

## *cis-trans* Isomerization of Unsaturated Fatty Acids: Cloning and Sequencing of the *cti* Gene from *Pseudomonas putida* P8

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**Transposon mutants of *Pseudomonas putida* P8 were generated by applying a mini-Tn5 mutagenesis system. The mutants obtained were checked for their ability to tolerate increased temperatures and elevated phenol concentrations. Approximately 5,800 transposon mutants were used to generate a pool of 600 temperature-sensitive strains; one of these strains was identified as being damaged in its ability to perform *cis-trans* isomerization of fatty acids. A gene library of *P. putida* P8 was constructed and screened by using as a probe sequences immediately adjacent to the mini-Tn5 insertion. A DNA fragment that complemented the mutation was isolated and cloned. The corresponding gene, termed *cti*, is located close to the methionine synthase locus (*metH*) in *P. putida* P8. A *cti*-carrying fragment integrated into a plasmid also conferred the ability for *cis-trans* isomerization to *Escherichia coli*; the *cti* gene was completely sequenced, and the amino acid sequence was deduced.**

Monounsaturated fatty acids with the *trans* configuration of the double bond have only recently been detected in membrane lipids of various aerobic bacteria. These unusual lipid constituents were first described for methane utilizers (17). Also, *trans* fatty acids have been reported to exist in several marine microorganisms, such as *Vibrio* species, and in different *Pseudomonas* strains which are known degraders of phenolic compounds (11, 13–15, 23, 33).

From investigations on *Pseudomonas putida* and *Vibrio* species it became evident that *trans* fatty acids are synthesized by direct isomerization of *cis* to *trans* unsaturated fatty acids without a shift in the position of the double bond (8, 19). The conversion of *cis* to *trans* fatty acids is independent of de novo fatty acid and lipid biosynthesis. Since isomerization takes place in isolated membrane vesicles (8), the acyl chains of the phospholipids must be the substrates for the reaction. The conversion of *cis* unsaturated fatty acids to *trans* unsaturated fatty acids is accompanied by a change in membrane fluidity. Thus, it has been postulated that bacteria growing in the presence of toxic substrates, like phenols, adapt to these membrane-active substances by modification of the double-bond configuration (9). Change in membrane fluidity is considered to be a general mechanism for protecting bacterial cells from being damaged by various environmental stress factors (e.g., by exposure to elevated temperatures and harmful compounds, such as phenol or ethanol) (12, 16).

*cis-trans* isomerization of fatty acid constituents of phospholipids is brought about by a hitherto unknown enzymatic system which has not been genetically characterized. In this study, we describe the isolation of a transposon mutant of the phenol-degrading bacterium *P. putida* P8 which is defective in the conversion of *cis* unsaturated fatty acids to *trans* unsaturated fatty acids. Moreover, the structural gene for the *cis/trans* isomerase (*cti*) of fatty acids was cloned, analyzed, sequenced, and expressed in *Escherichia coli* and *P. putida*.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *P. putida* P8 served as the source of DNA for construction of a gene bank and in transposon mutagenesis experiments. *E. coli* strains were grown at 37°C in complete medium (StI; Merck, Darmstadt, Germany). *P. putida* was grown in the minimal medium described by Löffeld and Keweloh (16) supplemented with 0.2% succinate as the carbon source at 30°C on a rotary shaker (model G76; New Brunswick Scientific). When necessary, *E. coli* was also grown under the same conditions. Solid media (1.5% agar added) and liquid media were supplemented, when required, with 50 µg of ampicillin per ml, 25 to 50 µg of kanamycin per ml, or 20 µg of tetracycline per ml.

**Tn5 transposon mutagenesis.** Plasmid pUT/Km (kindly provided by K. Timmis, Braunschweig, Germany) was transferred from donor strain *E. coli* SM10 (Apir) into *P. putida* P8 by mobilization with a filter mating technique (for details of the system used see reference 6). Filters with a mixture of donor and recipient strains at a 1:2 ratio were incubated for 12 to 15 h at 30°C on complete-medium plates. Cells were subsequently suspended in 10 mM MgSO<sub>4</sub>, and appropriate dilutions were plated on minimal medium containing kanamycin.

**Scoring of *cti*-carrying clones.** Colonies to be checked for production of *trans* fatty acids were transferred with toothpicks onto minimal medium. The resulting master plates were replica plated onto minimal medium, and the preparations were incubated at 30, 37, and 40°C. They were also replica plated onto minimal medium containing different phenol concentrations (0.5, 0.8, and 1 g/liter). Since the medium also contained succinate as a carbon source, it was possible to screen for cells which no longer tolerated phenol rather than those that had simply lost the ability to degrade it. All clones which did not grow or grew poorly both at temperatures above 37°C and on phenol concentrations of ≥0.8 g/liter were tested for their ability to isomerize *cis* to *trans* unsaturated fatty acids.

**Determination of fatty acids.** Cells were grown overnight in 7 ml of liquid minimal medium, ethanol was added to a final concentration of 10%, and the preparation was incubated for 2 h at 30°C. Cells were subsequently harvested by centrifugation (10 min, 7,000 × g). The supernatant was decanted, and 10 µl of the remainder was analyzed for cellular fatty acids by gas-chromatography by using trimethylsulfonium hydroxide as the methylating agent (20). The conditions used for gas chromatography have been described previously (9).

**Transformation techniques.** Plasmids were introduced into *E. coli* by transformation of CaCl<sub>2</sub>-treated cells with purified plasmid DNA (29).

*P. putida* was transformed by electroporation as described by Taghavi et al. (31), with the following modifications. *P. putida* strains were grown at 30°C in complete medium to an optical density at 600 nm of 0.8. Cells were collected by centrifugation (10 min, 7,000 × g) and washed twice with 1 volume of ice-cold buffer (10% [vol/vol] glycerol, 90% [vol/vol] H<sub>2</sub>O). Subsequently, the cells were concentrated 100-fold. To 40 µl of the suspension, 4 µl of plasmid DNA (total weight, 1 µg) was added, and the preparation was transferred into a 2-mm electroporation cuvette. An Elektroporator 2510 instrument (Eppendorf, Hamburg, Germany) was used, and the best results were obtained with the following settings: voltage, 2,400 V; capacitance, 10 µF; and resistance, 600 Ω. Following electroporation, cells were incubated in 1 ml of complete medium for 1 h at 30°C before they were plated onto selective medium.

**Isolation and manipulation of DNA.** *P. putida* chromosomal DNA was prepared by the protocol of Rodriguez and Tait (26). Lambda DNA was prepared

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TABLE 1. Bacteria and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype or characteristics	Reference
<b>Strains</b>		
<i>E. coli</i> JM107	<i>E. coli</i> K-12 <i>supE</i> $\Delta(lac-proAB)$ <i>hskR17</i> F' <i>tra</i> $\Delta$ 36 <i>proAB</i> <sup>+</sup> <i>lacIR</i> <i>lacZ</i> $\Delta$ M15	34
<i>E. coli</i> K803	F <sup>-</sup> <i>hsdR514</i> ( $r_K^+$ $m_K^+$ ) <i>supF58</i> <i>lacYI galT22 metB2 trpR55</i>	10
<i>E. coli</i> SM10( $\lambda$ pir)	<i>E. coli</i> K-12 Km <sup>r</sup> <i>thr leu tonA lacY</i> <i>supE recA::RP4-2-Tc::Mu</i> $\lambda$ pir	18
<i>P. putida</i> P8	Wild type	2
<i>P. putida</i> A9	Km <sup>r</sup> , <i>cti::Tn5</i>	This study
<b>Plasmids</b>		
pBHR26	Tc <sup>r</sup> , <i>oprH::59</i> , broad-host-range vector derived from pUC18	25
pBHR26cti	Tc <sup>r</sup> , <i>cti</i>	This study
pUCBM20	Ap <sup>r</sup>	4
pUCISA9-1	Ap <sup>r</sup> , carrying 1-kb <i>MluI-XhoI</i> frag- ment of <i>cti</i> with inserted Tn5-Km	This study
pUT mini-Tn5 Km	Ap <sup>r</sup> Km <sup>r</sup> , delivery plasmid for mini-Tn5 Km	7

essentially as described by Sambrook et al. (29). Plasmid DNA was isolated as described by Birnboim and Doly (3).

For construction of the DNA library, *P. putida* P8 chromosomal DNA was isolated and partially digested with *Sau3A*, and approximately 200  $\mu$ g of DNA was loaded onto a continuous sucrose gradient (10 to 30%) to fractionate the DNA by size. Fragments of 10 to 22 kb were collected, dialyzed, precipitated with ethanol, dissolved in TE buffer, and ligated to *Bam*HI-restricted  $\lambda$ EMBL4. Ligated DNA was packaged in vitro as described by Rosenberg et al. (28).

Phages were plated on *E. coli* K803, and recombinant Cti<sup>+</sup> phages were detected by plaque hybridization by using the cloned fragment as a probe.

Restriction endonuclease digestion, ligation, sucrose density gradient centrifugation, agarose electrophoresis, hybridization, and additional recombinant DNA techniques were carried out as described by Sambrook et al. (29), unless indicated otherwise.

**Sequencing.** Small fragments (1 to 1.5 kb) of the *cti* gene locus were subcloned in pUCBM20 and sequenced with IRD41-labelled universal and reverse primer by using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Buchler, Braunschweig, Germany) and an automatic LI-COR sequencer (LI-COR Inc., Lincoln, Nebr.).

**Nucleotide sequence accession number.** The nucleotide sequence of the *cti* gene has been deposited in the EMBL Data Library under accession no. AJ000978.

## RESULTS

**Transposon mutagenesis.** Mutants of *P. putida* P8 defective in *cis-trans* isomerization of fatty acids were generated by applying a mini-Tn5 transposon mutagenesis system as described in Materials and Methods. Of the ~5,800 clones tested, 600 did not grow or grew only poorly at 37°C; however, growth at 30°C was not affected. The temperature-sensitive clones were subsequently checked for their ability to tolerate elevated phenol concentrations ( $\geq 0.8$  mg/liter). Several mutants were identified in which growth was affected by phenol. Determinations of the fatty acid contents after exposure to ethanol (10%) confirmed that mutant A9 was damaged in its ability to perform *cis-trans* isomerization, because no *trans* fatty acids could be detected in membranes of this mutant strain (Table 2).

**Cloning and sequencing of the *cti* gene.** By making use of the Km<sup>r</sup> gene of Tn5 as a selectable trait, we cloned an *MluI-XhoI* fragment in *E. coli* by using pUCBM20 as the vector. The resulting hybrid plasmid was designated pUCISA9-1. Preliminary sequence analysis of the cloned fragment (data not shown) revealed an open reading frame that had no apparent similarity to any previously described open reading frame present in the GenBank database. Since the fragment did not contain the entire open reading frame, we used it as a probe to screen the EMBL4-based gene bank of *P. putida* P8. From a positive phage clone restriction fragments were subcloned, and their abilities to complement the mutation of strain A9 were checked. Based on the results of these experiments, a 4.5-kb *Bgl*II fragment was identified and sequenced completely. As

TABLE 2. Effect of ethanol on *cis-trans* isomerization of unsaturated fatty acids in different strains<sup>a</sup>

Strain	Ethanol addition	Fatty acid content (%) <sup>b</sup>						% Conversion of <i>trans</i> unsaturated fatty acids <sup>c</sup>	
		16:0	16:1 <i>cis</i>	16:1 <i>trans</i>	18:0	18:1 <i>cis</i>	18:1 <i>trans</i>	16:1	18:1
<i>P. putida</i> P8	-	33.6 $\pm$ 1.6	35.0 $\pm$ 3.3	6.5 $\pm$ 1.3	2.1 $\pm$ 0.3	20.9 $\pm$ 1.0	1.6 $\pm$ 0.8	15.7	7.1
	+	33.4 $\pm$ 1.2	24.6 $\pm$ 5.8	17.3 $\pm$ 5.2	2.4 $\pm$ 1.0	18.7 $\pm$ 2.2	3.3 $\pm$ 2.3	41.3	15.0
<i>P. putida</i> P8(pBHR26cti)	-	34.4 $\pm$ 0.9	25.9 $\pm$ 5.2	14.7 $\pm$ 4.9	5.3 $\pm$ 1.8	16.9 $\pm$ 2.4	2.7 $\pm$ 1.2	36.2	13.8
	+	34.6 $\pm$ 0.8	18.0 $\pm$ 2.6	24.5 $\pm$ 2.1	3.0 $\pm$ 0.8	15.7 $\pm$ 0.8	4.2 $\pm$ 0.8	57.6	21.1
<i>P. putida</i> A9	-	32.3 $\pm$ 0.8	44.7 $\pm$ 1.0	0	1.7 $\pm$ 0.2	20.2 $\pm$ 0.6	0		
	+	32.5 $\pm$ 1.5	43.6 $\pm$ 1.4	0	2.0 $\pm$ 0.7	21.5 $\pm$ 0.8	0		
<i>P. putida</i> A9(pBHR26cti)	-	35.1 $\pm$ 4.6	28.6 $\pm$ 4.8	11.1 $\pm$ 2.6	4.8 $\pm$ 3.8	18.3 $\pm$ 2.0	2.1 $\pm$ 1.0	27.9	10.3
	+	34.2 $\pm$ 3.7	20.2 $\pm$ 2.4	21.2 $\pm$ 5.6	2.9 $\pm$ 1.4	17.7 $\pm$ 1.6	4.0 $\pm$ 1.8	51.4	18.4
<i>E. coli</i> JM107	-	41.9 $\pm$ 1.3	27.9 $\pm$ 0.9	0	1.7 $\pm$ 0.8	28.4 $\pm$ 1.1	0		
	+	43.5 $\pm$ 2.2	26.9 $\pm$ 1.6	0	2.1 $\pm$ 0.7	27.3 $\pm$ 2.3	0		
<i>E. coli</i> JM107(pBHR26cti)	-	42.4 $\pm$ 0.7	28.8 $\pm$ 1.5	1.1 $\pm$ 1.4	2.2 $\pm$ 1.0	25.2 $\pm$ 1.6	0.2 $\pm$ 0.4	3.7	0.8
	+	42.5 $\pm$ 1.5	20.0 $\pm$ 3.2	7.4 $\pm$ 2.0	2.9 $\pm$ 2.0	24.3 $\pm$ 1.1	2.7 $\pm$ 0.6	27.0	10.0

<sup>a</sup> Strains were grown in minimal medium to an optical density at 600 nm of 0.3 at 30°C before ethanol was added to a final concentration of 10%.

<sup>b</sup> Fatty acids were assayed as described in Materials and Methods; values for only relevant fatty acids are given. The values are the means from at least quadruplicate experiments; standard deviations are also indicated.

<sup>c</sup> Percentages of conversion were calculated with the following formula: [(percentage, by weight, of *trans* unsaturated fatty acids)/(percentage, by weight, of *trans* unsaturated fatty acids plus *cis* unsaturated fatty acids)]  $\times$  100.

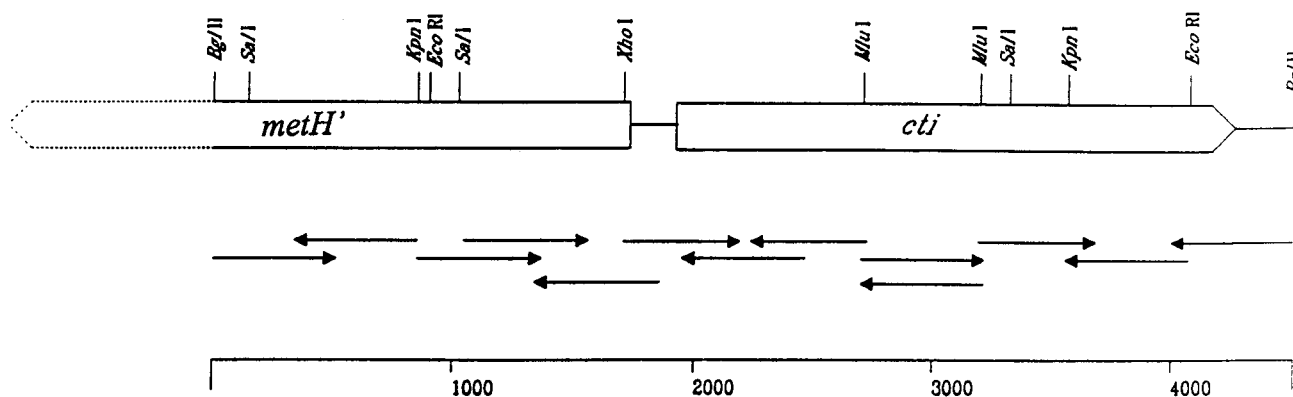


FIG. 1. Schematic representation of the 4.5-kb *Bgl*II DNA fragment from *P. putida* P8 containing the *cis/trans* isomerase gene (*cti*) and truncated open reading frame which putatively encodes a methionine synthase, *metH'*. The arrows indicate the sequencing strategy.

Fig. 1, shows, two open reading frames are located on this fragment, and one of them shows striking similarities to genes encoding methionine synthases (*metH'* in Fig. 1) (1, 32). The other open reading frame consists of 2,298 bp (Fig. 2), and the deduced polypeptide has 766 amino acid residues, corresponding to a molecular mass of 86.9 kDa; the protein is thought to encode the putative *cis/trans* isomerase, designated *cti* in Fig. 1. The first 22 amino acids (Fig. 2, boldface type) show striking similarities to signal sequences, as judged from computer alignments. We identified putative promoter sequences for both of the genes and a putative binding site of a regulator protein for the *metH* gene; the positions are indicated in the noncoding region between the two open reading frames in Fig. 2.

***cis-trans* isomerization of fatty acids in plasmid-carrying strains.** Since *E. coli* itself is not capable of isomerization of fatty acids, we studied whether strains carrying the putative *cti* gene acquired the ability for *cis-trans* isomerization. We also investigated whether the mutation in *P. putida* A9 is complemented by a fragment carrying only this gene. To do this, the *Xho*I-*Bgl*II fragment harboring the gene (Fig. 1 and 2) was cloned into broad-host-range plasmid pBHR26 (25), which resulted in hybrid plasmid pBHR26*cti*. *P. putida* P8 wild type, mutant A9, and *E. coli* JM107 were transformed, and their fatty acid contents were determined. Table 2 shows the data obtained. In contrast to the *E. coli* JM107 wild type, which never formed *trans* fatty acids, the bacterium carrying plasmid pBHR26*cti* was capable of *cis-trans* isomerization. *P. putida* P8 (wild type) with additional copies of the *cti* gene showed an increase in the basic level of *trans* fatty acids without activation by ethanol. The mutation in strain *P. putida* A9 was complemented by the plasmid containing the intact *cti* gene. The level of *trans* fatty acids in transformed mutant A9 was close to that in *P. putida* P8(pBHR26*cti*).

## DISCUSSION

Our determination of the nucleotide sequence of the *cti* gene revealed that the gene product may be a novel enzyme. No significant homologous regions were found when we compared the deduced amino acid sequence encoded by *cti* with

proteins for which sequences have been deposited in databases. The N terminus consists of a hydrophobic region which is similar to signal sequences; however, the proline immediately adjacent to this signal sequence prevents cleavage (24), and that is why we assume that the *cti* gene product is membrane associated. This localization is a prerequisite for accessibility of the enzyme to its substrates, the phospholipids that reside in the membrane. This is in accordance with results obtained previously by Diefenbach and Keweloh (8), who found that isolated vesicles of the cytoplasmic membrane retained a capacity for isomerization of unsaturated fatty acids. It has been suggested that the isomerase is bound to the outer surface of the inner membrane of the bacteria. Additional support that the enzyme is a peripheral membrane protein came from observations of Okuyama et al. (22). A membraneless cell preparation was still active in fatty acid isomerization, although changes in the substrate pattern occurred. When isolated membranes were added to an enzyme preparation, the ability to act on phospholipids was restored (22), thus providing evidence that the *cis/trans* isomerase is an enzyme bound to the inner membrane in intact bacterial cells. Since there is no extensive hydrophobic region except the N terminus, the isomerase may be anchored to the membrane rather than being an integral membrane protein.

Cloning of the *cti* gene in different hosts (i.e., in strains with and without the capacity of fatty acid isomerization) had a remarkable effect on *trans* fatty acid formation. After we cloned the gene in the wild-type strain from which it originated, *P. putida* P8, a higher basic level of *trans* fatty acids was detected in nonactivated cells than in the wild-type strain. Also, after activation with phenol (data not shown) or ethanol (Table 2) the cellular amount of *trans* fatty acids was significantly higher. The copy number of plasmid pBHR26 and its derivatives was approximately 5 to 10, as judged from plasmid preparations. Thus, it may be the increase in copy number which is responsible for the elevated levels of *trans* fatty acids in both treated and untreated cells. *E. coli*, which normally does not form *trans* fatty acids, was capable of *cis-trans* isomerization when the *cti* gene was introduced on a plasmid. It was necessary to grow *E. coli* at low temperatures (30°C) to observe

FIG. 2. Nucleotide sequence of the *Xho*I-*Bgl*II fragment derived from a recombinant phage in the gene library showing the coding region of the *P. putida* P8 *cis/trans* isomerase, and the deduced amino acid sequence. The putative signal sequence is indicated by boldface type. Putative -10 and -35 regions of *metH* and *cti* are underlined. +1 indicates the position of the *cti* transcription initiation site as determined by primer extension (data not shown). The asterisk indicates the site of Tn5 insertion in mutant A9. A possible *metH* regulatory (MetR) binding site is also shown. A region of dyad symmetry acting presumably as a terminator that could form a stem-loop structure downstream from the stop codon is also indicated. Putative ribosome binding sites are underlined and designated.



high levels of isomerization because this increased the overall level of unsaturated fatty acids. *cis-trans* isomerization after incubation with ethanol was not as effective in *E. coli* as in *P. putida*; this may have been due to the fact that ethanol is an end product in the metabolism of *E. coli* (i.e., cells may be adapted to this compound). With octanol, 50% conversion of 16:1 *cis* to 16:1 *trans* unsaturated fatty acids was obtained after 2 h (data not shown). Since *trans* fatty acids were also formed in *E. coli* carrying a *cti* gene, it became obvious that only one gene product (i.e., the *cti* isomerase) is sufficient for the process of fatty acid isomerization.

In the noncoding region between the divergently transcribed *metH* and *cti* genes putative promoter sequences of both open reading frames were identified. On the basis of a comparison of the 5' noncoding regions of corresponding genes (*metH*) from *E. coli* and *Salmonella typhimurium* a binding site of a regulatory protein (*metR* gene product in Fig. 2) was identified. This binding site has only one mismatch compared to the consensus sequence, TGAANNNTTCA (5, 32). The -35 and -10 sequences of *metH* are in accordance with promoter sequences of *P. putida* genes (27), as is their position with respect to the MetR binding site. Expression of *metH* must also be affected in the mutant, because the insertion site of Tn5 (Fig. 2, asterisk) affects the translational start site of *metH*. However, the mutant does not require methionine for growth on minimal medium (data not shown), and that is why there must be a functional equivalent locus (*metE*), as described for *E. coli* and *S. typhimurium* (5, 32).

Concerning the *cti* gene, preliminary primer extension experiments point to the transcriptional start point indicated in Fig. 2. A putative *Pseudomonas* constitutive *rpoD* promoter (27) is located immediately upstream of the mini-Tn5 insertion site (Fig. 2, asterisk). Thus, insertion of the transposon prevents expression of the *cti* gene, eventually resulting in the mutant phenotype. In previous studies it has been shown that isomerization of the double bond occurred in the presence of chloramphenicol, indicating that there is a constitutively operating enzymatic system (13). The position of mini-Tn5 in mutant A9 is consistent with these data.

Several *cis/trans* isomerases which shift the double bond position have been purified and characterized (21, 30). In contrast to these enzymes, the isomerase of *P. putida* and some other bacteria does not change the position of the double bond, and it is unique in the conversion of unsaturated fatty acids esterified to phospholipids (14). In this study, for the first time, the gene encoding this unidentified enzyme was isolated, sequenced, and cloned in *E. coli*, which concomitantly gained the capacity to isomerize fatty acids. Thus, the physiological role of this protection system against harmful environmental conditions can be studied in detail in an *E. coli* model system.

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