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Psychrophilic *Pseudomonas syringae* requires *trans*-monounsaturated fatty acid for growth at higher temperature

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Abstract A psychrophilic bacterium, *Pseudomonas syringae* (Lz4W) from Antarctica, was used as a model system to establish a correlation, if any, between thermal adaptation, *trans*-fatty acid content and membrane fluidity. In addition, attempts were made to clone and sequence the *cti* gene of *P. syringae* (Lz4W) so as to establish its characteristics with respect to the *cti* of other *Pseudomonas* spp. and also to in vitro mutagenize the *cti* gene so as to generate a *cti* null mutant. The bacterium showed increased proportion of saturated and *trans*-monounsaturated fatty acids when grown at 28°C compared to cells grown at 5°C, and the membrane fluidity decreased with growth temperature. In the mutant, the *trans*-fatty acid was not synthesized, and the membrane fluidity also decreased with growth temperature, but the decrease was not to the extent that was observed in the wild-type cells. Thus, it would appear that synthesis of *trans*-fatty acid and modulation of membrane fluidity to levels comparable to the wild-type cells is essential for growth at higher temperatures since the mutant exhibits growth arrest at 28°C. In fact, the *cti* null mutant-complemented strain of *P. syringae* (Lz4W-C30b) that was capable of synthesizing the *trans*-fatty acid was indeed capable of growth at 28°C, thus confirming the above contention. The *cti* gene of *P. syringae* (Lz4W) that was cloned and sequenced exhibited high sequence identity with the *cti* of other *Pseudomonas* spp. and exhibited all the conserved features.

Keywords *cti* cloning · *cti* mutants · Membrane fluidity · Psychrophilic *Pseudomonas syringae*

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Introduction

Changes in fatty acyl chains of membrane phospholipids is a general adaptive strategy to modulate membrane fluidity so as to overcome adverse environmental conditions caused due to low or elevated temperatures, increased salinity, presence of organic solvents, and other chemical pollutants (Diefenbach and Keweloh 1994; Diefenbach et al. 1992; Holtwick et al. 1997; Junker and Ramos 1999; Keweloh and Heipieper 1996; Ramos et al. 1997, 2001). Normally, membrane-fluidity modulation occurs due to changes in the ratio of the saturated to unsaturated fatty acid content, due to variation in the fatty acid chain length, variation in the ratio of iso- to anteisobranched-chain fatty acids, and the protonation and deprotonation status of the phospholipid head group (Denich et al. 2003; Suutari and Laasko 1994). *cis–trans* isomerization of fatty acids has also been implicated as an adaptive response to changes in ambient temperature and solvent stress in *Pseudomonas* and *Vibrio* spp. and some methylotrophs (Makula 1978; Morita et al. 1993; Okuyama et al. 1990), and such changes are also known to modulate membrane fluidity (Okuyama et al. 1991). Thus, it appears that the complex composition of the membrane of microorganisms is tightly regulated (Beney and Gervais 2001; Denich et al. 2003) so as to maintain the cohesion of the membrane structure and function, which appears to be crucial for the survival of organisms (Beney and Gervais 2001; Vigh et al. 1998). Vigh et al. (1998) suggested that changes in membrane fluidity due to environmental cues could serve as an input signal, which in turn could ultimately

regulate expression of specific genes like the desaturase genes, *cis–trans* isomerase gene, heat shock genes, etc. (Lehel et al. 1993; Vigh et al. 1998).

Despite all the above studies on the role of saturated and unsaturated fatty acids with respect to cold acclimation, our understanding of the role of *cis*- and *trans*-fatty acids with respect to adaptation to changes in ambient temperature is limited. As of now, high levels of *trans*-fatty acids have been correlated positively with solvent stress (Junker and Ramos 1999; Okuyama et al. 1991) and elevated temperatures (Okuyama et al. 1990, 1991; Yumoto et al. 1998). In fact, in psychrophilic *Vibrio* sp. (strain ABE-1) grown at 5°C, only traces of *trans*-fatty acids were present, implying that *trans*-fatty acids are probably not crucial for survival at low temperature. In the absence of more studies on similar lines using either mesophilic, psychrophilic, or psychrotolerant bacteria, it is difficult to generalize on the importance of *trans*-fatty acids in temperature acclimation. Further, unlike saturated and unsaturated fatty acids, which are present in all bacteria, *trans*-fatty acids are predominate only in some Gram-negative bacteria (Keweloh and Heipieper 1996).

The present study was undertaken to evaluate the changes in the saturated, unsaturated, *cis*-, and *trans*-fatty acid composition of a psychrophilic *Pseudomonas syringae* (Lz4W) from Antarctica, capable of growth between 2 and 30°C. Simultaneously, the fluidity of the membranes was also ascertained at different growth temperatures. The data indicated an increase in the proportion of saturated and *trans*-fatty acids in cells grown at higher temperatures and concomitantly, a decrease in membrane fluidity. Further, to establish the essentiality of *trans*-fatty acids at higher temperatures, a *cti* null mutant of *P. syringae* (Lz4W-30b) was constructed so as to establish a correlation, if any, with respect to the requirement of *trans*-fatty acid, membrane fluidity, and thermal adaptation.

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 *Pseudomonas 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, *E. coli* HB101 (pRK 600) was used as a helper strain in triparental mating, and *E. coli* S17-1 was used to mobilize the cosmid to *P. syringae* by biparental mating.

The plasmids used in this study (Table 1) were pUFR034 and pBSKS$^-$ as cloning vectors and pRK600 as a helper plasmid. Antibiotics when required were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; tetracycline, 25 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 100 μg/ml; and trimethoprim, 60 μg/ml. 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).

Extraction and analysis of total free fatty acids

Extraction and analysis of total free fatty acids were done essentially according to the method of Sato and Murata (1982). To about 10 mg of freshly harvested bacterial cells, 3 ml 5% methanolic-HCl was added and subjected to methanolysis at 80°C for 3 h, using $C_{13:0}$ as an internal standard. The resultant fatty acid methyl esters (FAME) were extracted with hexane and analyzed

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
Escherichia coli, DH 10B[a]	F$^-$, *mcr*A, Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80d *lac*Z 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, S17-1	RP4-2Tc::Mu-Kn::Tn7 *pro hsd*R *rec*A	Simon et al. (1983)
E. coli, HB 101	F$^-$, *hsd*S20 (*hsd*R *hsd*M), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *galK*2, *rpsL*20 (Sm^R):*xyL*-5, *mtl*-1, *sup*E44, λ^-	Lab collection
Pseudomonas syringae (Lz4W)	Wild type	Present study
P. syringae (Lz4W-30b)	Tet^r, *cti*::Tn5	
P. syringae (Lz4W-C30b)	Tet^r, Kan^r, *cti*::Tn5 pUFR034 + *cti* insert	
Plasmids		
PUFR034	Inc W, Nm^r, Tra$^-$, Mob$^+$, *mob*(P), *lac*Zα$^+$ Par$^+$, *cos*	DeFeyter et al. (1990)
pUFR034 + *cti*	Inc W, Nm^r, Tra$^-$, Mob$^+$ *mob*(p) *lac*Zα, Par$^+$, *cti* insert	Present study
pBSKS$^-$	Ap^r	Stratagene, La Jolla, Calif., USA
pBSKS$^-$ + *cti*	Ap^r + 1.8 kb *cti* insert	Present study
pRK600	Cm^r, Mob$^+$, Tra$^+$, *ColE1*, replicon	Lab collection

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

using a 5890 plus gas–liquid chromatography system (Hewlett Packard, Palo Alto, Calif., USA) equipped with a flame ionization detector and a capillary column (DB-23) (30 m × 0.246 mm × 0.25 μm) that was obtained from J&W Scientific (Folsom, Calif., USA). The injector and detector temperatures were maintained at 200 and 240°C, respectively. The column temperature was programmed from 175–220°C (4°C/min), with an initial time of 8 min. The carrier gas used was nitrogen/helium, and the flow rate was 1 ml/min.

Identification of *trans*-monounsaturated fatty acids

Trans-monounsaturated fatty acids were identified by Fourier transform infrared spectroscopy (FTIR), as described earlier (Okuyama et al. 1990, 1991). For this purpose, the FAME were extracted with hexane and chromatographed on a silver-nitrate thin-layer chromatographic plate, using a solvent system containing chloroform and ethanol (99:1, v/v). The plates were then dried and sprayed with 0.01% primuline in acetone:H_2O (4:1, v/v). The FAME spots were detected under UV light. The separated methyl esters were extracted from the silica gel with chloroform:methanol:water (1:1:0.9, v/v/v), and the *cis* and *trans* configurations of the individual esters were determined by FTIR, using the potassium-bromide method with authentic methyl esters of $C_{16:1(9t)}$ and $C_{16:1(9c)}$ (Sigma-Aldrich, St. Louis, Mo., USA) as standards (Okuyama et al. 1991).

Effect of toluene, NaCl, and starvation on the synthesis of $C_{16:1(9t)}$ of psychrophilic *P. syringae* (Lz4W)

In order to evaluate the effect of toluene and NaCl on the levels of $C_{16:1(9t)}$ in *P. syringae*, cells were grown to stationary phase, and then, toluene and NaCl were added separately at a final concentration of 2% and 0.75 or 2 M NaCl, respectively. Subsequently, samples were processed for fatty acid analysis. *P. syringae* grown to stationary phase was also subjected to starvation. For this purpose, cells were washed with sterile water (5,000 *g* for 10 min) and resuspended in minimal medium equivalent to the original volume. Subsequently, samples were harvested at regular intervals of time up to 2 h and processed for fatty acid analysis.

Statistical analysis

All the physiological studies are an average of a minimum of three independent experiments.

Construction of a partial gene bank of *P. syringae* (Lz4W)

To construct a partial gene bank of *P. syringae* (Lz4W), genomic DNA was isolated as described by us earlier (Reddy et al. 2000), digested with *Eco*RV, electrophoresed on 0.7% agarose gel, and hybridized with an heterologous *cti* gene from *P. psychrophila* (T. Kusano et al. 2002, unpublished data). The *Eco*R restricted fragments in the range of 7–9 kb, which hybridized with the *cti* gene, were purified from the agarose gel, using a Qiagen column (QIAGEN, Germany), ligated to a cosmid (pUFRO34) that was already digested with *Eco*RI, end filled with Klenow polymerase (New England Biolabs, Beverly, Mass., USA), and dephosphorylated using shrimp alkaline phosphatase (Boehringer–Mannheim, Germany). The cosmid with the ligated fragments was then used to transform *E. coli* DH10B by electroporation, and about 500 transformants were selected on LB agar medium supplemented with 50 μg/ml of kanamycin, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 1 mM of isopropyl-β-D-thiogalactopyranoside. Colony screening and hybridization were performed according to the method of Sambrook et al. (1989) to detect clones positive for the *cti* gene. The cosmid pUFR034 containing the 8-kb *cti* insert was digested with *Pst*I and hybridized using heterologous *cti* from *P. psychrophila* (T. Kusano et al. 2002, unpublished data). Fragments of 0.8 and 1.8 kb, which hybridized with the probe, were cloned at *Pst*I site of pBSKS$^-$. The pBSKS$^-$ carrying the 1.8-kb fragment was used for in vitro mutagenesis.

Plasmid isolation and DNA sequencing

Plasmids were isolated using an alkali lysis method as described by Sambrook et al. (1989) and the *cti* gene sequenced using M_{13}/pUC as the forward primer, (5′ CGCCAGGGTTTTCCCAGTCACGAC 3′), M_{13}/pUC as the reverse primer (5′ AGCGGATAACAATTTCACACAGGA 3′), and four other primers that were specifically designed namely KMD1 (5′ CCACAACGAAATCACACTGGC 3′), KMD3 (5′ ACCGATCAGCAATATCAAACCG 3′), TET-1FP1 (5′ GGGTGCGCATGATCCTCTAGAGT 3′), and TET-1RP-1 (5′ TAAATTGCACTGAAATCTAGAAATA 3′), using an automated DNA sequencer (ABI prism Model 3700, PE Biosystems, PerkinElmer, Boston, Mass., USA).

Nucleotide sequence accession number

The fatty acyl *cis–trans* isomerase gene of *P. syringae* (Lz4W) was sent to EMBL, Heidelberg, Germany, and it was assigned the accession number AJ 535703.

Sequence analysis

Sequences were analyzed and compared with BLASTX program available from the National Center for Biotechnology Information (NCBI, http://www.ncbi.

nlm.nih.gov/BLAST) Web server. Identification of the restriction enzyme sites, designing of PCR primers, other nucleic acid sequence analyses, and multiple alignments of all Cti proteins available in the protein database were done using SeWeR (http://iubio.bio.indiana.edu/webapps/SeWeR/).

Generation of *cti* null mutants

cti null mutants were generated by in vitro mutagenesis, using the EZ::TN <TET-1> Insertion kit from Epicenter Technologies (Madison, Wis., USA). The pBSKS$^-$ containing the truncated *cti* gene (1.8 kb) as an insert was used as the target DNA for transposition of Tn5, according to the manufacturer's protocol (Epicentre Technologies). The plasmid following in vitro transposon mutagenesis was electroporated into *E. coli* DH10B strain, and transformants were selected on LB agar plates containing tetracycline and ampicillin. The plasmids were then isolated, checked for transposon insertion in the *cti* insert by restriction analysis, and then electroporated into wild-type *P. syringae* (Lz4W). The conditions used for electroporation were: field strength 6.25 kV/cm, capacitance 50 μF, and resistance 186 Ω.

Complementation of *cti* null mutant

The cosmid clone (pUFR034 + *cti*) carrying the full length *cti* gene in *E. coli* DH10B strain was mobilized into *E. coli* S17-1, using the helper strain *E. coli* HB101 (pRK 600) by triparental mating by mixing the cells in a ratio of 1:1:1 (DeFeyter et al. 1990). Transconjugants were selected on spectinomycin, trimethoprim, and kanamycin plates. Subsequently, the cosmid clone from *E. coli* S17-1 was mobilized to *cti* null mutants of *P. syringae* (Lz4W-30b) by biparental mating (DeFeyter et al. 1990), which was achieved by mixing *E. coli* S17-1 and *P. syringae* (Lz4W) in the ratio of 1:4. Transconjugants were selected on tetracycline and kanamycin plates.

Growth analysis

A single bacterial colony was inoculated into a sterile tube containing 5 ml ABM, incubated at 22°C, and grown to stationary phase. This culture served as the inoculums (1%) for growth studies at 5, 22, and 28°C. At different time intervals, 1 ml of the culture was aliquoted, and the optical density at 600 nm (OD_{600}) was measured as a function of time using a Shimadzu 1601 UV-visible spectrophotometer (Tokyo, Japan).

Steady-state pyrene excimer/monomer studies

The ratio of the fluorescence emission intensity of the pyrene excimer peak at 470 nm (E) to the intensity of pyrene monomer peak at 372 nm (M) was used to monitor membrane fluidity, since the formation of the excimer is related to the lateral mobility of pyrene molecules in the lipid phase, and the E/M ratio is positively correlated with membrane fluidity (Galla and Sackmann 1974; Shivaji 1986; Vanderkooi and Callis 1974; Vijayasarathy et al. 1982). Fluorescence emission spectra of pyrene were recorded using a Hitachi F-4010 spectrofluorometer (Hitachi, Tokyo, Japan) and a 1-cm path-length quartz cuvette. Excitation and emission slits with a band pass of 3 nm were used for all the measurements. Pyrene (Vanderkooi and Callis 1974) was excited at 333 nm, and emission spectra were recorded from 360–530 nm.

When intact cells of *P. syringae* (Lz4W) or the *cti* null mutant of *P. syringae* (Lz4W-30b) were used for the determination of membrane fluidity, the culture was pelleted by centrifugation (4,000 *g* for 5 min), the pellet recovered and washed by centrifugation with 10 mM Tris-HCl buffer (pH 7.5), and then suspended in the above buffer to an OD_{600} equivalent to 0.5. To 1 ml of such cells, pyrene was added such that the final concentration was 5 µM and incubated at room temperature for 30 min prior to recording the fluorescence spectrum of pyrene.

Results

Temperature-dependent changes in the proportion of fatty acids in psychrophilic *P. syringae* (Lz4W)

In psychrophilic *Pseudomonas syringae* (Lz4W), the predominant fatty acids following gas–liquid chromatography were identified as $C_{12:0}$, $C_{16:0}$, $C_{16:1(7c)}$, $C_{16:1(9c)}$, and $C_{18:1(11c)}$ (Table 2). In addition, another prominent peak was visible between $C_{16:1(7c)}$ and $C_{16:1(9c)}$ and was identified as $C_{16:1(9t)}$ since it eluted at a retention time identical to authentic $C_{16:1(9t)}$, it co-eluted with authentic $C_{16:1(9t)}$, and the FTIR spectrum of the methyl ester fraction of psychrophilic *P. syringae* (Lz4W) showed the characteristic methyl ester peak at 1,170 cm^{-1} and the *trans*-double-bond peak at 966 cm^{-1}, indicating the presence of *trans*-fatty acid (data not shown; Guckert et al. 1987; Okuyama et al. 1990). The above fatty acids were consistently observed in *P. syringae* (Lz4W) irrespective of the growth temperature (5, 22, and 28°C; Table 1), but the proportion of these fatty acids appeared to be temperature dependent. For instance, in cells grown at 5°C, $C_{16:1(9c)}$ and $C_{18:1(11c)}$ were present at greater proportion than in cells grown at 28°C. In contrast, in cells grown at 28°C, increase in the proportion of saturated fatty acid $C_{16:0}$ and the *trans*-fatty acid $C_{16:1(9t)}$ was observed compared to cells grown at 5°C. Further, the proportion of short-chain fatty acids ($C_{12:0}$ and $C_{14:0}$), $C_{16:1(7c)}$, and $C_{17:0(cyclo)}$ remained constant irrespective of the growth temperature of *P. syringae* (Lz4W).

Table 2 Temperature-dependent changes in the fatty acid composition in *P. syringae* (Lz4W), *P. syringae* null mutant (Lz4W-30b), and the *cti* complemented strain of *P. syringae* (Lz4W-C30b) grown to stationary phase at 5, 22, and 28°C

Strain	Growth temperature (°C)	Fatty acid composition (mol%)[a]												
		$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0(OH)}$	$C_{16:0}$	$C_{16:1(7c)}$	$C_{16:1(9t)}$	$C_{16:1(9c)}$	$C_{17:0}$	$C_{17:0(cyclo)}$	$C_{18:0}$	$C_{18:1(9c)}$	$C_{18:1(9t)}$	$C_{18:1(11c)}$
P. syringae (Lz4W)	5	2.6 ± 0.3	*t*	*t*	1.6 ± 0.3	17.2 ± 0.5	2.2 ± 0.4	0.4 ± 0.1	44.4 ± 2.1	0.8 ± 1.2	1.4 ± 0.2	0.2 ± 0.4	0.7 ± 0.5	*t*	28.8 ± 2.7
	22	1.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	28.9 ± 2	2.3 ± 0.2	1.9 ± 0.5	41.7 ± 3.3	0.6 ± 0.2	1.4 ± 0.2	0.6 ± 0.3	0.3 ± 0.2	*t*	18.5 ± 0.8
	28	1.3 ± 0.3	0.6 ± 0.2	0.33 ± 0.1	1.1 ± 0.5	31.3 ± 2.1	2.3 ± 1.1	5 ± 1.5	37.8 ± 1.7	0.3 ± 0.1	1.3 ± 0.3	0.7 ± 0.3	0.6 ± 0.2	*t*	17.9 ± 1.9
P. syringae (Lz4W-30b)	5	1.7 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	1.2 ± 0.2	19.4 ± 1.2	2.6 ± 0.2	0	49.3 ± 2.1	*t*	1.2 ± 0.4	0.3 ± 0.3	0.2 ± 0.2	0	23.3 ± 3.2
	22	1.1 ± 0.1	0.6 ± 0.2	0.2 ± 0.2	1.1 ± 0.3	29.4 ± 1.5	2.9 ± 0.4	0	42.9 ± 2.2	0.7 ± 0.6	1.1 ± 0.6	0.9 ± 1.1	0.2 ± 0.34	0	19 ± 3.7
	28	1.4 ± 0.4	0.7 ± 0.3	0.4 ± 0.1	0.7 ± 0.3	33.7 ± 2.1	2.7 ± 0.3	0	41.2 ± 1.4	*t*	0.5 ± 0.5	0.9 ± 0.2	0.2 ± 0.2	0	17.6 ± 2.3
P. syringae (Lz4W-C30b)	5	2.1 ± 0.1	0.5 ± 0.1	*t*	1.4 ± 0.1	19.4 ± 0.1	2.6 ± 0.1	1 ± 0.2	49 ± 0.3	*t*	0.7 ± 0.1	0.2 ± 0.1	*t*	*t*	23.2 ± 1.1
	22	1.0 ± 0.1	0.5 ± 0.1	*t*	1.3 ± 0.1	30.6 ± 0.1	2.7 ± 0	4.3 ± 0.1	39 ± 0	0.3 ± 0	1.4 ± 0.1	0.4 ± 0	0.3 ± 0.1	*t*	18.2 ± 0.2
	28	0.7 ± 0.1	1.0 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	38.3 ± 0.1	2.5 ± 0.2	5.5 ± 0.1	36.9 ± 0.2	0.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.4 ± 0.3	*t*	12.3 ± 0.3

[a]Values indicate mol% fatty acid (mean ± SD) and *t* is less than 0.2 mol%

Effect of toluene and NaCl on the proportion of $C_{16:1(9t)}$ in psychrophilic *P. syringae* (Lz4W)

Trans-fatty acids are also known to increase when bacteria are subjected to solvent, salt, or starvation stress. The results indicated a substantial increase in the level of $C_{16:1(9t)}$ only in the presence of 2% toluene, but treatment with 0.75 M or 2 M NaCl or starvation for 2 h did not bring about any change (Fig. 1a). It was also observed that the increase in mol% $C_{16:1(9t)}$ in the presence of toluene was very rapid and reached a peak with in 2 min of exposure (Fig. 1b). Cell viability in the presence of 2% toluene remained unaltered until 5 min of treatment.

Isolation and characterization of the *cti* gene of psychrophilic *P. syringae* (Lz4W)

$C_{16:1(9t)}$ increases when grown at higher temperature or when the cells are exposed to solvent stress, thus implying that the *trans*-fatty acid may be crucial for the growth and survival of the bacterium at higher temperature. One way of demonstrating the essentiality of the *trans*-fatty acid would be to generate a *cti* null mutant and to demonstrate that the mutant is incapable of growth at higher temperatures. This would be possible by identifying the *cti* gene and subsequently mutagenizing the same. Therefore, attempts were made to clone the *cti* gene from *P. syringae* (Lz4W).

A partial genomic library of *P. syringae* in pUFR034 yielded a single clone with an 8-kb insert, which, following digestion with *Pst*I and Southern hybridization using the heterologous *cti* gene as a probe, revealed two positive fragments of 0.8 and 1.8 kb, respectively (data not shown). These two fragments, following subcloning in pBSKS$^-$ and sequencing, yielded the entire sequence of the *cti* gene of psychrophilic *P. syringae* (Lz4W) (AJ 535703). The *cti* gene has an ORF of 2,295 bp, coding for a protein with a molecular mass of about 86,000 Da and 765 amino acids. Further, an incomplete ORF of *met*H (methionine synthase) is located upstream of *cti* in the opposite orientation. Downstream to *cti* is an incomplete ORF coding for a yhgI (a hypothetical cytoplasmic protein) and was identified to be in the same orientation as *cti*. The stop codon of *cti* and the start codon of the yhgI protein were separated by 111 bp. The arrangement and orientation of genes at the *cti* locus is conserved in all *Pseudomonas* spp. (data not shown).

ClustalW analysis of the Cti from *P. syringae* (Lz4W) exhibited 90% identity with *P. psychrophila*, 77–78% with three strains of *P. putida* (KT2440, DOT-T1E and P8), and 66% with *P. aeruginosa* (PA01), but with the Cti from *Vibrio cholerae* the identity was observed to be only 37%. Further, it possessed the characteristic heme-

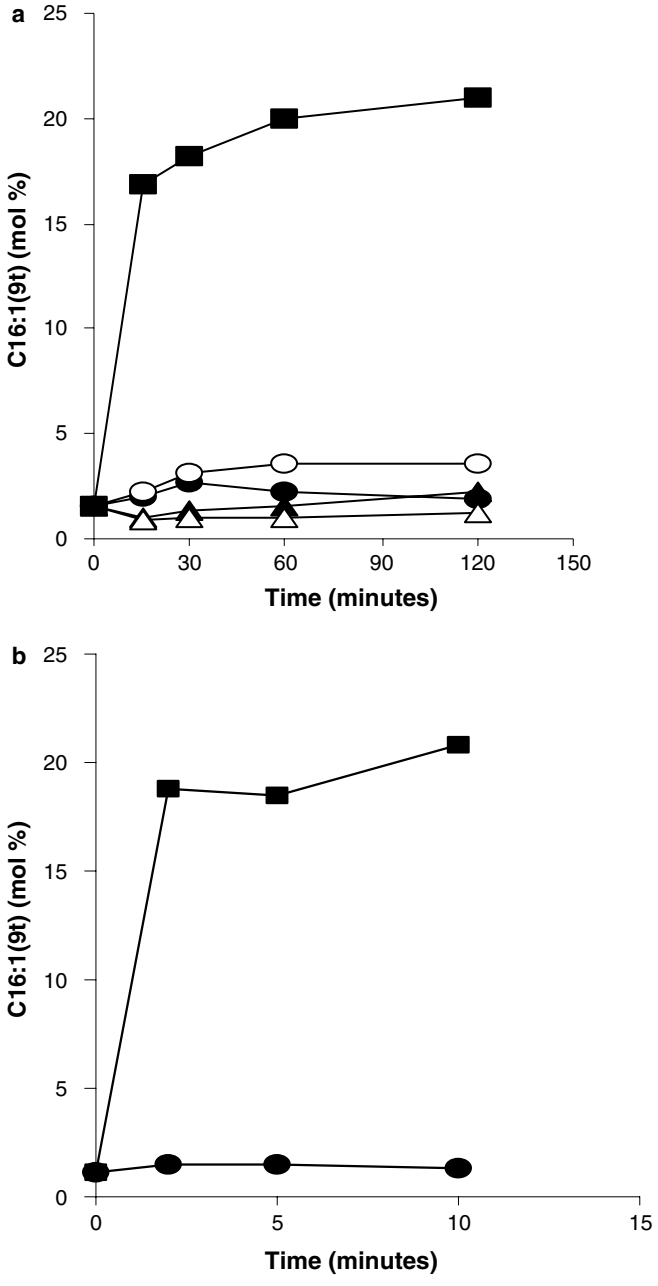

Fig. 1 **a** Time-dependent effect of toluene, NaCl and starvation on the mol% $C_{16:1(9t)}$ in *Pseudomonas syringae* (Lz4W) grown to stationary phase at 22°C. *P. syringae* (*filled circle*), *P. syringae* with 0.75 M NaCl (*open triangle*), *P. syringae* with 2 M NaCl (*filled triangle*), *P. syringae* after starvation (*open circle*), *P. syringae* with 2% toluene (*filled square*). **b** Time course of induction of $C_{16:1(9t)}$ in *P. syringae* (Lz4W) in the absence (*filled circle*) and presence of 2% toluene (*filled square*)

binding site CVACHA, a conserved proline, after the membrane signal sequence and conserved sequences such as PGSTEAL, SRTPSG, DMNRYENL, RWLYEHLFL, GPVCRGQ, and FDSASV (whose functional significance is yet to be elucidated). In addition, ClustalW analysis shows that the first stretch of 22 amino acids, which represents the putative signal sequence (data not shown), is conserved in *P. syringae* (Lz4W), *P. psychrophila*, and *P. putida* strains.

Generation of a *cti* null mutant of *P. syringae* (Lz4W-30b) and the complemented strain of the *cti* null mutant of *P. syringae* (Lz4W-C30b)

The truncated *cti* gene of 1.8 kb was mutagenized in vitro, and transformants harboring an insertion in the *cti* gene were selected based on restriction digestion and sequencing of the mutagenized insert. By this approach, it was possible to identify one transformant of *P. syringae* (Lz4W-30b) that had an insertion in the *cti* gene between 1,059 and 1,060 bp (Fig 2a). Southern analysis of the genomic DNA of *P. syringae* (Lz4W-30b) and wild-type *P. syringae* (Lz4W), using the 1.8-kb *cti* gene as a probe, picked up a DNA fragment of 3.5 kb in the wild type and two fragments of 2.3 and 2.7 kb in the *cti* null mutant, indicating that in the mutant, the *cti* gene is disrupted [Fig. 2b(A)]. Southern hybridization with $pBSKS^-$ did not pick up any signal in the null mutant of *P. syringae* (Lz4W-30b), implying that the null mutant is a result of a double crossover event [Fig. 2b(B)]. This null mutant of *P. syringae* (Lz4W-30b) could be successfully complemented with the intact *cti* gene cloned at *Eco*R site of the cosmid pUFR034 as evidenced by the fact that the complemented strain was now resistant to tetracycline and kanamycin and also synthesized $C_{16:1(9t)}$.

Fatty acid composition of *P. syringae cti* null mutant (Lz4W-30b) and the null mutant-complemented strain of *P. syringae* (Lz4W-C30b)

That the mutant of *P. syringae* (Lz4W-30b) is indeed a *cti* null mutant was further confirmed by fatty acid analysis data that indicated that the predominant fatty acids present in the null mutant were similar to that observed in the wild-type *P. syringae* (Lz4W) (Table 2), except that $C_{16:1(9t)}$ was not detected even when the mutant was grown at 5, 22, or 28°C. In the null mutant, the levels of $C_{16:1(9c)}$ and $C_{18:1(11c)}$ were high in cells grown at 5°C and $C_{16:0}$ in cells grown at 28°C. It appears that the increased level of $C_{16:0}$ at 28°C in the mutant compensates for the absence of $C_{16:1(9t)}$ in the mutant (Table 2).

The complemented *P. syringae* null mutant (Lz4W-C30b) synthesized $C_{16:1(9t)}$, and the fatty acid profile was similar to the wild type (Table 2). Further, like the wild-type cells, the complemented strain of *P. syringae* (Lz4W-C30b) showed increased levels of $C_{16:1(9c)}$ and $C_{18:1(11c)}$ at 5°C and $C_{16:0}$ at 28°C. Further, $C_{16:1(9t)}$ level in the complemented strain was higher in cells grown at 28°C compared to cells grown at 5°C (Table 2). The proportions of short chain fatty acids ($C_{12:0}$, $C_{14:0}$), $C_{16:1(9c)}$, and $C_{17:0(cyclo)}$ remained constant irrespective of their growth temperature.

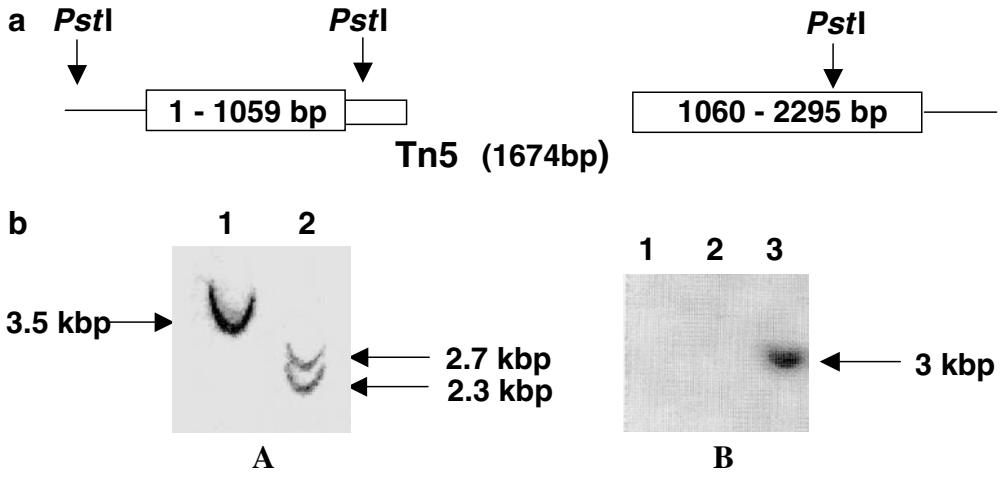

Fig. 2a, b Construction of a *cti* null mutant of psychrophilic *P. syringae* (Lz4W). **a** Map of the manipulated *cti* gene that was in vitro mutagenized by Tn5 mutagenesis. The sites of Tn5 transposition are indicated. **b** Southern hybridization of the wild-type *P. syringae* (*lane 1*), the mutant of *P. syringae* (Lz4W-30b) (*lane 2*), and pBSKS$^-$ (*lane 3*) with the *cti* gene (A) and pBSKS$^-$ (B)

Growth characteristics of *P. syringae* (Lz4W), *P. syringae cti* null mutant (Lz4W-30b), and the null mutant-complemented strain of *P. syringae* (Lz4W-C30b)

Assuming that the *trans*-fatty acid is required for growth at higher temperatures, the three *P. syringae* strains were analyzed for their ability to grow at three different temperatures. The assumption was that the *cti* null mutant would be sensitive to growth at higher temperatures, unlike the wild type and the complemented strain that synthesizes the *trans*-fatty acid) and therefore would grow better at higher temperatures. *P. syringae* (Lz4W) and the *cti* null mutant of P. *syringae* (Lz4W-30b) exhibited similar growth characteristics at 5 and 22°C; whereas at 28°C the growth of the mutant was retarded and arrested by 10 h, and the maximum cell density was significantly decreased (Fig. 3a–c). However, the null mutant-complemented strain of *P. syringae* (Lz4W-C30b), when grown at 5 and 22°C, exhibited a slight decrease in the growth, which could be ascribed to the presence of pUFRO34, since even wild-type cells in the presence of pUFR034 showed similar decrease in growth. At 28°C the complemented strain exhibited retarded growth compared to the wild type, but recovered with time and attained the control cell density (Fig. 3c). The retarded growth of the complemented strain is also attributed to the presence of pUFRO34. But, the fact that the growth of the complemented strain recovered to control characteristics at all the three temperatures and especially at 28°C in contrast to the mutant that still exhibited arrest in growth, is an indication that the *cti* gene is important for growth at higher temperature.

Growth temperature and membrane fluidity

Pyrene monomers diffuse in the membrane to form excimers, and the ease with which the excimers are formed reflects the fluidity of the hydrophobic phase, which can be assessed from the ratio of the pyrene excimer to monomer ratio (470/372 nm) (Fig. 4). The results indicated that, as anticipated, both in the wild-type and the mutant cells, the fluidity of the membrane decreased significantly with increase in growth temperature (Fig. 4; Table 3). Cells growing at 5°C exhibited maximum fluidity compared to cells growing at 22 and 28°C, which exhibited significantly decreased fluidity. Further, it is interesting to note that the fluidity of the membranes of mutant cells and the wild-type cells both grown at 5°C was similar, indicating that the absence of $C_{16:1(9t)}$ did not influence the fluidity of the membrane since at this temperature of growth, even in the wild type the $C_{16:1(9t)}$ was only 0.4 mol%. However, in cells grown at 22 and 28°C, the membrane fluidity was higher in the mutant compared to the wild-type cells, implying that the absence of *trans*-fatty acids in the mutant at these temperatures makes the membrane more fluid.

Discussion

The present study is directed towards understanding the role of fatty acids in thermal adaptation of an Antarctic psychrophilic bacterium *Pseudomonas syringae* (Lz4W). The fatty acid analysis of *P. syringae* indicated that palmitic acid ($C_{16:0}$), palmitoleic acid [$C_{16:1(9c)}$], and *cis*-vaccenic acid [$C_{18:1(11c)}$] are the predominant fatty acids. The presence of *cis*-vaccenic acid [$C_{18:1(11c)}$], which accounts for 18–25 mol%, is indicative of an anaerobic pathway of fatty acid biosynthesis in *P. syringae* (Lz4W), during which C2 elongation of palmitoleic acid [$C_{16:1(9c)}$] leads to *cis*-vaccenic acid synthesis (Keweloh and Heipieper 1996; Russel 1990). Further, the observed increase in the level of saturated fatty acid $C_{16:0}$ (palmitic acid) and the concurrent decrease in the level of unsaturated fatty acids [$C_{16:1(9c)}$] and [$C_{18:1(11c)}$] (palmitoleic and *cis*-vaccenic acid) with increase in growth temperature confirms the involvement of β-keto-acyl-ACP synthase II (*fab*F) in the thermal regulation of fatty acid composition in *P. syringae* as observed in *Escherichia coli* (de Mendoza et al. 1983; Suutari and Laasko 1994). These results are also in agreement with earlier

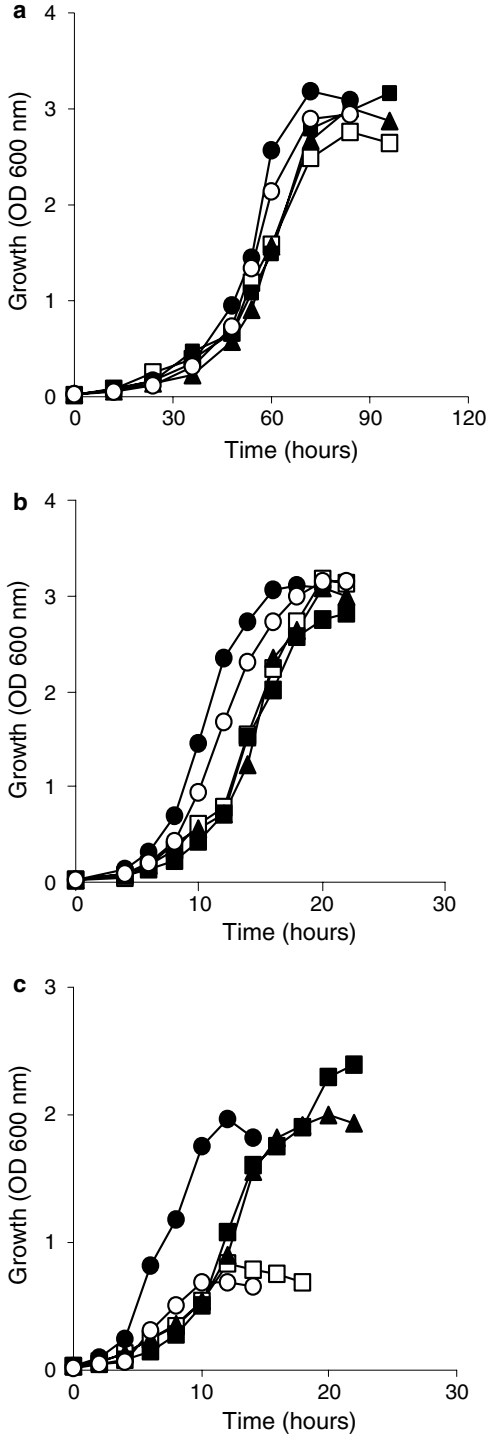

Fig. 3a–c Growth curve of psychrophilic *P. syringae* (Lz4W) (*filled circle*), the *cti* null mutant of *P. syringae* (Lz4W-30b) (*open circle*), the *cti* null mutant-complemented strain of *P. syringae* (Lz4W-C30b) (*filled square*), the cti null mutant of *P. syringae* (Lz4W-30b) containing pUFRO34 (*open square*), and the *P. syringae* (Lz4W) containing pUFR034 (Lz4W) (*filled triangle*), cultured at 5 (**a**), 22 (**b**) and 28°C (**c**)

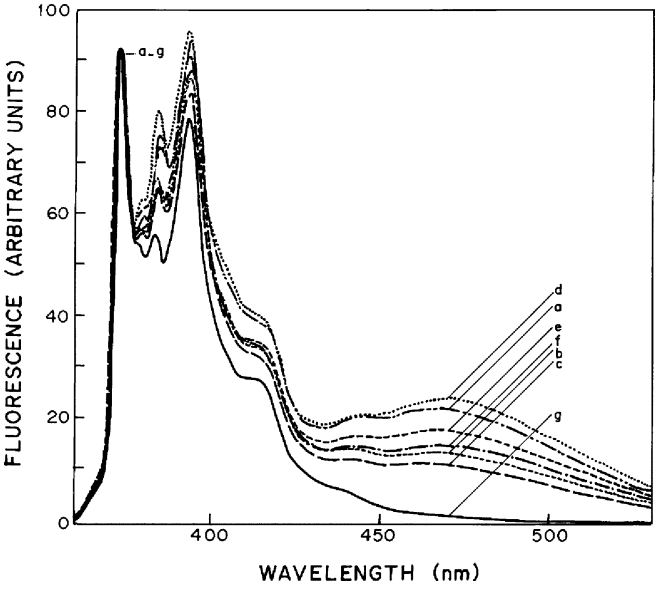

Fig. 4a–g Fluorescence emission spectra of pyrene (5 μM) incorporated into intact cells of *P. syringae* (Lz4W) and *cti* null mutant of *P. syringae* (Lz4W-30 b) grown at 5, 22, and 28°C and recorded at 22°C. **a–c** *P. syringae* (Lz4W) grown at 5, 22, and 28°C, respectively. **d–f** *cti* null mutant of *P. syringae* (Lz4W-30b) grown at 5, 22, and 28°C, respectively. **g** Free pyrene (5 μM)

Table 3 Membrane fluidity of the intact cells of *P. syringae* (Lz4W) and the *cti* null mutant of *P. syringae* (Lz4W-30b) monitored, using the excimer/monomer ratio of the fluorescent probe pyrene

Strain	Pyrene excimer (470 nm)/monomer (372 nm)		
	5°C	22°C	28°C
P. syringae (Lz4W)	0.23 ± 0.01^{a}	0.14 ± 0.01^{b}*	0.12 ± 0.02^{c}*
P. syringae (Lz4W-30b)	0.24 ± 0.02^{a}	0.18 ± 0.01^{b}*	0.15 ± 0.01^{c}*

[a,b,c]The values are significantly different ($P \leq 0.05$) when cells grown at 5, 22 and 28°C were compared
*Significance value: *$P \leq 0.05$ when the fluidity of wild-type and mutant cells grown at the same temperature were compared

studies, which demonstrated that one of the modes by which Gram-negative bacteria respond to decrease in temperature is to increase the proportion of *cis*-vaccenic acid [$C_{18:1(11c)}$] and concomitantly decrease the proportion of palmitic acid ($C_{16:0}$) (Suutari and Laasko 1994). The fatty acid data of psychrophilic *P. syringae* (Lz4W) also indicated an increase in the mol% of the *trans*-fatty acid $C_{16:1(9t)}$, with increase in growth temperature from 0.4 mol% at 5°C to 5 mol% at 28°C. These results confirm earlier studies, which indicated that in both mesophilic and psychrophilic bacteria high levels of *trans*-fatty acids, correlated with the high growth rate of the bacterium at elevated temperatures (Cronan 2002; Keweloh and Heipieper 1996; Morita et al. 1993; Okuyama et al. 1990, 1991, 1998; Yumoto et al. 1998). In the present study, increase in the levels of $C_{16:1(9t)}$ was also observed in *P. syringae* (Lz4W) exposed to 2% toluene, thus confirming earlier observations in *P. putida* (Junker and Ramos 1999; Pedrotta and Witholt 1999;

Ramos et al. 1997). High salt has also been demonstrated to increase the level of *trans*-fatty acid, but in *P. syringae* (Lz4W) no significant increase was observed.

As of now, the role of *cis–trans* isomerization with respect to adaptation to changes in ambient temperature is yet not clearly understood. One approach to understanding the role would be to generate *cti* null mutants for which it would be essential to clone the gene, sequence the gene, and then in vitro mutagenize same so that it could be used for homologous recombination. With this in mind, the *cti* gene of *P. syringae* (Lz4W) was cloned and sequenced. The gene exhibited 66–90% sequence identity with the *cti* gene of various species of *Pseudomonas* (Heipieper et al. 2003; von Wallbrunn et al. 2003), and the gene retained several other conserved features (Holtwick et al. 1997; see also Results). The amino acid sequence analysis of Cti of *P. syringae* (Lz4W) indicated that the protein has an N-terminal signal sequence of 22 amino acids similar to that of other Cti from *P. putida* strain DOT-T1E, *P. psychrophila*, *P. aeruginosa* strain PA01, and *P. oleovorans*, in which the Cti protein was identified as a periplasmic protein (Junker and Ramos 1999; Okuyama et al. 1998; Pedrotta and Witholt 1999). Recent reports clearly indicate that Cti is a periplasmic protein and has a *sec*-dependent signal sequence for transport to periplasm (Heipieper et al. 2003).

Having established the sequence and the identity of the *cti* gene, it was then in vitro mutagenized and used to generate a *cti* null mutant of *P. syringae* (Lz4W-30b), which was deficient in the *trans*-fatty acid. The growth of the *cti* null mutant was not different from that of the wild type at 5 and 22°C, but the growth was arrested at 28°C, indicating that *trans*-fatty acid is crucial for growth at higher temperature (28°C) but not at 5 and 22°C. This was further confirmed using the *cti* null mutant-complemented strain of *P. syringae* (Lz4W-C30b), which exhibited a slower growth at 28°C; but unlike the mutant strain, it eventually reached the stationary phase with a cell density comparable to the wild-type cells, clearly indicating that high levels of *trans*-fatty acid are required for the sustenance of growth at higher temperatures.

The present *cti* null mutant of *P. syringae* (Lz4W-30b) represents the first report on a psychrophile and emphasizes that bacteria, irrespective of whether their psychrophiles (present study) or mesophiles, require *trans*-fatty acids for growth at higher temperatures. For instance, in mesophilic *P. putida*, mutants defective in *cis–trans* isomerization grew poorly at 37 or 38.5°C, respectively; but growth was not affected at 30°C. In addition, complementation of the *cti* gene in the mutants restored its ability to grow like the wild type (Holtwick et al. 1997; Junker and Ramos 1999). Junker and Ramos (1999) also demonstrated that the *cti* null mutant of *P. putida* not only exhibited poor growth at elevated temperature, but was also more sensitive to toluene. These results imply that the primary effect of *cti* is to decrease membrane fluidity, so as to decrease permeability to organic solvents, which is a secondary consequence (Cronan 2002). Therefore, there is a need to correlate *cis–trans* isomerization with thermal growth characteristics and membrane fluidity so as to understand the role of *trans*-fatty acids with respect to growth and survival of microorganisms. In fact, it was earlier proposed that conversion of *cis*- to *trans*-fatty acid is probably an immediate control mechanism to modulate the fluidity of the membrane that has been subjected to abrupt changes in temperature (Okuyama et al. 1998).

Membrane-fluidity studies indicated that the fluidity of mutant cells was significantly higher than that of wild-type cells, implying that the absence of *trans*-fatty acids in the mutant increases the membrane fluidity. It is known that *trans*-fatty acids decrease the fluidity of membranes (Cronan and Gelman 1975; Keweloh and Heipieper 1996; Okuyama et al. 1991; Weber et al. 1994). Therefore, the increase in membrane fluidity in the mutant is obviously due to its inability to synthesize the *trans*-fatty acid because of inactivation of the *cti* gene. Such an increase in membrane fluidity—apart from destabilizing the membrane as a physical barrier—could also alter certain crucial membrane functions, such as ion permeability and energy transducing properties (van de Vossenberg et al. 1995, 1999), and thus affect growth of the cells.

Taken together, these results indicate a role for *trans*-fatty acids in adaptation of bacteria to temperatures close to the maximum growth temperature. To the best of our knowledge, this is the first single comprehensive study on a *trans*-monounsaturated fatty acid in a psychrophilic bacterium and includes identification of the *trans*-fatty acid, its quantification during growth at different temperatures, cloning and sequencing of the *cti* gene, its identity with other *cti* genes, characteristics of the enzyme, and the generation of the *cti* null mutant and the complemented strain for functional studies. Most importantly, it also provides evidence, using biophysical studies, that *trans*-fatty acid synthesis is crucial to the survival of the microbe at higher temperatures due to its ability to decrease the fluidity of the membrane and stabilize the membrane.

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