was heat inactivated at 65°C for 10 min. The ligation mixture was then transformed into *E. coli* DH10B and selected on LB agar plates containing the X-gal and chloramphenicol (20 μg/ml). The recombinant plasmid pPR9TT harboring the putative *cti* promoter was isolated and nearly 5 μg of DNA was electroporated into *P. syringae* (Lz4W) electrocompetent cells by subjecting the cells to a field strength of 6.25 kV/cm, resistance of 183 Ω and capacitance of 25 μF. Further, after the electroporation, the cells were suspended in 1 ml of ABM medium and incubated for 3–4 h and spread on AB agar plates containing chloramphenicol (100 μg/ml) and incubated at 22°C for 36–48 h. The transformants were again screened on AB agar plates containing chloramphenicol (100 μg/ml) and X-gal (40 μg/ml) at 22°C.

β-galactosidase assay

Pseudomonas syringae (Lz4W) containing the putative *cti* promoter on a promoter fusion vector pPR9TT was streaked on AB agar plate containing 100 μg/ml of chloramphenicol and 40 μg/ml of X-gal and incubated for 16–24 h and visually analyzed for the development of blue color.

Primer extension analysis

Transcription start site mapping was carried out using Avian Myeloma Virus Reverse Transcriptase (AMV-RT). For this purpose, nearly 10 pmoles of the appropriate primer (Apa1 5′ GCTGTATGTCCCGGGTA-TAGGAAATA 3′; Apa2 5′ GGGTATAGGAAATA-GCCGGACTTACCA3′; Apa3 5′ GAGAACCGAGT-AAAAGCCCTTGTTGCG 3′; Apa4 5′ TTCAGCTGG-CAGGCGGCATCGT 3′; Apa5 5′ ACACCGGGAC-TTTTGCTTGCTCC 3′) was labeled at the 5′ end with γ-p^{32} ATP using T4 polynucleotide kinase (PNK) in a total reaction volume of 10 μl containing 1x PNK buffer. The reaction was terminated after 10 min at 37°C by inactivating the enzyme by heating the reaction mixture at 90°C for 2 min in a boiling water bath. The end-labelled primer was then separated from the unincorporated labeled ATP using a G-10 sephadex spin column. For this purpose, 10 μl of the end labeling reaction mix to be purified was loaded and the column was centrifuged at 2,500 *g* to elute the labeled primer.

For primer extension analysis the equivalent of 10^6 cpm of the labeled primer was mixed with 20 μg of RNA and the reverse transcription reaction was reconstituted by the addition of 5 mM $MgCl_2$, 1 mM dNTP mix, 1x RT buffer and 10 U of AMV-RT in a final volume of 25 μl. The RT reaction mix was incubated at 42°C for 1 h and subsequently, the nucleic acid in the mixture was precipitated with two volumes of ethanol after the addition of 0.1 volume of 3 M sodium acetate (pH 5.2). The pellet was washed once with 70% ethanol, dried and dissolved in 10 μl of water. Sequencing-gel loading dye (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue) was then added to the samples. After a heat denaturation step (90°C for 2 min followed by rapid cooling on ice), the RT reaction products were loaded onto a 8% urea-polyacrylamide sequencing gel and electrophoresed using 1x TBE. A sequence ladder on an appropriate DNA template was also generated (employing the same radiolabelled primer) on the polyacrylamide gel to estimate the size of the RT reaction product. The gel was then dried and the bands were visualized by autoradiography.

Results

RT-PCR analysis of *cti* gene expression

RT-PCR analysis of *cti* gene was done on total RNA of *P. syringae* (Lz4W) cells grown to stationary phase at 5, 22 and 28°C using the primers RTCTR and RTCTF. The expected product size of 105 bp was observed in RNA isolated from the cells grown at 5, 22 and 28°C indicating that *cti* gene is expressed at all the three temperatures of growth (Fig. 1a) and the level of transcript was similar (Fig. 1a1). Further, in order to find out whether the gene is constitutively expressed during growth or not, RT-PCR analysis was done on RNA isolated from cells grown at 22°C at different growth stages (optical density measured at 600 nm equivalent to 0.12, 0.36, 0.82, 1.4, 2.4, 2.7, 3.3, 3.5 and 3.45). The results showed presence of *cti* transcript in RNA isolated from different growth stages implying constitutive expression of *cti* gene (Fig. 1b) during growth and the level of the transcript did not appear to be significantly different during the various phases of growth (Fig. 1b1).

RT-PCR analysis was also done on RNA isolated from *P. syringae* (Lz4W) subjected to temperature stress. In these experiments *P. syringae* (Lz4W) was grown upto an OD_{600} of 0.8 at 22°C, aliquoted into three flasks and the incubation was continued at 5, 22 and 28°C. Subsequently, at regular intervals of time RT-PCR analysis was performed. RT-PCR analysis showed no marked increase in the *cti* transcript level upon temperature shift (Fig. 1c, c1).

Fig. 1 RT-PCR analysis of *cti* gene expression of *Pseudomonas syringae* (Lz4W). **a** and **a1**, grown at 5, 22 and 28°C; **b** and **b1** grown to different growth stages (OD$_{600}$ 0.12, 0.36, 0.82, 1.4, 2.4, 2.7, 3.3, 3.5 and 3.45); **c** and **c1**, during temperature shift from 22°C to 5°C, 22°C to 22°C and 22°C to 28°C at 5, 10, 15 and 30 min after shift; **d** and **d1**, in cells treated with toluene (2%) for 1, 2 and 5 min. **a1** to **d1** depict quantitation of the results in **a** to **d**, respectively
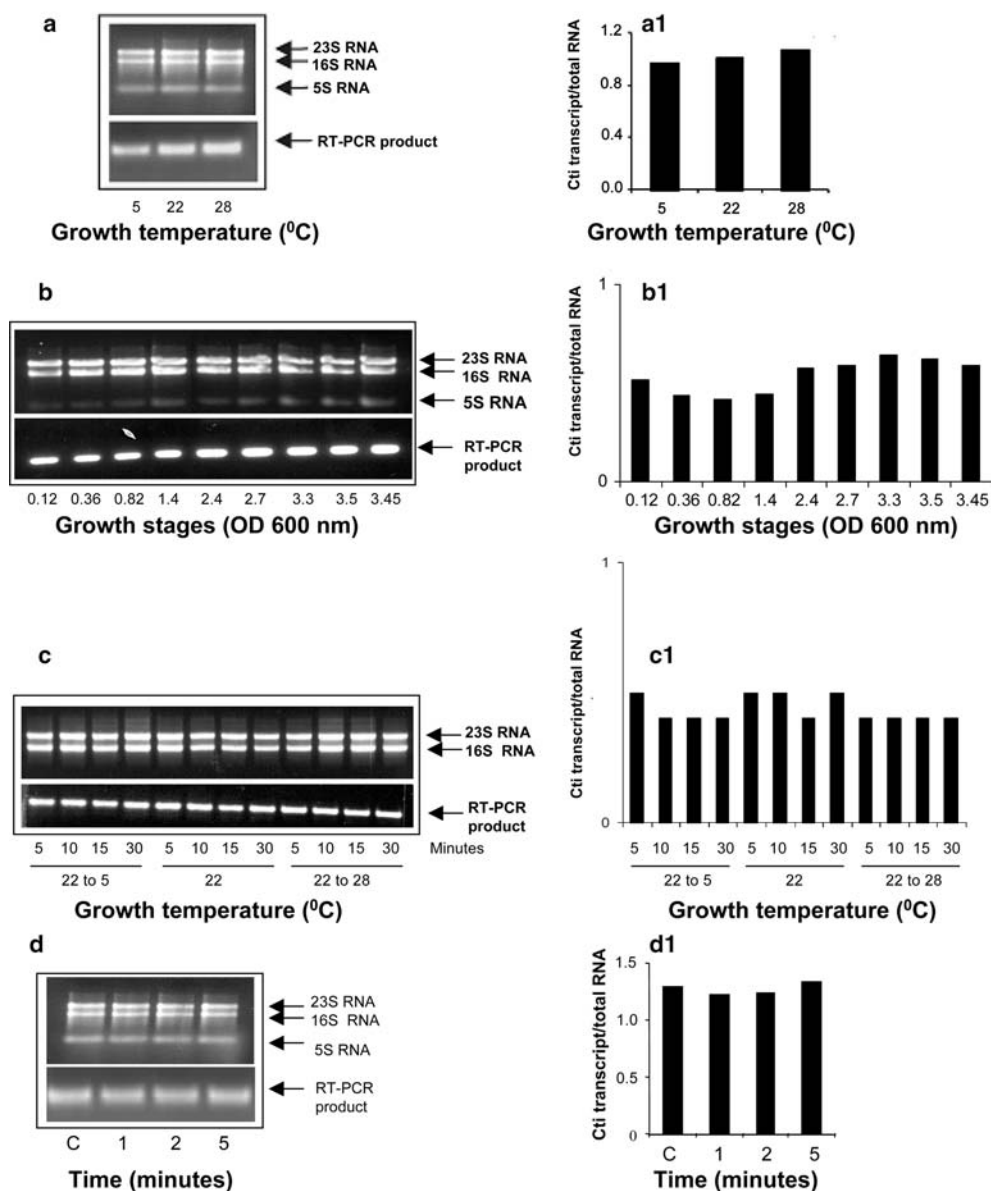
The ability of toluene to induce *cti* expression was also studied. Toluene-treated cells grown at 22°C and treated with toluene for 1, 2 and 5 min (Fig. 1d, d1) did not show any changes in the transcript level. Under similar conditions, the mol% of $C_{16:1\ (9t)}$ increased almost ten times (Kiran et al. 2004).

cti gene expression by quantitative real time PCR

To confirm that the *cti* gene is constitutively expressed when grown at 5, 22 and 28°C or when cells grown at 22°C were shifted to either 5 or 28°C real time reverse transcription PCR was carried out. The results clearly indicated that *cti* gene is expressed in *P. syringae* (Lz4W) grown at the above-mentioned three temperatures and the $2^{-\Delta\Delta C_T}$, which indicates the fold change in gene expression related to the untreated control (that is cells growing at 22°C in the present study) was 1 implying that *cti* transcript levels are similar. In fact, even when *P. syringae* (Lz4W) was grown at 22°C and then shifted to either 5 or 28°C it was observed that even after 2 h of shift, the levels of *cti* transcript did not vary and the $2^{-\Delta\Delta C_T}$, value was approximately 1 indicating that temperature stress does not induce the expression of *cti* (Table 2).

cti promoter analysis using *β*-galactosidase as a reporter gene

Sequence analysis of intergenic region of *cti* and *metH* using the prokaryotic promoter prediction by neural network software (NNPP/Prokaryotic) available in

Table 2 *cti* gene expression of *Pseudomonas syringae* (Lz4W) as determined by real time reverse transcription PCR

Time after shift (min)	Fold increase/decrease in *cti* of *P. syringae* ($2^{-\Delta\Delta C_T}$)[a] grown at 22°C and shifted to 5 or 28°C	
	5°C	28°C
0	1.07	1.14
15	1.05	0.90
30	1.26	0.88
60	1.10	0.88
120	1.05	0.86

[a] $\Delta\Delta C_T = \Delta C_T$ of 5 or 28°C $-\ \Delta C_T$ of 22°C at the same time point

Fig. 2 Putative *cti* promoter activity in *Escherichia coli* DH10B (**a**, **b**) and *Pseudomonas syringae* (Lz4W) (**c**, **d**) transformed with promoter probe vector pPR9TT containing β-galactosidase as the reporter gene. **a** *E. coli* DH10B, containing only the promoter probe vector pPR9TT; **b** *E. coli* DH10B containing putative *cti* promoter in pPR9TT; **c** *P. syringae* (Lz4W) containing only pPR9TT; **d** *P. syringae* (Lz4W) containing *cti* promoter in pPR9TT

BCM launcher indicated the presence of a promoter of length 50 bp (5′ GGTAGAATTGCGACATCTATTG-ATATCAGGACAATGGCACATGTTTTTTC 3′) with a probability of 0.97 in the intergenic region between *cti* and *metH*. The intergenic region was amplified by PCR and cloned in a PCR cloning vector, pGEM-T-Easy and transformed into *E. coli* DH10B. Restriction digestion of recombinant plasmid with *Nco* I and *Sma* I released a 142 bp fragment, which was cloned at *Sma* I site of a translational fusion vector pPR9TT containing a reporter gene β-galactosidase. The construct thus generated was used to transform *E. coli* DH10B and transformants were selected on LB agar plate containing chloramphenicol and X-gal. *E. coli* DH10B containing putative *cti* promoter turned blue on X-gal plate indicating that *cti* promoter was active in *E. coli* DH10B (Fig. 2a, b). The plasmid was then isolated from *E. coli* DH10B and electroporated into *P. syringae* (Lz4W) and β-galactosidase activity was monitored on an X-gal plate at 22°C (Fig. 2c, d). *P. syringae* (Lz4W) transformants with the putative promoter and the reporter gene exhibited β-galactosidase activity indicating that the promoter is active. *P. syringae* (Lz4W) transformed with pPR9TT alone showed no β-galactosidase activity (Fig. 2c, d).

Transcription start site mapping

Primer extension analysis was carried out to locate the transcription start site of *cti* gene in *P. syringae* (Lz4W). For this purpose RNA was isolated from cells of *P. syringae* (Lz4W) grown at 22°C and the primer extension reaction was carried out using the primers Apa 1, 2, 3, 4, and 5. A band of 210 bp was detected (arrowhead) when primer Apa 2 was used (Fig. 3a) but the other primers (Apa 1, 3, 4 and 5) did not show any product. The translation start site is shown as ATG and the Shine-Dalgarno sequence AGGA was identified. Further, examination of DNA sequence around the transcription start site C (indicated as +1) showed the −10 and −35 regions as CAAAA and TAGGACT, respectively (Fig. 3b).
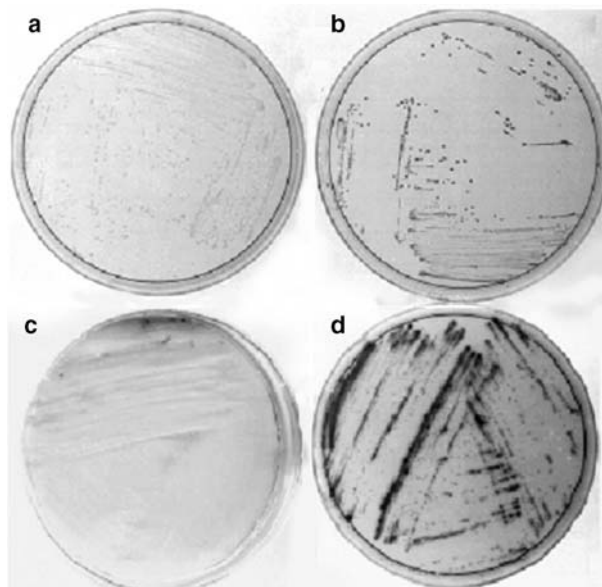
Discussion

In a recent study using psychrophilic *P. syringae* (Lz4W), we demonstrated an increase in the level of $C_{16:1\ (9t)}$, a *trans*-monounsaturated fatty acid in cells grown at 28°C compared to 5°C. In addition, it was demonstrated that the corresponding *cti* gene was highly conserved and cells in which the gene was mutated showed arrest of growth at 28°C implying that the *trans*-monounsaturated fatty acid is required for growth (Kiran et al. 2004). The reason for the growth arrest at 28°C was attributed to the fact that in the *cti* null mutant the fluidity of the membrane was higher compared to the wild type cells which synthesized the *trans*-monounsaturated fatty acid (Kiran et al. 2004). Therefore, there is a need to understand the regulation of the gene, which would ultimately influence the level of the *trans*-monounsaturated fatty acid. This increase may arise either due to upregulation of the *cti* gene or due to increase in the activity of the enzyme *cis–trans* isomerase. As of now, neither of the above possibilities has been addressed. With this in view, the present study was undertaken to study the expression of the *cti* gene by RT-PCR under normal growth conditions, under adverse conditions of temperature and also under solvent stress. The results indicated that the *cti* gene is constitutively expressed during growth and did not seem to vary depending on the growth phase of the culture. It is interesting to note that conversion of *cis*- to *trans*-monounsaturated fatty acid also occurs when bacteria are in the stationary
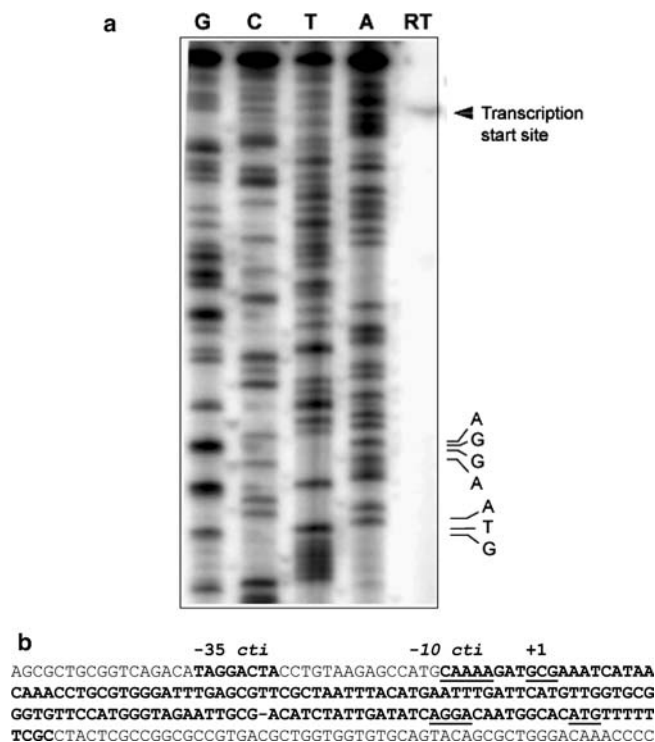
Fig. 3 a Primer extension analysis of *cti* gene transcript. RNA prepared from cells grown at 22°C was used for primer extension and the primer extension reaction was done with primer Apa2. The reaction products were analyzed by 8 M urea-6% polyacrylamide gel electrophoresis alongside a sequence ladder (G, C, T, A), which were generated with the same primer Apa2. The primer extension product is shown by an *arrowhead* (transcription start site). The translation start site (ATG) and Shine-Dalgarno sequence (AGGA) are shown. **b** Sequence of intergenic region between *metH* and *cti* as identified by automated sequencing and from primer extension sequencing reaction (*underlined*). The −10 region (CAAAA), the transcription start site (+1, "C"), the translation start site (ATG) and Shine-Dalgarno sequence (AGGA) of *cti* are shown in *bold* and *underlined*

phase of growth implying that it is a post-biosynthetic event occurring even in the absence of fatty acid biosynthesis. In fact conversion of *cis-* to *trans*-monounsaturated fatty acid which occurs even in non-growing cells has been proposed as an adaptive mechanism to overcome the disadvantage of bringing about a change in the degree of fatty acid desaturation, which strictly occurs only during the growth phase of the bacterium (Heipieper et al. 2003).

The expression of the *cti* gene in psychrophilic *P. syringae* remained unaltered when cells were grown at different growth temperatures or even when cultures were shifted from 22°C to 5 or 28°C or when treated with toluene for 1, 2 and 5 min indicating that under these conditions the gene is constitutive in its expression. However, this result is in contrast to the increased levels of *trans*-monounsaturated fatty acids observed in psychrophilic *P. syringae* grown at 28°C or when exposed to toluene (Kiran et al. 2004). Taken together, these results would imply that the lack of correlation between the *cti* transcript level (present study) and the increased level of

trans-monounsaturated fatty acids under adverse condition is indicative of post-transcriptional regulation of Cti, possibly occurring due to upregulation in the activity of the Cti enzyme. Earlier studies using *P. putida* S12 and *P. putida* DOT-T1E and *Vibrio* sp. ABE-1 (Okuyama et al. 1991) also provided indirect evidence that the increase in *trans*-fatty acid immediately following exposure to toluene (Heipieper and de Bont 1994; Pinkart and White 1997; Ramos et al. 1997; Pedrotta and Witholt 1999; Okuyama et al. 1991) is under post-transcriptional control. In these latter studies the transcript levels were not monitored.

Regulation of Cti

It is still not clear as to how this upregulation in activity occurs. But, the possibilities are that it may occur due to post-translational modifications such as phosphorylation and glycosylation. The possibility of glycosylation could be ruled out since the Cti enzyme of *P. syringae* or other bacteria does not have any putative glycosylation sites (Kiran et al. 2004). But, the Cti enzyme does have a number of conserved putative serine and tyrosine phosphorylation sites (Kiran et al. 2004). Thus, it is not illogical to assume that the activity of the enzyme is regulated by the phosphorylation status of the enzyme since we had earlier demonstrated that psychrophilic *P. syringae* (Lz4W) exhibits differential phosphorylation of proteins in response to temperature and in addition, protein kinases have been implicated in cold adaptation (Ray et al. 1994a–c).

However, based on the available information it may be speculated that the activity of Cti may be dependent on the degree of membrane fluidity or the phosphorylation status of the enzyme or both. For instance, when the fluidity of the membrane changes due to increase in growth temperature or because of the presence of membrane perturbing agents such as toluene, Cti, which is present in the periplasm may get translocated so as to reach the double bond of *cis*-monounsaturated fatty acyl chains. This could happen either by deeper penetration of Cti into the membrane bilayer so as to get a better access to the substrate namely *cis*-monounsaturated fatty acid or by the substrate approaching the membrane surface more frequently thus facilitating interaction between Cti and its substrate (Heipieper et al. 2003; von Wallbrunn et al. 2003). This hypothesis is supported by previous observations, which showed that a decrease in the acyl chain order increased the penetration and translocation of proteins in the membrane (Killian et al. 1992).

Identification of the putative promoter of *cti*

Studies were also done to identify the putative promoter of the *cti* gene by using a translational fusion construct of a putative *cti* promoter amplified upstream to the *cti*

gene with a reporter gene *β*-galactosidase. Results indicated that the intergenic region of *metH* and *cti* of 142 bp exhibited promoter activity both in *Escerhichia coli* and *P. syringae*. Similar studies done in *P. putida* DOT-T1E strain using a fusion construct consisting of a putative *cti* promoter and *phoA* (*cti*:: *phoA*), which encodes for alkaline phosphatase (Junker and Ramos 1999) indicated that *cti* promoter activity was constitutive and the presence of solvent did not increase the activity of the promoter (Junker and Ramos 1999).

In a recent study, nucleotide alignment of 11 putative promoters from a cold-adapted *Pseudoalteromonas haloplanktis* (strain TAC125) led to the identification of a cold-adapted consensus sequence representing the putative −35 (TA/GGA/GTAT) and −10 (TATA/GAT/C) regions and it was proposed that these were consensus promoter sequences for *P. haloplanktis* (strain TAC125) (Duilio et al. 2004). The −35 and −10 regions of *P. syringae* (Lz4W) *cti* gene (TAGGAC AND CA-AAAG, respectively) are not identical to that of *P. haloplanktis* (strain TAC125) but are similar to the corresponding regions (TA/GGA/GTA/T and TATA/GAT/C). Further, these regions in *P. syringae* (Lz4W) are less similar to that of *E. coli*. Duilio et al. (2004) also demonstrated that in two of the 11 promoters, the presence of an AT-rich region between −59 and −38 bp from the transcriptional start site and demonstrated that presence of this region activates downstream promoter activity by 3.8-fold at 4°C and 2.3-fold at 15°C, indicating that it positively regulates transcription at low temperature. Such an AT-rich region is absent in *P. syringae* (Lz4W) and this may explain the lack of induction in *cti* at low temperature.

Transcription start site mapping

In the present study, attempts were also made to map the *cti* gene transcription start site by primer extension. The translation start site ATG and the Shine-Dalgarno sequence AGGA were identified upstream to the *cti* gene at positions +1 to +3 and −9 to −13 of *cti*. Further, sequence analysis at the transcription start "C" site indicated presence of −10 and −35 sites of *cti* promoter containing the unique sequences CAAAAG and TAG-GAC respectively. CAAAAG was earlier identified as a unique −10 sequence in the regulatory element of the *hut* gene of *P. syringae* (Lz4W) (Janiyani and Ray 2002). In contrast, the −10 and −35 sites of *cti* promoter in *P. putida* P8 were identified as AGACAT and CTTGGA respectively (Holtwick et al. 1997).

Conclusions

This paper describes for the first time the expression of the *cti* gene in a psychrophilic bacterium under normal conditions of growth and under conditions of temperature and solvent stress. The results indicate that the gene is constitutively expressed during different stages of growth and the transcript level is unaltered under the above-mentioned conditions and also imply that the observed increase in trans-monounsaturated fatty acids under adverse environmental conditions is not under transcriptional control. A putative promoter of the *cti* gene has also been characterized and the transcription start site identified.

References

Duilio A, Madonna S, Tutino ML, Pirozzi M, Sannia G, Marino G (2004) Promoters from a cold adapted bacterium: definition of a consensus motif and molecular characterization of UP regulative elements. Extremophiles 8:125–132

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H and Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O (2000) DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. Nature 406:477–483

Heipieper HJ, De Bont JAM (1994) Adaptation of Pseudomonas putida S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl Environ Microbiol 60:4440–4444

Heipieper HJ, Meinhardt F, Segura A (2003) The cis-trans isomerase of unsaturated fatty acids in Pseudomonas and Vibrio: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiol Lett 229:1–7

Holtwick R, Meinhardt F, Keweloh H (1997) cis-trans isomerization of unsaturated fatty acids: cloning and sequencing of the cti gene from Pseudomonas putida P8. Appl Environ Microbiol 63:4292–4297

Janiyani KL, Ray MK (2002) Cloning, sequencing and expression of the cold-inducible hutU gene from the Antarctic psychrotrophic bacterium, Pseudomonas syringae. Appl Environ Microbiol 68:1–10

Junker F, Ramos JL (1999) Involvement of the cis/trans isomerase Cti in solvent resistance of Pseudomonas putida DOT-T1E. J Bacteriol 181:5693–5700

Keweloh H, Heipieper HJ (1996) trans-unsaturated fatty acids in bacteria. Lipids 31:129–137

Killian JA, Fabrie CHJP, Baart W, Morein S, de Kruijff B (1992) Effects of temperature variation and phenethyl alcohol addition on acyl chain order and lipid organization in Escherichia coli derived membrane systems. A 2H- and 31P-NMR staudy. Biochim Biophys Acta 1105:253–262

Kiran MD, Prakash JS, Annapoorni S, Dube S, Kusano T, Okuyama H, Murata N, Shivaji S (2004) Psychrophilic Pseudomonas syringae requires trans-monounsaturated fatty acid for growth at higher temperature. Extremophiles 8:401-410

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408

Makula RA (1978) Phospholipid composition of methane-utilizing bacteria. J Bacteriol 134:771–777

Morita N, Shibahara A, Yamamoto K, Shinkai K, Kajimoto G, Okuyama H (1993) Evidence for cis–trans isomerization of a double bond in the fatty acids of the psychrophilic bacterium Vibrio sp. strain ABE-1. J Bacteriol 175:916–918

Okuyama H, Sasaki S, Higashi S, Murata N (1990) A *trans*-unsaturated fatty acid in a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. J Bacteriol 172:3515–3518

Okuyama H, Okajima N, Sasaki S, Higashi S, Murata N (1991) The *cis/trans* isomerization of the double bond of a fatty acid as a strategy for adaptation to changes in ambient temperature in the psychrophilic bacterium, *Vibrio* sp. strain ABE-1. Biochim Biophys Acta 1084:13–20

Pedrotta V, Witholt B (1999) Isolation and characterization of the *cis-trans*- unsaturated fatty acid isomerase of *Pseudomonas oleovorans* GPo12. J Bacteriol 181:3256–3261

Pinkart HC, White DC (1997) Phospholipid biosynthesis and solvent tolerance of *Pseudomonas putida* strains. J Bacteriol 179:4219–4226

Ramos JL, Duque E, Rodriguez-Herva JJ, Godoy P, Haidour A, Reyes F, Fernandez-Barrero A (1997) Mechanisms for solvent tolerance in bacteria. J Biol Chem 272:3887–3890

Ramos JL, Gallegos MT, Marques S, Ramos-Gonzalez MI, Espinosa-Urgel M, Segura A (2001) Responses of Gram-negative bacteria to certain environmental stressors. Curr Opin Microbiol 4:166–171

Ray MK, Seshu Kumar G, Shivaji S (1994a) Phosphorylation of membrane proteins in response to temperature in an Antarctic *Pseudomonas syringae*. Microbiology 140:3217–3223

Ray MK, Seshu Kumar G, Shivaji S (1994b) Phosphorylation of lipopolysaccharides in the Antarctic psychrotroph *Pseudomonas syringae*: a possible role in temperature adaptation. J Bacteriol 176:4243–4249

Ray MK, Seshu Kumar G, Shivaji S (1994c) Tyrosine phosphorylation of a cytoplasmic protein from the Antarctic psychrotrophic bacterium *Pseudomonas syringae*. FEMS Microbiol Lett 122:49–54

Santos PM, Blatny JM, Di Bartolo I, Valla S, Zennaro E (2000) Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl Environ Microbiol 66:1305–1310

Shivaji S, Rao NS, Saisree L, Sheth V, Reddy GSN, Bhargava PM (1989) Isolation and identification of *Pseudomonas* spp. from Schirmacher Oasis, Antarctica. Appl Environ Microbiol 55:767–770

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406:959–964

von Wallbrunn A, Richnow HH, Neumann G, Meinhardt F, Heipieper HJ (2003) Mechanism of *cis–trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*. J Bacteriol 185:1730–1733

Weber FJ, Isken S, de Bont JA (1994) *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology 140:2013–2017