

# Coordinated response of phospholipids and acyl components of membrane lipids in *Pseudomonas putida* A (ATCC 12633) under stress caused by cationic surfactants

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The present study assessed the role of membrane components of *Pseudomonas putida* A (ATCC 12633) under chemical stress conditions originated by treatment with tetradecyltrimethylammonium bromide (TTAB), a cationic surfactant. We examined changes in fatty acid composition and in the fluidity of the membranes of cells exposed to TTAB at a specific point of growth as well as of cells growing with TTAB. The addition of 10–50 mg TTAB l<sup>-1</sup> promoted an increase in the saturated/unsaturated fatty acid ratio. By using fluorescence polarization techniques, we found that TTAB exerted a fluidizing effect on *P. putida* A (ATCC 12633) membranes. However, a complete reversal of induced membrane fluidification was detected after 15 min of incubation with TTAB. Consistently, the proportion of unsaturated fatty acids was lower in TTAB-treated cells as compared with non-treated cells. In the presence of TTAB, the content of phosphatidylglycerol increased (120%), whilst that of cardiolipin decreased (60%). Analysis of the fatty acid composition of *P. putida* A (ATCC 12633) showed that phosphatidylglycerol carried the major proportion of saturated fatty acids (89%), whilst cardiolipin carried an elevated proportion of unsaturated fatty acids (18%). The increase in phosphatidylglycerol and consequently in saturated fatty acids, together with a decrease in cardiolipin content, enabled greater membrane resistance, reversing the fluidizing effect of TTAB. Therefore, results obtained in the present study point to changes in the fatty acid profile as an adaptive response of *P. putida* A (ATCC 12633) cells to stress caused by a cationic surfactant.

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## INTRODUCTION

Bacteria are commonly exposed to a wide range of stress-inducing conditions and their membranes are the first target able to cope with such alterations. The structural characteristics and fluidity of the membrane change in response to fluctuating environments through changes in the biochemical properties of lipid acyl components. Thus, the predominant response of most bacteria to environmental perturbations is the alteration of the saturated/unsaturated fatty acid ratio (Cronan & Gelmann, 1975; Ingram, 1976; Härtig *et al.*, 1999; Ramos *et al.*, 2002; Mrozik *et al.*, 2004; Bisbiroulas *et al.*, 2011; Paulucci *et al.*, 2011). Other mechanisms, such as isomerization of *cis*/

*trans* double bonds (Gillan *et al.*, 1981; Okuyama *et al.*, 1991; Diefenbach *et al.*, 1992; Heipieper *et al.*, 2003; Bernal *et al.*, 2007b; Kiran *et al.*, 2005), shortening or elongation of fatty acid chain lengths (Cronan & Gelmann, 1975; Russell, 1984; Diefenbach *et al.*, 1992) and changes in the ratio of terminally branched *iso/anteiso* fatty acids (Kaneda, 1977; Konopásek *et al.*, 2000), have been found to play a role in the fluidity balance within bacteria. Modifications in the polar heads of anionic phospholipids have also been associated with the response to certain types of stress. It has been shown that exposure to stress, such as solvents, cationic surfactants, changes in pH, high temperature and high salinity, causes modifications in the content of phosphatidic acid, phosphatidylglycerol and cardiolipin (Matsumoto, 2001; von Wallbrunn *et al.*, 2002; Catucci *et al.*, 2004; López *et al.*, 2006; Bernal *et al.*, 2007a, Boeris *et al.*, 2007; Ghosh *et al.*, 2008).

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; FAME, fatty acid methyl ester; TTAB, tetradecyltrimethylammonium bromide; QAC, quaternary ammonium compound.

Quaternary ammonium compound (QAC)-based surfactants are commercially available chemicals frequently used as biocides or disinfectants in a variety of products, including cosmetics, antiseptic solutions, textile finishes, domestic cleaning products and fabric softeners (McDonnell & Russell, 1999; Gilbert & Moore, 2005; Zhao & Sun, 2007). The mode of action of QACs in bacteria relies on the interaction of the surfactant with the lipidic bilayer of the membrane, which leads to the loss of membrane integrity, disruption of transduction energy, disturbance of membrane barrier functions, inhibition of protein action, and subsequent deleterious effects on the growth and viability of microbial cells (McDonnell & Russell, 1999; Gilbert & Moore, 2005). At the molecular level, the action of QACs involves an association between the positively charged quaternary nitrogen and the head groups of acidic phospholipids within the membrane. At high concentrations, QACs solutions form mixed micellar aggregates that solubilize hydrophobic membrane components (i.e. lipid A, phospholipids, etc.) (Gilbert & Moore, 2005). However, some bacterial species, especially species within the genus *Pseudomonas*, have high resistance to QACs and are thought to be capable of degrading these surfactants in activated sludge (Dean-Raymond & Alexander, 1977; van Ginkel *et al.*, 1992; Nishihara *et al.*, 2000; Takenaka *et al.*, 2007). It has been demonstrated that the adaptation of many microorganisms to high concentrations of QACs may include changes in fatty acid composition (Jones *et al.*, 1989; Méchin *et al.*, 1999; Loughlin *et al.*, 2002; To *et al.*, 2002), changes in the acidic phospholipid content of membranes (Nikaido, 1994; Boeris *et al.* 2007, 2009) and the acquisition or hyperexpression of multidrug efflux pumps (Aase *et al.*, 2000; To *et al.*, 2002; Gilbert & Moore, 2005).

*Pseudomonas putida* A (ATCC 12633) is a ubiquitous Gram-negative bacterium and a potent pollutant degrader that utilizes the QAC tetradecyltrimethylammonium bromide (TTAB) as a carbon, nitrogen and energy source, and was found to be capable of growing in media containing up to 50 mg TTAB l<sup>-1</sup> (Liffourrena *et al.*, 2008). In *P. putida* A (ATCC 12633), the adaptive response and resistance to the bactericidal activity of TTAB involves a mechanism that initiates changes to membrane composition, including specific variations in the content of phosphatidic acid, phosphatidylglycerol and cardiolipin (Boeris *et al.*, 2007). In a previous survey, we found that in the presence of 50 mg TTAB l<sup>-1</sup>, phosphatidic acid and phosphatidylglycerol levels increased 140 and 120 %, respectively, whilst cardiolipin decreased ~60 %. Similar changes were detected in TTAB-adapted bacteria, indicating that these phospholipids were initially and consecutively damaged by TTAB, and needed to be replaced rapidly (Boeris *et al.*, 2009). As we detected such relevant changes in the *P. putida* A (ATCC 12633) phospholipid membrane composition in response to TTAB, our next work aimed at evaluating the change in the content and composition of membrane fatty acids, and its relation with the stability of cell membranes of *P. putida* A (ATCC 12633) under treatment with TTAB.

## METHODS

**Micro-organisms and culture conditions.** *P. putida* A (ATCC 12633) cells were grown aerobically at 30 °C with shaking in a rich Luria–Bertani (LB) medium as well as in a basal salt liquid medium (HPI-BSM) (Lucchesi *et al.*, 1989) with 20 mmol glucose l<sup>-1</sup> and 18 mmol NH<sub>4</sub>Cl l<sup>-1</sup> or 0.15 mmol TTAB l<sup>-1</sup> (50 mg l<sup>-1</sup>) as carbon and nitrogen sources. Growth was evaluated through measurement of culture OD<sub>660</sub> using a Beckman DU 640 spectrophotometer. In experiments where *P. putida* A (ATCC 12633) was exposed to TTAB, cells were grown in HPI-BSM plus glucose and NH<sub>4</sub>Cl until the culture reached late exponential phase (OD<sub>660</sub> 0.8–1.00). At this point, the culture was divided in two parts: one was kept as a control and the other was treated with 10–50 mg TTAB l<sup>-1</sup>. After 15 min of exposure to TTAB, cells were harvested by centrifugation at 8000 g for 10 min in a Sorvall RC5C refrigerated centrifuge. The pellets obtained were washed twice with 0.9 % NaCl (w/v) and used for further studies.

**Lipid extraction and transesterification.** Lipids were extracted from the cells following the Bligh & Dyer (1959) method and separated by TLC (Silica Gel HLF, 250 µm; Analtech) according to Boeris *et al.* (2007). Separated lipids were visualized with iodine vapour and identified by comparison with purified standards (Sigma). All of the different phospholipid classes were scraped from the silica plates and the fractions were quantified by phosphorus determination as described by Fiske & Subbarow (1925).

Fatty acid methyl esters (FAMEs) were prepared from total phospholipid extracts using 10 % BF<sub>3</sub> in methanol (Morrison & Smith, 1964); they were then extracted with hexane and finally concentrated under a stream of N<sub>2</sub> gas.

To determine the fatty acid composition of every specific phospholipid class, the phospholipids were separated as described above, and each spot was scraped from the plates and then methyl-esterified. The resulting FAMEs were resolved according to number of double bonds on TLC plates impregnated with AgNO<sub>3</sub> (10 %, w/v) using hexane/ethyl ether/acetic acid (94 : 4 : 2, by vol.) as solvent. FAME bands were visualized under UV light after spraying the plates with 0.1 % 2,7-dichlorofluorescein in ethanol. Identified spots were scraped from the plates, eluted with hexane and finally dried within conical tubes (Henderson & Tocher, 1992).

**Fatty acid analysis.** FAMEs were analysed by using a Hewlett Packard 5890 II gas chromatograph equipped with a methyl silicone column (length 50 m; inner diameter 0.2 mm; film thickness 0.33 µm) and with a flame ionization detector. GC conditions were: injector temperature 250 °C; detector temperature 300 °C; nitrogen as carrier gas. Temperature program: 180 °C, 25 min isothermal; 3 °C min<sup>-1</sup> to 250 °C. Peak areas of carboxylic acids in total ions were used to determine their relative amounts (Paulucci *et al.*, 2011). Fatty acids were identified by comparison with retention times of purified standards (Sigma).

Replicate determinations indicated that the relative error [(SD/mean) × 100 %] of the values was 2–5 %. Three independent determinations were performed in each case. The SD was <5 %.

The degree of saturation of the extracts was defined as the ratio between the saturated fatty acids and the *cis*-unsaturated fatty acids.

**Determination of membrane fluidity.** Membrane fluidity was determined by measuring fluorescence polarization of a 1,6-diphenyl-1,3,5-hexatriene (DPH) probe inserted into the cytoplasmic membranes. Fluorescence polarization methods quantify the degree of depolarization of the light emitted by the embedded fluorescence probe, as a measurement of membrane state (Mykytczuk *et al.*, 2007). The relationship between the probe polarization ratio and cytoplasmic membrane fluidity is an inverse one. As the bacterial cytoplasmic

membrane fluidity decreases, the polarization ratio increases and vice versa (Litman & Barenholz, 1982). Following procedures described by Trevors (2003), cells of *P. putida* A (ATCC 12633) grown in HPI-BSM with glucose and  $\text{NH}_4\text{Cl}$  to  $\text{OD}_{660}$  0.8–1.00 were harvested, washed in sterile Tris/HCl buffer (pH 7.0;  $15 \text{ mmol l}^{-1}$ ) and resuspended in the same buffer up to  $\text{OD}_{660}$  0.2. Then,  $1 \mu\text{l}$  fluorescent probe (stock solution  $12 \text{ mmol l}^{-1}$  in tetrahydrofuran) was added to each 3 ml resuspended culture obtaining a final probe concentration of  $4 \mu\text{mol l}^{-1}$ . Cultures were incubated with a magnetic stirrer at 200 r.p.m. for 10 min in the dark, at room temperature, to allow DPH incorporation into cytoplasmic membranes. After this time, the culture was divided in two parts: one part was maintained as a control and the other was treated with  $50 \text{ mg TTAB l}^{-1}$ . Aliquots were collected at different times (1, 5, 15, 30 and 60 min) and their degree of polarization was determined.

Similarly, cells of *P. putida* A (ATCC 12633) grown in HPI-BSM medium with  $50 \text{ mg TTAB l}^{-1}$  as the carbon and nitrogen source until the beginning of the stationary phase ( $\text{OD}_{660}$  0.4) were incubated with DPH and measurements were performed.

Fluorescence polarization measurements were performed in a Hitachi 2500 spectrofluorometer with a Glam–Thomson polarizer. The excitation wavelength for the DPH probe was 358 nm and the emission wavelength was 428 nm. The slit widths for the excitation and emission beams were 12 and 10 nm, respectively.

The degree of polarization ( $P$ ) was calculated from the polarization ratio using the expression  $P = (I_{VV} - I_{VH}G) / (I_{VV} + I_{VH}G)$ , where  $I_{VV}$  and  $I_{VH}$  are the vertically and horizontally polarized components of light intensities emitted after excitation by vertically polarized light, respectively, and  $G$  is the sensitivity factor of the detection system (Lakowicz, 1999).

## RESULTS

### Changes in fatty acid composition of *P. putida* A (ATCC 12633) cells exposed to TTAB

We demonstrated previously that cells of *P. putida* A (ATCC 12633) grown in basal medium with glucose and  $\text{NH}_4\text{Cl}$  were differentially sensitive to the addition of TTAB:  $150 \text{ mg TTAB l}^{-1}$  killed 99 % of the cells, at  $100 \text{ mg TTAB l}^{-1}$  the number of cells decreased five to six orders of magnitude compared with the initial number of bacteria, whereas the addition of  $10\text{--}50 \text{ mg TTAB l}^{-1}$  resulted in survival of  $>99\%$  of the cells (Boeris *et al.*, 2007). As the mechanisms of adaptation of *P. putida* A (ATCC 12633) to TTAB indicated that the tolerance to  $50 \text{ mg TTAB l}^{-1}$  is related to changes in phospholipid composition (Boeris *et al.*, 2007, 2009), whole fatty acid profiles of cells grown in glucose and  $\text{NH}_4\text{Cl}$  medium either exposed or not exposed to  $50 \text{ mg TTAB l}^{-1}$  for 15 min were compared to assess the influence of the surfactant on the fatty acid composition of *P. putida* A (ATCC 12633) cells.

Fatty acid patterns of *P. putida* A (ATCC 12633) growing exponentially in basal medium with glucose and  $\text{NH}_4\text{Cl}$  as carbon and nitrogen sources are shown in Table 1. The most abundant saturated fatty acid was stearic acid (18:0), representing up to 60 % of the total fatty acids, followed by palmitic acid (16:0), which represented up to 20 % of the total fatty acid content. Palmitoleic acid (16:1 $\Delta$ 9) and

vaccenic acid (18:1 $\Delta$ 11) were dominant unsaturated fatty acids within the cells (6.27 and 5.83 %, respectively) (Table 1, column 1). All unsaturated fatty acids detected were in the *cis* configuration.

Total fatty acid composition after exposure to TTAB compared with control samples is listed in Table 1 (columns 2). When cells were exposed to  $50 \text{ mg TTAB l}^{-1}$ , 16:1 $\Delta$ 9 fatty acid decreased from 6.27 to 0.34 % and 18:1 $\Delta$ 11 fatty acid decreased from 5.83 to 0.14 %, whereas 15:0 and 16:0 fatty acids increased 87 and 24 %, respectively. These results indicated that the degree of saturation of fatty acids was affected. In this sense we also observed that the content of unsaturated fatty acids in TTAB-exposed cells was  $\sim 83\%$  lower than in control cells, increasing the saturated/unsaturated fatty acid ratio up to 49.74, whereas in control cells this value was only 7.92. We also detected a TTAB dose-dependent compensatory change in fatty acid composition of *P. putida* A (ATCC 12633) cells in response to increasing TTAB concentrations ( $10\text{--}50 \text{ mg l}^{-1}$ ). For all TTAB concentrations analysed, the decrease in the proportion of both 16:1 $\Delta$ 9 and 18:1 $\Delta$ 11 fatty acids was accompanied by a significant increase in the proportion of 16:0 fatty acid (Fig. 1). Although the saturated/unsaturated fatty acid ratio always showed an increase in treated cells in response to TTAB (12.46 and 17.21 for 10 and 25  $\text{mg TTAB l}^{-1}$ , respectively), a significantly higher ratio was obtained in cells exposed to  $50 \text{ mg TTAB l}^{-1}$  (49.74). These results suggested that the reduced content of unsaturated fatty acids in membranes promoted higher rigidity and this modification constituted an adaptive mechanism of *P. putida* A (ATCC 12633) cells to TTAB exposure.

When we evaluated the fluidity of membranes we found that immediately after the addition of  $50 \text{ mg TTAB l}^{-1}$ , the DPH fluorescence polarization value decreased from  $0.12 \pm 0.01$  to  $0.08 \pm 0.01$  after 1 min exposure to TTAB (Fig. 2), indicating that the surfactant had a fluidizing effect on the hydrophobic core region of the cell membrane. However, as shown in Fig. 2, after 15 min of exposure to TTAB, the DPH fluorescence polarization value returned to a similar value as that obtained in the absence of TTAB ( $0.11 \pm 0.01$ ). Therefore, the early effect of TTAB was an increase in the fluidity of the membrane; *P. putida* A (ATCC 12633) cells seemed to respond by decreasing the degree of unsaturated fatty acids in order to counteract the action of this compound.

### Changes in *P. putida* A (ATCC 12633) fatty acid composition during TTAB degradation

*P. putida* A (ATCC 12633) is able to degrade up to  $50 \text{ mg TTAB l}^{-1}$  when used as a sole carbon, nitrogen and energy source (Liffourrena *et al.*, 2008). To identify changes in the cellular fatty acid composition during bacterial degradation of TTAB, fatty acid profiles of control (growing with glucose and  $\text{NH}_4\text{Cl}$ ) and TTAB-treated (growing with  $50 \text{ mg TTAB l}^{-1}$ ) cells of *P. putida* A (ATCC 12633) were

**Table 1.** Effect of TTAB on the fatty acid composition of *P. putida* A (ATCC 12633)

Lipids were extracted and fatty acids of total lipids were converted to FAMES and analysed by GC. Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent mean  $\pm$  SD of three independent experiments.

Fatty acid	<i>P. putida</i> A (ATCC 12633)		
	Control*	Exposed to TTAB (15 min)*	Grown with TTAB†
<b>Saturated (%)</b>			
14:0	1.28 $\pm$ 0.55 <sup>a</sup>	1.65 $\pm$ 0.23 <sup>a</sup>	1.98 $\pm$ 0.28 <sup>a</sup>
15:0	0.07 $\pm$ 0.02 <sup>a</sup>	0.52 $\pm$ 0.21 <sup>b</sup>	0.15 $\pm$ 0.01 <sup>c</sup>
16:0	24.88 $\pm$ 1.63 <sup>a</sup>	32.66 $\pm$ 0.99 <sup>b</sup>	31.69 $\pm$ 3.09 <sup>b</sup>
17:0	2.63 $\pm$ 0.87 <sup>a</sup>	0.84 $\pm$ 0.12 <sup>b</sup>	1.08 $\pm$ 0.43 <sup>b</sup>
18:0	62.92 $\pm$ 8.33 <sup>a</sup>	62.84 $\pm$ 2.08 <sup>a</sup>	60.35 $\pm$ 8.32 <sup>a</sup>
Total	88.77 $\pm$ 5.02 <sup>a</sup>	97.99 $\pm$ 0.87 <sup>b</sup>	91.78 $\pm$ 7.87 <sup>a,b</sup>
<b>Unsaturated (%)</b>			
16:1 $\Delta$ 9	6.27 $\pm$ 0.14 <sup>a</sup>	0.34 $\pm$ 0.17 <sup>b</sup>	5.55 $\pm$ 1.03 <sup>a</sup>
18:1 $\Delta$ 9	1.99 $\pm$ 0.62 <sup>a</sup>	1.96 $\pm$ 0.48 <sup>a</sup>	2.9 $\pm$ 1.04 <sup>a</sup>
18:1 $\Delta$ 11	5.83 $\pm$ 0.4 <sup>a</sup>	0.14 $\pm$ 0.04 <sup>b</sup>	2.89 $\pm$ 0.46 <sup>c</sup>
Total	11.20 $\pm$ 5.04 <sup>a</sup>	1.97 $\pm$ 0.87 <sup>b</sup>	8.22 $\pm$ 1.2 <sup>c</sup>
<b>Saturated/unsaturated fatty acid ratio</b>	<b>7.92<sup>a</sup></b>	<b>49.74<sup>b</sup></b>	<b>11.16<sup>c</sup></b>

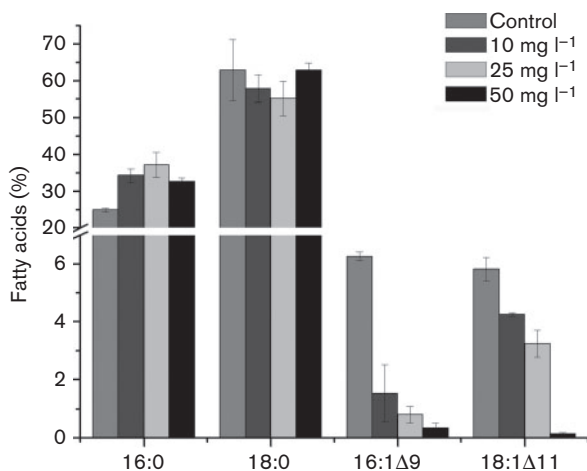
Values with the same letter within a row are not significantly ( $P \leq 0.05$ ) different according to the ANOVA test.

\*Cultures were grown with glucose and  $\text{NH}_4\text{Cl}$  up to late exponential phase. At this point, the cells were either not exposed (control) or exposed to 50 mg TTAB  $\text{l}^{-1}$ . After 15 min, the cells were collected.

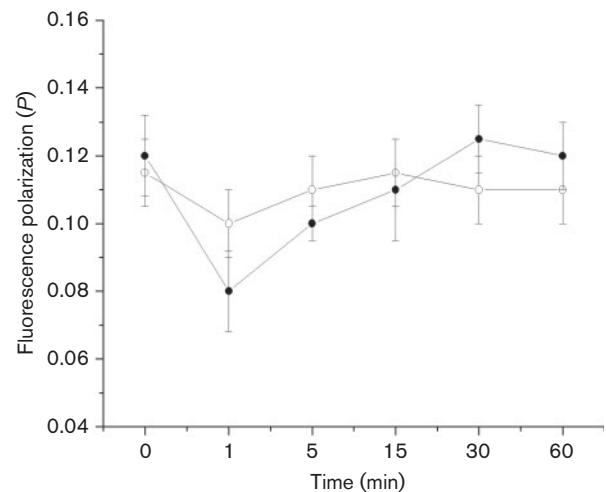
†Cultures were grown with 50 mg TTAB  $\text{l}^{-1}$  as the carbon and nitrogen source.

compared. No differences were observed in specific saturated fatty acids and unsaturated fatty acids of *P. putida* A (ATCC 12633) cells grown up to late exponential

phase either with or without TTAB. However, in cells grown with TTAB, the content of 15:0 and 16:0 fatty acids increased 50 and 20%, respectively. The saturated fatty acids represented up to 91.78% of total fatty acids, a value



**Fig. 1.** Changes in fatty acids of *P. putida* A (ATCC 12633) cells following exposure to different TTAB concentrations. Cultures were grown with glucose and  $\text{NH}_4\text{Cl}$  up to late exponential phase. At this point, the cells were exposed or not to 10, 25 and 50 mg TTAB  $\text{l}^{-1}$ . After 15 min, the cells were collected. Lipids were extracted and fatty acids of total lipids were converted to FAMES and analysed by GC. Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent mean  $\pm$  SD of three independent experiments.



**Fig. 2.** Fluorescence polarization of DPH in *P. putida* A (ATCC 12633) cells exposed or not to TTAB. Cells were harvested, washed, resuspended in Tris/HCl buffer to  $\text{OD}_{660}$  0.2 and then incubated in the absence (○) or presence (●) of 50 mg TTAB  $\text{l}^{-1}$  after DPH was added. Aliquots were collected at different times and their degree of polarization was determined. Results are the mean  $\pm$  SD of three independent determinations.

slightly higher than that detected in cells grown with glucose and  $\text{NH}_4\text{Cl}$  (88.77%) (Table 1, column 3). The total level of unsaturated fatty acids decreased from 11.20% in the control condition to 8.22% in the TTAB medium, mainly by decreasing the content of 18:1 $\Delta$ 11 from 5.83 (control value) to 2.89% (TTAB). Consequently, the proportion of the characterized fatty acids expressed as the saturated/unsaturated fatty acid ratio changed in response to TTAB, showing an increase of ~30% in treated cells. For the results obtained after 15 min of exposure of cells to the surfactant (Table 1, column 2), a decrease was observed in the content of unsaturated fatty acids, mostly due to a reduced content of 18:1 $\Delta$ 11.

Fluorescence polarization assays showed no differences in membrane states between cells grown with or without TTAB ( $P=0.14\pm 0.01$  and  $0.12\pm 0.01$ , respectively). The results indicated that the fluidizing effect of TTAB (Fig. 2) was counteracted in the membranes of the cells. In spite of the fact that in both assays that included contact of *P. putida* A (ATCC 12633) cells with TTAB (exposure or growing with TTAB) we found an increase in the saturated/unsaturated fatty acid ratio, a significantly higher ratio was obtained in cells exposed for 15 min to TTAB (49.74) compared with cells grown with TTAB (11.16). This suggested that other mechanisms may also be involved in the maintenance of *P. putida* A (ATCC 12633) membrane fluidity, enabling bacterial growth in the presence of TTAB.

#### Fatty acid composition of specific *P. putida* A (ATCC 12633) phospholipids

We analysed the fatty acid composition in each of the four main phospholipids of *P. putida* A (ATCC 12633), i.e. phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and phosphatidylcholine, after 15 min of exposure to 50 mg TTAB  $\text{l}^{-1}$ . In each phospholipid, the fatty acid pattern was qualitatively the same as that observed in the absence of TTAB (Table 2). Under both conditions (absence or presence of TTAB) and in each phospholipid class, the identified saturated fatty acids detected were 16:0 and 18:0, whilst the unsaturated fatty acids were 16:1 $\Delta$ 9 and 18:1 $\Delta$ 11.

It should be noted that phosphatidylglycerol, which represents 6.25% of the total *P. putida* A (ATCC 12633) phospholipid, carries the greater proportion of saturated fatty acids (Table 2). However, cardiolipin and phosphatidylcholine, representing 5.85 and 1.76%, respectively, of total phospholipid carry an elevated proportion of unsaturated fatty acids (18.33 and 21.44% of total unsaturated fatty acids, respectively). The content of saturated fatty acids and unsaturated fatty acids in phosphatidylglycerol and cardiolipin did not change significantly when the cells were exposed to TTAB (Table 2). However, in cells exposed to this surfactant, phosphatidylcholine had ~11% more saturated fatty acids than control cells and also their levels of unsaturated fatty acids decreased ~48% compared with unexposed cells (Table 2).

## DISCUSSION

In this work, we studied the modifications in lipid composition of *P. putida* A (ATCC 12633) in relation to TTAB tolerance. In previous studies, we found that this strain was able to modify the phospholipid composition of its membranes in response to stress produced by the presence of 50 mg TTAB  $\text{l}^{-1}$  (Boeris *et al.*, 2007, 2009). Here, we evaluated in detail the parameters that alter fluidity, as the lipid composition is tightly linked to the state of the membranes. This evaluation was performed through analysis of the degree of saturation in fatty acid alkyl chains of membrane phospholipids. Fatty acid patterns detected in *P. putida* A (ATCC 12633) are consistent with those determined for other *Pseudomonas* species where the predominant fatty acids are saturated (16:0 and 18:0) and monounsaturated (16:1 $\Delta$ 9 and 18:1 $\Delta$ 11) (Ramos *et al.*, 1997; Schweizer, 2004). However, in contrast to earlier reports related to fatty acid analysis within *P. putida* strains, such as *P. putida* KT2440, *P. putida* DOTT1E, *P. putida* P8 and *P. putida* NYC10936, we have not detected either the fatty acid with a cyclopropane ring (*cis*-9,10-methylenehexadecanoic acid) known as C17:cyclopropane nor the hydroxy fatty acid. Remarkably, *P. putida* A (ATCC 12633) presented a lower proportion of unsaturated fatty acids than found in previously analysed strains (Diefenbach *et al.*, 1992; Loffeld & Keweloh, 1996; Pinkart *et al.*, 1996; Ramos *et al.*, 1997; Neumann *et al.*, 2003; Schweizer 2004; Härtig *et al.*, 2005; Pini *et al.*, 2009). In addition, *trans* unsaturated fatty acids were not found in *P. putida* A (ATCC 12633) during its exponential growth phase, regardless of the presence or absence of QACs in the medium.

The overall results obtained in this work indicate that the content of unsaturated fatty acids in TTAB-exposed cells decreases as a response to an increasing concentration of TTAB in the medium (Table 1, Fig. 1). As a result, the saturated/unsaturated fatty acid ratio always showed an increase in treated cells in response to different TTAB concentrations, although major changes were evident with 50 mg  $\text{l}^{-1}$ . When *P. putida* A (ATCC 12633) cells were exposed to 50 mg TTAB  $\text{l}^{-1}$  the content of unsaturated fatty acids was ~83% lower than control values and consequently a 6.3-fold increased saturated/unsaturated fatty acid ratio was obtained under this condition (Table 1). The same response was obtained when cells were grown with 50 mg TTAB  $\text{l}^{-1}$  as the sole carbon and nitrogen source, although under this condition the saturated/unsaturated fatty acid ratio increased only 1.4-fold compared with the control. Although the addition of this non-lethal concentration of TTAB resulted in the immediate fluidification of *P. putida* A (ATCC 12633) cell membranes, we detected a complete reversal of this fluidification induced by TTAB after long-term incubation with the surfactant. After 15 min of incubation of *P. putida* A (ATCC 12633) cells with TTAB, DPH fluorescence polarization values were similar to those registered in the absence of TTAB (Fig. 2). In this sense, we found a decrease in the content of membrane unsaturated fatty acids that confers a higher rigidity to membranes

**Table 2.** Fatty acid composition of individual phospholipids of *P. putida* A (ATCC 12633) after 15 min of exposure to TTAB

Phospholipids were resolved by TLC and levels of inorganic phosphorus were determined (Boeris *et al.*, 2007). The fatty acids of total lipids were converted to FAMES, separated according to degree of unsaturation and detected under UV light. The spots identified were scraped off the plates and analysed by GC, as described in Methods.

	Phosphatidylethanolamine		Phosphatidylglycerol		Cardiolipin		Phosphatidylcholine	
	Control	+TTAB	Control	+TTAB	Control	+TTAB	Control	+TTAB
<b>Content [nmol phospholipid (mg protein)<sup>-1</sup>]*</b>	5.70 ± 0.25 (77.55)	4.95 ± 0.44 (71.84)	0.46 ± 0.14 (6.25)	0.90 ± 0.15 (13.06)	0.43 ± 0.07 (5.85)	0.16 ± 0.011 (2.32)	0.13 ± 0.04 (1.76)	0.11 ± 0.02 (1.59)
<b>Fatty acids (%)</b>								
16:0	36.19 ± 0.89	40.29 ± 0.40	38.09 ± 0.86	40.16 ± 0.97	33.09 ± 0.98	38.07 ± 1.50	30.3 ± 1.18	37.96 ± 2.03
18:0	50.55 ± 1.42	45.02 ± 1.23	50.68 ± 0.95	49.66 ± 0.87	48.57 ± 0.92	43.6 ± 1.20	48.79 ± 1.06	50.78 ± 1.95
Total saturated fatty acids	86.74 ± 1.20	85.31 ± 1.60	88.77 ± 0.69	89.82 ± 0.90	81.66 ± 1.30	81.67 ± 1.36	79.09 ± 1.43	88.74 ± 1.36
16:1Δ9	6.47 ± 0.30	6.22 ± 0.45	3.92 ± 0.29	3.83 ± 0.81	7.80 ± 0.40	8.03 ± 1.02	9.81 ± 1.23	5.27 ± 0.52
18:1Δ11	6.79 ± 0.01	8.46 ± 0.52	7.32 ± 0.68	6.33 ± 1.02	10.53 ± 1.05	10.29 ± 1.05	11.63 ± 1.40	5.98 ± 0.60
Total unsaturated fatty acids	13.26 ± 0.36	14.68 ± 0.65	11.24 ± 0.96	10.16 ± 1.05	18.33 ± 1.03	18.32 ± 1.05	21.44 ± 1.20	11.25 ± 1.24

Control, cultures were grown with glucose and NH<sub>4</sub>Cl up to late exponential phase.

+TTAB, cultures were grown with glucose and NH<sub>4</sub>Cl up to late exponential phase and 50 mg TTAB l<sup>-1</sup> was added. After 15 min, the cells were collected and lipids were extracted and analysed.

\*Numbers in parentheses indicate the percentage of the total phospholipid content (mol phosphorus).

(Mykytczuk *et al.*, 2007). This response constitutes an adaptive mechanism of *P. putida* A (ATCC 12633) in the presence of TTAB. As reported by Keweloh *et al.* (1991), the decrease in the content of unsaturated fatty acids probably helps not only to compensate for the fluidizing effect of TTAB, but also to block access of TTAB molecules within the membrane interior due to the greater packing of the saturated chains.

Different reports point out the increasing degree of lipid saturation in membranes as one of the major adaptive mechanisms of bacterial cells to the presence of many toxic compounds (Keweloh *et al.*, 1991; Heipieper *et al.*, 1994; Gutierrez *et al.*, 1999; Cronan, 2002; Denich *et al.*, 2003). It has been reported that aromatic compounds such as benzene, phenol and toluene bring fatty acid alkyl chains of the phospholipid in the cell membrane closer together, increasing the rigidity of the membrane and allowing tolerance to aromatic substrates (Sikkema *et al.*, 1994, 1995). A similar response was activated in *P. putida* A (ATCC 12633) due to the presence of the cationic surfactant TTAB.

Given that all of the unsaturated fatty acids extracted from *P. putida* A (ATCC 12633) cells, either exposed to or grown with TTAB, were in the *cis* configuration, we assume that TTAB did not induce the enzyme responsible for the *cis/trans* isomerization of unsaturated fatty acids – one of the adaptive mechanisms that allows micro-organisms to survive in toxic environments (Heipieper *et al.*, 2003; Bernal *et al.*, 2007b).

Studies on microbial mechanisms of adaptation to QACs include the analysis of changes in the composition of fatty acids and of changes in the polar heads of the phospholipids, separately (Jones *et al.*, 1989; Guérin-Méchin *et al.*, 1999; Méchin *et al.*, 1999; Loughlin *et al.*, 2002; To *et al.*, 2002; Boeris *et al.* 2007, 2009; Bisbiroulas *et al.*, 2011). The purpose of our study was to integrally analyse the adaptation responses of *P. putida* A (ATCC 12633), including the link with the alterations of membranes. It is known that cells of *P. putida* A (ATCC 12633) exposed to TTAB tend to respond by increasing phosphatidic acid and phosphatidylglycerol levels, and decreasing cardiolipin (Boeris *et al.*, 2007, 2009). Consequently, one of the major bacterial responses in TTAB-containing media is to produce a highly negatively charged membrane by increasing the amount of anionic phospholipids, probably to neutralize the positive charge found in the trimethylammonium moiety of TTAB. The fatty acid composition in each of the four main phospholipids of *P. putida* A (ATCC 12633) showed that phosphatidylglycerol carries the greater proportion of saturated fatty acids (Table 2). The content of phosphatidylglycerol increased sixfold when the cells were exposed to TTAB or when they were grown on TTAB as the sole carbon and nitrogen source (Boeris *et al.*, 2009), allowing greater rigidity of membranes. Thus, this alteration resulted in less mobile lipids within the phospholipid bilayer and in a more rigid structure of membranes that is

able to counteract the fluidizing effect of TTAB. However, cardiolipin carried an elevated proportion of unsaturated fatty acids (18.33% of total unsaturated fatty acids) and when *P. putida* A (ATCC 12633) was exposed to TTAB, the levels of cardiolipin decreased up to 60%. Therefore, the decrease detected in the content of unsaturated fatty acids (Table 1) can be associated with the decrease in the content of cardiolipin registered when cells were exposed to TTAB.

Moreover, the presence of TTAB reduced the content of unsaturated fatty acids in phosphatidylcholine (48%) (Table 2). As biosynthesis of unsaturated fatty acids in *Pseudomonas* is associated with membrane-localized desaturase enzymes (Baysse & O'Gara, 2007), such a reduction of the unsaturated fatty acids in phosphatidylcholine could be explained by inhibition of a  $\Delta^9$  desaturase (DesA) that was detected in *P. putida* A (ATCC 12633) and is capable of catalysing the oxygen-dependent desaturation at the *sn*-2 position of existing phospholipids (Nelson *et al.*, 2002; Zhu *et al.*, 2006). Whilst further research is required to elucidate the participation of  $\Delta^9$  desaturase in the response to TTAB, this assumption is supported by the decrease in the level of 16:1 $\Delta^9$  fatty acid and the concomitant increase in the content of 16:0 fatty acid detected under TTAB stress conditions. However, independent of the desaturase acting on different phospholipid species, it is clear that phosphatidylcholine constituted <2% of the total phospholipids of *P. putida* A (ATCC 12633) cells and its content did not change when cells were exposed to TTAB, indicating its minor participation in the response to QAC.

In summary, both the decrease in the content of unsaturated fatty acids due to the reduction in cardiolipin levels and the increase in phosphatidylglycerol levels, with its major content of saturated fatty acids, contribute to *P. putida* A (ATCC 12633) tolerance to stress resulting from the addition of TTAB. Both responses indicate a mutual dependency and make cells more resistant to the fluidizing effect of the cationic surfactant, and probably allow *P. putida* A (ATCC 12633) to survive in TTAB-contaminated sites.

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