



# **The bacterial protective armor against stress: The cis-trans isomerase of unsaturated fatty acids, a cytochrome-c type enzyme**

Mickaël Mauger, Carla Ferreri, Myriam Seemann, Chrysostomos Chatgililoglu

## **► To cite this version:**

Mickaël Mauger, Carla Ferreri, Myriam Seemann, Chrysostomos Chatgililoglu. The bacterial protective armor against stress: The cis-trans isomerase of unsaturated fatty acids, a cytochrome-c type enzyme. *Journal of Inorganic Biochemistry*, 2021, 224, pp.111564. <10.1016/j.jinorgbio.2021.111564>. <hal-03445076>

**HAL Id: hal-03445076**

**<https://hal.science/hal-03445076v1>**

Submitted on 23 Nov 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

# The bacterial protective armor against stress: The *cis-trans* isomerase of unsaturated fatty acids, a cytochrome-c type enzyme.

Mickaël Mauger <sup>(a)</sup>, Carla Ferreri <sup>(b)</sup>, Chryssostomos Chatgililoglu <sup>(b)</sup>, and Myriam Seemann<sup>\*(a)</sup>

- 
- [a] M. Mauger, Dr. M. Seemann  
Equipe Chimie Biologique et Applications Thérapeutiques  
Institut de Chimie UMR 7177  
Université de Strasbourg/CNRS  
4, rue Blaise Pascal, 67070 Strasbourg (France)  
email : [mseemann@unistra.fr](mailto:mseemann@unistra.fr)
- [b] Dr. C. Ferreri, Dr. C. Chatgililoglu  
Consiglio Nazionale delle Ricerche - ISOF  
Via Piero Gobetti 101  
40129 Bologna (Italy)

## Abstract:

Bacteria have evolved several outstanding strategies to resist to compounds or factors that compromise their survival. The first line of defense of the cell against environmental stresses is the membrane with fatty acids as fundamental building blocks of phospholipids. In this review, we focus on a periplasmic heme enzyme that catalyzes the *cis-trans* isomerization of unsaturated fatty acids to trigger a decrease in the fluidity of the membrane in order to rapidly counteract the danger. We particularly detailed the occurrence of such *cis-trans* isomerase in Nature, the different stresses that are at the origin of the double bond isomerization, the first steps in the elucidation of the mechanism of this peculiar metalloenzyme and some aspects of its regulation.

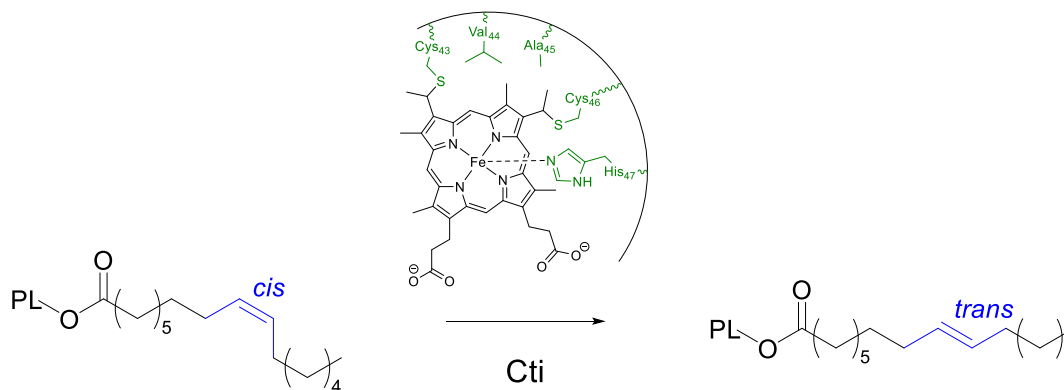
## 1. Introduction

The formation of *trans*-unsaturated fatty acids (UFA) in some bacteria including *Pseudomonas* strains plays an important role as adaptation response to the increase in temperature, the presence of organic solvents, heavy metals, osmotic stress, and the addition of antibiotics acting on the membrane properties. In these bacteria, the amount of *trans*-UFA raises by the simultaneous decrease of their *cis* form. This transformation contributes significantly to cell

survival by decreasing membrane fluidity when a rapid response to environmental stress is required. The isomerization reaction is catalyzed by a periplasmic *cis-trans* isomerase (Cti) that contains the CXXCH motif responsible of the covalent attachment of a heme nucleus (cytochrome-c type) to the protein (Figure 1). The formation of *trans*-UFA is independent from *de novo* fatty acid biosynthesis, protein synthesis, ATP, or any other cofactor and is a rapid adaptation mechanism to rigidify the membrane in response to severe environmental conditions where growth is inhibited. The key steps on the investigation of the *cis-trans* isomerization of fatty acids in bacteria are presented in this review.

## 2. Occurrence of *trans*-UFA in bacteria

Saturated fatty acids (SFA) and unsaturated fatty acids (UFA) are major components of the bacterial envelop. Until the 1980s, the *cis* configuration of the double bond was considered as the only naturally present in the UFA of bacteria, in analogy with the eukaryote world. With the introduction and improvement of capillary columns in gas chromatographic analyses, the *trans* isomers of UFA were first reported in aerobic methylotrophic bacteria and in a marine bacterial isolate [1,2]. To date, *trans*-UFA have also been described in membrane lipids of various bacteria, mainly in Gram-negative bacteria [3]. As for other bacterial fatty acids, the relative amounts of *trans*-UFA are highly dependent on the physiological state of the cells, such as the stage of growth, medium composition, or environmental factors.



**Figure 1: *cis-trans* Isomerization of UFA, palmitoleic acid residue (16:1 *cis*-Δ<sup>9</sup>), catalyzed by the Cti enzyme.**

The amino acid residues described refer to the *Pseudomonas putida* P8 enzyme (NCBI reference: CAA04438). PL: phospholipid.

In *Pseudomonas putida* P8 and *Colwellia maris* (*Vibrio* sp. ABE-1) [4], *trans*-UFA are synthesized by direct isomerization of the *cis*-UFA without modification of the position of the double bond (Figure 1). The conversion of *cis* to *trans*-UFA is considered as a mechanism to modify membrane fluidity and permeability properties, in response to the presence of toxic organic compounds in the mesophilic bacterium *P. putida* P8, and to withstand a temperature increase (from 5 °C to 20 °C) in the psychrophilic bacterium *C. maris* (*Vibrio* sp. ABE-1) [5-8]. The advantage of this conversion comes from the steric hindrance differences between *cis* and *trans*-UFA and the consequent membrane organization. The acyl chains of SFA in the membranes are linear and assemble in a tightly packed manner, to form an optimal hydrophobic interaction between them, leading to a rigid, compact, and low permeable membrane. In contrast, the *cis* double bond of the UFA determines an immovable bending in the acyl chain. This curvature leads to increased steric hindrance compared to SFA, creating a larger space between each hydrophobic tail, thus resulting in relatively high fluidity and permeability of the assembly. *Trans*-UFA, display a structure that resembles that of SFA [3]. Although the shift from *cis* to *trans* of the double bond does not have the same effect on the membrane fluidity as a conversion to SFA, it nevertheless has a substantial effect on the decrease of membrane fluidity, and contributes significantly to cell survival when a rapid response to environmental stress is required [9-11].

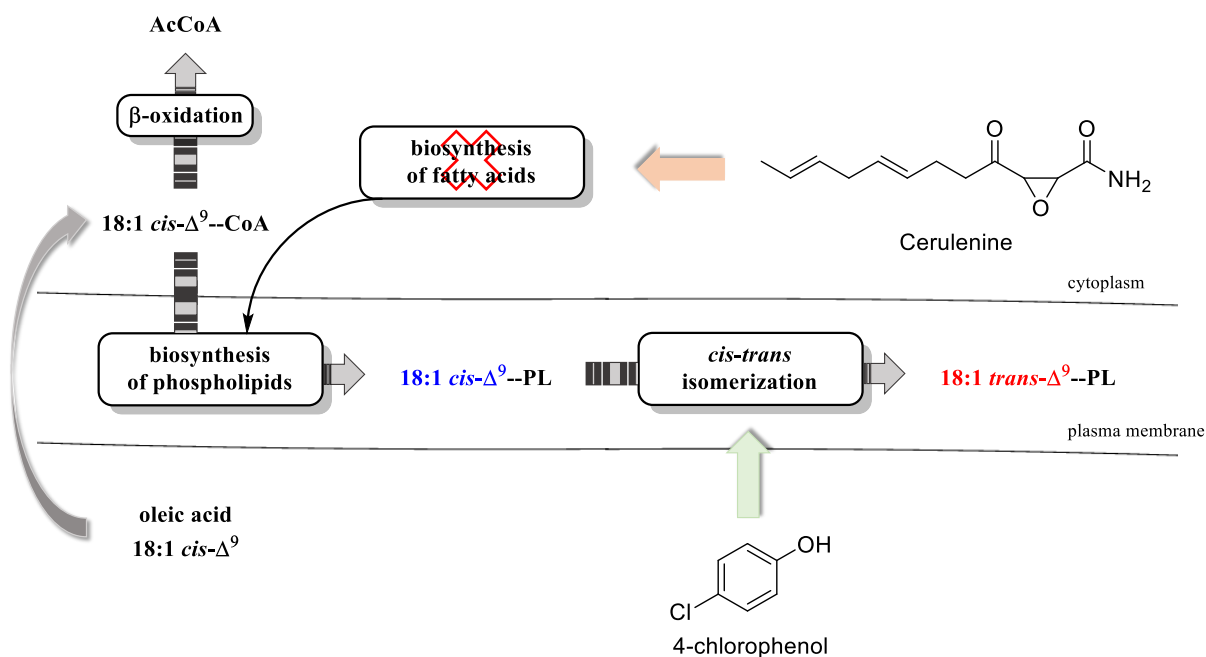
### **3. Environmental factors influencing the *cis-trans* isomerization**

#### *3.1. Effect of organic solvents*

The influence of phenolic compounds has been thoroughly studied on *P. putida* P8 cells, which acquired the capacity to degrade these toxic compounds.

When various concentrations of organic solvents were added to the cells, the fatty acid composition was changed by a marked increase in the amount of *trans*-UFA. In a concentration-dependent manner, cells reacted by increasing the amount of *trans*-UFA and by decreasing the amount of *cis*-UFA concomitantly, while the total amount of fatty acids was maintained constant [5]. Oleic acid (18:1

*cis*- $\Delta^9$ ), which cannot be synthesized by *P. putida* P8, was added to the culture media of this bacterium, and membrane lipid analysis revealed that oleic acid has been converted after exposure of the cells to 4-chlorophenol into its *trans* isomer elaidic acid (18:1 *trans*- $\Delta^9$ ) by isomerization without shifting of the double bond (Figure 2) [12]. *cis-trans* Isomerization was independent from *de novo* biosynthesis of fatty acids and proteins, as the reaction was unaffected by the presence of cerulenin (an inhibitor of the ketoacyl-ACP synthase activity of FabB and FabF) and chloramphenicol (an inhibitor of the peptidyl transferase activity of the bacterial ribosome), respectively [5]. In addition, the highest amount of *trans*-UFA was observed 30 minutes after stress initiation [12] demonstrating that the *cis-trans* isomerization is a rapid adaptive response.



**Figure 2: *In vivo* formation of elaidic acid (18:1 *trans*- $\Delta^9$ ) from oleic acid (18:1 *cis*- $\Delta^9$ ) induced by the addition of 4-chlorophenol in *Pseudomonas putida* P8.** Adapted from [3].  
Ac: acetyl; CoA: coenzyme A; PL: phospholipid.

The *cis-trans* isomerization of UFA can be induced by the accumulation of phenolic compounds in the membrane but also hydrocarbons [9,13,14]. The antimicrobial action of a solvent depends on its hydrophobic properties, expressed by the octanol/water partition coefficient ( $\text{LogP}_{\text{ow}}$ ) of a compound.  $\text{LogP}_{\text{ow}}$  is expressed as the logarithm of the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system. Hydrophobic solvents,

with a  $\text{LogP}_{\text{ow}}$  greater than 4 do not reach a high concentration in the membrane and are not toxic due to their low water solubility. In contrast, solvents with  $\text{LogP}_{\text{ow}}$  between 1 and 4 have higher water solubility values, and can accumulate in biological membranes, resulting in relatively high concentrations of these solvents in the membranes causing an increase in membrane fluidity, leading to non-specific permeabilization and hence cellular toxicity [15].

Organic solvent	$\text{LogP}_{\text{ow}}$	$\text{IC}_{50}$ (mM)	$\text{TC}_{50}$ (mM)
Methanol	-0.76	1480.0	1700.0
Ethanol	-0.28	345.0	600.0
1-Butanol	0.88	30.1	41.2
Phenol	1.45	8.6	9.1
1-Hexanol	1.87	5.8	5.5
<i>p</i> -Cresol	1.98	3.8	4.1
4-Chlorophenol	2.40	2.4	2.8
Toluene	2.48	2.1	2.6
1-Octanol	2.92	1.1	1.3
2,4-Dichlorophenol	3.20	0.4	0.6

**Table 1: Hydrophobicity, toxicity of several organic compounds and their effect on the *cis-trans* isomerization in *Pseudomonas putida* S12 cells.** Adapted from [9,13,14].

$\text{LogP}_{\text{ow}}$ : octanol/water partition coefficient;  $\text{IC}_{50}$ : concentration of toxin resulting in 50% inhibition of cell growth;  $\text{TC}_{50}$ : concentration of toxin resulting in 50% increase in *trans/cis* ratio of UFA.

A number of studies have shown a direct correlation between the hydrophobic nature of organic substances ( $\text{LogP}_{\text{ow}}$ ), the concentration of toxin leading to a 50% inhibition of cell growth ( $\text{IC}_{50}$ ) and the concentration of toxin leading to a 50% increase in the *trans/cis* ratio ( $\text{TC}_{50}$ ) of UFA (Table 1) [9,13,14]. In most cases, the concentration leading to maximum *cis-trans* isomerase activity was slightly higher than the concentration of solvent leading to total growth inhibition, and was independent of the structure of the molecules but rather strongly related to their ability to accumulate in the membrane [16].

### 3.2. Effect of the temperature

Temperature is one of the most important environmental factors that affect all aspects of growth and development of living organisms. An increase in temperature, in addition to affect the activity and stability of enzymes, weakens the membrane as a physical barrier and also alters specific relevant membrane functions such as ion permeability and energy transduction properties [17].

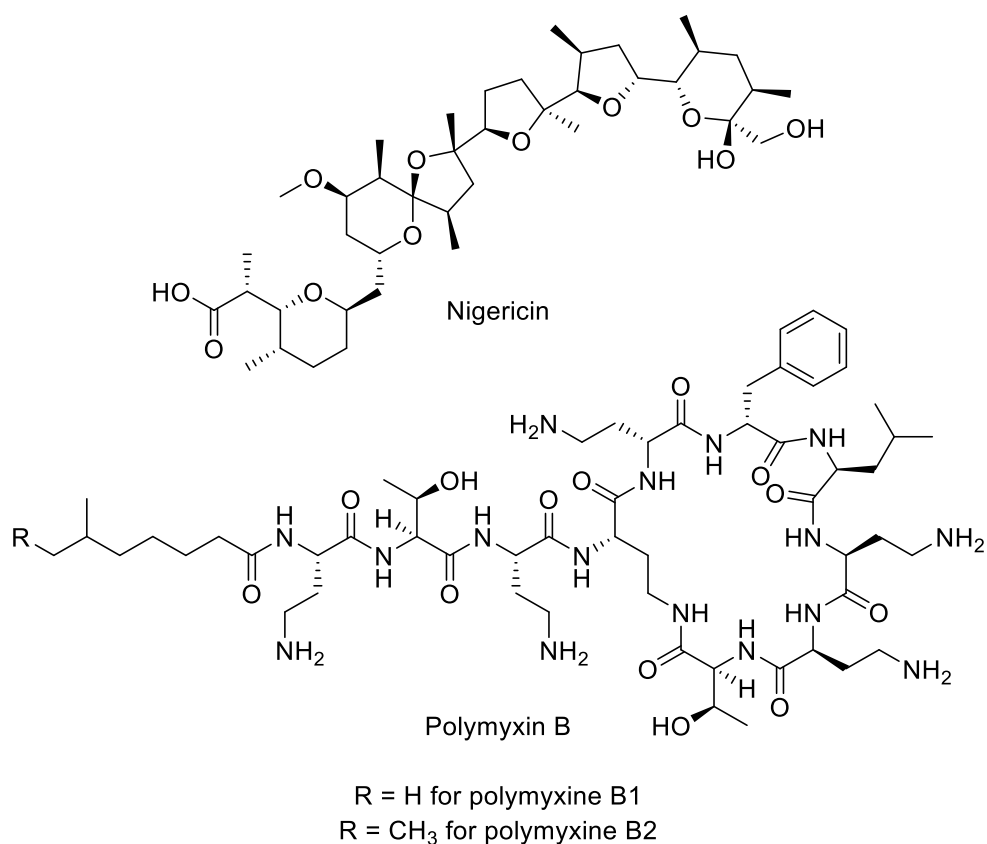
Studies on the effects of temperature on *P. putida* P8 cells during the transition from exponential growth phase to stationary growth phase showed that the production of *trans*-UFA plays an important role in bacterial adaptation to temperature increase (from 30 °C to 37 °C) when *de novo* synthesis of fatty acids was blocked by cerulenin. However, when *de novo* synthesis was active, the cells increased the proportion of SFA while maintaining a constant *trans/cis* ratio (TC) [18]. In response to a raise of external temperature, increasing the degree of saturation (SFA/UFA ratio) *via de novo* synthesis of SFA is a common adaptation mechanism in Gram-negative bacteria [17]. However, *de novo* fatty acid synthesis occurs only in growing cells [19,20]. Thus, *trans*-UFA synthesis appears as an alternative mechanism to regulate membrane fluidity, which is, opposite to the increase of degree of saturation, independent from *de novo* lipid synthesis.

The *cis-trans* isomerization of UFA has also been reported in some psychrophilic bacteria, such as *C. maris* (*Vibrio* sp. ABE-1) and *Pseudomonas* sp. Lz4W (*P. syringae* Lz4W). In response to an increase in ambient temperature, psychrophilic bacteria increased the amount of *trans*-UFA by increasing the proportion of palmitelaidic acid (16:1 *trans*- $\Delta^9$ ) [6,7,21]. Only traces of *trans*-UFA were detected when these bacteria are grown at their optimum growth temperature (5 °C). In order to better understand the role of *trans*-UFA, a mutant of *Pseudomonas* sp. Lz4W (*P. syringae* Lz4W) carrying an insertion in the *cis-trans* isomerase gene (*cti*) was constructed. The growth of the mutant strain ( $\Delta cti$ ) was inhibited at 28 °C in contrast to the growth of the wild-type strain. The growth capacity of this mutant was restored upon supplementation with the *cti* gene [21]. Similar observations were reported with mesophilic strains of *P. putida* that show impair in cell growth at elevated temperatures when the *cis-trans* isomerization reaction is defective [22,23]. In exponentially growing *P. putida* cells, temperature variations resulted in a decreased growth rate, and Heipieper and coworkers observed an increase in the *trans/cis* ratio (TC) only at elevated temperatures (> 30 °C) [24]. These results emphasize that bacteria, regardless of whether they are mesophilic or psychrophilic, require *trans*-UFA for growth at temperatures higher than their optimal one. Fluidity studies have also shown that the  $\Delta cti$  mutant strain of *Pseudomonas* sp. Lz4W (*P. Syringae* Lz4W) has a significantly higher fluidity than the wild-type strain and is also more sensitive to toluene due to the absence of *trans*-UFA [21]. High temperatures increase membrane fluidity while shift from *cis* to *trans*-UFA significantly increases

the membrane stiffness [25]. Hence, *cis-trans* isomerization of fatty acid provides an immediate control mechanism for modulating membrane fluidity under abrupt temperature changes.

### 3.3. Effect of other environmental stresses

In addition to organic solvents and high temperatures, most membrane-affecting stimuli such as toxic organic compounds, heavy metals, osmotic stress, and nanoparticles were found to activate the isomerization of *cis*-UFA into *trans*-UFA [21,24,26-28].



**Figure 3: Structure of nigericin and polymyxin B.**

The antibiotic nigericin, an ionophore binding to potassium ( $K^+$ ) or proton ( $H^+$ ) ion, triggers import of protons inside the plasma membrane leading to acidification of the cytoplasm, and exports potassium outside the cell. Polymyxin B, a cationic cyclic peptide, displays antibiotic activity through disruption of the structure of the membrane by incorporation and pore formation, leading to an increase



in cell permeability. Both membrane active antibiotics nigericin and polymyxin B were shown to also induce an increase in the proportion of *trans*-UFA (Figure 3) [29].

Heavy metal	Effect on microorganisms	MIC (mM) <sup>a</sup>	TC <sub>max</sub> (mM) <sup>b</sup>
<b>Zinc</b> (Zn <sup>2+</sup> )	Inhibits growth and causes cell death	3	6
<b>Cadmium</b> (Cd <sup>2+</sup> )	Denatures proteins, destroys nucleic acids and impairs cell division and transcription	8	10
<b>Chrome</b> (Cr <sup>3+</sup> )	Inhibits oxygen uptake, growth and elongates the latency phase	8	10
<b>Copper</b> (Cu <sup>2+</sup> )	Leads to oxidative stress altering enzymatic and physiological processes	6	8
<b>Nickel</b> (Ni <sup>2+</sup> )	Causes oxidative stress, alters enzymatic processes, and disrupts cell membrane functions	6	8

**Table 2: Effects of heavy metals on growth and *cis-trans* isomerization of *Pseudomonas putida*.** Adapted from [24,30].  
<sup>a</sup> MIC: lowest concentration resulting in no increase in colony forming unit (CFU) 2 h after metal addition; <sup>b</sup> TC<sub>max</sub>: metal concentration that gave the highest value for the *trans/cis* ratio.

Heavy metal toxicity involves several mechanisms such as alteration of enzymatic and physiological processes, induction of oxidative stresses causing severe damage to deoxyribonucleic acid (DNA), lipids and proteins, cell membrane impairment and formation of unbalance ion gradients by interacting with microbial membrane proteins or penetrating by ion channels [30]. Heipieper and coworkers have shown that the *trans/cis* ratio (TC) of UFA increases sharply following the addition of toxic concentrations of metals, with the highest ratio observed at concentrations above the minimum inhibitory concentration (MIC) (Table 2) [24].

Osmotic stress induced by sodium chloride (NaCl) and sucrose was also reported to cause an increase in the *trans/cis* ratio (TC) of UFA in *P. putida* S12, but no significant increase was observed in *Pseudomonas* sp. Lz4W (*P. Lz4W syringae*) [21,24]. Due to the heterogeneity of the genus *Pseudomonas*, activation of *cis-trans* isomerization of UFA in *P. putida* S12 and *Pseudomonas* sp. Lz4W (*P. Syringae* Lz4W) may differ [31].

The effect of pH on *P. putida* S12 was also studied, and it revealed that an increase in the TC ratio occurs at low pH range ( $3.5 \leq \text{pH} \leq 5.0$ ) while high pH ( $\text{pH} > 5.0$ ) had no influence on the TC ratio [24].

Antibacterial properties of AgNO<sub>3</sub> and AgNP are not completely understood but interactions between silver ions and components of the cell wall or of the cytoplasmic membrane, or with bacterial

DNA, proteins and enzymes involved in ATP formation, such as the electron transport chain, have been reported. It was shown that the presence of silver nitrate (AgNO<sub>3</sub>) and silver nanoparticles (AgNP) results in a strong increase in the TC ratio of UFA in *P. putida* cells [26].

Nanoscale zerovalent iron (nZVI) nanoparticles have significant oxidoreduction properties allowing them to process or degrade a wide range of toxic organic contaminants, anions, and inorganic metals, as well as special physical-chemical properties for removal by absorption or adsorption. Despite benefits of nZVI for environmental remediation, adverse effects on microorganisms through direct contact between nanoparticles and cell membranes have been reported. Kotchaplai *et al.* have shown that *P. putida* F1 cells have adapted to nZVI exposure by activating *cis-trans* isomerization of UFA to stiffen the membrane and maintain cell survival [28].

#### 3.4. Correlation between activation of the *cis-trans* isomerization of UFA and potassium influx

Potassium (K<sup>+</sup>), with concentrations between 0.1 and 0.6 mol L<sup>-1</sup> is the most abundant cation in bacterial cells. It participates in the adjustment of the membrane potential and is necessary for activity of the respiratory chain. Potassium uptake acts as a second messenger, activating enzymes or transport systems, and as a regulatory element in the control of transcription allowing cells to adapt to high osmolarity [32].

Stress	<i>cis-trans</i> Isomerization	Potassium influx
Organic solvents	Phenol	+
	4-Chlorophenol	+
	Toluene	+
	Ethanol	+
Temperature	↑	+
	↓	-
Heavy metals	Zinc (Zn <sup>2+</sup> )	+
	Cadmium (Cd <sup>2+</sup> )	+
Osmotic stress	NaCl	+
	Sucrose	+
	Glycerol	-
pH	↑	-
	↓	+
Antibiotics	Polymyxin	+
	Nigéricin	+

Table 3: Correlation between activation of *cis-trans* isomerization of UFA and activation of potassium influx caused by

One of the first changes that occur with increased fluidity is an intracellular potassium leakage [34]. In order to maintain high intracellular potassium concentrations, bacteria generally express several specific absorption systems [32].

A very strong correlation has been demonstrated between activation of potassium influx pumps and *cis-trans* isomerization of UFA (Table 3) [33]. Interestingly, all factors that activate *cis-trans* isomerization are also activators or inducers of the potassium absorption system. These factors include organic solvents, temperature increase (> 30 °C), heavy metals, sodium chloride (NaCl) and sucrose, low pH (pH = 3.5 to 5.0) and membrane-active antibiotics. These results reflect the importance of *cis-trans* isomerization as a general stress response mechanism for microorganisms. On the other hand, stress conditions, which are not known to be activators of potassium uptake, such as decreasing temperature, osmotic stress induced by glycerol and basic pH did not cause activation of Cti [24,33].

### 3.5. The *trans/cis* ratio as a biomarker?

For bacteria in which *cis-trans* isomerization of UFA occurs, the value of the TC ratio has been proposed as a biomarker for the analysis of changes in a microbial population. As measurement of membrane fatty acid profile has become a common method in many laboratories, Guckert *et al.* have suggested that a TC ratio greater than 0.1 (the normal index reported for most environmental samples) can be used to assess environmental effects on natural samples or during *in situ* bioremediation processes [35]. However, a potential problem in the use of the TC ratio is that it may not be an appropriate benchmark for assessing the long-term environmental effects of persistent organic contaminant [36]. Indeed, *cis-trans* isomerization of UFA is a rapid adaptive response that ensures the survival of the bacterium until prolonged membrane adaptation mechanisms, such as an increase in fatty acid saturation, take place [17,37,38]. In batch culture experiments, the TC ratio can be measured directly after a contamination event. For soil samples used in ecological microflora studies, the TC ratio decreases when long-term adaptations occur or when there is a change in the species composition of the microbial population

## 4. The *cis-trans* isomerase of UFA

### 4.1. Discovery, identification, and localisation of the *cis-trans* isomerase

In 1997, Holtwick and coworkers, using transposon mutagenesis experiments, isolated the *cti* gene from *P. putida* P8 responsible for the *cis-trans* isomerization. The gene was further sequenced and cloned into *E. coli*, making this bacterium able of producing *trans* fatty acids from some of its natural occurring *cis* fatty acids [22]. The *cti* nucleotide sequence of *P. putida* P8 was subsequently cloned by adding a sequence encoding a polyhistidine tag (His<sub>6</sub>), recombinantly expressed in *E. coli*, and the corresponding Cti protein was isolated and purified [39]. *P. putida* P8 Cti was shown to be a monomeric protein of 766 amino acids, corresponding to a molecular weight of 86.9 kDa. The protein displayed no significant similarity with homologous peptides when the amino acid sequence was compared with other proteins deposited in the GenBank database [22].

Based on assays to determine the activity of Cti in different cell compartments [12], Cti was initially localized in the cytoplasm or anchored to the outer surface of the cytoplasmic membrane [22,40]. This localization was consistent with the accessibility of the enzyme to its substrates. Subsequently, the periplasmic localization of Cti was demonstrated in *P. putida* DOT-T1E and *P. putida* S12 [23,41]. For this purpose, Junker *et al.* constructed a fusion protein between the N-terminal region of Cti, containing its putative signal peptide (encoding the 22 first amino acids plus additional 172 amino acids) and *E. Coli* alkaline phosphatase (PhoA), an enzyme only active once transported to the periplasm as four of its cysteines need to be oxidized for its activity (Figure 4). When 5-bromo-4-chloro-3-indolyl phosphate (BCPI, an artificial chromogenic substrate used for the sensitive colorimetric detection of PhoA activity) was added, the mutant strain containing the fusion protein produced a blue color upon hydrolysis, meaning that the N-terminal sequence of Cti transferred the fusion protein to the periplasmic space [23]. Heipieper and coworkers showed that adding octanol to spheroplasts instead of whole *P. putida* S12 cells causes a decrease in the final *trans*-UFA content. This decrease in the amount of *trans*-UFA was attributed to a significant loss of the Cti enzyme upon removal of the outer membrane and peptidoglycan [41]. Cti localization was confirmed upon purification of the enzyme from the periplasmic

fraction of *P. oleovorans* GPo12. Analysis of the N-terminal sequence of Cti from *P. oleovorans* GPo12 revealed sequence homology with the protein sequence coded by the *cti* gene from *P. putida* P8 starting from the 21<sup>st</sup> residue showing that the first 20 amino acids were cleaved off [42].

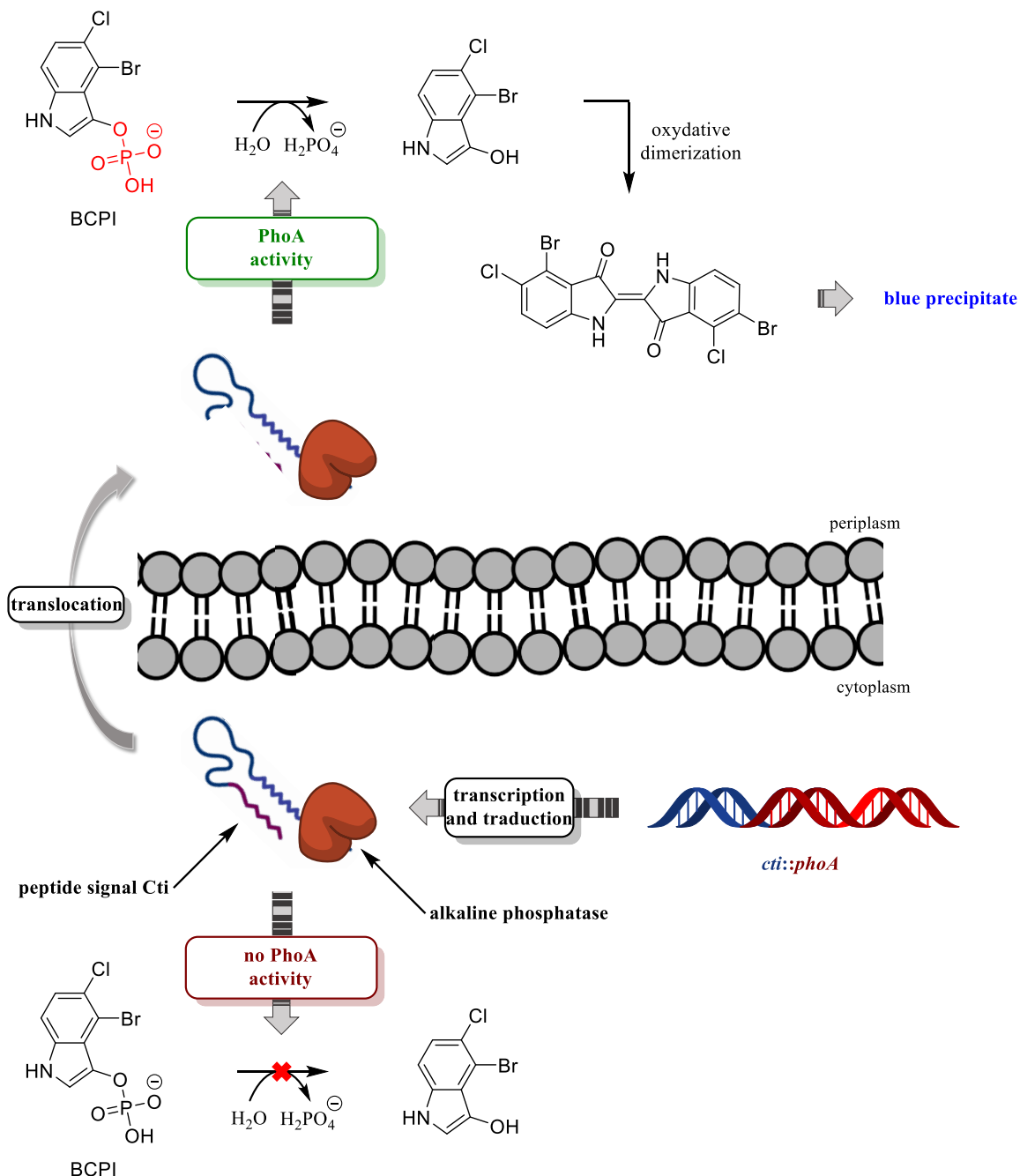
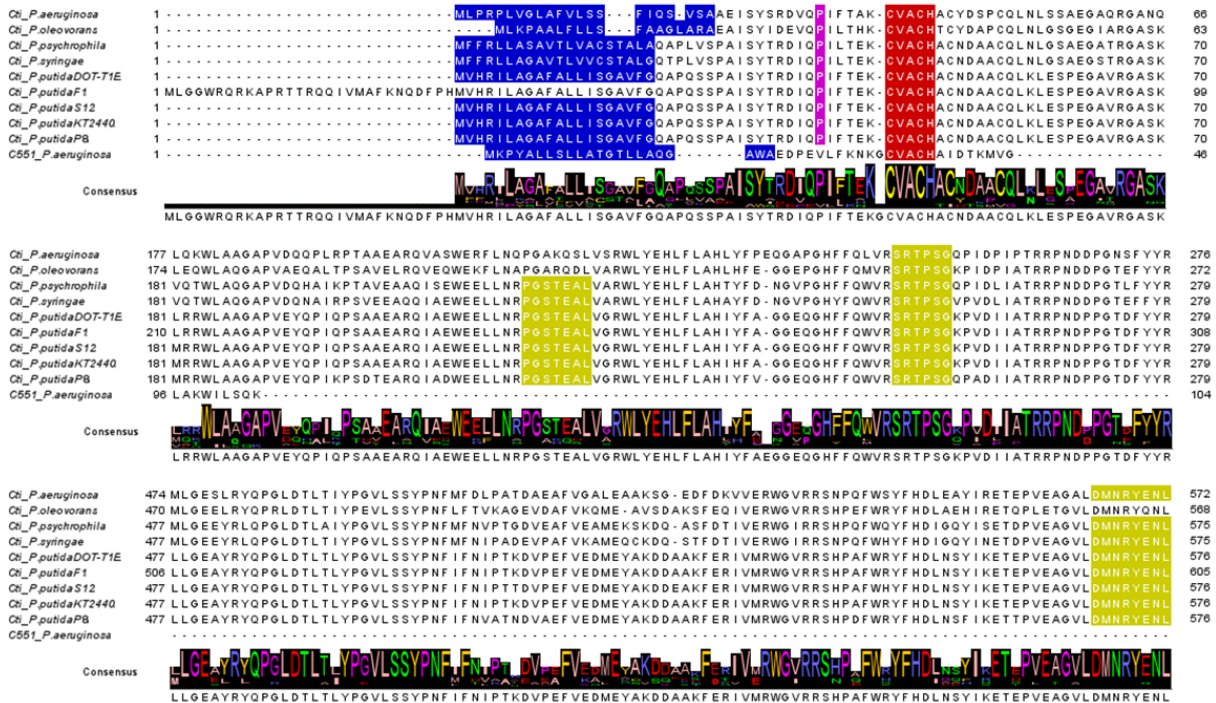


Figure 4: Evidence for the periplasmic localization of Cti in *Pseudomonas putida* DOT-T1E.

These results confirm the presence of an N-terminal hydrophobic signal sequence, which is removed after translocation of the protein into the periplasm [22,23].

#### 4.2. Cti, a cytochrome-c type enzyme

A multiple sequence alignment of Cti proteins indicates that the enzyme is highly conserved [43]. In the genus *Pseudomonas*, the protein sequence is about 766 amino acids and has common characteristics such as the heme binding site (CXXCH), a conserved proline after the N-terminal signal peptide sequence and three putative phosphorylation sites (PGSTEAL, SRTPSG and DMNRYENL) [44].



**Figure 5: Multiple sequence alignment of the Cti proteins as well as a representative of the bacterial cytochrome c family.**

The N-terminal signal sequence, conserved proline, heme binding site and putative phosphorylation sites are shown in blue, purple, red and yellow, respectively. This figure displays the sequence alignment of Cti from *Pseudomonas aeruginosa* PAO1 (NP\_250537), *Pseudomonas oleovorans* (WP\_045733337), *Pseudomonas psychrophila* (*Pseudomonas* sp. E-3) (WP\_019827282), *Pseudomonas* sp. Lz4W (*Pseudomonas syringae* Lz4W) (CAD59690), *Pseudomonas putida* DOT-T1E (AAD41252), *Pseudomonas putida* F1 (ABQ79445), *Pseudomonas putida* S12 (AJA16735), *Pseudomonas putida* KT2440 (NP\_744525), *Pseudomonas putida* P8 (CAA04438) and the cytochrome-*C*<sub>551</sub> from *Pseudomonas aeruginosa* (WP\_134604471).

Cti is a cytochrome-c type protein (Figure 1) [39]. Figure 5 displays the sequence alignment of different Cti enzymes from *Pseudomonas* spp. as well as a representative of the prokaryotic cytochrome-c family: the cytochrome-*C*<sub>551</sub> from *P. aeruginosa*. It can be observed that both periplasmic enzyme families have the CXXCH motif in common showing that Cti carries a heme covalently

attached to the protein by two thioether bonds formed between a vinyl group of the heme and the sulfur atom of a cysteine residue of the highly conserved CXXCH motif. The histidine of this motif is also known to be the proximal ligand of the heme into cytochrome-c type proteins.

The importance of the heme has then been confirmed by inactivation of genes involved in the maturation and installation of the heme into cytochrome-c type proteins using transposon mutagenesis as well as by site-directed mutagenesis experiments involving the cysteines of the heme binding motif CXXCH of Cti from *P. putida* P8. Both types of mutagenesis experiments revealed a loss of enzyme activity, therefore providing evidence that the periplasmic Cti is a cytochrome-c type protein with the heme being crucial for enzyme activity [39].

## 5. Activity and kinetics of the *cis-trans* isomerase of UFA

A cell-free extract of *P. psychrophila* (*Pseudomonas* sp. E-3) was shown to convert palmitoleic acid (16:1 *cis*- $\Delta^9$ ) into palmitelaidic acid (16:1 *trans*- $\Delta^9$ ) in its free acid form and esterified to phosphatidylethanolamine (PE) with a maximal specific activity of 20 and 122 nmol h<sup>-1</sup> mg<sup>-1</sup>, respectively. After separation of the cytosolic and membrane fractions of the acellular extract, Okuyama and coworkers showed that the cytosolic fraction exhibits isomerase activity only when UFA is in its free acid form with a maximal specific activity of 15-20 nmol h<sup>-1</sup> mg<sup>-1</sup>. The isomerization activity of phospholipid fatty acids was restored when the membrane fraction is added. In contrast, the membrane fraction had a low isomerization capability for both free and esterified fatty acids [40]. The Cti enzyme from *P. psychrophila* (*Pseudomonas* sp. E-3) was further purified and showed a K<sub>m</sub> value of 117.6  $\mu$ M and a maximal specific activity of 22.8  $\mu$ mol h<sup>-1</sup> mg<sup>-1</sup> when palmitoleic acid (16:1 *cis*- $\Delta^9$ ) was used as substrate [45].

Subsequently, Cti was isolated from the periplasmic fraction of *P. oleovorans* GPo12 and showed a specific activity of 216  $\mu$ mol h<sup>-1</sup> mg<sup>-1</sup>. The activity of both enzymes was maximum between pH = 7 and 8, and at a temperature of 30 °C [40,42].

*In vitro* the isomerase was active on UFA as free acid form without addition of ATP or external cofactor. No isomerization could be demonstrated on esterified fatty acids such as phospholipids or

methyl esters, even in the presence of organic solvents known to activate *cis-trans* isomerization *in vivo* [9,13,14]. Cti showed a different substrate specificity depending on its bacterial origin and based on the position of the double bond in the UFA rather than the length of the carbon chain. For *P. oleovorans* GPo12, *cis*-UFA having seven carbon atoms after the position of the double bond ( $\omega$ -7) are the preferential substrates [42]. Conversely, Cti from *P. psychrophila* (*Pseudomonas* sp. E-3) exhibits preferential activity towards *cis*-UFA with double bond at the position  $\Delta^9$  and no activity was found for *cis*-UFA with chain lengths of more than seventeen carbon atoms [43]. These results are in line with the analysis of the composition of the membrane fatty acids of *P. oleovorans* GPo12 and *P. psychrophila* (*Pseudomonas* sp. E-3). Indeed, the membrane lipids of *P. oleovorans* GPo12 contain palmitelaidic acid (16:1 *trans*- $\Delta^9$ ) and *trans*-vaccenic acid (18:1 *trans*- $\Delta^{11}$ ), whereas only palmitelaidic acid (16:1 *trans*- $\Delta^9$ ) has been detected in the membrane of *P. psychrophila* (*Pseudomonas* sp. E-3) [6,18].

Anoxic conditions, chelating agents [1,10-phenanthroline, ethylene diamine tetraacetic acid (EDTA), aminopolycarboxylic acid (EGTA)], metal ions ( $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$ ) and thiol compounds ( $\beta$ -mercaptoethanol and dithiothreitol) do not influence Cti activity [42,43]. On the other hand, the addition of 1 mM of antioxidants such as nordihydroguaiaretic acid and  $\alpha$ -tocopherol strongly inhibited Cti activity [43]. Although Cti contains a type-c heme, the isomerization reaction was not influenced by the addition of EDTA suggesting that the iron atom is strongly bound or weakly accessible to chelating agents. The enzyme is active in the absence of cofactors such as ATP, glutathione, NAD(P)H, dioxygen which differentiates it from other known heme iron enzymes acting on fatty acids [cytochromes P450 4A (CYP4A)] [44].

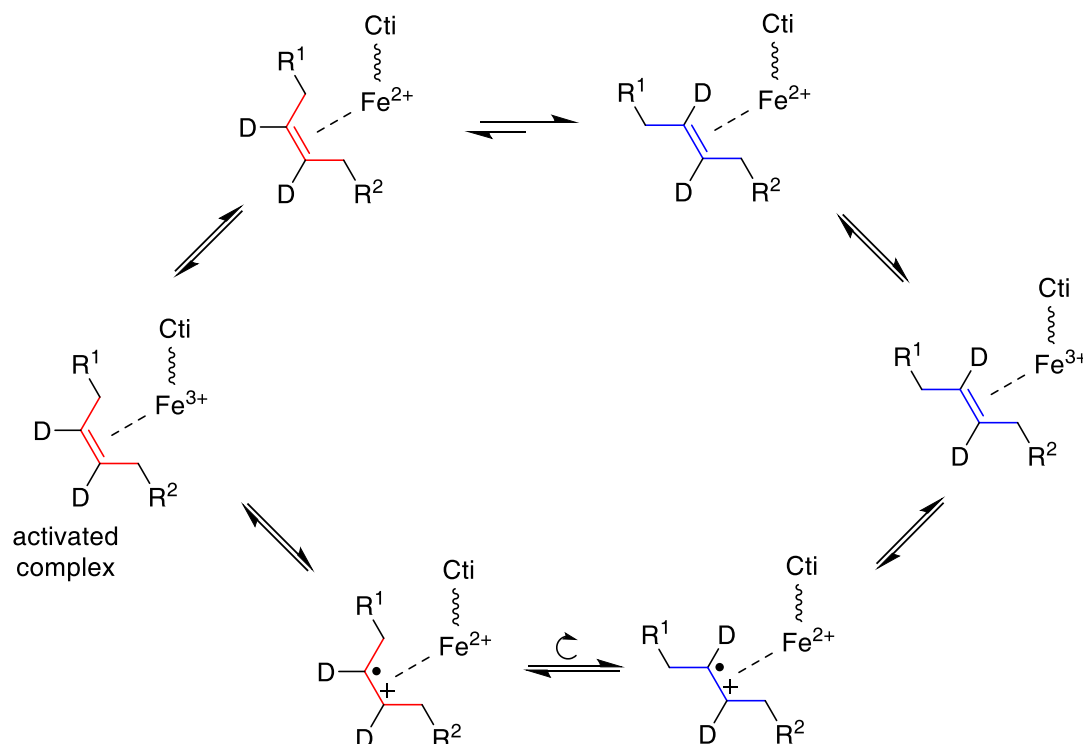
## 6. Catalytic mechanism of the *cis-trans* isomerase of UFA

Cti is a cytochrome-c type protein as it is bearing a heme-c binding motif. The molecular mechanism of *cis-trans* isomerization by Cti is still under debate and particularly, the iron oxidation state remains to be investigated. However, some structural information has been obtained based on labelling with deuterium on both carbons of the double bond [46] and the carbon isotope fractionation of the *cis-trans* isomerization [47]. Indeed, incorporation experiments of oleic acid labelled with deuterium on both



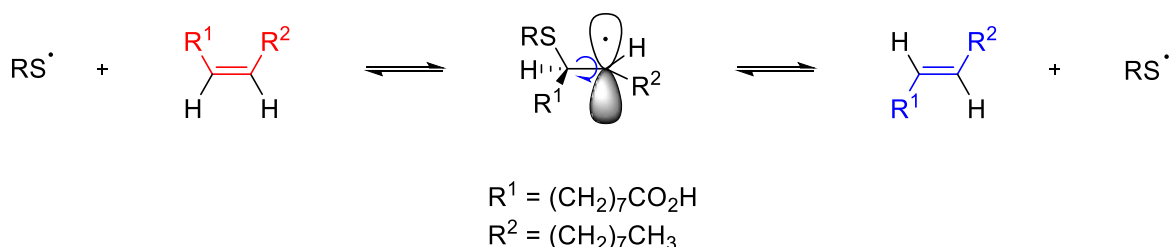
carbons of the double bond ([9,10-D]-18:1 *cis*- $\Delta^9$ ) using either *P. putida* P8 or *C. maris* (*Vibrio* sp. ABE-1), revealed that oleic acid ([9,10-D]-18:1 *cis*- $\Delta^9$ ) was converted exclusively into deuterium labelled elaidic acid ([9,10-D]-18:1 *trans*- $\Delta^9$ ) with retention of both deuterium atoms after activation of the isomerization by organic solvents [8,46]. Since no deuterium loss was observed, a transient saturation of the double bond by a coupled hydration/dehydration reaction in the Cti mechanism can be excluded. The carbon isotope fractionation of the *cis-trans* isomerization in *P. putida* S12 cells was determined by inducing the *cis-trans* isomerase using increasing amount of 3-nitrotoluene and measuring the carbon isotopic composition of the C16:1 fatty acids. The results indicated that during the isomerization process, the *cis* fatty acids were enriched in  $^{13}\text{C}$  whether the *trans* fatty acids were depleted in  $^{13}\text{C}$ . The reported isotopic fractionation factor ( $\alpha_c = 1.0011$ ) and the intrinsic fractionation factor ( $\alpha_{\text{intrinsic}} = 1.020 \pm 0.0024$ ) revealed a significant primary kinetic isotope effect that was attributed to a change from the  $\text{sp}^2$  carbon linkage of the double bond to a  $\text{sp}^3$  linkage (or *vice versa*) corresponding to the formation or the breaking of the intermediate  $\sigma$  bond of the enzyme-substrate complex [47].

Based on these results, it was proposed that  $\text{Fe}^{3+}$  is essential for this catalytic reaction [43]. Since  $\text{Fe}^{2+}$  of the cytochrome-c is known to be present in Cti, an activation complex involving oxidized iron ( $\text{Fe}^{3+}$ ) provided by the heme domain present in the enzyme has been invoked. Figure 6 illustrates the proposed mechanism: upon the formation of activated enzyme-substrate complex, an electron temporarily moves from the *cis*-double bond to the electrophilic iron allowing the  $\text{sp}^2$  hybridization to  $\text{sp}^3$  thus permitting the rotation of the C–C bond in a radical cation-like intermediate; the electron is transferred back reforming the double bond in the *trans* configuration; the location of metal center is also unknown [11,46-47]. It is worth underlining that little is known regarding the electronic structure or ligand-coordination state of the heme iron in the Cti and future research is necessary to elucidate these aspects.



**Figure 6. Proposed mechanisms of *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida* [11,46,47].** Upper part shows the deuterium labelling experiments indicating that the position of double bond remains the same after isomerization; Lower part shows the rotation of the C–C bond in the proposed intermediate. The oxidation state of the heme remains to be investigated.

It is also worth mentioning the case of carotenoid enzyme Z-ISO that controls carotenoid biosynthesis through a heme-based *cis-trans* isomerase [48]. Interestingly, carotenoids are other lipids with diverse structures and geometrical isomers that also regulate membrane fluidity. Z-ISO is a plant *cis-trans* isomerase responsible for the isomerisation of the 15-15'-*cis* carbon double bound in 9,15,9'-tri-*cis*- $\zeta$ -carotene to form 9,9'-di-*cis*- $\zeta$ -carotene that was shown to be essential to respond to environmental stress such as temperature fluctuation. The hypothetical catalytic mechanism described above for Cti is different from that of Z-ISO. Indeed, the isomerization by Z-ISO depends upon a ferrous heme-b cofactor that undergoes redox-regulated ligand switching between the heme iron and alternate Z-ISO amino acid residues (replacement of an axial cysteine by a histidine upon reduction) [48]. Taking into account the profound difference between the delocalized  $\pi$  electrons of multi-diene structure of carotenoid (reduction potential:  $E^\circ=1.06$  V) and the monounsaturated fatty acid structure, this suggests two distinct molecular mechanisms.



**Figure 7: Addition and elimination step of *cis-trans* isomerization of double bonds catalyzed by thiyl radicals.**

Another possibility, which remains to be demonstrated, is the involvement of free radicals during the enzymatic isomerization process. In chemistry, thiyl-type radicals are known to catalyze the *cis-trans* isomerization of UFA [49]. Double bond isomerization occurs *via* the addition, rotation, and fragmentation cycle, leading to the formation of more than 80% *trans* isomer (Figure 7) [50-54]. Two factors have been found to play a role in driving the thiyl-type radical catalyzed *cis-trans* isomerization: the thermodynamically more stable *trans* configuration of the double bond and the kinetically favored  $\beta$ -fragmentation from the radical intermediate to give *trans* double bonds, as demonstrated by targeted mechanistic studies [50-54]. It is often forgotten that the *cis* configuration of the double bonds in natural lipids is the less thermodynamically stable isomer, however Nature needs such a molecular requisite in order to keep physiologically compatible membrane biophysical properties of fluidity and permeability. The “price” that cells pay for this requisite is the need of coding desaturase enzymes that create double bonds in stereo- and regio- specific manner. It is interesting to note that, so far, in eukaryotes the *cis-trans* isomerase activity is not present and the formation of *trans* double bond containing lipids can occur only *via* chemical transformations, either endogenously or exogenously produced [49].

This catalytic isomerization is characterized by the reversibility of all steps [49]. *In vitro*, the Cti enzyme was able to convert *trans* to *cis* double bond, suggesting a reversible mechanism. However, the activity was 400 to 450 times greater in the direction of *trans* isomer formation than in the opposite direction, suggesting that *trans* to *cis* isomerization does not occur *in vivo* [45]. Although it is difficult to propose a mechanism in the absence of structural data of Cti, a mechanism involving thiyl radical resulting from the oxidation of a cysteine residue in the active site of the enzyme remains possible. The addition of a thiyl radical to the *cis* double bond would generate a radical intermediate thus permitting the free rotation around the C-C bond. After elimination of the thiyl radical, the *trans* double

bond can be formed (Figure 7). If this mechanism would hold true, one remaining question would be the role of the heme. We could hypothesize that it would be involved in the formation of the catalytic thiyl radical by oxidation of a cysteine residue or by one electron reduction of a disulfide bound.

## 7. Regulation of the *cis-trans* isomerase of UFA

*Trans*-UFA are formed when a change in the cell environment affecting the fluidity of the membrane is too abrupt to be compensated by *de novo* fatty acid synthesis. Thus, the formation of *trans*-UFA can be viewed as an adaptive response in order to increase membrane stiffness, *i.e.*, an emergency response rather than a means of adjusting membrane fluidity during growth and multiplication [55].

One remaining question regarding *cis-trans* isomerization of UFA is the regulation of the Cti enzyme. It can be regulated at DNA level (transcriptional regulation), at the messenger ribonucleic acid (mRNA) level (post-transcriptional regulation) or at the protein level (post-translational regulation).

### 7.1. Transcriptional regulation

The *cti* gene has been isolated from six species of *Pseudomonas*, namely *P. putida* P8, *P. putida* DOT-T1E, *Pseudomonas* sp. Lz4W (*P. syringae* Lz4w), *P. putida* KT2440, *P. aeruginosa* PAO1 and *P. putida* F1 [22,23,44,56-58].

Based on the study of the *cti* gene sequence of *P. putida* DOT-T1E, it was initially concluded that the gene was transcribed into a monocistronic mRNA (mRNA encoding only one protein chain) [23]. However, recent analysis has showed that *cti* is co-transcribed with downstream genes in *P. aeruginosa* PAO1 and *P. putida* F1 that are not involved in *trans*-UFA synthesis [56].

Several studies on *Pseudomonas* spp. to define the transcriptional start site (TSS) have been performed [22,23,44,59], and two TSS of *cti* were found located at positions -77 and -279 upstream of the translation start site (+1) in *P. putida* F1 [56]. Similar data were reported for *P. putida* DOT-T1E where the *cti* TSS was placed at position -294 [59]. It was recently shown, by Cronan and coworkers,

that predominant expression of *cti* is initiated from the P<sup>2</sup> promoter (*cti* -279) which is highly conserved in *P. putida* species, and that the Cti protein is moderately expressed in the absence of stressors [56].



**Figure 8: Nucleotide sequence of the *cti* promoter region of *Pseudomonas putida* F1.**

The deduced -10 and -35 sequences are shown in yellow and green respectively. The transcription initiation sites *cti* -77 and *cti* -279 are represented by black arrows (NCBI reference: CP000712.1). Adapted from [56].

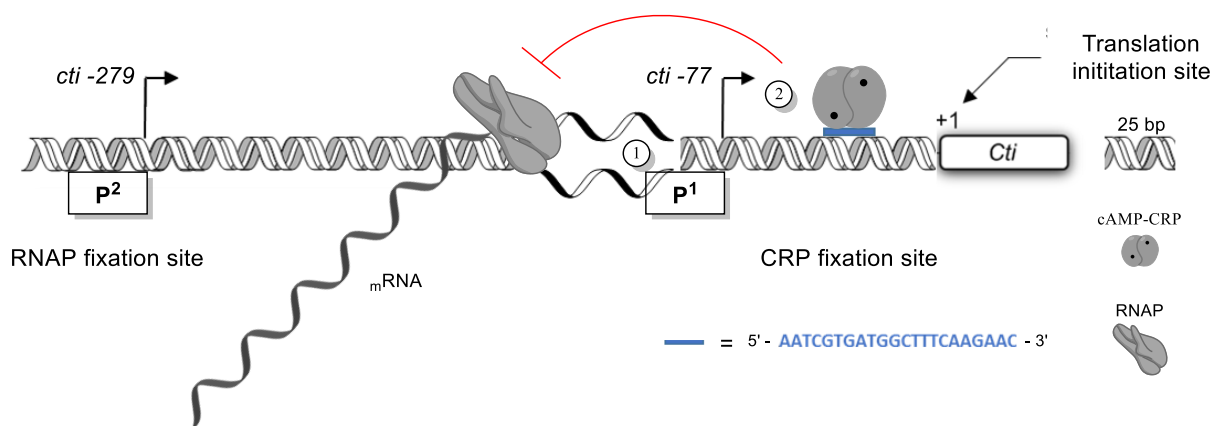
Within the bacteria cell, transcription requires a protein cofactor called sigma factor ( $\sigma$ ) that associates with the catalytic core of RNA polymerase (RNAP) [60]. The  $\sigma^{70}$  factor, also known as the primary factor, initiates transcription of thousands of essential genes during the exponential phase of growth, while other  $\sigma$  factors are used to express genes in response to environmental stress such as heat shock ( $\sigma^{32}$ ) or for specific purposes such as flagella synthesis ( $\sigma^{28}$ ) [61]. Promoters are specifically recognized by the RNAP via the  $\sigma^{70}$  factor which binds to two relatively conserved hexanucleotide promoter sequences. Their consensus sequences are 5' - TATAAT - 3' (Pribnow box) and 5' - TTGACA - 3', although it is important to note that almost no natural promoter has the consensus sequence described.

In *P. putida* F1, these sequences are located respectively at positions -10 and -35 upstream of the TSS and are separated by fifteen to nineteen base pairs (bp) (consensus - 17 bp) (Figure 8). This spacing is generally well preserved in contrast to their sequence [60]. The *cti* promoter in *Pseudomonas* spp. appears to be a  $\sigma^{70}$  factor-dependent promoter since both promoter sequences at positions -10 and -35 upstream of the TSS have been found and could hence explain the moderate expression of Cti in the absence of stressors [22,44,56,59].

#### 7.1.1. Regulation via the cAMP-CRP complex

Transcription factors (TF) are proteins capable of interacting with DNA and modulate the activity of RNAP. Cyclic adenosine monophosphate (cAMP) can induce a change in the conformation of the cAMP receptor protein (CRP), thus forming an active complex able to bind to a specific site on DNA.

This latter binding site is a semi-palindromic sequence of twenty-two base pairs, whose consensus sequence is 5' - AAATGTGATCTAGATCACATT - 3' [62]. The cAMP-CRP complex acts as a TF able to promote or repress the transcription of a gene.



**Figure 9: Proposed mechanism for the repression of *cti* transcription mediated by the cAMP-CRP complex in *Pseudomonas putida* F1.**

The cAMP-CRP complex binds to its binding site located downstream of the P1 promoter (*cti* -77) and represses *cti* transcription by ① inhibition of DNA double helix opening or by ② blocking the progression of RNAP along the DNA.

Cronan and coworkers reported that cAMPc-CRP complex represses *cti* expression in *P. putida* F1, binding only on the downstream region of the P<sup>1</sup> promoter (*cti* -77) [56]. Since the initiation of *cti* gene transcription is mainly initiated from the P<sup>2</sup> promoter (*cti* -279), direct inhibition of the binding of RNAP to its promoter by steric hindrance due to the cAMP-CRP complex can presumably be excluded. However, repression of transcription by the cAMP-CRP complex could occur by inhibiting the transition from the closed to open complex of the DNA double-strand or by blocking the progression of RNAP along the DNA by collision with downstream cAMP-CRP repressor (Figure 9) [63].

#### 7.1.2. Correlation between *cti* gene expression and the amount of trans-UFA produced in cells

Studies with *P. putida* P8 cells and *C. maris* (*Vibrio* sp. ABE-1) cells provided indirect evidence of constitutive expression of the Cti protein since *cis-trans* isomerization of UFA occurs in cells in which *de novo* protein synthesis is inhibited by chloramphenicol [5,7]. Then the recent analysis of *cti* gene expression in *P. putida* F1 cells has shown that the gene is moderately expressed in the absence of

stress factors and that the level of transcription is significantly higher in the stationary growth phase compared to the exponential growth phase [56].

Factors		Transcription level	Amount of <i>trans</i> -UFA
Organic solvent		↑ moderate dose-dependent	↑
Increase in temperature <sup>a</sup>		↑ heat-dependent	Low ↓
Growth phase	Exponential	Constant	↑
	Stationary	↑	↓

**Table 4: Correlation between *cti* expression level and *trans*-UFA amount in *Pseudomonas putida* F1.**

<sup>a</sup> Temperature increase from 30 °C to 37 °C.

When *P. putida* F1 cells were treated with different octanol concentrations, the level of transcription was moderately increased in a dose-dependent manner demonstrating that the *cti* gene is transcriptionally regulated (Table 4). The increase in *cti* expression 20 min after stress initiation led to an overproduction of *trans*-UFA required to maintain membrane fluidity [56]. These results are similar to those obtained for *P. putida* DOT-T1E but in contrast with an investigation in *Pseudomonas* sp. Lz4W (*P. Syringae* Lz4W) using RT-PCR experiments reported by Shivaji and coworkers, who ruled out expression of the *cti* gene at the transcriptional level and suggested a post-transcriptional control [44,59]. Given that genotypes of the genus *Pseudomonas* are characterized by significant genetic heterogeneity, *cti* expression in *P. putida* and *Pseudomonas* sp. Lz4W (*P. syringae* Lz4W) could differ remarkably due to selection pressures [31].

In response to an increase in external temperature, analysis of *cti* gene expression in *P. putida* F1 showed a heat-dependent increase (Table 4). The highest level of *cti* expression was found in cultures incubated at 37 °C, while the level of expression was lower when cells were incubated at 20 °C and 30 °C [56]. However, the increase in temperature had no effect on *trans*-UFA production in agreement with earlier work of Diefenbach *et al.* who showed that *trans*-UFA production plays an important role in the adaptation of *P. putida* P8 to temperature increase when *de novo* fatty acid synthesis is impaired [18]. At all temperatures tested, *cti* gene expression in *P. putida* F1 was higher in the stationary phase of growth compared to the exponential phase of growth (Table 4). In contrast, the amount of *trans*-UFA decreased significantly after 24 h of growth followed by an increase in the proportion of cyclopropane fatty acids (CFA) [56]. Although Cfa synthase and Cti use *cis*-UFA as substrate, and *trans*-UFA and CFA have a decreasing effect on membrane fluidity, no competition

between these two enzymes was shown in *P. putida* KT2400 and *P. putida* F1 [56,57]. However, the discrepancy between *cti* gene expression and *trans*-UFA production suggests post-transcriptional or post-translational regulation [44,56,59].

## 7.2. Post-translational regulation

Since the periplasmic enzyme Cti is moderately expressed in the absence of stressors, it may be spatially separated from its substrate or exist in a non-active state.

### 7.2.1. Regulation via phospholipase

*In vitro*, no activity was detected when Cti was incubated with phospholipids as a substrate [9,13,14]. Nevertheless, it was shown by Witholt and coworkers that the isomerization of phospholipid fatty acids by Cti from *P. oleovorans* GPo12 is possible when crude membranes isolated from *Pseudomonas* or *E. coli* cells are used as phospholipid source. In this case, the *cis-trans* conversion of phospholipid acyl chains is significantly increased in the presence of organic solvents. Furthermore, in membranes isolated in the presence of solvents, EDTA strongly inhibited the *cis-trans* isomerization of palmitoleic acid (16:1 *cis*- $\Delta^9$ ), which was restored when calcium ( $\text{Ca}^{2+}$ ) ions were added [40,42]. Based on these results of Witholt, a model has been proposed in which membrane phospholipid UFA would be cleaved by phospholipase during environmental stress and then Cti would catalyze the isomerization of the free UFA which can then be reincorporated into lysophospholipids [42]. Another possibility proposed by Okuyama is that specific interactions of one or more membrane components would interact with Cti or membrane lipids to catalyze the direct isomerization of phospholipids [40].



### 7.3. Regulation via membrane fluidity

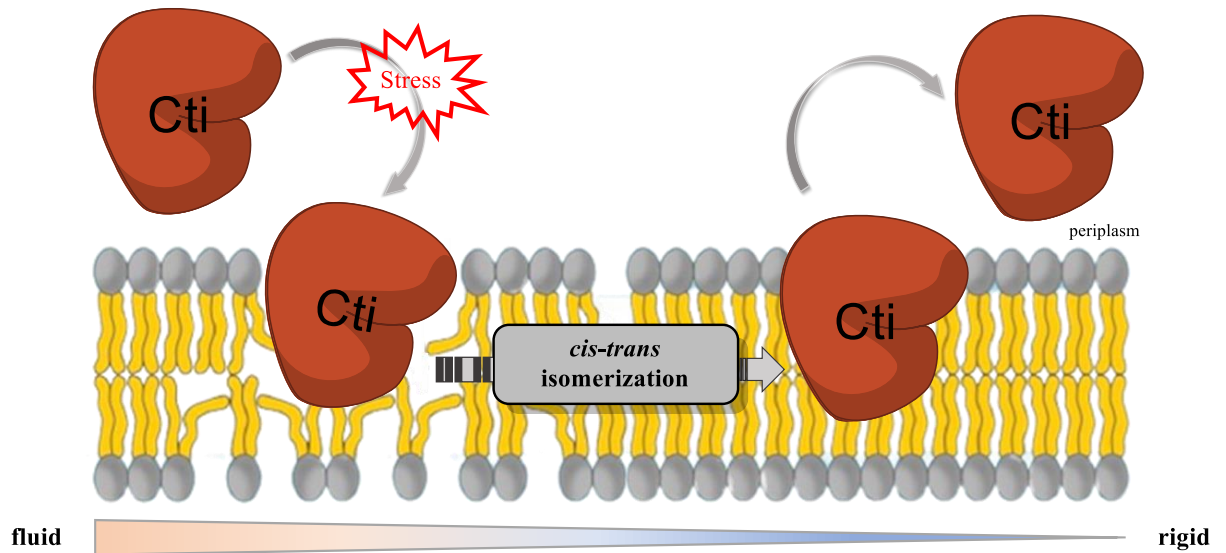


Figure 10: Proposed regulation of Cti mediated by membrane fluidity according to [11].

Based on earlier observations, a complete picture of the currently accepted regulation model based on the fluidity state of the membrane was summarized by Heipieper and coworkers (Figure 10) [11]. Under unstressed conditions, the membrane is relatively rigid and *cis*-UFA residues of phospholipids are ordered and embedded at a certain depth in the membrane. The periplasmic localization of Cti prevents the relatively more hydrophilic protein from accessing its substrate. During increased fluidity, caused by hydrocarbons or high temperatures, gaps form in the phospholipid arrangement allowing either Cti to penetrate deeper into the membrane to access the *cis*-UFA or Cti to move closer to the membrane surface more frequently, which would facilitate the interaction between the enzyme and its substrates. As *cis*-UFA residues are converted to *trans*-UFA, the acyl chains are tighter and assemble efficiently to form an optimal hydrophobic interaction between them. This leads to a reduction in fluidity and could lead to Cti being carried outside the membrane. This mechanism would be strictly controlled without the involvement of indirect signaling mechanisms or pathways. When the environment becomes viable for bacterial growth, the *trans/cis* ratio (TC) of the UFA can be rapidly shifted to a basal level by *de novo* synthesis of *cis*-UFA, followed by removal of *trans*-UFA containing phospholipids and integration of *de novo* synthesized *cis*-UFA into

lysophospholipids [9-11].

## 8. Distribution of the *cis-trans* isomerase of UFA in the bacterial kingdom

The *cis-trans* isomerization of UFA is not restricted to the genus *Pseudomonas* since *trans*-UFA have been detected in various bacteria [3], and *cis-trans* conversion has been demonstrated in other Gram-negative bacteria such as *Alcanivorax borkumensis* SK2 and *Methylococcus capsulatus* [64,65].

Based on the Cti protein sequence of *P. aeruginosa* PAO1, the presence of Cti in *Colwellia psychrerythraea* and *Vibrio cholerae* was predicted using the basic local alignment search tool (BLAST) [66]. Although *trans*-UFA in membrane phospholipids were found in these bacteria, no physiological or biochemical evidence of the presence of Cti was demonstrated [35,67].

Almost all Cti proteins carry an N-terminal signal sequence allowing targeting to the periplasmic space. However, a signal peptide characteristic of the general secretion pathway (sec) is not present in the Cti protein of *V. cholerae* [9-11].

A study of the Cti protein distribution in the bacterial kingdom revealed that only 5.5% of the sequenced bacterial strains would have proteins orthologous to Cti from *P. putida* F1 [68]. Most of these bacteria are present in marine environments with high osmolarity environments contaminated by hydrocarbons, heavy metals, or other toxic compounds, suggesting that the environment could be a major factor driving the distribution of Cti [68]. This analysis showed that proteins orthologous to Cti would only be found in Gram-negative bacteria, and that the Cti family is divided into three major groups: *Pseudomonas*, *Vibrio* and *Pseudoalteromonas*. About 50% amino acid sequence identity exists between the *Pseudoalteromonas* and *Vibrio* groups, whereas the *Pseudomonas* group had only 35-38% sequence identity with the *Pseudoalteromonas* and *Vibrio* groups [68].

The Cti protein sequence of the *Vibrio* group would show significant similarity with the Cti protein of *Alteromonas* spp. and *Shewanella* spp. However, these two bacteria are phylogenetically closer to the genus *Pseudoalteromonas* (order *Alteromonadales*) than to the genus *Vibrio* (order *Vibrionales*) [68]. This suggests that the Cti enzyme could be acquired by horizontal gene transfer through the

association of several bacteria in the same habitat [69,70].

*Enterococcus faecalis* is a commensal Gram-positive bacterium found in the gastrointestinal tract of mammals. *E. faecalis* belongs to the genus *Enterococcus* which are opportunistic pathogens which can induce bacteremia, infective endocarditis, intra-abdominal infections, wound infections, and urinary tract infections [71]. Although Cti orthologous proteins are reported only in Gram-negative bacteria, *E. faecalis* FA2-2 has been shown to synthesize *trans*-UFA during growth *via* a mechanism analogous to that of *Pseudomonas* sp [68]. As reported for *P. putida* F1, the amount of *trans*-UFA in the membrane of *E. faecalis* FA2-2 increased with increasing temperature (37 °C to 42 °C, Table 5). The amount of *trans*-UFA also increased during the exponential phase of growth when *E. faecalis* FA2-2 and *P. putida* F1 were grown under standard conditions of growth temperature (37 °C and 30 °C, respectively, Table 5). Nevertheless, organic solvents (octanol and toluene), chelating agents (EDTA) and oxidative stress (paraquat) had no effect on the production of *trans*-UFA in *E. faecalis* FA2-2 unlike *P. putida* F1 (Table 5). These results suggest that the enzyme involved in the *cis-trans* isomerization of *cis*-UFA of *E. faecalis* FA2-2 does not have the same characteristics as Cti from *P. putida* F1, and that *trans*-UFA may have another role in *E. faecalis* FA2-2 [68].

Stress		Amount of <i>trans</i> -UFA	
		<i>Enterococcus faecalis</i> FA2-2	<i>Pseudomonas putida</i> F1
Organic solvents		No effect	↑
Chelating agents		No effect	↓
Oxidative stress		No effect	↓
Increase in temperature		↑ <sup>a</sup>	↑ <sup>b</sup>
Growth phase	Exponential	↑	↑
	Stationary	↓	↓

**Table 5: Effect of different stresses on the amount of *trans*-UFA of *Enterococcus faecalis* FA2-2 and *Pseudomonas putida* F1.**

<sup>a</sup> Temperature increase from 37 °C to 42 °C; <sup>b</sup> Temperature increase from 30 °C to 37 °C.

## 9. Conclusion

The *cis-trans* isomerase of fatty acids is a critical metalloenzyme for several bacteria allowing them to resist to environmental stresses. Even if most aspects of the *cis-trans* isomerization have been addressed, some unsolved questions remain. What is the molecular mechanism of the Cti enzyme? Why pure Cti is not active on its natural substrates: phospholipids? Why this mechanism is

not found in human? As matter of facts, the absence of *cis-trans* isomerase in humans had only once an initial explanation based on evolutive selection principles, since the *trans* geometry produces, together with the saturated fatty acid structures, a shrinkage of the cell diameter that is not compatible with the large compartments needed by eukaryotic cells. Such different compartmentalization was indeed observed with saturated, *cis* and *trans* fatty acids as components of unilamellar vesicles made by methanol injected methodology [72]. Also, the variation of membrane transition temperatures shown in the case of vesicles made by *trans* fatty acids [73], could be thought as evolutive barriers that worked for the exclusion of the *trans* geometry from the membrane structures of multicellular organisms. More work is needed in evolutionary biology to fully explain the intriguing exclusion of the *trans* geometry in mammals.

The *cis-trans* isomerization of fatty acids has already attracted great interest in biotechnology for the production of biorenewable products using microorganisms. Indeed, product toxicity *via* membrane damage of the microbial factory is a common issue and membrane engineering using *trans* unsaturated fatty acids production has revealed to be a promising solution to enhance the membrane robustness and increase the tolerance of the microorganism to industrial valuable chemicals such as octanoic acid, but also hexanoic acid, butanol, hexanol, styrene, succinate, toluene [58]. Further research will be necessary to tackle the remaining questions and the exploitation of the results might lead to new applications that could span from food industry to medicine.

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Acknowledgments**

This work was supported by a grant from “Centre International de Recherche aux Frontieres de la Chimie” and from Region Grand Est, by COST Action 1201, by the Centre National de la Recherche Scientifique (CNRS) and University of Strasbourg. This work of the Interdisciplinary Thematic Institute

InnoVec, as part of the ITI program of the University of Strasbourg, CNRS and Inserm, was supported by IdEx Unistra (ANR-10-IDEX-0002), and by SFRI-STRAT'US project (ANR-20-SFRI-0012) under the framework of the French Investments for the Future Program.

## Keywords

Heme enzyme; Enzyme catalysis; Cti, Metalloenzyme; *trans*-Unsaturated fatty acids; Isomerase.

## References

- [1] R.A. Makula, Phospholipid composition of methane utilizing bacteria, *J Bacteriol*, 134 (1978) 771-777.
- [2] F.T. Gillan, R.B. Johns, T.V. Verheyen, J.K. Volkman, H.J. Bavor, *trans*-Monounsaturated acids in a marine bacterial isolate, *Appl Environ Microbiol*, 41 (1981) 849-856.
- [3] H. Keweloh, H.J. Heipieper, *trans*-Unsaturated fatty acids in bacteria, *Lipids*, 31 (1996) 129-137.
- [4] I. Yumoto, K. Kawasaki, H. Iwata, H. Matsuyama, H. Okuyama, Assignment of *Vibrio* sp. strain ABE-1 to *Colwellia maris* sp. nov., a new psychrophilic bacterium, *Int J Syst Bacteriol*, 48 Pt 4 (1998) 1357-1362.
- [5] H.J. Heipieper, R. Diefenbach, H. Keweloh, Conversion of *cis*-unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol degrading *Pseudomonas putida* P8 from substrate toxicity, *Appl Environ Microbiol*, 58 (1992) 1847-1852.
- [6] H. Okuyama, S. Sasaki, S. Higashi, N. Murata, A *trans*-unsaturated fatty acid in a psychrophilic bacterium, *Vibrio* sp. strain ABE-1, *J Bacteriol*, 172 (1990) 3515-3518.
- [7] H. Okuyama, N. Okajima, S. Sasaki, S. Higashi, N. Murata, 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 (1991) 13-20.
- [8] N. Morita, A. Shibahara, K. Yamamoto, K. Shinkai, G. Kajimoto, H. Okuyama, 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 (1993) 916-918.
- [9] H.J. Heipieper, F. Meinhardt, A. Segura, 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 (2003) 1-7.
- [10] H.J. Heipieper, J. Fischer, F. Meinhardt, *cis-trans* Isomerase of unsaturated fatty acids: an immediate bacterial adaptive mechanism to cope with emerging membrane perturbation caused by toxic hydrocarbons, in: K.N. Timmis (Ed.) *Handbook of Hydrocarbon and Lipid Microbiology*, Springer Berlin Heidelberg, Berlin, Heidelberg, 2010, pp. 1605-1614.
- [11] C. Eberlein, T. Baumgarten, S. Starke, H.J. Heipieper, Immediate response mechanisms of Gram-negative solvent tolerant bacteria to cope with environmental stress: *cis-trans* isomerization of unsaturated fatty acids and outer membrane vesicle secretion, *Appl Microbiol Biot*, 102 (2018) 2583-2593.
- [12] R. Diefenbach, H. Keweloh, Synthesis of *trans*-unsaturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids, *Arch Microbiol*, 162 (1994) 120-125.
- [13] H.J. Heipieper, J.A. de Bont, Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes, *Appl Environ Microbiol*, 60 (1994) 4440-4444.
- [14] H.J. Heipieper, B. Löffel, H. Keweloh, J.A.M. De Bont, The *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida* S12 - an indicator for environmental stress due to organic compounds, *Chemosphere*, 30 (1995) 1041-1051.
- [15] J. Sikkema, J.A. de Bont, B. Poolman, Mechanisms of membrane toxicity of hydrocarbons,

Microbiol Rev, 59 (1995) 201-222.

[16] J. Sikkema, J.A. de Bont, B. Poolman, Interactions of cyclic hydrocarbons with biological membranes, J Biol Chem, 269 (1994) 8022-8028.

[17] M. Suutari, S. Laakso, Microbial fatty acids and thermal adaptation, Crit Rev Microbiol, 20 (1994) 285-328.

[18] R. Diefenbach, H.J. Heipieper, H. Keweloh, The conversion of *cis* into *trans*-unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity, Appl Microbiol Biot, 38 (1992) 382-387.

[19] K. Magnuson, S. Jackowski, C.O. Rock, J.E. Cronan, Jr., Regulation of fatty acid biosynthesis in *Escherichia coli*, Microbiol Rev, 57 (1993) 522-542.

[20] H.J. Janssen, A. Steinbuchel, Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels, Biotechnol Biofuels, 7 (2014) 7.

[21] M.D. Kiran, J.S. Prakash, S. Annapoorni, S. Dube, T. Kusano, H. Okuyama, N. Murata, S. Shivaji, Psychrophilic *Pseudomonas syringae* requires *trans*-monounsaturated fatty acid for growth at higher temperature, Extremophiles, 8 (2004) 401-410.

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

[23] F. Junker, J.L. Ramos, Involvement of the *cis-trans* isomerase (Cti) in solvent resistance of *Pseudomonas putida* DOT-T1E, J Bacteriol, 181 (1999) 5693-5700.

[24] H.J. Heipieper, G. Meulenbeld, Q. van Oirschot, J. de Bont, Effect of environmental factors on the *trans/cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12, Appl Environ Microbiol, 62 (1996) 2773-2777.

[25] Y.M. Zhang, C.O. Rock, Membrane lipid homeostasis in bacteria, Nat Rev Microbiol, 6 (2008) 222-233.

[26] N. Hachicho, P. Hoffmann, K. Ahlert, H.J. Heipieper, Effect of silver nanoparticles and silver ions on growth and adaptive response mechanisms of *Pseudomonas putida* mt-2, FEMS Microbiol Lett, 355 (2014) 71-77.

[27] A. Piotrowska, A. Syguda, L. Chrzanowski, H.J. Heipieper, Toxicity of synthetic herbicides containing 2,4-D and MCPA moieties towards *Pseudomonas putida* mt-2 and its response at the level of membrane fatty acid composition, Chemosphere, 144 (2016) 107-112.

[28] P. Kotchaplai, E. Khan, A.S. Vangnai, Membrane alterations in *Pseudomonas putida* F1 exposed to nanoscale zerovalent iron: effects of short-term and repetitive nZVI exposure, Environ Sci Technol, 51 (2017) 7804-7813.

[29] S. Isken, P.M.A.C. Santos, J.A.M. deBont, Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12, Appl Microbiol Biotechnol, 48 (1997) 642-647.

[30] B.E. Igiri, S.I.R. Okoduwa, G.O. Idoko, E.P. Akabuogu, A.O. Adeyi, I.K. Ejiogu, Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: A Review, J Toxicol, 2018 (2018) 2568038.

[31] A.J. Spiers, A. Buckling, P.B. Rainey, The causes of *Pseudomonas* diversity, Microbiology (Reading, Engl), 146 ( Pt 10) (2000) 2345-2350.

[32] W. Epstein, The roles and regulation of potassium in bacteria, Prog Nucleic Acid Res Mol Biol, 75 (2003) 293-320.

[33] G. Neumann, N. Kabelitz, H.J. Heipieper, The regulation of the *cis-trans* isomerase of unsaturated fatty acids in *Pseudomonas putida*: correlation between *cti* activity and K<sup>+</sup> uptake systems, Eur J Lipid Sci Tech, 105 (2003) 585-589.

[34] P.A. Lambert, S.M. Hammond, Potassium fluxes, first indications of membrane damage in microorganisms, Biochem Biophys Res Commun, 54 (1973) 796-799.

[35] J.B. Guckert, M.A. Hood, D.C. White, Phospholipid ester linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids, Appl Environ Microbiol, 52 (1986) 794-801.

[36] J. Fischer, F. Schauer, H.J. Heipieper, The *trans/cis* ratio of unsaturated fatty acids is not applicable as biomarker for environmental stress in case of long term contaminated habitats, Appl Microbiol Biot, 87 (2010) 365-371.

[37] F.J. Weber, J.A. de Bont, Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes, Biochim Biophys Acta, 1286 (1996) 225-245.

- [38] S. Isken, J.A. de Bont, Bacteria tolerant to organic solvents, *Extremophiles*, 2 (1998) 229-238.
- [39] R. Holtwick, H. Keweloh, F. Meinhardt, *cis-trans* isomerase of unsaturated fatty acids of *Pseudomonas putida* P8: evidence for a heme protein of the cytochrome-c type, *Appl Environ Microbiol*, 65 (1999) 2644-2649.
- [40] H. Okuyama, D. Enari, A. Shibahara, K. Yamamoto, N. Morita, Identification of activities that catalyze the *cis-trans* isomerization of the double bond of a monounsaturated fatty acid in *Pseudomonas* sp. strain E-3, *Arch Microbiol*, 165 (1996) 415-417.
- [41] H.J. Heipieper, P. de Waard, P. van der Meer, J.A. Killian, S. Isken, J.A. de Bont, G. Eggink, F.A. de Wolf, Regiospecific effect of 1-octanol on *cis-trans* isomerization of unsaturated fatty acids in the solvent tolerant strain *Pseudomonas putida* S12, *Appl Microbiol Biot*, 57 (2001) 541-547.
- [42] V. Pedrotta, B. Witholt, Isolation and characterization of the *cis-trans* isomerase of unsaturated fatty acid of *Pseudomonas oleovorans* GPo12, *J Bacteriol*, 181 (1999) 3256-3261.
- [43] A.M. Waterhouse, J.B. Procter, D.M. Martin, M. Clamp, G.J. Barton, Jalview version 2: a multiple sequence alignment editor and analysis workbench, *Bioinformatics*, 25 (2009) 1189-1191.
- [44] M.D. Kiran, S. Annapoorni, I. Suzuki, N. Murata, S. Shivaji, *cis-trans* Isomerase gene in psychrophilic *Pseudomonas syringae* is constitutively expressed during growth and under conditions of temperature and solvent stress, *Extremophiles*, 9 (2005) 117-125.
- [45] H. Okuyama, A. Ueno, D. Enari, N. Morita, T. Kusano, Purification and characterization of 9-hexadecenoic acid *cis-trans* isomerase from *Pseudomonas* sp. strain E-3, *Arch Microbiol*, 169 (1998) 29-35.
- [46] A. von Wallbrunn, H.H. Richnow, G. Neumann, F. Meinhardt, H.J. Heipieper, Mechanism of *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*, *J Bacteriol*, 185 (2003) 1730-1733.
- [47] H.J. Heipieper, G. Neumann, N. Kabelitz, M. Kastner, H.H. Richnow, Carbon isotope fractionation during *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*, *Appl Microbiol Biot*, 66 (2004) 285-290.
- [48] J. Beltrán, B. Kloss, J.P. Hosler, J. Geng, A. Liu, A. Modi, J.H. Dawson, M. Sono, M. Shumskaya, C. Ampomah-Dwamena, J.D. Love, E.T. Wurtzel, Control of carotenoid biosynthesis through a heme based *cis-trans* isomerase, *Nat Chem Biol*, 11 (2015) 598-605.
- [49] C. Chatgililoglu, C. Ferreri, M. Melchiorre, A. Sansone, A. Torreggiani, Lipid geometrical isomerism: from chemistry to biology and diagnostics, *Chem Rev*, 114 (2014) 255-284.
- [50] C. Chatgililoglu, A. Altieri, H. Fischer, The kinetics of thiyl radical induced reactions of monounsaturated fatty acid esters, *J Am Chem Soc*, 124 (2002) 12816-12823.
- [51] C. Chatgililoglu, C. Ferreri, *trans* Lipids: the free radical path, *Acc Chem Res*, 38 (2005) 441-448.
- [52] C. Chatgililoglu, A. Samadi, M. Guerra, H. Fischer, The kinetics of Z/E isomerization of methyl oleate catalyzed by photogenerated thiyl radicals, *ChemPhysChem*, 6 (2005) 286-291.
- [53] I.N. Lykakis, C. Ferreri, C. Chatgililoglu, The sulfhydryl radical (HS(·)/S(·)): a contender for the isomerization of double bonds in membrane lipids, *Angew Chem Int Ed Engl*, 46 (2007) 1914-1916.
- [54] C. Chatgililoglu, V.W. Bowry, Why Not *trans*? Inhibited radical isomerization cycles and coupling chains of lipids and alkenes with alkane thiols, *J Org Chem*, 83 (2018) 9178-9189.
- [55] C. Hartig, N. Loffhagen, H. Harms, Formation of *trans* fatty acids is not involved in growth linked membrane adaptation of *Pseudomonas putida*, *Appl Environ Microbiol*, 71 (2005) 1915-1922.
- [56] T. Kondakova, J.E. Cronan, Transcriptional regulation of fatty acid *cis-trans* isomerization in the solvent tolerant soil bacterium *Pseudomonas putida* F1, *Environ Microbiol*, 21 (2019) 1659-1676.
- [57] C. Pini, P. Godoy, P. Bernal, J.L. Ramos, A. Segura, Regulation of the cyclopropane synthase *cfaB* gene in *Pseudomonas putida* KT2440, *FEMS Microbiol Lett*, 321 (2011) 107-114.
- [58] Z. Tan, J.M. Yoon, D.R. Nielsen, J.V. Shanks, L.R. Jarboe, Membrane engineering *via trans*-unsaturated fatty acids production improves *Escherichia coli* robustness and production of biorenewables, *Metab Eng*, 35 (2016) 105-113.
- [59] P. Bernal, A. Segura, J.L. Ramos, Compensatory role of the *cis-trans* isomerase and cardiolipin synthase in the membrane fluidity of *Pseudomonas putida* DOT-T1E, *Environ Microbiol*, 9 (2007) 1658-1664.
- [60] A. Mazumder, A.N. Kapanidis, Recent advances in understanding sigma70 dependent transcription initiation mechanisms, *J Mol Biol*, 431 (2019) 3947-3959.
- [61] H. Cook, D.W. Ussery, Sigma factors in a thousand *E. coli* genomes, *Environ Microbiol*, 15 (2013)

3121-3129.

- [62] C.L. Lawson, D. Swigon, K.S. Murakami, S.A. Darst, H.M. Berman, R.H. Ebright, Catabolite activator protein: DNA binding and transcription activation, *Curr Opin Struct Biol*, 14 (2004) 10-20.
- [63] F. Rojo, Mechanisms of transcriptional repression, *Curr Opin Microbiol*, 4 (2001) 145-151.
- [64] D.J. Naether, S. Slawtschew, S. Stasik, M. Engel, M. Olzog, L.Y. Wick, K.N. Timmis, H.J. Heipieper, Adaptation of the hydrocarbonoclastic bacterium *Alcanivorax borkumensis* SK2 to alkanes and toxic organic compounds: a physiological and transcriptomic approach, *Appl Environ Microbiol*, 79 (2013) 4282-4293.
- [65] C. Löffler, C. Eberlein, I. Mausezahl, U. Kappelmeyer, H.J. Heipieper, Physiological evidence for the presence of a *cis-trans* isomerase of unsaturated fatty acids in *Methylococcus capsulatus* Bath to adapt to the presence of toxic organic compounds, *FEMS Microbiol Lett*, 308 (2010) 68-75.
- [66] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, *J Mol Biol*, 215 (1990) 403-410.
- [67] M. Hashimoto, Y. Orikasa, H. Hayashi, K. Watanabe, K. Yoshida, H. Okuyama, Occurrence of *trans*-monounsaturated and polyunsaturated fatty acids in *Colwellia psychrerythraea* strain 34H, *J Basic Microbiol*, 55 (2015) 838-845.
- [68] T. Kondakova, S. Kumar, J.E. Cronan, A novel synthesis of *trans*-unsaturated fatty acids by the Gram-positive commensal bacterium *Enterococcus faecalis* FA2-2, *Chem Phys Lipids*, 222 (019) 23-35.
- [69] M.F. Polz, E.J. Alm, W.P. Hanage, Horizontal gene transfer and the evolution of bacterial and archaeal population structure, *Trends Genet*, 29 (2013) 170-175.
- [70] P.H. Oliveira, M. Touchon, J. Cury, E.P.C. Rocha, The chromosomal organization of horizontal gene transfer in bacteria, *Nat Commun*, 8 (2017) 841.
- [71] M. Beganovic, M.K. Luther, L.B. Rice, C.A. Arias, M.J. Rybak, K.L. LaPlante, A review of combination antimicrobial therapy for *Enterococcus faecalis* bloodstream infections and infective endocarditis, *Clin Infect Dis*, 67 (2018) 303-309.
- [72] C. Ferreri, C. Chatgililoglu, Membrane lipidomics and the geometry of unsaturated fatty acids from biomimetic models to biological consequences, *Methods Mol Biol*, 579 (2009) 391-411.
- [73] C. Ferreri, S. Pierotti, C. Chatgililoglu, A. Barbieri, F. Barigelletti, Probing the influence of *cis-trans* isomers on model lipid membrane fluidity using *cis*-parinaric acid and a stop flow technique, *Chem Commun*, (2006) 529-531.