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Transcriptional regulation of fatty acid *cis*–*trans* isomerization in the solvent-tolerant soil bacterium, *Pseudomonas putida* F1

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Summary

One key to the success of *Pseudomonas* spp. is their ability to reside in hostile environments. *Pseudomonas* spp. possess a *cis*–*trans* isomerase (Cti) an enzyme that converts the *cis*-unsaturated fatty acids (FAs) of the membrane lipids to their *trans*-isomers to rigidify the membrane and thereby resist stresses. Whereas the posttranslational Cti regulation has been previously reported, transcriptional *cti* regulation remains to be studied in more details. Here, we have studied *cti* transcriptional regulation in the solvent-tolerant strain *Pseudomonas putida* F1. Two *cti* transcriptional start sites (*cti*-279 and *cti*-77) were identified with *cti*-279 transcript being dominant. Expression of *cti* was found to increase with temperature increase, addition of the organic solvent, octanol and in the stationary growth phase. We found that *cti* expression was repressed by the cyclic-AMP receptor protein (Crp) and repression required the cyclic-AMP ligand of Crp. Production of *trans*-unsaturated FAs was found to decrease after 24 h of growth. Although this decrease was accompanied by an increase in cyclopropane FA content, this was not at the expense of *trans*-unsaturated FAs demonstrating the absence of competition between Cti and Cfa in FA modification.

Introduction

Pseudomonas spp. are ubiquitous bacteria that thrive in a wide variety of environments (Mena and Gerba, 2009; De las Heras and De Lorenzo, 2011). The remarkable ecological success of *Pseudomonas* spp. can be attributed to the metabolic versatility encoded by their large genomes and the presence of sophisticated mechanisms to adjust membrane fluidity. Adjustment of membrane fluidity is accomplished by changes in the fatty acid (FA) composition of membrane lipids. For instance, *Pseudomonas* spp. are capable to post-synthetically transform *cis*-unsaturated FA (*cis*-UFA) into their *trans* isomers (*trans*-UFA) without changing the position of the double bond (von Wallbrunn et al., 2003). Based on their stereochemistry *trans*-UFA mimic, saturated FA (SFA) imparts rigidity and a tight packing of the membrane lipids, whereas *cis*-UFA gives membranes having relatively high fluidity (Okuyama et al., 1990). Enzymatic isomerization of *cis*-UFA to *trans*-UFA is

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Conflict of interest

We have no conflicts of interest to declare.

catalysed by Cti and does not depend on the *de novo* FA and protein synthesis, ATP or any other cofactor (Pedrotta and Witholt, 1999; Heipieper et al., 2003), presenting an efficient mean to rigidify the membrane in the response to changing environments. Cti, which located in the cellular periplasm (Pedrotta and Witholt, 1999), is a cytochrome *c*-type protein, bearing a heme-binding motif essential for the *cis* to *trans* isomerization reaction (Holtwick et al., 1999). Moreover, the highest levels of the *trans*-UFA were found 30 min after the initiated stress, demonstrating *cis*–*trans* isomerization to be a fast adaptive response (Heipieper et al., 1992; Eberlein et al., 2018).

Cyclopropane FA synthase Cfa also uses phospholipid bound *cis*-UFA as a substrate to replace the double bond with a methylene bridge to give cyclopropane FA (cyclo-FA) (Pohl et al., 1963; Wang et al., 1992). As *trans*-UFA, cyclo-FA stabilizes membranes against adverse conditions such as acidic conditions (Chang and Cronan, 1999; Poger and Mark, 2015). Cfa is present in most bacteria, whereas Cti is much rarer and to date is found only in a few proteobacterial species (Heipieper et al., 2010), among which *Pseudomonas putida* seems one of the more interesting to study due to its ability to grow in soils and sediments contaminated with high concentrations of heavy metals and organic contaminants (Ramos et al., 1995; Wu et al., 2011). *P. putida* is of particular interest in biotechnology because of its high potential in bioremediation and biodegradation processes (Loh and Cao, 2008; Samuel et al., 2014). Thus, several reports report that *trans*-UFA plays an important role in the adaptation of diverse *P. putida* strains to the increase in temperature, the presence of organic solvents and heavy metals, as well as osmotic stress and addition of membrane-active antibiotics (Heipieper et al., 1996; Isken et al., 1997; Neumann et al., 2003). It is generally accepted that *cti* is constitutively expressed in *Pseudomonas* spp. (Kiran et al., 2004) and proposed to be regulated only post-translationally by the membrane fluidity (Eberlein et al., 2018). However, Bernal and colleagues (2007) reported the upregulation of *P. putida cti* expression in response to the toluene addition, raising the question about a possible transcriptional *cti* regulation in this bacterium. Here, we report a detailed assessment of the *cti* transcriptional regulation in *P. putida* F1, a soil isolate of known genomic sequence that is resistant to organic solvents (Zylstra et al., 1988). We have characterized the *cti* promoter region and defined two *cti* transcriptional start sites (TSS). The effect of stress factors on *cti* expression was accessed. Hence, we found that *cti* expression is upregulated by temperature increases, solvent addition, as well as in stationary growth phase. We also found that the cyclic-AMP receptor protein (Crp)–cyclic-AMP complex represses *cti* expression and that *cti* is co-transcribed with downstream genes. In addition, this study resolved the question of competition between Cti and Cfa in bacterial membrane modification.

Results

Formation of *trans*-UFA occurs in *P. putida* F1 during growth

The production of *trans*-UFA in *P. putida* during growth has been documented (Diefenbach et al., 1992; Heipieper et al., 1996). However, several studies reported that some *P. putida* strains produce *trans*-UFA only in stress or nongrowing conditions, such as during sample preparation (Härtig et al., 2005; Loffhagen et al., 2007). To ask if *P. putida* F1 produces *trans*-UFA in the absence of stress, cultures were grown at the optimal growth temperature

of 30°C for 24 h and treated with the powerful chaotropic agent 10% trichloroacetic acid (TCA) to immediately stop bacterial metabolism (Supporting Information Fig. S1A). FA methyl esters (FAME) were using a CP-Sil 88 column which gave a well-separated peak corresponding to C16:1-*trans* (Supporting Information Fig. S1B). To test if the formation of C16:1-*trans* is not the artefact of TCA treatment, FAME of *Escherichia coli* K-12 MG1655, known to be unable to form *trans*-UFA, were tested using the same protocol. No *trans*-UFA was detected in MG1655 samples. Altogether, these data demonstrated that *P. putida* F1 produces *trans*-UFA during the growth rather than during the stress of handling.

The pattern of *cti* transcription in *P. putida* F1

We have dissected the *cti* promoter region in *P. putida* F1 to investigate the response of *cti* expression to environmental stimuli. Multiple studies in *Pseudomonas* spp. have sought to define the *cti* TSS (Junker and Ramos, 1999; Kiran et al., 2005; Bernal et al., 2007). However, these studies targeted *Pseudomonas* spp. strains of undetermined genome sequence and each identified a different *cti* promoter. To more systematically characterize *cti* promoter region in *P. putida* F1, we performed RLM-RACE analysis. Two *cti* TSS regions were found located at positions -77 and -279 upstream of the *cti* translational start site (+1) (Fig. 1A and C). Similar data were reported in *P. putida* DOT-T1E, where the *cti* TSS was placed at position -294 (Bernal et al., 2007). Hexamer sequences resembling those proposed to be recognized by the housekeeping σ^{70} RNA polymerase of *Pseudomonas* spp. (Ojangu et al., 2000; Domínguez-Cuevas and Marqués, 2004; Vakulskas et al., 2009) were found within the -10 and -35 regions of each of the in *P. putida* F1 TSS we detected (Fig. 1D).

To investigate the activity of the two *cti* promoters, P₁ (TSS at -77) and P₂ (TSS at -279), we constructed two translational fusions in which one of the promoters drove β -galactosidase expression (*cti*'-'*lacZ* fusions). The pFCtiPP1 'long' promoter region fusion contained both P₁-77 and P₂-279, whereas the pFCtiPP2 fusion corresponded to the 'short' promoter region containing only P₁-77 (Fig. 1E). The β -galactosidase activity of the long pFCtiPP1 fusion was 26-fold higher than that of the pFCtiPP2 fusion, which showed a very low activity (7.1 Miller units) (Fig. 1B), indicating that in the tested conditions expression from the P₂-279 promoter is dominant. To test if the low β -galactosidase activity of pFCtiPP2 fusion was not an experimental artefact, we constructed the pFCtiPP0 plasmid, which had P₁-77 and P₂-279 promoters but lacked *cti* ribosome-binding site (RBS) and *cti* translational start site. This construct showed significantly lower activity (0.5 Miller units) than seen in pFCtiPP2, demonstrating that the P₁-77 promoter is able to express the *cti* gene. We next constructed deletions within P₁-77 and P₂-279 promoters. Deletion of the putative P₁-77 -10 region (pFCtiPP1KO) resulted in an insignificant decrease in β -galactosidase activity. In contrast, deletion of putative -10 region of the P₂-279 promoter (pFCtiPP2KO) showed a significant drop in *cti* expression.

To ask if P₁-77 and P₂-279 are conserved in *P. putida* strains, we aligned *cti* sequences in all *P. putida* strains having genomes available in NCBI database (last accessed 27 December 2018). All *P. putida* strains possessed the *cti* coding sequences. In agreement with previously reported high level of sequence conservation of *P. putida* the core genome (Udaondo et al., 2016), *cti* and its 5'UTR were well conserved. For instance, *cti* of *P. putida* F1 and DOT-T1E

strains (Udaondo et al., 2012), *cti* of which was previously described (Bernal et al., 2007), has 99% identity, although the lowest *cti* identity of 77% was found between the F1 and JBC17 strains. Whereas P₂-279 promoter was highly conserved in all tested strains, P₁-77 was missing in strains E46, E41, PC2, NX-1, IEC33019 and W619 strains (Supporting Information Fig. S2).

Altogether, these data indicate that the predominant *cti* expression initiates from the well-conserved P₂-279 promoter and *cti* is moderately expressed without stressors addition.

Addition of octanol activates *cti* expression

The effect of addition of organic solvents to cultures of *Pseudomonas* spp. on *trans*-UFA production has been the subject of multiple studies (Junker and Ramos, 1999; Kiran et al., 2005; Heipieper and Fischer, 2010) and showed that addition of various organic solvents activated *trans*-UFA production. Thus, the ability of organic solvents to activate *trans*-UFA production was positively correlated with solvent hydrophobicity (Heipieper et al., 1995; Weber and de Bont, 1996; Ramos et al., 2002). Highly hydrophobic solvents partition preferentially into the membranes, where they were supposed to cause an increase of membrane fluidity, which would be offset by increased *trans*-UFA production. Based on these reports, a model of Cti regulation by membrane fluidity has been proposed (Heipieper et al., 2010). According to this model, an increase in membrane fluidity engendered by addition of organic solvents allows the Cti active centre to reach the double bond to catalyse *trans*-UFA production (Heipieper et al., 2010). However, studies of the effect of organic solvents on *cti* expression are few. To test if the addition of organic solvents modulates *cti* expression in *P. putida* F1, the pFCtiPP1 and pFCtiPP2 fusions were assayed in octanol-treated cultures (Fig. 2A). In *P. putida* F1, treatment with different concentrations of this solvent gave a moderate dose-dependent activation of the expression of the pFCtiPP1 and the pFCtiPP2 fusions (Fig. 2B). The *cti* expression from P₂-279 and P₁-77 was 1.4- and 1.6-fold higher in 2.5 mM octanol-treated cultures relative to the untreated cultures. These data agree with previously reported results (Bernal et al., 2007) and parallel others (Junker and Ramos, 1999; Kiran et al., 2005). The basal level of *trans*-UFA in untreated *P. putida* F1 carrying pFCtiPP1 was already high, due to the tetracycline needed to maintain the *'lacZ* fusions (Isken et al., 1997) and did not change significantly with octanol treatment (Fig. 2C). However, when tetracycline was omitted, a dose-dependent increase of *trans*-UFA production was seen with addition of octanol in agreement with previously obtained results (Heipieper et al., 1995). Importantly, the concentrations of octanol used in this study allowed bacterial survival after 1 h of exposure (Fig. 2D).

Previously, *P. putida* P8 cells were found to reach their highest *trans* to *cis* ratio 30 min after addition of the membrane-toxic agent 4-chlorophenol (Heipieper et al., 1992). To ask if octanol treatment produced the similar effect, we assayed the time course of formation of *trans*-UFA in *P. putida* F1 treated with 2.5 mM octanol. In agreement with (Heipieper et al., 1992), the highest *trans*-UFA levels were found 30 min after solvent addition (Fig. 2E). The *cti* expression was activated 20 min after addition of 2.5 mM octanol to bacterial cultures (Fig. 2F), indicating the possible link between octanol-dependent activation of *cti* expression and increase in *trans*-UFA production.

Altogether, these results show that the treatment with at least 2.5 mM octanol activates *cti* expression. Even if the *trans*-UFA production increased when cultures were treated with lower than 2.5 mM octanol concentrations, the highest amount of *trans*-UFA was detected in 2.5 mM octanol treated cells. This indicates a possible correlation between solvent-dependent activation of *cti* expression and *trans*-UFA production in *P. putida* F1. The activation of *cti* expression 20 min after octanol addition followed by the formation of highest *trans*-UFA levels 30 min after solvent addition supports the hypothesis of correlation between *cti* expression and *trans*-UFA production during the solvent addition stress.

Temperature increase and stationary growth phase active the *cti* expression

Previous studies of the effects of temperature on *trans*-UFA production in *P. putida* P8 showed that *trans*-UFA plays an important role in bacterial adaptation to temperature increase when *de novo* FA synthesis is blocked. However, when *de novo* FA synthesis was active, no significant changes in *trans*-UFA production were detected in response to temperature raise (Diefenbach et al., 1992). We used the *P. putida* F1 pFCtiPP1 and pFCtiPP2 fusions to assess the effect of growth temperature on *cti* expression. Given that the production of *trans*-UFA is considered a fast-adaptive response, we first tested the temperature shift effect. As reported by Diefenbach and colleagues (1992), cultures were grown at optimal growth temperature 30°C until the mid-exponential growth phase and then the growth temperature was shifted to 20 or 37°C for 3 h. The *cti-279* and *cti-77* expression levels increased with the temperature increase to 37°C and decreased with temperature decrease to 20°C (Fig. 3A). However, the temperature shift produced the opposite effect on the *trans*-UFA synthesis; the amount of *trans*-UFA in cultures growing at 20°C was slightly higher than seen in cultures growing at 37°C (Fig. 3B). The same trend was seen in cultures growing with or without tetracycline addition, allowing us to use tetracycline to maintain the reporter plasmids. To ask if this temperature-dependent *cti* activation is maintained at different growth stages, cultures were grown at 20, 30 and 37°C and the samples were taken at different growth stages for β -galactosidase and FA analyses (Fig. 3C). In agreement with previously reported results, the highest *cti-279* and *cti-77* expression levels were found in cultures growing at 37°C, whereas at 20°C, *cti-279* and *cti-77* expression levels were significantly lower than seen in cultures grown at the higher temperatures (30 and 37°C) (Fig. 4D). This temperature-dependent induction was particularly high in stationary growth phase. At all tested temperatures, *cti* expression was significantly higher in stationary growth phase comparing to exponential growth phase. This agrees with results reported for *P. putida* DOT-T1E (Bernal et al., 2007), showing an increase in *cti* expression in stationary growth phase, and parallel others reporting constitutive *cti* expression in *Pseudomonas* spp. (Junker and Ramos, 1999; Kiran et al., 2005).

In agreement with temperature shift results (which corresponded to point 2 in Fig. 3C–E), the *trans*-UFA content was slightly lower in cultures growing at 37°C relative to cultures growing at 20°C in all growth phase, except exponentially growth phase. Interestingly, at all tested temperatures, the *trans*-UFA content of the cells significantly decreased after 24 h of bacterial growth (Fig. 3E). This decrease was followed by a significant increase of cyclo-FA. It is, consistent with previous reports, found the cyclo-FA to be predominantly produced in stationary phase of growth (Wang and Cronan, 1994; Grogan and Cronan, 1997). Taken

together these data indicated that in *P. putida* F1, *cti* expression is activated by temperature rise and, in stationary phase of growth, conditions in adaptation to which *trans*-UFA were reported to play an important role. The mismatch between the fraction of *trans*-UFA and *cti* expressions suggested the possible post-transcriptional control of *trans*-UFA production in *P. putida* F1. In addition, the decrease in *trans*-UFA production after 24 h of bacterial growth in favour of cyclo-FA overproduction suggested the possibility of competition between Cti and Cfa.

The absence of competition between Cti and Cfa

Previous studies tested the competition between Cti and Cfa in early stationary growth phase (corresponding to point 3 in Fig. 3C–E) and suggested the absence of competition between those two enzymes in *P. putida* KT2440 (Pini et al., 2011, 20,100). We observed decreased *trans*-UFA and increased cyclo-FA production after 24 h of growth (starting at point 4 in Fig. 3C–E), which raised a question about the possibility of competition between Cti and Cfa after 24 h of growth. To test this possibility, we first made the knockout mutants of the corresponding genes in *P. putida* F1 and compared the FA composition of the mutant strains with that of the WT strain at different stages of growth. The *P. putida* F1 *cti* (PPut3319) strain produced a level of cyclo-FA indistinguishable from that of the WT strain (Fig. 4A). However, after 24 h of growth the *P. putida* F1, *cfaB* (PPut5273) strain produced twice the level of *trans*-UFA seen in the WT strain. Complementation with *cfaB* gene in *trans* (PPut5273C) was effective and the complemented strain produced less *trans*-UFA than the mutant. These results supplement those obtained in a previous report (Pini et al., 2011) and have multiple interpretations. The competition between Cti and Cfa aside, the inability of the *cfaB* strain to synthesize cyclo-FA can alter membrane fluidity. Given that both cyclo-FA and *trans*-UFA are able to order the membrane and decrease membrane fluidity (Loffhagen et al., 2007), *P. putida* F1 *cfaB* may overproduce *trans*-UFA to compensate for the absence of cyclo-FA.

To dissect this possibility, we made an *E. coli fabA cfa* mutant strain (TK411) that is unable to synthesize *cis*-UFA (and hence cyclo-FA) and expressed *P. putida* F1 *cti* in this strain (*fabA cfa cti*, TK422). As a control, we used the *fabA cfa cti* G23 strain (TK436), which was unable to form *trans*-UFA due to a frame-shift (deletion of base G23) in the *cti* sequence. The culture medium was supplemented with either a *cis*-UFA or a cyclo-FA to allow growth of the strain. When fed with *cis*-UFA, *fabA cfa cti* strain formed *trans*-UFA. However, the fraction of *trans*-UFA did not exceed 9.6% of the total FA and remained quite stable for 13 h of *cti* induction (Fig. 4B). Contrary to our expectations, the fraction of *cis*-UFA increased with *cti* induction, suggesting the absence of competition between Cti and Cfa, as well as an important role of *cis*-UFA in *trans*-UFA-forming bacteria. At 6 h of *cti* induction, strain *fabA cfa cti* contained a significantly lower SFA content compared to the *cti* frameshift strain *fabA cfa cti* G23. This difference was most striking at 13 h of *cti* induction, when the strain *fabA cfa cti* contained 2.5-fold more *cis*-UFA (thus 2.5-fold less SFA) than *fabA cfa cti* G23 strain. When fed with the cyclo-FA, no *trans*-UFA was formed and no large change in membrane FA composition was found. However, at 13 h of *cti* induction *fabA cfa cti* had 10% more cyclo-FA and 10% less SFA than *fabA cfa cti* G23, whereas the FA compositions of strains *fabA cfa cti* G23 and *fabA cfa* were

similar. These data indicated the absence of competition between Cti and Cfa within the bacterial membrane and suggested that *E. coli* can adapt the membrane FA composition to *trans*-UFA production by increasing the fraction of *cis*-UFA into the membrane.

Cti toxicity

Interestingly, the growth of *cti* expressing strain *fabA cfa cti* was completely inhibited after induction of the arabinose pBAD promoter driving its expression whereas *fabA cfa cti G23* grew as well as *fabA cfa* (Fig. 4C). This growth defect was independent of *trans*-UFA production, as shown when *fabA cfa cti* was fed with cyclo-FA. Hence, toxicity seemed due to the Cti protein *per se* rather than its action. Given that Cti is a periplasmic protein (Pedrotta and Witholt, 1999), its overexpression could result in a lethal jamming of the *E. coli* secretion machinery (Shuman and Silhavy, 2003). To test this possibility, we deleted the *cti* signal peptide sequence (*cti* SP) and assayed the growth of *fabA cfa cti* SP strain (TK455). This strain failed to produce any detectable amount of *trans*-UFA and had a membrane FA composition identical to that of strains *fabA cfa* and *fabA cfa cti G23* (Fig. 4B), indicating that the periplasmic location of Cti is necessary to produce *trans*-UFA. Moreover, this bacterium grew as well as the *fabA cfa* and *fabA cfa cti G23* strains, arguing that Cti toxicity in *E. coli* is very likely due to the lethal jamming of secretion system.

Crp represses *cti* transcription in *P. putida* F1

Upon binding cAMP, Crp is one of the most important transcription factors in *E. coli* (Deutscher, 2008; Fic et al., 2009). When the cAMP–CRP complex acts on a binding site for gene regulation, it has one of the two opposing regulatory roles: activation or repression of gene transcription (Mori and Aiba, 1985; Yang et al., 2014). We analysed the *P. putida* F1 *cti* promoter region and identified two putative Crp-binding sequences (Crp1 and Crp2, Fig. 5A). The Crp1-binding region was located downstream of the *cti*-77TSS, close to the RBS sequence. The Crp2-binding region was located just downstream of the RNA polymerase –35 binding site of *cti*-279 promoter. Similar Crp-binding sites were predicted by PredCRP-model, which assumes that when Crp binds to these regions, it blocks the transcription process (Tsai et al., 2018).

Previous studies showed that the properties of *E. coli* Crp regarding its interaction with target DNA sequences have been preserved in the *P. putida* counterpart. Moreover, threading of the *P. putida* Crp sequence on the crystal structure of *E. coli* Crp strengthens the notion that they are not just two related proteins but are essentially the same protein with minor changes, which do not affect its key functions (Milanesio et al., 2011). Given the difficulties reported in the production of pure and high-activity *P. putida* Crp protein (Arce-Rodríguez et al., 2012), we purified *E. coli* Crp and performed electrophoretic mobility shift assays (EMSA) to test if Crp binds the *cti* promoter sequence *in vitro* (Fig. 5B). The cAMP–CRP complex bound the *P. putida* F1 *cti* promoter region (P*cti*), whereas binding was not observed in the absence of cAMP or to the *P. putida* F1 *cfa* promoter region (P*cfa*). We next deleted the Crp-binding sites from P*cti* (giving P*cti* crp1 and P*cti* crp2) and tested Crp binding to obtained DNA fragments. Crp bound P*cti* crp2 as well as P*cti*, whereas no binding was observed to P*cti* crp1, indicating that the Crp1 site is necessary for Crp

binding. In addition, both Crp1- and Crp2-binding sites were conserved in *P. putida* strains (Supporting Information Fig. S2), indicating the possible Crp-dependent *cti* regulation in several *P. putida* strains.

To test *in vivo* if Crp regulates *P. putida* F1 *cti* transcription, we made a *P. putida* F1 *crp* mutant strain (PPut0457) and assayed the activity of the pFCtiPP1 fusion in this strain. This mutant showed a significant increase in β -galactosidase activity comparing to WT (Fig. 5C) indicating that Crp represses *cti* expression in *P. putida* F1. Complementation with *crp* gene was effective and the complemented strain (PPut0457C) showed lower expression of the pFCtiPP1 fusion than did the mutant strain. We also assayed the activity of the pFCtiPP1crp1KO strain and pFCtiPP1crp2KO fusion strains containing *Pcti* crp1 and *Pcti* crp2 DNA fragments, respectively, in *P. putida* F1 WT (Fig. 5D). The activity of pFCtiPP1crp1 KO was modest and growth stage independent, suggesting that Crp1-binding site may play a dual and important role in *cti* expression. The activity of pFCtiPP1crp2KO was also growth stage independent but significantly higher than that of pFCtiPP1 in exponential growth phase and similar to it in stationary growth phase. This indicated that Crp2-binding site may play a role in *cti* regulation in exponential growth phase by a Crp independent mechanism. In agreement with PredCRP-model (Tsai et al., 2018), our data showed that Crp represses *cti* expression. However, our EMSA results suggested that only the Crp1 site located distal downstream of *cti* promoter sequences is necessary for Crp binding, highlighting the importance of validation of the *in-silico* studies by experimental assays.

***cti* is co-transcribed with downstream genes in *Pseudomonas* spp.**

Previous studies assumed that *cti* is transcribed as a monocistronic unit (Junker and Ramos, 1999; Heipieper et al., 2003). We analysed the *cti* genomic environment in *Pseudomonas* spp. using the MaGe platform. In all available *Pseudomonas* spp., *cti* was the first gene downstream of which one or several genes transcribed at the same direction were located (Fig. 6A). To ask if these genes are transcribed in operon with *cti*, we used RT-PCR in octanol-treated *P. putida* F1 and *P. aeruginosa* PAO1 cells. In both strains, cDNA amplifications using primers specific for *cti* and downstream genes were obtained (Fig. 6B and C), indicating that in tested experimental conditions, *cti* is co-transcribed with the downstream genes. Gene PA1847 in *P. aeruginosa* PAO1, as well as Pput_3317 in *P. putida* F1, is annotated as coding for NfuA, a protein involved in Fe/S biogenesis in *E. coli* (Angelini et al., 2008) and *Acinetobacter baumannii* (Zimblet et al., 2012). The gene Pput_3318 in *P. putida* F1 is annotated as acyltransferase 3 with unknown function. To ask if these genes are involved in *trans*-UFA synthesis in *Pseudomonas* spp., we made nonpolar knockout mutants of both genes and assayed FA composition of obtained mutant strains. Neither mutant strain showed a significant difference in *trans*-UFA content compared to the WT strain (Fig. 6D), indicating that these genes are not involved in *trans*-UFA synthesis.

Discussion

The *Pseudomonas* spp. comprise one of the most widely distributed groups of bacteria. Consistent with this wide distribution, these bacteria developed several resistance

mechanisms to respond to diverse environmental stresses (Rainey, 1999; Monteil et al., 2016; Moradali et al., 2017). One mechanism is membrane FA modification by the conversion of *cis*-UFA to their *trans* isomers (Heipieper and Fischer, 2010). Although the first report of *trans*-UFA in *Pseudomonas* was 31 years ago (Guckert et al., 1987), the regulation and mechanism of their formation remain a puzzle. We have examined *cti* transcriptional regulation in the soil-borne solvent degrader *P. putida* F1, competition between its Cti and Cfa enzymes, as well as the toxicity of expression of *P. putida* F1 Cti in *E. coli*.

First, our data demonstrate that *P. putida* F1 produces *trans*-UFA during the growth. This agrees with several previous reports (Diefenbach and Keweloh, 1994; Heipieper et al., 1996) and disagrees with other reports (Härtig et al., 2005; Loffhagen et al., 2007). This could be due to technical issues or possibly differences in the strains studied. This is the first report of *cti* action and regulation in the otherwise heavily studied *P. putida* F1 strain.

We identified two *P. putida* F1 *cti* TSS. Our data showed that in *P. putida* F1, *cti* is mainly expressed from the P₂-279 promoter, which is highly conserved in all available *P. putida* strains. Whereas *cti* was expressed at basal level without stressors addition, promoter P₂ is moderately activated upon addition of organic solvent octanol, as well as in stationary growth phase. These results are similar to those obtained in a previous report (Bernal et al., 2007) and are in contrast to two other studies reporting constitutive *cti* expression (Junker and Ramos, 1999; Kiran et al., 2005). In the first report, the Ramos research group used a Cti::PhoA fusion. PhoA becomes active only in the periplasmic space (Junker and Ramos, 1999), which raises the possibility that effects on *cti* expression could have been masked by effects on secretion. In our work as well as in that reported by Bernal and colleagues (2007) expression of a Cti::LacZ fusion was monitored. The second report was focused on *cti* expression in *P. syringae* Lz4W strain. Given the genetic heterogeneity of the genus *Pseudomonas* (Bodilis et al., 2012), *cti* expression in *P. putida* and in *P. syringae* species could well differ markedly.

The previously reported constitutive *cti* expression in solvent-treated bacteria led to the establishment of a Cti regulation model, according to which Cti activity is not controlled at the transcriptional level, but rather by simply allowing or prohibiting the active center of the enzyme to reach the double bond, which would depend only on membrane fluidity (Heipieper et al., 2010; Eberlein et al., 2018). The activation of *cti* expression in octanol-treated *P. putida* F1 cultures observed in our studies demonstrates that in response to solvent addition *cti* is regulated at the transcriptional level. The quick activation of *cti* expression after solvent addition followed by overproduction of *trans*-UFA allows us to propose that *cti* overexpression is activated to respond to high solvent concentrations and leads to the production of highest *trans*-UFA levels necessary to maintain the optimal membrane fluidity. Thus, our study adds an additional level of Cti regulation to the previous model, showing the complexity of the mechanisms that regulate Cti action. However, Cti overexpression in *E. coli* led to stable but modest production of *trans*-UFA. Given that *P. putida* S12 was found to have only 2–3 copies of Cti per cell (Neumann et al., 2003) and Cti expression in *E. coli* would be expected to result in greater *trans*-UFA production than we observed. Thus, our data agree with Heipieper *et al.* model, indicating possible postsynthetic Cti regulation.

However, future studies are necessary to test this possibility, as well as the role of membrane fluidity in Cti regulation.

We found that *cti* expression is induced by increased growth temperature. The effect of growth temperature on *trans*-UFA production is well documented in *P. putida* (Diefenbach et al., 1992) and in *Vibrio* spp. (Okuyama et al., 1990, 1991). However, to the best of our knowledge, this is the first report of modulation of *cti* expression by growth temperature. However, production of *trans*-UFA did not increase with increasing temperature in agreement with a previous report (Diefenbach et al., 1992). This mismatch between *cti* expression and production of *trans*-UFA suggests posttranscriptional Cti regulation in *Pseudomonas* spp. and requires future study. Posttranscriptional Cti regulation could also explain the decreased *trans*-UFA production in stationary growth phase, the stage of bacterial growth at which we found the highest *cti* expression. Previously, the effect of growth phase on the production of *trans*-UFA in *P. putida* was only tested at the transition from exponential to stationary growth phase (Diefenbach et al., 1992).

The decrease in *trans*-UFA production followed by the increase in cyclo-FA raised the question of possible competition between Cti and Cfa since they modify the same substrate. Previous studies suggested the absence of competition between those two enzymes in early stationary growth phase (Pini et al., 2011, 2010), whereas we assayed the competition at different stages of the growth of *P. putida* F1 cultures and also in an *E. coli* model we constructed. First, the *P. putida* F1 *cfaB* strain produced more *trans*-UFA than the WT strain after 24 h of growth. However, the *E. coli* model indicated the absence of competition between Cti and Cfa. In contrast to our expectations, the fraction of *cis*-UFA in *E. coli* membrane increased with the induction of *cti*, indicating that *cis*-UFA are incorporated into the bacterial membrane in higher quantities to offset the *trans*-UFA production and optimize membrane fluidity. Hence, we propose that the increase in *trans*-UFA formation in *P. putida* F1 *cfaB* is not due to the competition between Cti and Cfa, but to the increased production of this FA perhaps to optimize membrane fluidity in the absence of cyclo-FA. These data complement previous report (Pini et al., 2011) and add additional information about Cti and Cfa competition. In addition, these assays led us to discover a rationale for Cti toxicity in *E. coli* cells, which is probably caused by lethal jamming of *E. coli* secretion machinery. However, the observed decrease in SFA content in *E. coli* expressing Cti could also explain Cti toxicity. Given that *E. coli* does not encode a Cti enzyme, FabR-dependent membrane regulation (Zhu et al., 2009), as well as the functioning of several membrane proteins (Bogdanov et al., 2008; Dowhan and Bogdanov, 2011), could be impaired when *trans*-UFA are produced leading to the observed toxic effect.

In *E. coli* and many other bacteria, Crp plays a role of catabolite repressor (Brückner and Titgemeyer, 2002; Deutscher, 2008), whereas Crp ortholog in *P. aeruginosa* (named Vfr) is mostly associated with virulence factors' expression (Fuchs et al., 2010). In *P. putida*, Crp is not involved in metabolic control (Milanesio et al., 2011) and to date its role in this bacterium remains unelucidated. Here, for the first time, we showed that Crp represses *cti* expression in *P. putida* F1, thereby demonstrating one of the first Crp targets in *P. putida*. Interestingly, most phenotypes of *P. putida* *crp* mutant were previously shown to be traced to envelope-associated functions (peptide permeases, membrane-disrupting agents)

(Milanesio et al., 2011). Our study adds an additional envelope-associated Crp target in *P. putida* and indicates that *P. putida* Crp could be associated with the regulation of cell envelope composition. In addition, our EMSA studies complemented previously reported PredCRP model (Tsai et al., 2018), by showing that even in the presence of two putative Crp-binding sites, only the downstream site is necessary for Crp binding and repression of *cti* expression (Fig. 5E). Despite the general assumption that repressors of prokaryotic operons act exclusively by preventing the onset of transcription, Crp seems to bind only Crp1 site located distal downstream of *cti* promoters and not to the Crp2 site located within the DNA sequence covered by promoter-bound RNA polymerase. Similar results were shown for *lac* operon regulation, where a *lac* repressor-operator complex located downstream of the promoter sequence can directly interfere with gene expression by efficiently terminating transcription (Deuschle et al., 1986).

The results presented here provide the most detailed assessment of *cti* transcriptional regulation in any bacteria to date. This study brings a stone in understanding of *Pseudomonas* spp. adaptation to environmental stimuli and can be useful to develop the strategies for biodegradation of toxic compounds in natural environments.

Experimental procedures

Strains and growth conditions

Strains and bacterial plasmids used in this study are listed in Supporting Information Table S1, and details of their construction are in the Supporting Information. *Escherichia coli* K-12 derivatives were grown at 37°C in LB, SOC or M9 medium. Diaminopimelic acid (1 mM) was added to the media for the growth of *E. coli* WM6029. *P. aeruginosa* PAO1 and *P. putida* F1 were grown at 37 and 30°C, respectively, in LB or *Pseudomonas* minimal medium (PMM) with sodium succinate as carbon source (Kirner et al., 1996). Given that the growth conditions are relevant for the *cis-trans* isomerization, all assays were performed in PMM with vigorous shaking (250 r.p.m.) and the constant aeration (the volume of cultures represented 10% of the total flask volume). For plasmid maintenance, PMM was supplemented with 20 µg ml⁻¹ tetracycline hydrochloride (Tet). Oligonucleotides used in this study are listed in Supporting Information Table S2. For plasmid maintenance in *E. coli*, the medium was supplemented with (in µg ml⁻¹) kanamycin sulfate (Km) and gentamicin sulfate (Gm), 50 or Tet, 10. The antibiotic concentration (in µg ml⁻¹) used in LB medium for *P. putida* F1 was Km, Tet and Gm, 50 whereas the concentrations for *P. aeruginosa* PAO1 were Km, 1000 and Gm, 50. The detailed media composition is in the Supporting Information.

Extraction of total RNA, cDNA synthesis and amplification

Total RNA was purified using RNease Mini Kit (Qiagen). Details of this procedure are provided in SI Appendix, Supplemental Materials and Methods. RNAs were non-specifically converted to single stranded cDNAs using the ProrScript First Stand cDNA Synthesis Kit (NEB Biolabs). The control samples, RT⁻ (lacking reverse transcriptase) were made during cDNA synthesis from the total RNA using the ProrScript First Stand cDNA

Synthesis Kit without the addition of reverse transcriptase. All experiments were performed in three biological replicates.

RLM-race

The 5' ends of *cti* mRNA in *P. putida* F1 were mapped using RLM-RACE according to the manufacturer's instructions. Detailed procedure is reported in the Supporting Information. To identify the 5' ends of the *cti* mRNA, the PCR products were cloned into vector pMTL23P (Chambers et al., 1988) and sequenced. All experiments were performed in three biological replicates.

Monitoring of *cti* transcription

To construct the *cti*'-'*lacZ* translational fusions in *P. putida* F1, 5' fragments of *cti* (*Pcti*') were fused in the correct translational reading frame to a large 3' fragment of *lacZ* ('*lacZ*') gene in vector pSRKTc (Shuman and Silhavy, 2003; Khan et al., 2008). Detailed procedure is reported in the Supporting Information. Then, the deletions into *cti* promoter regions were made using Site Directed Mutagenesis Kit (NEB Biolabs).

β -Galactosidase assays

Bacterial cultures (2 ml) were centrifuged, and the pelleted cells were assayed for β -galactosidase activity after chloroform/sodium dodecyl sulfate lysis as described by (Miller, 1972). The cell debris in the assay mix was removed by centrifugation before absorbance was read at 420 and 550 nm. All experiments were performed at least in three biological replicates.

Construction of *Pseudomonas* spp. knockout mutants

To make *P. putida* F1 knockout mutants, the suicide plasmids were constructed as described previously (Choi and Schweizer, 2005). Briefly, the sequences' upstream and downstream of target gene, as well as the Gm cassette flanked by FRT sites, were amplified from pFGm1 vector (Choi et al., 2005) and assembled into the EcoRI BamHI digested pMTL23P vector using the Gibson Assembly Kit (NEB). The obtained insert was ligated into BamHI EcoRI digested pK19*mobsacB* suicide vector (Schäfer et al., 1994). A rapid electroporation method described elsewhere (Choi et al., 2006) was used to transfer the pK19*mobsacB*-borne deletion mutations to *Pseudomonas* spp. strains. When indicated the Gm cassette was removed from the chromosomal DNA with help of flippase encoded by plasmid pFLP3 (Choi et al., 2005) according (Choi and Schweizer, 2005). The presence of the correct mutations was verified by colony PCR and sequencing. To complement the mutations, the corresponding genes were PCR amplified with their native promoters from genomic DNA. The obtained fragments were ligated into vector pUC18R6KT-mini-Tn7T-Km and inserted into *P. putida* F1 chromosome using pTNS2 transposase expression vector (Choi et al., 2005). The detailed protocol can be found in the Supporting Information.

FA analysis

Phospholipids were extracted as described by Bligh and Dyer (1959), and FA methyl esters were prepared according the lab protocol (Zhu and colleagues (2010)). FA methyl esters and

their standards were measured by GC–MS on highly polar chiral (CP-Sil88) column. When indicated, FA methyl esters were prepared according to a prior report (Härtig et al., 2005) and analysed using a standard non-polar (ZB-5MS) column. All experiments were performed in three biological replicates (for more details see Supporting Information).

Crp expression and purification

Hexahistidine-tagged *E. coli* Crp protein was produced in *E. coli* BL21 (DE3) carrying the expression plasmid pET28-*crp* (Supporting Information Table S1) as previously reported by Feng and Cronan (2012). The detailed protocol can be found in the Supporting Information. *P. putida* F1 Crp (Pput_0457) is not only 63% identical and 80% similar to the *E. coli* counterpart but also keeps the known motifs for the activation of RNA polymerase through direct protein-protein interactions to maintain the same helix-turn-helix (HTH) motif for DNA binding (identity HTH domains: 72%) and to preserve the basic architecture of its cyclic nucleotide (cNMP)-binding site (identity cNMP domains 55%) (Milanesio et al., 2011; Arce-Rodríguez et al., 2012).

Electrophoretic mobility shift assays

These assays of the interaction between the cAMP-CRP complex and *P. putida* F1 *cti* promoter region were done essentially as previously reported (Herrera et al., 2012; Henke and Cronan, 2016). The DNA-binding reaction contained 200 µg ml⁻¹ of bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM dithiothreitol (DTT), 100 µM cAMP and 1 mM EDTA, 40 nM DNA and the indicated concentrations of Crp. Reactions were incubated at room temperature for 30 min and run in a 6% DNA retardation gel. The gel was stained with SYBR Green I nucleic acid gel stain and visualized using BioRad Chemidoc XRS and Quantity One software.

Heterologous *cti* expression in *E. coli*

The essays were carried out in M9 medium supplemented with 0.01% oleic acid (*cis*-C18:1) or 0.01% *cis*-9,10-methyleneoctadecanoic acid (cyclo-C19:0). After 11 h of growth (OD₆₀₀ ≈ 0.5) in M9 supplemented with 0.4% glucose and 0.8 mM D-fucose, the cells were washed and resuspended in M9 medium supplemented with 0.8% glycerol and 0.1% arabinose to induce the P_{BAD} promoter responsible for *cti* expression. FA compositions were assayed at 0, 3, 6 and 13 h after induction. After 13 h of induction, 10-fold serial dilutions of the bacterial cultures were plated and incubated at 37°C for 16 h. All experiments were performed in three biological replicates.

Bioinformatic analyses

Analysis of the *cti* operons of *Pseudomonas* spp. was performed with the ‘Synteny Line Plot’ tool available from the MaGe Platform (<http://www.genoscope.cns.fr/agc/mage>, last accessed 10 June 2018), which carried out a global comparison of 63 *Pseudomonas* spp. genomes on the basis of synteny results. Analysis of *cti* promoter regions was carried out by PRODORIC® Database (Münch et al., 2003) with standard settings and *P. aeruginosa* PAO1 as a query. To align *cti* and its 5′ UTR sequences of *P. putida* strains, all available *P. putida* *cti* orthologs were extracted from the NCBI Platform (last accessed 27 December 2018),

which carries out the Blast analysis using *P. putida* F1 *cti* nucleic acid sequence (*Pput_3319*) and 507 bp of *cti* 5' UTR as a query in BLAST-N searches. The resulting sequences were aligned using CLC Sequence Viewer 7 (CLC bio).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Characterization of the *cti* promoter region.

A. Sequencing profiles of the amplified fragments obtained by RLM-RACE experiments demonstrating the two *cti* transcriptional start sites (top: *cti*-77; bottom: *cti*-279). The 5' ends of *cti* mRNAs are shown by orange lines, and the blue lines show the 5' RACE RNA adaptor sequence. The transcriptional start sites are shown by asterisk.

B. β -Galactosidase activity. The bacteria were grown in PMM for 25 h ($OD_{600} = 2$). $N = 3$.

C. Schematic representation of the *cti* promoter region. *cti*-77 and *cti*-279 transcriptional start sites are shown as black arrows. Their positions are relative to *cti* translational initiation start (+1). The ORFs are based on *P. putida* F1-annotated genome sequence. The schema is drawn in scale.

D. DNA sequence of the *cti* promoter region. Transcriptional start sites are shown as black arrows. The deduced -10 and -35 sequences are underlined.

E. Construction of translational *cti*'-lacZ fusions. Black lines show long (CtiPP1) and short (CtiPP2) 5' *cti* fragments. Their 5' end positions shown under the lines are relative to the *cti* translational initiation start (+1). The fragments were fused in-frame with 3' lacZ (light

grey) to give pFCtiPP1 and pFCtiPP2 plasmids. The pFCtiPP1-based pFCtiPP1KO and pFCtiPP2KO fusions were constructed by 18-bp deletion within putative -10 region of P_1-77 and P_2-279 respectively. The pFCtiPP0 fusion used as a negative control contains an 88-bp deletion within *cti* RBS and translational start preventing *lacZ* expression. For all panels, mean $\pm$ SEM is shown. *N* indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). ns, not significant.

Fig. 2.

Addition of octanol activates *cti* expression and production of *trans*-UFA in *P. putida* F1.

A. Workflow. Prior to octanol treatment, bacterial cultures were grown in PMM at 30°C with vigorous shaking (250 r.p.m.) and constant aeration for OD₆₀₀ of 0.6. After 1 h of treatment with different octanol concentrations, bacterial samples were taken for β-galactosidase activity and FAME assays.

B. β-Galactosidase activity of pFCtiPP1 (left y-axis) and of pFCtiPP2 (right y-axis). *N* = 3.

- C. Percentages of *trans*-UFA from total bacterial FA. *P. putida* F1 + pFCtiPP1 cultures were grown in the presence of 20 $\mu\text{g ml}^{-1}$ Tet. $N=3$.
- D. Visualization of bacterial susceptibility to octanol by plating serially diluted bacterial cultures on LB plates. $N=3$.
- E. Time-dependent activation of *trans*-UFA production in *P. putida* F1 treated with 2.5 mM octanol. $N=3$.
- F. Time-dependent β -galactosidase activity of pFCtiPP1 in *P. putida* F1 treated with 2.5 mM octanol, showing that octanol activates *cti* expression within 20 min after octanol addition. $N=3$. For all panels, mean $\pm$ SEM is shown. N indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). ns, not significant.

Fig. 3.

Effect of growth temperature and phase on *cti* expression and production of *trans*-UFA in *P. putida* F1.

A. Effect of temperature shift on β -galactosidase activity of pFCtiPP1 and pFCtiPP2 showing the increasing *cti* expression when the temperature increases. Bacterial cultures grown in PMM at 30°C with vigorous shaking (250 r.p.m.) and constant aeration for OD₆₀₀ of 0.6 were incubated for 3 h at 20, 30 and 37°C. *N*= 3.

- B. Percentages of *trans*-UFA from total bacterial FA. *P. putida* F1 WT + pFCtiPP1 cultures were grown in the presence of 20 $\mu\text{g ml}^{-1}$ Tet. $N=3$.
- C. Growth curves of *P. putida* F1 at 20°C (left), 30°C (centre) and 37°C (right) in PMM. The arrows show the growth stages at which samples of cultures were taken for assays. $N=3$.
- D. β -Galactosidase activity of pFCtiPP1 (top) and pFCtiPP2 (bottom). $N=4$. P -values are calculated by comparing with 30°C.
- E. Percentages of total bacterial FA for *trans*-UFA and cyclo-FA. $N=3$. For all panels, mean $\pm$ SEM is shown. N indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). ns, not significant.

Fig. 4.

Cti does not compete with Cfa.

A. Left panel: growth curves of *P. putida* F1 strains in PMM at 30°C. The arrows show the growth stages at which samples of cultures were taken for assays. $N=3$. Right panel: percentages of *trans*-UFA and cyclo-FA in total bacterial FA. The numbers on x-axis show the growth stages at which samples of cultures were taken for GC-MS analyses. $N=3$. Strain names: *P. putida* F1 *cti*, PPut 3319; *P. putida* F1 *cti* compl. *cti*, PPut 3319C; *P. putida* F1 *cfaB*, PPut 5273; *P. putida* F1 *cfaB* compl. *cfaB*, PPut 5273C.

B. Left panel: fractions of *cis*-UFA and SFA from total bacterial FA in *E. coli* strains growing with the addition of *cis*-UFA. Right panel: fractions of cyclo-FA and SFA from total bacterial FA in *E. coli* strains growing with the addition of cyclo-FA. The numbers on the *x*-axis show the times after *cti* induction at which samples of cultures were taken for GC–MS analyses (see Fig. 3C). *N* = 3. Strain names: *fabA cfa*, TK411; *fabA cfa cti*, TK422; *fabA cfa cti* G23, TK436; *fabA cfa cti* SP, TK455. Sp, signal peptide.

C. Expression of *cti* leads to *E. coli* growth inhibition. Left panel: growth curves in M9 supplemented with *cis*-C18:1 or cyclo-C19:0. The black arrows show the time (h) after induction of *cti* expression at which samples of cultures were taken for GC–MS analyses (*N* = 3). Right panel: visualization of bacterial susceptibility to Cti production by plating serially diluted bacterial cultures on LB + *cis*-C18:1 plates. *N* = 3. For all panels, mean ± SEM is shown. *N* indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison).

Fig. 5.

Crp binds a region downstream of the *P. putida* F1 *cti* P₁ promoter and represses *cti* expression.

A. DNA sequence of *cti* promoter region. Transcriptional start sites *cti*-77 and *cti*-279 are shown as black arrows. The deduced -10 and -35 sequences are underlined. Two putative Crp-binding sequences identified using the PRODORIC Database (Münch et al., 2003) are in grey boxes.

B. EMSA showing cAMP–Crp binding to 5' *cti* fragment. Left top: cAMP–Crp binding to 5' *cti* fragment (*Pcti*). Note the shifts seen for cAMP–Crp complex versus the absence of shift seen for the Crp alone or cAMP alone samples. Right top: The absence of cAMP–Crp binding to 5' *cfbB* fragment (*Pcfb*). Left bottom: cAMP–Crp binding to *Pcti* containing the deletion of Crp 2 binding sequence (*Pcti* Δ crp2). Right bottom: The absence of cAMP–Crp binding to *Pcti* containing the deletion of Crp 1 binding sequence (*Pcti* Δ crp1).

C. β -Galactosidase activity of pFCtiPP1 showing that Crp represses *cti* expression in *P. putida* F1. Bacteria were grown in PMM at 30°C. The numbers on x-axis show the growth stages at which samples of cultures were taken for β -galactosidase analyses (see Fig. 2B). $N = 3$. Strain names: *crp*, PPut0457; *crp* compl. *crp*, PPut0457C.

D. β -Galactosidase activity showing the expression from *Pcti* Δ crp1, *Pcti* Δ crp2 and *Pcti*. $N = 3$.

E. Proposed mechanism of Crp–cAMP-dependent repression of *cti* transcription in *P. putida* F1. Crp is shown in grey as a dimer and cAMP is shown as a rhomb. Crp–cAMP complex binds Crp1 site (black rectangle) located near ribosome-binding site (RBS) and represses *cti* transcription. The schema is drawn in scale. For all panels, mean $\pm$ SEM is shown. N indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). ns, not significant.

B. Top: physical map of *cti* and genes located downstream in *P. putida* F1. Predicted Rho-independent transcriptional terminator is indicated with lollipop shapes. The location of primers along the DNA sequence is indicated by light grey arrows with their names given. Bottom: genomic DNA and cDNA amplicons separated in 1% agarose gel electrophoresis ($N=3$). RT- lines show the no-reverse-transcriptase-negative controls. (–) negative PCR control with H₂O. (+) Genomic DNA amplicons.

C. Left: physical map of *cti* and genes located downstream in *P. aeruginosa* PAO1. Right: genomic DNA and cDNA amplicons separated in 1% agarose gel electrophoresis ($N=3$).

D. Deletion of genes located downstream from *cti* does not affect the production of *trans*-UFA in *P. putida* F1 (left) and *P. aeruginosa* PAO1 (right). Bacteria were grown in PMM until OD₆₀₀ 0.6 for *P. putida* F1 ($N=6$) and OD₆₀₀ 0.4 for *P. aeruginosa* PAO1 ($N=3$). *P*-values are calculated by comparing with WT. Mean $\pm$ SEM is shown. For all panels, *N* indicates biological replicates corresponding to independent experiments. Statistical significance was determined by using one-way ANOVA (Turkey multiple comparison). ns, not significant. Strain names: 3317, PPut3317; 3318, PPut3317; PA1847, PAO1847.