

# Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E

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## Summary

**Bacterial membranes constitute the first physical barrier against different environmental stresses. *Pseudomonas putida* DOT-T1E accumulates cyclopropane fatty acids (CFAs) in the stationary phase of growth. In this strain the *cfaB* gene encodes the main cyclopropane synthase responsible of the synthesis of CFAs, and its expression is mediated by RNA polymerase with sigma factor  $\sigma^{38}$ . We generated a *cfaB* mutant of *P. putida* DOT-T1E and studied its response to solvents, acid pH and other stress conditions such as temperature changes, high osmolarity and the presence of antibiotics or heavy metals in the culture medium. A CfaB knockout mutant was more sensitive to solvent stress than the wild-type strain, but in contrast to *Escherichia coli* and *Salmonella enterica*, the *P. putida cfaB* mutant was as tolerant to acid shock as the wild-type strain. The *cfaB* mutant was also as tolerant as the parental strain to a number of drugs, antibiotics and other damaging agents.**

## Introduction

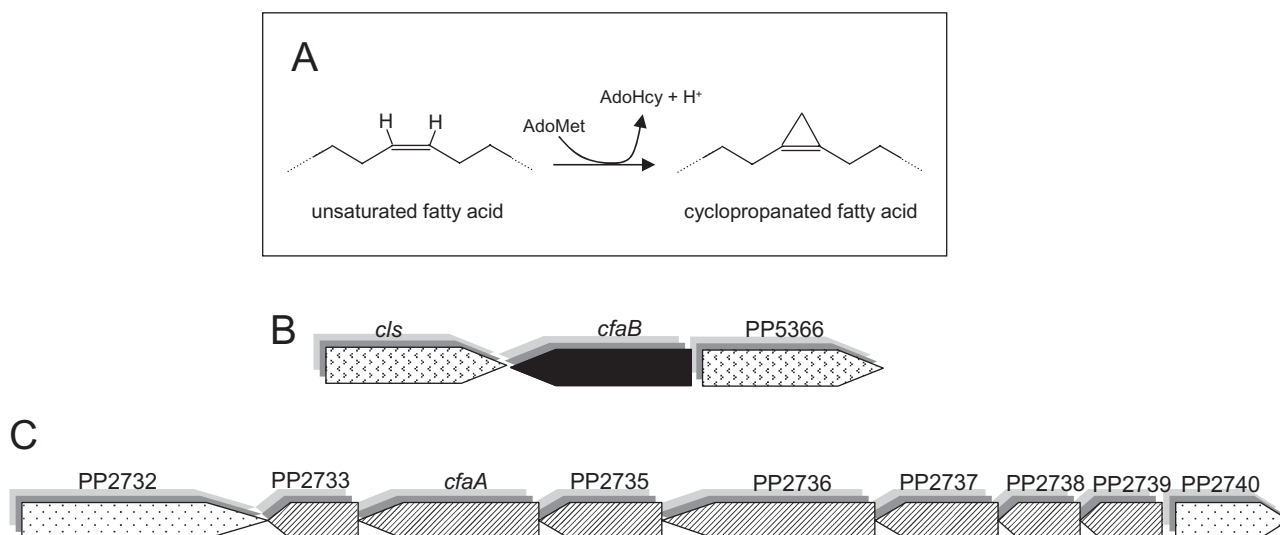
Bacterial life in the environment is threatened by predators, and by many abiotic factors such as temperature changes, presence of toxic and chaotropic compounds, exposure to ultraviolet radiation, desiccation conditions and many other physical and chemical agents (Ramos *et al.*, 2002). Cellular membranes constitute the first physical barrier against physicochemical challenges, and

are provided with a series of sensors that trigger a succession of responses to overcome damage caused by noxious agents. One such response is the alteration of membrane lipid composition, an observation made more than 30 years ago by Sinensky (1974), who proposed the term 'homeoviscous adaptation' to refer to these changes.

A number of environment protection agencies have declared organic solvents as hazardous pollutants (<http://www.epa.gov>; European Union's Guideline 1999/13/EG). These chemicals are inherently toxic, and when they reach soils and waters, they affect the biodiversity of specific niches (Huertas *et al.*, 2000). A limited number of microbes survive exposure to solvents, and the molecular mechanisms of solvent resistance have been partially elucidated in Gram-negative bacteria (Sikkema *et al.*, 1995; Segura *et al.*, 1999; Ramos *et al.*, 2002). Some bacterial strains belonging to the genus *Pseudomonas* (as *P. putida* DOT-T1E or *P. putida* S12) are extremely tolerant to organic solvents and extrusion of toxic compounds via a series of efflux pumps has been proposed to be the main mechanism of solvent tolerance (Isken and de Bont, 1996; Kieboom *et al.*, 1998; Kim *et al.*, 1998; Ramos *et al.*, 1998; Rojas *et al.*, 2001; Rodríguez-Hervá *et al.*, 2007); however, membrane modifications play an important role in the response to toluene and other solvents as well as the response to other environmental stresses such as acid stress or temperature shifts (Pinkart *et al.*, 1996; Chang and Cronan, 1999; Junker and Ramos, 1999; Loffhagen *et al.*, 2001; Kim *et al.*, 2005; Bernal *et al.*, 2007a).

Changes in the overall degree of fatty acid saturation or in the *cis-trans* isomerization of unsaturated fatty acids have been directly implicated in the cellular responses to fluidizing agents such as temperature or organic solvents (Heipieper *et al.*, 1992; Sikkema *et al.*, 1995; Weber and de Bont, 1996; Ramos *et al.*, 1997; Junker and Ramos, 1999; Härtig *et al.*, 2005). The amount of *trans* unsaturated fatty acids in the *P. putida* membrane increased up to 10-fold just 5 min after toluene addition, enhancing membrane rigidity (Junker and Ramos, 1999; Bernal *et al.*, 2007b). However, less is known about the role in the response to these environmental challenges of other membrane phenomena such as the modification of phospholipid head groups or the formation of cyclopropane

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**Fig. 1.** A. Reaction catalysed by the cyclopropane synthase.

B. Schematic representation of the ORFs surrounding the *cfaB* gene in *P. putida* DOT-T1E. *cls* stands for the cardiolipin synthase gene, PP5366 encoded for a putative dihydroliipoamide dehydrogenase 3.

C. Schematic representation of the ORFs surrounding the *cfaA* gene in *P. putida* DOT-T1E. PP2732, PP2733, PP2735 and PP2736 are hypothetical proteins; PP2737 encode for a putative oxidoreductase (short-chain dehydrogenase/reductase family), PP2738 for a putative transcriptional regulator, PP2739 for a putative sensory box protein and PP2740 for a putative transcriptional regulator of the MerR family.

fatty acids (CFA) (Ramos *et al.*, 1997; Bernal *et al.*, 2007a). Cyclopropane fatty acids have been proposed to be involved in the resistance of some  $\gamma$ -proteobacteria to certain stresses such as low pH (Chang and Cronan, 1999; Kim *et al.*, 2005) or freeze-drying conditions (Muñoz-Rojas *et al.*, 2006). However, little is known about the effect of changes in cyclopropane levels in response to organic solvents. The CFAs are produced by a postsynthetic modification of *cis* unsaturated fatty acids, which involves the addition of a methylene group across the double bond of these unsaturated fatty acids (Grogan and Cronan, 1997). This reaction is catalysed by the enzyme cyclopropane synthase (Fig. 1A). In *Escherichia coli* and *P. putida*, CFAs accumulated during the late-exponential and stationary phases of bacterial growth (Grogan and Cronan, 1997; Muñoz-Rojas *et al.*, 2006). In the solvent-sensitive *P. putida* KT2440 genome, two different putative cyclopropane synthase genes (*cfaA* and *cfaB*) have been annotated (Nelson *et al.*, 2002). A *P. putida* KT2440 *cfaB* mutant was shown to be unable to synthesize CFA and that a *cfaA* mutant had only slightly lower levels of CFAs than the parental strain (Muñoz-Rojas *et al.*, 2006).

In this study, we identified the *cfaA* and *cfaB* genes in *P. putida* strain DOT-T1E. *Pseudomonas putida* DOT-T1E is a highly solvent-tolerant strain that was isolated from a waste water treatment plant in Granada (Ramos *et al.*, 1995). This strain is able to grow in the presence of high concentrations of toluene (up to 90% toluene [v/v]) and it is also able to degrade toluene via the toluene dioxygenase pathway (Mosqueda *et al.*, 1999). This is a model

strain regarding solvent tolerance not only because of its high tolerance but because of the accumulated data about the molecular mechanisms of solvent tolerance (Ramos *et al.*, 2002; Rodríguez-Hervá *et al.*, 2007). The *cfaB* gene product seems to be the only one responsible for the synthesis of CFA. Expression of the *cfaB* gene was shown to be RpoS-dependent. We generated a *cfaB* mutant of *P. putida* DOT-T1E and studied its response to solvents, low pH and other stresses such as temperature shifts, high osmolarity and the presence of antibiotics or heavy metals in the medium. The mutant was more sensitive to toluene shocks than the wild-type strain, but in contrast to *E. coli* and *Salmonella enterica*, the *P. putida* *cfaB* mutant was as tolerant to acid shock as the wild-type strain.

## Results

### *CfaB* is the main cyclopropane synthase in *P. putida* DOT-T1E

Total chromosomal DNA of *P. putida* DOT-T1E was hybridized against *cfaA* and *cfaB* gene probes generated by PCR amplification using oligonucleotides based on the *cfaA* and *cfaB* gene sequence of *P. putida* KT2440. We found that the *cfaA* gene hybridized to a single 20 kb fragment PpuMI or 3.5 kb XcmI fragment, whereas the *cfaB* gene hybridized to an EcoRI fragment of about 14 kb or a BamHI fragment of about 12 kb in the DNA of the solvent-tolerant DOT-T1E strain. We subsequently amplified and sequenced the complete *cfaA* and *cfaB* genes from the chromosome of *P. putida* DOT-T1E. Translation of the nucleotide sequences into amino acid sequences

**Table 1.** Fatty acid composition of *P. putida* DOT-T1E and its *cfaA* and *cfaB* mutant derivatives.

Fatty acid	Wild-type (exp)	Wild-type (st)	<i>cfaA</i> <sup>-</sup> (exp)	<i>cfaA</i> <sup>-</sup> (st)	<i>cfaB</i> <sup>-</sup> (exp)	<i>cfaB</i> <sup>-</sup> (st)	<i>cfaB</i> <sup>-</sup> (pCEC-3) (exp)	<i>cfaB</i> <sup>-</sup> (pCEC-3) (st)
C14:0	1.52	0.9	1.57	0.46	0.00	0.73	0.00	0.00
C15:0	0.08	0.822	0.11	0.33	0.00	0.69	0.00	0.00
C16:1,9 <i>cis</i>	21.7	7.09	21.12	5.46	25.5	34.1	14.63	2.85
C16:1,9 <i>trans</i>	0.28	0.0	0.70	0	0.00	0	0.00	0.00
C16:0	40.38	50.8	41.59	51.9	39.6	51.5	35.35	40.15
<sup>a</sup> C17:cyclo	0.79	25.8	1.49	24.3	0.00	1.9	2.45	24.10
C17:0	0.0	0.49	0.06	0	0.00	0.39	0.00	0.00
C18:1,11 <i>cis</i>	12.24	9.55	8.93	12.8	12.9	7.38	10.50	15.06
C18:1,11 <i>trans</i>	0.00	0.00	0.00	0	0.00	0	0.00	0.00
C18:0	22.96	4.257	24.42	5.35	21.8	3.28	32.52	12.35
<sup>a</sup> C19:cyclo	0.00	0.46	0.00	0.32	0.00	0	0.00	0.00

a. C17:cyclo, 9,10-methyl hexadecanoic acid; C19:cyclo, 11,12-methyl octadecanoic acid.

Data are given as percentages of the total fatty acid content. Fatty acids were extracted at the exponential (exp) and late-stationary phase after 24 h of incubation (st). Shaded numbers corresponded with the CFAs.

revealed 98.7% identity between CfaB proteins of *P. putida* KT2440 and DOT-T1E, and 99% identity between CfaA proteins in the two organisms, although the translated *cfaA* in DOT-T1E is 26 amino acid longer than that deduced from the KT2440 genome sequence. An *in silico* analysis of the chromosomal region around the *cfaA* and *cfaB* genes in *P. putida* KT2440 suggested that the *cfaB* gene is transcribed as a monocistronic unit, as it is divergent from the 3' downstream gene and convergent with the gene located in 5' (Fig. 1B). In contrast, the originally annotated *cfaA* gene is in a cluster of seven open reading frames that are transcribed in the same direction (Fig. 1C). This gene organization is identical in both the solvent-sensitive *P. putida* KT2440 and solvent-tolerant *P. putida* DOT-T1E strains. We have shown with RT-PCR that cluster in *P. putida* DOT-T1E is transcribed as a single operon (data not shown). The functions of the gene products of this operon are unknown.

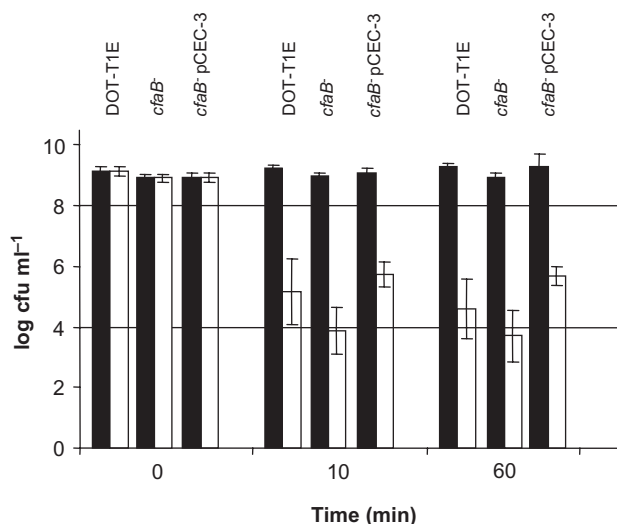
To assign a function to each of the genes in *P. putida* DOT-T1E, knockout mutants of the two putative *cfa* genes were constructed as described in *Experimental procedures*, and fatty acid profiles were established in cells in the early-exponential and stationary phases. In the wild-type strain cyclopropane levels were higher in the stationary phase of growth than in the exponential phase (Table 1). As expected, relative levels of the precursor unsaturated fatty acids (C16:1.9 *cis* for C17:cyclo and C18:1.11 *cis* for C19:cyclo) diminished as cyclopropane levels increased. The *cfaA* mutant contained almost the same amount of CFA as the wild-type strain, whereas CFA levels in the *cfaB* mutant were undetectable (Table 1). A double *cfaA**cfaB* mutant had the same fatty acid profile as the *cfaB*<sup>-</sup> strain (data not shown). To verify that *cfaB* was responsible for CFA synthesis we complemented the *cfaB* mutant with plasmid pCEC-3, which carries the *cfaB* gene. The results showed that in the complemented strain the pattern of fatty acids in the sta-

tionary phase was similar to that in the parental strain. These data support that the *cfaB* gene product is the one responsible for CFA synthesis in *P. putida* DOT-T1E.

#### *The cfaB* mutant is more sensitive to organic solvent shocks than the wild-type strain

Cyclopropane fatty acids have been implicated in the resistance to different stresses in *E. coli*, *S. enterica* serovar Thyphimurim and *P. putida* KT2440 (Chang and Cronan, 1999; Kim *et al.*, 2005; Muñoz-Rojas *et al.*, 2006). To study the role of CfaB in stress resistance in *P. putida* DOT-T1E, cultures in the stationary phase (OD ≈ 2.5–3.0) of the wild-type and *cfaB* mutant were exposed to different stress conditions and survival was compared. We found that survival of the *cfaB* mutant strain was nearly 1.5 orders of magnitude lower than in the wild type when exposed to 0.3% (v/v) toluene (Fig. 2). Complementation of the *cfaB* mutation by pCEC-3 restored survival to levels similar to those in the wild-type strain (Fig. 2). Increased organic solvent sensitivity of the *cfaB* mutant was confirmed when solvent shock was triggered with 0.3% (v/v) xylene, ethylbenzene or propylbenzene instead of toluene. Survival rate of the *cfaB* mutant was correlated with the theoretical toxicity of the hydrocarbons (that is related with the log  $P_{ow}$  value) and was lower than that of the wild-type strain (Table 2).

Aromatic organic acids such as *p*-hydroxybenzoate or protocatechuate are tolerated up to a concentration of 25 mM without affecting survival rate in the wild type (Ramos-González *et al.*, 2001). This was also the case for the *cfaB* mutant strain with other aromatic organic acids such as benzoate or salicylate. In *E. coli* CFAs are involved in acid stress resistance in the stationary phase. To test whether this was the case in *P. putida* DOT-T1E, HCl (to 20 mM) was added to cultures of the parental and *cfaB* mutant strains grown in Luria–Bertani (LB) until the



**Fig. 2.** Survival of *P. putida* DOT-T1E, its *cfaB*<sup>-</sup> derivative strain and the *cfaB*<sup>-</sup> pCEC-3 complemented strain at 0, 10 and 60 min after the addition of 0.3% (v/v) toluene. Black bars, survival of control cultures; white bars, survival of cultures with toluene.

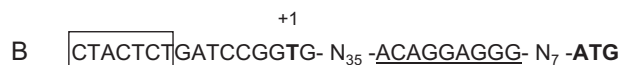
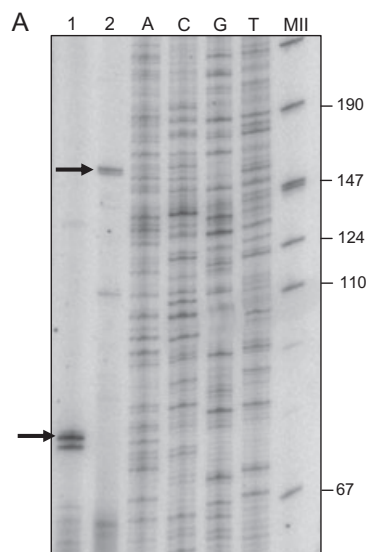
stationary phase. We found that viability of both strains decreased by two orders of magnitude after 24 h. Then we run an experiment where cells grown on minimal medium supplemented with glucose until stationary phase were harvested and HCl was added to obtain pHs of 6, 5.5, 5, 4.5, 4 and 3.5. There was no difference between the wild type and mutant (100% survival at pH 6, 5.5, 5 and 4, and no survival at pH 3.5).

Mutant *cfaB* cells showed no differences in survival rates compared with the wild type when cultures at the late-exponential growth phase were exposed to temperature shifts from 30°C to 16°C or from 30°C to 42°C. The mutant was also not more sensitive to a number of antibiotics and heavy metals than the wild type.

The series of results presented above suggested that the *cfaB* gene product is specifically involved in organic solvent stress, but not in a general stress response.

#### Transcriptional analysis of the *cfa* genes

The higher levels of CFA during the stationary phase suggested that *cfaB* expression might be governed by the



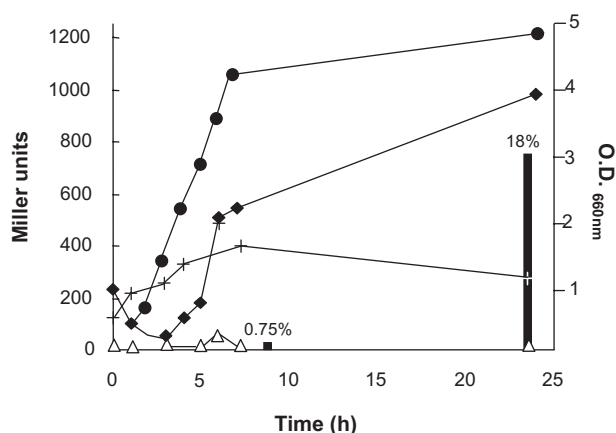
**Fig. 3.** Mapping of the transcription start point of the *cfaB* gene. A. Primer extension analysis. Lane 1, primer extension using primer p100; lane 2, primer extension using primer p180. B. The +1 position in the promoter region is in boldface; the putative  $\sigma^{38}$  recognition sequence is boxed.

RpoS sigma factor. To test this hypothesis we first determined the transcriptional start point of the *cfaB* gene, which was found 53 nucleotides upstream from the ATG start codon (Fig. 3). Around the -10 region we found a sequence that resembled that proposed to be recognized by RNA polymerase/ $\sigma^{38}$  (5'-CTACTCT-3'). To further study the expression of the *cfaB* gene we fused the *cfaB* promoter region to *'lacZ* to yield plasmid pMPcfaB. Figure 4 shows that expression of the *cfaB* gene was clearly dependent on the growth phase, with a sharp increase in expression when the culture entered the late-exponential phase and persistently high levels throughout the stationary phase. The response of the *cfaB* promoter was totally different to that of the promoter of the efflux pump TtgGHI (Fig. 4) in which no increase in expression was observed

**Table 2.** Survival of the *P. putida* DOT-T1E and *cfaB*<sup>-</sup> cultures after the addition of 0.3% (v/v) *m*-xylene, ethylbenzene or propylbenzene.

	log $P_{ow}$	DOT-T1E	<i>cfaB</i> <sup>-</sup>	<i>ttgGHI</i>	<i>ttgABC</i> <sup>-</sup>
None		9.3 ± 0	9.3 ± 0.6	9.4 ± 0.1	9.2 ± 0.1
<i>m</i> -xylene	3.2	9.4 ± 0.7	7.0 ± 0.4	7.5 ± 0.3	6.6 ± 0.2
Ethylbenzene	3.4	8.8 ± 0.1	8.0 ± 0.2	6.3 ± 0.1	5.9 ± 0.3
Propylbenzene	3.6	9.3 ± 0.2	8.8 ± 0.1	8.9 ± 0.1	8.5 ± 0.1

Solvents were added when cultures reached the stationary phase, and cells were plated 10 min after the solvent was added. Survival is expressed as log cfu ml<sup>-1</sup>. Logarithm of the partition coefficient of an organic solvent in a mixture of octanol/water (log  $P_{ow}$ ) was used as a toxicity indicator. Organic solvents with log  $P_{ow}$  between 1 and 4.5 are very toxic for microorganisms. Within this range, organic solvents with lower log  $P_{ow}$  values are more toxic than those with higher values.



**Fig. 4.** Expression of the *cfaB* promoter (measured as  $\beta$ -galactosidase activity) along the *P. putida* DOT-T1E growth curve. Circles indicate culture growth. Black diamonds, white triangles and crosses indicate  $\beta$ -galactosidase activity of pMcfab, pMP220 without promoter and pMP220 with  $P_{ttgG}::lacZ$  (promoter of *ttgGH1* efflux pump; Rojas *et al.*, 2003) respectively. Black bars show the percentage of CFAs in the membranes.

during stationary phase. This increase in expression, together with the finding of a  $-10$  region with high similarity to a consensus sequence for  $\sigma^{38}$ , indicated that expression of the cyclopropane synthase gene was tightly controlled by RpoS. To verify the role of RpoS in the expression of *cfaB* we introduced plasmid pMPcfaB into a *P. putida rpoS*-deficient strain. Expression of the *cfaB* promoter was completely shut down in this genetic background (not shown), confirming that RpoS is the sigma factor responsible for transcription of the *cfaB* gene of *P. putida* DOT-T1E. At the same time as expression of  $\beta$ -galactosidase was measured, we determined CFA levels in the cells, and found that in the wild-type strain CFAs became detectable as *cfaB* expression increased, whereas no C17:CFA was detected in the *rpoS* mutant background at any time.

#### *cfaB* expression under different stress conditions

To determine whether the expression of *cfaB* was influenced by the above stress conditions, we used a  $P_{cfaB}::lacZ$  fusion. Table 3 shows that in the stationary phase *cfaB* expression occurred at around 500 Miller units, a level of expression that was not significantly influenced by the presence of toluene or propylbenzene. The addition of HCl to 20 mM did not affect the level of  $\beta$ -galactosidase either (not shown). However, the addition of 15 mM benzoate led to a 2.5-fold increase in  $\beta$ -galactosidase levels (Table 3). No differences in expression were observed when the plasmid was introduced in the *rpoS* mutant or when the assay was done with the plasmid without promoter; that is, activity levels were always below 40 Miller units (not shown). These

results suggested that organic acids rather than inorganic acids were able to induce *cfaB* expression. To further test this hypothesis we analysed the expression of the promoter when different organic acids were added to the culture medium. None of the linear organic acids (mono- and di-carboxylic) increased expression from the *cfaB* promoter; however, several aromatic acids such as phenylacetate, salicylate and 2- or 3-toluate led to a modest increase in expression (Table 3). *cfaB* expression did not change after shifting cells from 30°C to 42°C or to 16°C.

Based on the expression results, we decided to test whether cells growing in the presence of benzoate or phenylacetate would exhibit higher levels of CFA than cells growing only on LB. The results revealed that the modest increase in *cfaB* expression in the presence of organic acids did not translate into higher levels of CFA, probably because under these conditions the *cis-trans* isomerase was also competing for the same substrate (Table 4).

#### Membrane fluidity

As described above, lipid membrane composition is an important determinant in tolerance towards different stresses, especially in response to those that affect membrane fluidity. The role of CFA in membrane fluidity has not been studied in detail. To determine membrane fluidity in the wild-type and *cfaB* mutant strains, we analysed fluorescence polarization values when the cultures were incubated with the dye 1,6-diphenyl-1,3,5-hexatriene. The *P* values for all cultures were  $0.25 \pm 0.04$ , indicating that there were no significant differences in membrane fluidity between the wild-type and mutant strains.

**Table 3.** Transcription from the *cfaB* promoter determined as  $\beta$ -galactosidase activity using a fusion of the *cfaB* promoter to  $'lacZ$ .

Compound	Miller units
None	520 $\pm$ 15
Acetate	500 $\pm$ 10
Citrate	350 $\pm$ 5
Succinate	500 $\pm$ 20
Lactate	260 $\pm$ 5
Protocatechuate	460 $\pm$ 30
Benzoate	1170 $\pm$ 20
Phenylacetate	1290 $\pm$ 30
Salicylate	1050 $\pm$ 40
2-Toluate	1230 $\pm$ 45
3-Toluate	1210 $\pm$ 35
4-Toluate	750 $\pm$ 5
3-Hydroxybenzoate	880 $\pm$ 35

Bacterial cultures were grown until they reached a turbidity at an 660 nm of 2.5–3.0.  $\beta$ -Galactosidase assays were determined as described by Miller (1972) 3 h after acid addition. Data are average values of three independent assays. All compounds were added to reach a final concentration of 15 mM except for protocatechuate, which was used at a final concentration of 5 mM.

**Table 4.** Fatty acid composition of *P. putida* DOT-T1E membranes grown in the presence of benzoate or phenylacetate.

Fatty Acid	LB	LB + benzoate	LB + phenyl acetate
C14:0	0.37	0	0.5
C15:0	0	0.35	0
C16:1.9 <i>cis</i>	7.78	3.72	2.79
C16:1.9 <i>trans</i>	0	7.14	4.05
C16:0	43.4	46.4	45.6
C17:cyclo	23.3	16.9	20.07
C17:0	0	0	0
C18:1.11 <i>cis</i>	18.6	12.56	11.78
C18:1.11 <i>trans</i>	0	0	1.47
C18:0	5.9	11.6	12.8
C19:cyclo	0.5	1.23	1.13

Cells were grown until they reached OD<sub>660</sub> ≈ 2 and then 15 mM of benzoate or phenylacetate was added. Lipids were extracted 16 h after the acid was added. Data are given as percentages of the total fatty acid content. Shaded entries highlight the increases in *trans*-UFAs and CFAs in response to benzoate and phenyl acetate.

## Discussion

Although CFAs are present in bacterial cytoplasmic membranes at high levels during the stationary phase of growth (when they can represent up to 25% of the total fatty acid content), their physiological role has not been clearly established. The CFAs are nonessential components of the membranes, at least under laboratory conditions, as mutants unable to synthesize CFAs are viable. Although in *P. putida* DOT-T1E two putative open reading frames may encode a cyclopropane synthase, our results suggest that the *cfaB* gene product is the main enzyme involved in cyclopropane synthesis. Although we subjected the *cfaA* mutant to the same stresses as the *cfaB* mutant we, did not obtain any distinctive phenotype for the *cfaA* mutation. Our results also show that the lack of CFA in the membranes of the *P. putida* DOT-T1E *cfaB* mutant increased toluene sensitivity as determined by the diminished viability (by 1.5 orders of magnitude) of mutant cultures with respect to the wild type. The decrease in solvent tolerance has also been documented when other enzymes responsible for the synthesis of membrane components (such as *cis-trans* isomerase or cardiolipin synthase) were knocked out (Junker and Ramos, 1999; Bernal *et al.*, 2007a). The fact that the *cfaB* mutant cultures did not totally lose viability after solvent shock indicates that CFAs are involved in, but not essential for, solvent tolerance. Thus far the viability of mutant cultures after exposure to toluene (log  $P_{ow}$  2.4) has been reported to be lost completely only when some of the efflux pumps directly involved in solvent extrusion have been knocked out (Rojas *et al.*, 2001). Other organic solvents with log  $P_{ow}$  values higher than that of toluene are less toxic for bacterial cells and viability of cultures exposed to a sudden shock with these solvents remained high even in the efflux pump mutants (Table 2). Our results confirmed

the toxic effect of these solvents, with a higher decrease in solvent tolerance in the *cfaB* mutant when *m*-xylene was added to the culture, subtle effects on *cfaB* survival with ethylbenzene and almost no differences with propylbenzene.

The involvement of CFA in solvent tolerance has been described in the anaerobic bacteria *Clostridium acetobutylicum* ATCC 824 (Zhao *et al.*, 2003); overexpression of cyclopropane synthase in this microorganism increased butanol tolerance; however, it decreased butanol production. In *C. acetobutylicum* butanol was able to increase the CFA content in membranes (Vollherbst-Schneck *et al.*, 1984), whereas in *P. putida* DOT-T1E no increase in CFA content was detected after toluene was added, and *cfa* gene expression was not induced in the presence of the solvent.

We found no other differences between the wild type and the *cfaB* mutant with regard to resistance to other stresses. Brown and colleagues (1997) reported a correlation between *E. coli* strains with a high degree of intrinsic acid tolerance and higher CFA content in the membranes of these strains. In this connection it was also found that *cfa* null mutants of *E. coli* and *S. enterica* were more sensitive to acid stress than the corresponding wild-type strain (Chang and Cronan, 1999; Kim *et al.*, 2005). We found no pH at which survival of the *P. putida* DOT-T1E *cfaB* mutant was compromised in comparison with the wild-type strain, suggesting that in *P. putida* CFA may not be related with survival after acid stress. In *E. coli* and *S. enterica* expression of the *cfa* gene is driven from two promoters (one dependent on  $\sigma^{70}$  and another on  $\sigma^{38}$ ); however, in *P. putida* DOT-T1E *cfaB* gene expression is mediated by RNA polymerase only with  $\sigma^{38}$ . Expression of the *cfa* gene in *E. coli* increased in response to short-chain carboxylic acids such as acetate, which is present in its natural gut niche (Arnold *et al.*, 2001); in contrast with *E. coli*, short-chain organic acids did not induce *cfaB* gene of *P. putida* DOT-T1E, although aromatic carboxylic acids did induce *P. putida* DOT-T1E *cfaB* gene expression. Whether the response to organic and inorganic acids reflects different regulatory mechanisms of cyclopropane synthase in pseudomonadaceae and enteric bacteria remain to be elucidated. The role of CFA in survival of *P. putida* under environmental conditions is an area that deserves further research, in particular in the rhizosphere of plants, a niche in which *P. putida* proliferates and in which a large number of aromatic carboxylic acids derived from lignin breakdown are present.

Interestingly, although the *cfaB* gene in pCEC-3 was expressed from a plasmid promoter, the CFA levels observed during the exponential phase were only slightly higher than when *cfaB* was expressed from its own promoter. This indicates that some other regulatory mechanisms operate to control the levels of CFAs in the

membranes. In *E. coli* CFA synthase is unstable because of RpoH-dependent proteolysis (Chang *et al.*, 2000), and in *P. putida* *rpoH* expression is induced in the presence of alkylbenzoates (Domínguez-Cuevas *et al.*, 2006), so increased cyclopropane synthase degradation might explain the discrepancy between *cfaB* expression and CFA content in the membranes. Proteolysis of cyclopropane synthase has been suggested to be an important mechanism to avoid consumption of S-adenosyl-methionine in the exponential phase during which this compound is required for the synthesis of other molecules. The complexity of the regulation of CFA might be related not only to membrane physical properties, but it might also respond to a general metabolic regulation.

## Experimental procedures

### Bacterial strains and growth conditions

*Pseudomonas putida* DOT-T1E and its mutant derivatives were routinely grown on LB or M9 minimal medium supplemented with the carbon sources indicated in the text (Abril *et al.*, 1989). Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per minute. The stationary phase was normally reached after 5 h of growth ( $OD_{660} \approx 2.5-3$ ).

### Construction of plasmid and mutant strains

The *P. putida* DOT-T1E *cfaB* gene was amplified from chromosomal DNA using oligonucleotides Kpncfa2b (5'-TA GGTACCGTAGGCGTCGTGGCCAATTA-3') and EcoRIcfa (5'-TGAATTTCGGCCGCTCCTGCACAACC-3'), and digested with EcoRI and KpnI. This DNA was ligated to pBBR1MCS-5 (Kovach *et al.*, 1995), which had been previously cut with the same enzymes. The resulting plasmid, named pCEC-3, was transformed in the *P. putida* DOT-T1E and *P. putida* DOT-T1E *cfaB*<sup>-</sup> strains. The resulting gentamicin-resistant colonies were checked for the presence of the plasmid, and one clone of each transformation was selected for further analysis.

To construct a *cfaB* mutant strain, a small fragment (706 bp) of the *cfaB* gene was amplified using oligonucleotides CFA1 (5'-ATGCTTGCTCAACTTCCACC-3') and CFA2-6 (5'-GTATGGGTGCGACTGTTGGA-3'). This fragment was cloned into the pMBL-T vector (Dominion MBL, Spain) to obtain pANA247, which was cut with BamHI, and the 2.2 kb BamHI fragment (containing the  $\Omega::km$  cassette) of pHP45 $\Omega$ -Km (Prentki and Krisch, 1984) was ligated in the linearized plasmid. The resulting plasmid (pANA247K) was introduced into *P. putida* DOT-T1E by electroporation (Enderle and Farwell, 1998), and cultures were plated onto LB medium plus kanamycin to select for the single homologous recombination event that disrupts *cfaB* by incorporating the plasmid into the chromosome. A *cfaA* mutant strain was constructed as in Muñoz-Rojas *et al.* (2006), but using *P. putida* DOT-T1E as a recipient strain. To construct the double mutant (*P. putida* DOT-T1E *cfaA*<sup>-</sup>*cfaB*<sup>-</sup>) we excised the  $\Omega::Km$  cassette of plasmid pANA247 with BamHI and replaced it with the Gm<sup>R</sup> cassette of pMS255 (Ko and Park, 2000) pre-

viously recovered with BamHI. The new plasmid (pCEC-1) was electroporated into *P. putida* DOT-T1E *cfaA*<sup>-</sup> strain and gentamicin-resistant clones were selected. All mutants were checked by Southern blot (not shown).

### Survival after stress treatment

Cultures of the wild-type and mutant strains were grown in LB medium until the stationary phase ( $OD_{660} \approx 2.5$ ). Then cultures were divided into two halves, one of which was treated with the stress agent [temperature shift from 30°C to 42°C or 16°C, addition of 15 mM benzoate, 0.7 M NaCl, 20 mM HCl or 0.3% (v/v) organic solvent] while the other one was kept as a control. Serial dilutions of the cultures were plated onto LB after 30 min, 1 h and 4 h to determined survival rates. In the assays with organic solvents, cells were plated after 10, 30 and 60 min; data represent the media of at least three independent experiments. Acid resistance was also tested as described by Chang and Cronan (1999). Briefly, cells of cultures growing in M9 minimal medium supplemented with glucose in the stationary phase were harvested and resuspended in M9 minimal medium. Hydrochloric acid was added until pH of the cultures reached 6, 5.5, 5, 4.5, 4 or 3.5 and survival was measured after incubation for 1 and 3 h at 30°C.

### Fatty acid analysis

Phospholipids were extracted as described by Bligh and Dyer (1959) and *trans*-esterified as described before (Bannon *et al.*, 1982). After gas chromatographic separation, the fatty acids were identified by mass spectrometry. Data presented in tables are representative of three experiments.

### $\beta$ -Galactosidase assays

The *cfaB* promoter was amplified using oligonucleotides PstIcfaB2 (5'-AACTGCAGAGCGGCAGATGCAA-3') and EcoR1cfaB2 (5'-GGAATTTCGCTGGCAGCTTCGT-3'). The fragment was then cut with PstI and EcoRI and cloned into pMP220 (Spaink *et al.*, 1987) previously cut with the same enzymes to construct plasmid pMPcfaB. This plasmid was electroporated into *P. putida* DOT-T1E and *P. putida* DOT-T1E *rpoS*. Cultures were grown overnight on LB medium plus tetracycline, and on the following morning they were diluted to an  $OD_{660}$  of 0.1. When the cultures reached the stationary phase ( $OD \approx 3.0$ ) they were exposed to different stressors, for example, 37°C or 16°C, 20 mM HCl, 15 mM benzoate, 15 mM phenylacetate, 25 mM protocatechuate, 25 mM *p*-hydroxybenzoate, 5 mM propylbenzene, 15 mM succinate, 15 mM acetate and 15 mM citrate, and 0.075% (v/v) toluene.  $\beta$ -Galactosidase activity was assayed 1, 3 and 20 h after stress treatment.

### Fluorescence anisotropy measurements

Membrane fluidity was determined by measuring fluorescence polarization of the dye 1,6-diphenyl-1,3,5-hexatriene probe inserted into the lipid bilayer of the cytoplasmic membranes. Experiments were done as described by Bernal and colleagues (2007a) except that cultures were harvested during the early stationary phase.

### Primer extension analysis

Cells of *P. putida* DOT-T1E grown overnight in LB medium were diluted 1:100 in the same medium. After 12 h of incubation, 15 ml samples were harvested by centrifugation and RNA was extracted as described before (Aranda-Olmedo *et al.*, 2005). Primer extension analysis of 100 µg of total RNA samples was carried out as described previously (Marqués *et al.*, 1993) using <sup>32</sup>P-end-labelled oligonucleotides. For this step we used oligonucleotides p180 (5'-GAATGGTGACCTGAGGACTA-3') and p100 (5'-CAATGCA GGTGGAAGTTGAG-3'), which were complementary to the coding strands within the *cfbB* gene. cDNAs were run in urea sequencing gels, and gels were exposed to a phosphor-imaging screen (Fuji Photo Film) for 5–12 h. Phosphor-imaging screens were scanned with a phosphor-imaging instrument (Molecular imager FX; Bio-Rad).

### Gene accession number

The sequence of the *P. putida* DOT-T1E *cfbB* gene has been deposited under number DQ665843. The sequence of the *P. putida* DOT-T1E *cfbA* and surrounding genes has been deposited under number DQ665844.

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