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Release of outer membrane vesicles in *Pseudomonas putida* as a response to stress caused by cationic surfactants

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Pseudomonas putida A (ATCC 12633), a degrader of cationic surfactants, releases outer membrane vesicles (OMVs) when grown with tetradecyltrimethylammonium bromide (TTAB) as the sole carbon, nitrogen and energy source. The OMVs exhibit a bilayer structure and were found to be composed of lipopolysaccharides, proteins and phospholipids (PLs) such as cardiolipin, phosphatidylcholine, phosphatidic acid and phosphatidylglycerol (PG). The OMVs showed a marked increase in the PG content, approximately 43 % higher than the amount registered in the parent cells from which the vesicles were derived. After growth of *P. putida* with TTAB, the amount of lipoprotein covalently cross-linked to the peptidoglycan showed a twofold decrease when compared with values found after growth without the surfactant [16 ± 2 and 28 ± 3 μg (mg cell envelope protein)$^{-1}$, respectively]. This decrease in the amount of lipoprotein can be related to areas of loss of contact between the outer membrane and the peptidoglycan and, therefore, to OMV production. In addition, due to its amphiphilic nature, TTAB can contribute to OMV biogenesis, through a physical mechanism, by induction of the curvature of the membrane. Taking into account that OVMs were produced when the cells were grown under external stress, caused by the surfactant, and that TTAB was detected in the vesicles [48 nmol TTAB (nmol PL)$^{-1}$], we concluded that this system of TTAB elimination is a mechanism that *P. putida* A (ATCC 12633) would utilize for alleviating stress caused by cationic surfactants.

INTRODUCTION

Quaternary ammonium compounds (QACs) are molecules with at least one long hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom. The other alkyl groups are mostly short-chain substituents, such as methyl or benzyl groups. Among the QACs, the generic term cetrimide relates to mixtures of n-alkyltrimethyl ammonium bromides, where the n-alkyl group is between 8 and 18 carbons long, whereas cetrimide USP is tetradecyltrimethylammonium bromide (TTAB). Another predominant QAC found in engineered and natural systems is benzalkonium chloride (BAC) (Kreuzinger *et al.*, 2007; Hajaya & Pavlostathis, 2012). Unlike TTAB, BAC contains a quaternary N bound to two methyl groups, a benzyl group and a straight-chained alkyl group that varies in length (typically C12, C14 and C16).

Despite the global trend for the use of non-ionic surfactants, QACs will remain the most widely used surfactants in pharmaceutical and fabric-softener formulations, cosmetics, commercial disinfectants, industrial sanitizers, food preservatives and phase-transfer catalysts (Zhang *et al.*, 2015). After usage, the residual products are discharged to sewage treatment plants or surface waters and finally to coastal waters, in which the biodegradability of QACs is limited, depending on their concentration, chemical structure and antimicrobial activity. Therefore, QACs may persist in natural and engineered biological systems, where they can be toxic for life (van Ginkel & Kolvenbach,

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Abbreviations: BAC, benzalkonium chloride; CL, cardiolipin; DPH, 1,6-diphenyl-1,3,5-hexatriene; OMV, outer membrane vesicle; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid; QAC, quaternary ammonium compound; RBC, red blood cell; SFA, saturated fatty acid; TEM, transmission electron microscopy; TTAB, tetradecyltrimethylammonium bromide; UFA, unsaturated fatty acid.

A supplementary table is available with the online Supplementary Material.

1991; Nishiyama *et al.*, 1995; Oh *et al.*, 2014; Tezel & Pavlostathis, 2015; Zhang *et al.*, 2015), and for this reason, the effective degradation of cationic surfactants is necessary to protect the environment. In particular, *Pseudomonas putida* A (ATCC 12633) is able to mineralize TTAB and BAC, and offers promising opportunities for the efficient biological removal of these and other predominant QACs found in engineered and natural systems (Liffourrena *et al.*, 2008; Lucchesi *et al.*, 2010; Bergero & Lucchesi, 2013, 2015).

The adaptation of bacteria to a change of environmental conditions constitutes a key survival strategy. The bacterial membrane plays an important role in this process because it is very sensitive to stress. 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, inhibition of protein action and subsequent deleterious effects on growth and viability (McDonnell & Russell, 1999; McBain *et al.*, 2004; Gilbert & Moore, 2005). Although the connection between QAC degradation and tolerance is not well known, we have shown that in *P. putida* A (ATCC 12633) the adaptive response and resistance to the bactericidal activity of the QACs TTAB involves a mechanism that initiates changes to membrane composition (Boeris *et al.*, 2007, 2009; Heredia *et al.*, 2014). The addition of a non-lethal concentration of TTAB resulted in the immediate fluidification of *P. putida* A (ATCC 12633) cell membranes. However, after 15 min of incubation with TTAB, a reversal of membrane fluidification was detected. This behaviour was related to a decrease in the proportion of the unsaturated fatty acids (UFAs) that help to compensate the fluidizing effect of TTAB, and also to block the access of TTAB molecules within the membrane's interior. Also, when the cells were exposed to TTAB, the content of phosphatidylglycerol (PG) increased sixfold, and the levels of cardiolipin (CL), with its elevated proportion of UFAs (18.33 % of total UFAs), decreased up to 60 % (Boeris *et al.*, 2007, 2009; Heredia *et al.*, 2014). Thus, the decrease in the content of UFAs, due to the reduction of CL levels, together with an increase of PG levels, with the major content of saturated fatty acids (SFAs), contributes to the tolerance of *P. putida* A (ATCC 12633) to the stress caused by TTAB (Heredia *et al.*, 2014).

Gram-negative bacteria have outer and inner membranes with different compositions and essential biological functions. Consequently, both membranes have to remain functional even under variable conditions. The best-investigated mechanism that involves changes in the outer membrane of Gram-negative bacteria is the formation of outer membrane vesicles (OMVs) (Kulp & Kuehn, 2010; Kulkarni & Jagannadham, 2014; Klimentová & Stulík, 2015). The OMVs are released during all growth phases of the bacterial cultures (Kadurugamuwa & Beveridge, 1995; Manning & Kuehn, 2011), although their amount and composition may vary according to the growing conditions. Structural studies of OMV invariably showed that they are small spherical particles, from 50 to 250 nm, enclosed by a membrane bilayer, that contain phospholipids (PLs), LPSs, proteins and in some cases nucleic acids. They are also involved in the transport of metabolites, toxins, signalling molecules and other small molecules (Horstman & Kuehn, 2000, 2002; Dutta *et al.*, 2004; Keenan *et al.*, 2008; Bomberger *et al.*, 2009; Schertzer & Whiteley, 2012; Biller *et al.*, 2014; Klimentová & Stulík, 2015). Furthermore, the formation of OMVs is a crucial part of the bacterial envelope stress response, protecting the bacteria from internal and external stressors (Manning & Kuehn, 2011; Baumgarten *et al.*, 2012; MacDonald & Kuehn, 2012; Schwechheimer *et al.*, 2013, 2014). In the present study, we investigated the formation of OMVs in *P. putida* A (ATCC 12633) in the presence of the QAC TTAB. We show that the release of OMVs containing TTAB is part of the response of *P. putida* to the presence of the surfactant. This mechanism enables bacteria to eliminate detergent molecules attached to the membrane.

METHODS

Micro-organisms and culture conditions. *P. putida* A (ATCC 12633) cells were grown aerobically at 30 °C, with shaking in a basal salt liquid medium (HPi-BSM) (Lucchesi *et al.*, 1989) with 20 mmol glucose l^{-1} and 18 mmol NH_4Cl l^{-1} or 0.15 mmol TTAB l^{-1} (50 mg l^{-1}) as carbon and nitrogen source. Growth was evaluated through measurement of culture optical density at 660 nm by using a light visible spectrophotometer (Beckman DU 640). In experiments where *P. putida* was exposed to TTAB, cells were grown in HPi-BSM plus glucose and NH_4Cl until the culture reached late-exponential phase (OD_{660} 0.8–1.00). At this point, the culture was divided into two parts: one was kept as a control and the other was treated with 50 mg TTAB l^{-1}. After different times, cells were harvested by centrifugation at 10 000 ***g*** for 15 min in a Sorvall RC5C refrigerated centrifuge, and the pellets and supernatants were used for further studies.

Isolation of vesicles. Vesicles were obtained as described by Grenier & Mayrand (1987). Briefly, 500 ml bacterial culture in early stationary growth phase was centrifuged at 10 000 ***g*** for 15 min. The supernatants obtained were treated with ammonium sulfate (40 % saturation). After 2 h of stirring at 4 °C, the suspensions were centrifuged at 20 000 ***g*** for 40 min, and the pellets obtained were suspended in 2 ml of 50 mmol Tris/HCl buffer l^{-1} (pH 9.5) plus 0.5 mmol DTT l^{-1}. The suspensions were dialysed against the same buffer and the vesicles were recovered by centrifugation at 27 000 ***g*** for 40 min at 4 °C, washed with 10 ml of 50 mmol Tris/HCl buffer l^{-1} (pH 7.2) plus 0.5 mmol DTT l^{-1} and finally suspended in 1.5 ml of 50 mmol Tris/HCl bufferl^{-1} (pH 7.2). The vesicles were stored at −20 °C.

Isolation of the peptidoglycan–lipoprotein complex. Cells of *P. putida* A (ATCC 12633) grown in HPi-BSM with TTAB or glucose and NH_4Cl were harvested at exponential phase (OD_{660} 0.45 and 0.85, respectively), washed and resuspended with HPi-BSM. The cells were disrupted by sonication at 20 000 Hz using a Vibra-Cell ultrasonic processor (10 cycles, 10 s per cycle) and, after centrifugation (16 000 ***g*** for 10 min at 4 °C), the soluble fraction containing the cell envelope was collected. The protein concentration of the cell envelope was determined by the Bradford method (Bradford, 1976). Then, a suspension containing 200 mg protein ml^{-1} was treated with 4 % SDS, boiled for 2 h and then kept overnight at room temperature. The suspension was spun down at 78 000 ***g*** at 4 °C for 20 min, washed at least three times with water and finally the peptidoglycan–lipoprotein

complex obtained was suspended in buffer 25 mmol Tris/HCl l^{-1} (pH 8.0).

To solubilize the lipoproteins, the peptidoglycan–lipoprotein complex was digested with 0.5 μmol lysozyme l^{-1}, for 3 h at room temperature. The reaction was stopped by the addition of 1 % SDS and the protein concentration was determined by the Bradford method (Bradford, 1976). The amount of solubilized Lpp protein was expressed as μg (mg cell envelope protein)$^{-1}$. After adding Laemmli solution (Laemmli, 1970), the suspension was boiled for 3 min and subsequently submitted to 15 % SDS-PAGE (denaturing PAGE). The protein bands were visualized by staining with Coomassie blue R-250. After visualization, the bands of interest were excised from the gel and sent for analysis by MALDI-TOF.

Echinocytosis experiments. Fresh blood samples were collected from rabbits with a syringe, and CPD buffer (26.3 g trisodium citrate dihydrate l^{-1}, 3.27 g citric acid monohydrate l^{-1}, 2.22 g sodium dihydrogen phosphate l^{-1}, 23.2 g glucose l^{-1}) was added to the samples (1 : 10). The red blood cells (RBCs) were washed three times and diluted 400 times in PBS [10 mmol Tris l^{-1}, 146 mmol NaCl l^{-1} (pH 7.5)] to give working solutions of ~0.1 % haematocrit (Schertzer & Whiteley, 2012). The RBCs were incubated for 30 min at 4 °C with TTAB (5 and 10 g l^{-1}) and were then observed using a microscope (model H600L; Nikon) with a ×40 objective, and multiple fields were photographed. After TTAB treatment, chlorpromazine (0–5 μmol l^{-1}) was added, when necessary. Micrographs were subsequently analysed by manually counting and recording the total number of cells and the number of echinocytes observed.

Extraction of LPS. Extraction of LPS was carried as described by Westphal & Jann (1965). Briefly, 100 μl vesicle suspension obtained as described above were mixed with 3 ml phenol : H_2O (45 : 55, v/v). The mixture was incubated at 68 °C in an orbital shaker at 160 r.p.m. for 15 min and then centrifuged (9600 ***g***). The aqueous phase was collected. A second extraction was made on the mixture of phenol and vesicles by the addition of 3 ml distilled water and centrifuged again. Both aqueous phases were combined and dialysed against distilled water until the phenol was completely eliminated. The dialysed LPS was incubated overnight at 37 °C with RNase (15 μg ml^{-1}), DNase (15 μg ml^{-1}) and proteinase K (15 μg ml^{-1}) in 50 mmol Tris/HCl (pH 7) l^{-1}/10 mmol $MgSO_4$ l^{-1}. Samples were dried in a Rotavapor and subsequently submitted to 14 % SDS-PAGE. The gel was fixed overnight in fixing solution (40 % ethanol, 5 % acetic acid and 0.005 % Alcian blue) and then washed three times with deionized water. Finally, the SDS-PAGE-fractionated LPSs were stained by silver staining (Gromova & Celis, 2006).

Lipid analysis. Lipids were extracted from the cells and vesicles following the method of Bligh & Dyer (1959); they were later separated using 1D TLC (silica gel HLF, 250 microns; Analtech) and 2D TLC was carried out, when necessary, to discard the co-migration of different lipids (Boeris *et al.*, 2007). The separated lipids were detected with iodine vapours and identified based on comparison to purified standards purchased from Sigma. The spots were scraped off the plates, and the different fractions were quantified by phosphorus determination (Fiske & Subbarow, 1925).

Fatty acid methyl esters were prepared from total PL extracts using 10 % BF_3 in methanol (Morrison & Smith, 1964), then extracted with hexane, concentrated under an N_2 gas stream and analysed 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 according to Heredia *et al.* (2014).

The percentages of lipids and fatty acids reported are relative to the respective total amount, defined as 100 %. Replicate determinations indicated that the relative error [(SD/mean) × 100 %] of the values was between 2 and 5 %. Three independent determinations were performed in each case. The SD was less than 5 %.

Determination of membrane fluidity. Membrane fluidity was determined by measuring the fluorescence polarization of a 1,6-diphenyl-1,3,5-hexatriene (DPH) probe inserted into the membranes of the vesicles, following procedures described by Trevors (2003). Fluorescence polarization measurements were performed with 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 width for the excitation and emission beams was 12 and 10 nm, respectively. The degree of polarization was calculated from the polarization ratio (P) as described by Lakowicz (1999).

TTAB determination. TTAB was determined in either the supernatants of the batch cultures or the vesicles using a colorimetric method based on the reaction of this QAC with Patent Blue Violet (Sigma) (Tezel *et al.*, 2006). According to this method, an anionic dye–TTAB pair is formed, which is then solvent extracted and the colour intensity in the extracted phase is spectrophotometrically measured at 620 nm. The concentration of TTAB was calculated using calibration graphs that were previously constructed.

Transmission electron microscopy (TEM). Bacterial pellets were fixed in 2.5 % glutaraldehyde, *S*-collidine buffer solution (0.2 mol l^{-1}) (pH 7.4) and post-fixed in 1 % osmium tetroxide, *S*-collidine buffer solution (0.2 mol l^{-1}) (pH 7.4). Cell pellets were then dehydrated in increasing concentrations of acetone (1 × 50 %, 1 × 70 %, 1 × 90 % and 3 × 100 %), embedded in EMbed 812 resin and finally sectioned with an ultra-microtome. The ultra-thin sections (±60 nm) were cut and placed on copper grids, counterstained with 50 % saturated uranyl acetate for 8 min and aqueous lead citrate solutions for 30 min (Cristofolini *et al.*, 2012). Vesicles were resuspended in 10 mmol $MgCl_2$ l^{-1} and negatively stained with a 2 % phosphotungstic acid solution (pH 7.3) (Deatherage *et al.*, 2009). The sections of the cells or vesicles were examined with an Elmiskop 101 transmission electron microscope (Siemens) and the acquisition of images was performed with Digital Micrograph software (Gatan).

RESULTS AND DISCUSSION

TTAB stress and vesicle production

Using TEM, we analysed the integrity of the membranes when cells of *P. putida* A (ATCC 12633) were grown in a basal medium with and without 50 mg TTAB l^{-1}. As shown in Fig. 1(a, b), the cells possessed intact cell envelopes, regardless of the presence or absence of this QAC. It should be noted that the cells grown with TTAB exhibited several membrane blebs, emanating from the cell surfaces or free in the environment (Fig. 1b, c). The OMVs presented a refractive envelope that was characteristic of a bilayer structure. Its diameter was approximately 200 nm (Fig. 1d).

The analysis of the structural components of the OMVs showed that LPS, proteins, PL and TTAB were present. After SDS-PAGE of the OMVs fraction of *P. putida* A (ATCC 12633), revealed by silver staining, we found bands of LPS (Fig. 2), characteristic of Gram-negative cell envelopes (Kadurugamuwa & Beveridge, 1995).

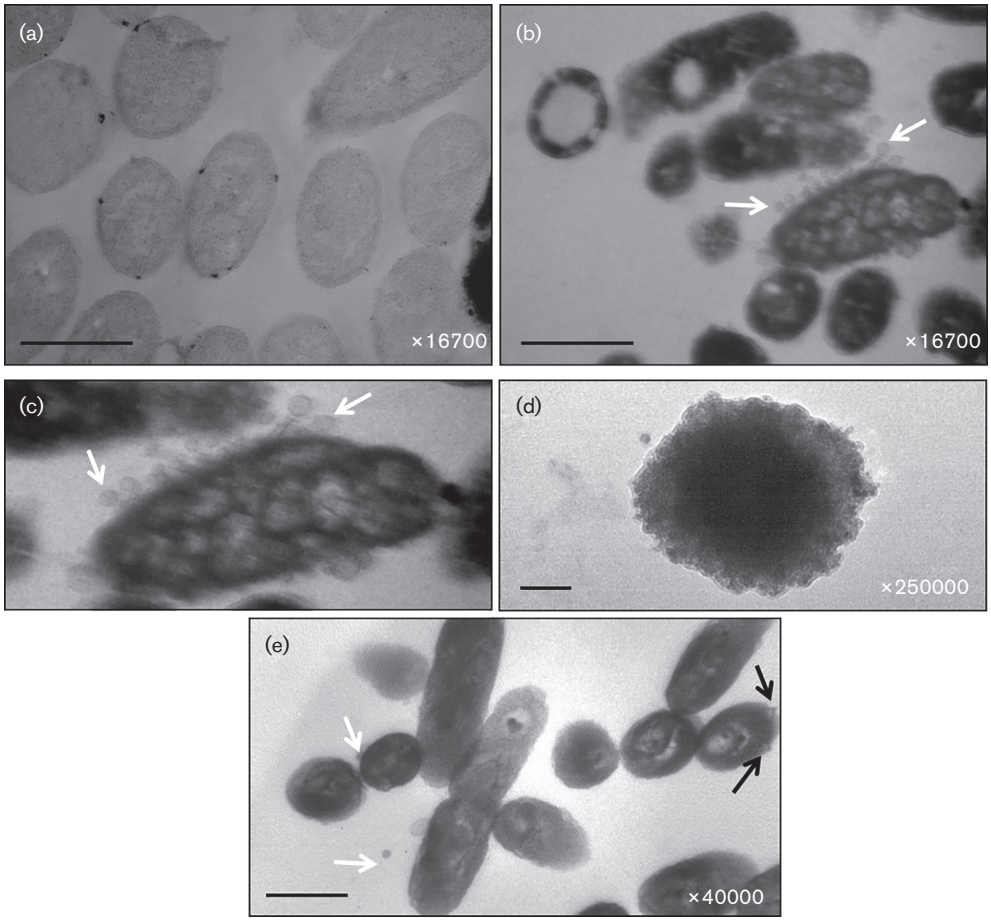

Fig. 1. TEM images of *P. putida* A (ATCC 12633) and its released vesicles. *P. putida* cells were grown until late-exponential phase in HPi-BSM with TTAB (OD_{660} 0.4) or with glucose and NH_4Cl (OD_{660} 0.8) exposed and not exposed to TTAB. The cells were harvested by centrifugation and the vesicles were obtained from the supernatant, as described in Methods. (a) Thin section of *P. putida* A (ATCC 12633) grown with glucose/NH_4Cl. (b) Thin section of *P. putida* A (ATCC 12633) grown with TTAB. The white arrows indicate OMVs. (c) An enlargement of part of the image in (b) showing the development of OMVs, while they were being released from the cells grown with TTAB. The white arrows indicate OMVs. (d) OMVs isolated and negatively stained. (e) Thin section of *P. putida* A (ATCC 12633) grown with glucose/NH_4Cl and exposed to TTAB for 60 min. The white arrows indicate OMVs and black arrows indicate spicule-containing cells. Bars, 1 μm in (a), (b) and (e); 0.1 μm in (d).

In the OMVs, 0.076 mg total proteins ml^{-1} and 0.56 nmol total PL ml^{-1} were registered, equivalent to 7.4 nmol PL (mg protein)$^{-1}$. The analysis of PLs revealed the presence of PG, phosphatidylcholine (PC), phosphatidic acid (PA) and CL (Table 1). PLs are one of the major components of the OMVs produced by different bacteria. In spite of the fact that the relative amount of each PL may change in the OMVs, numerous reports have described that the PL found in OMVs generally resembles the PL composition of the outer membrane from which the vesicles are derived (Horstman & Kuehn, 2000; Kato *et al.*, 2002; Tashiro *et al.*, 2011; Chowdhury & Jagannadham, 2013; Kulkarni *et al.*, 2014). Similarly to results found by Tashiro *et al.* (2011) when studying the OMVs of *Pseudomonas aeruginosa*, we observed that the OMVs produced by *P. putida* A (ATCC 12633) did not contain PE and that PG was the major glycerophospholipid detected (42.17 % of total PL; Table 1). Different facts indicated PG as the main component of the OMVs released by *P. putida* in the presence of TTAB: the sixfold increase (up to 120 %) in the content of PG (Table S1, available in the online Supplementary Material; Boeris *et al.*, 2007, 2009) and the fact that PG has been associated with the attainment of the proper curvature for the release of membrane vesicles (Tashiro *et al.*, 2012).

The results of the fatty acid methyl ester analysis showed that, similarly to previously obtained data of the membranes of whole cells grown with TTAB (Table S1), stearic acid (C18 : 0) and palmitic acid (C16 : 0) were the predominant SFAs of the vesicles (Table 1). We also found that the amount of UFAs in the vesicles was 10 % higher with

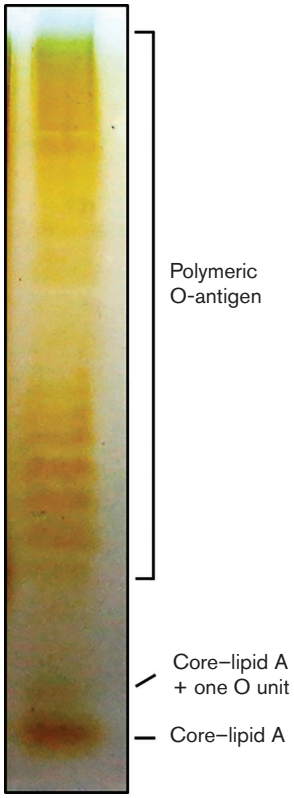

Fig. 2. Profiles of LPS of the OMVs. The purified LPS of the OMVs was separated on SDS-PAGE (14 %) and stained with a silver stain, as described in Methods. The gel displayed different bands corresponding to: LPS with incomplete O-antigen, the core region attached to lipid A and the core region attached to lipid A plus one O-antigen unit.

respect to the content detected in TTAB-grown cells (Table S1; Heredia *et al.*, 2014). Based on these findings, a higher fluidity can be expected in the vesicles with respect to the parent cells. However, when we evaluated the fluidity of the OMVs by means of DPH fluorescence polarization, a probe that penetrates the hydrophobic core orienting itself parallel to the fatty acid side chains (Trevors, 2003; Mykytczuk *et al.*, 2007), we found a *P*-value of 0.16 ± 0.01, while a similar *P*-value (0.14 ± 0.01) was registered for *P. putida* cells grown with TTAB.

One of the most interesting observations was the presence of TTAB in the vesicles. When the cells were grown with 50 mg TTAB l^{-1} as a sole source of carbon and nitrogen, 18 mg TTAB l^{-1} was detected in the supernatant of the culture after 48 h. Within this total surfactant content, 9 mg TTAB l^{-1} was detected in the isolated OMVs [equivalent to 48 nmol TTAB (nmol PL)$^{-1}$].

No production of OMVs was observed when *P. putida* A (ATCC 12633) cells were grown in saline medium with glucose and NH_4Cl as carbon and nitrogen sources, respectively (Fig. 1a). To evaluate the relationship between OMV formation and the stress caused by TTAB,

Table 1. PL and fatty acid composition of *P. putida* A (ATCC 12633) OMVs

P. putida A (ATCC 12633) was grown in HPi-BSM with TTAB up to late-exponential phase (OD_{660} 0.4). At this point, cells were harvested; the vesicles were obtained from the supernatant. PLs and fatty acid were extracted from OMVs and were analysed, as described in Methods. Percentages of PL and fatty acid are relative to the respective total amount, defined as 100 %. Results are means ± SDs (n=3).

PL/fatty acid	OMVs (%)
Specific PL	
CL	15.03 ± 2.92
PG	42.17 ± 2.95
PC	24.62 ± 1.98
PA	18.16 ± 2.87
Specific fatty acids	
SFA	
16:0	35.31 ± 4.44
18:0	51.85 ± 5.12
Total	87.16 ± 2.34
UFA	
18:1Δ9	12.66 ± 1.20
SFA/UFA ratio	6.88

P. putida A (ATCC 12633) cells were grown until late-exponential phase with glucose and NH_4Cl in the absence of TTAB, and they were then treated with 50 mg TTAB l^{-1}. Under these culture conditions, the evaluation of the resistance of *P. putida* to the bactericidal activity of TTAB was possible, since the surfactant was present in the medium but it was not incorporated as a nutrient by the cells. At different times, cells were harvested from the culture. We determined the content of proteins and PLs of the cells, the amount of TTAB adherent to the cells, the amount of free TTAB in the culture medium and the TTAB content of the OMVs. Fig. 3 shows that the amount of PL in the cells increased from 21 to 24 nmol PL (mg protein)$^{-1}$ after 30 min of contact with the detergent, and reached 26 nmol PL (mg protein)$^{-1}$ after 120 min of contact with TTAB. Also, it can be seen in Fig. 3 that in the period 15 to 120 min after the addition of TTAB, the amount of surfactant adherent to the cells decreased from about 2.16 to 1.72 nmol TTAB (nmol PL)$^{-1}$. In agreement with this decrease, the amount of TTAB detected in the medium increased from 30 mg l^{-1} at 60 min to 35 mg l^{-1} at 120 min post-exposure to surfactant (Fig. 3). Taking into account that detection of the OMVs free in the culture started 60 min after the addition of TTAB (Fig. 1e) and that the surfactant was found in the OMVs (4 ± 0.2 mg TTAB l^{-1} at 120 min), we conclude that the TTAB molecules adsorbed had been removed from the cells, likely due to their elimination through the production of OMVs. Thus, the production of OMVs constitutes a protective mechanism used by *P. putida* A (ATCC 12633) to eliminate detergent molecules attached to the membrane.

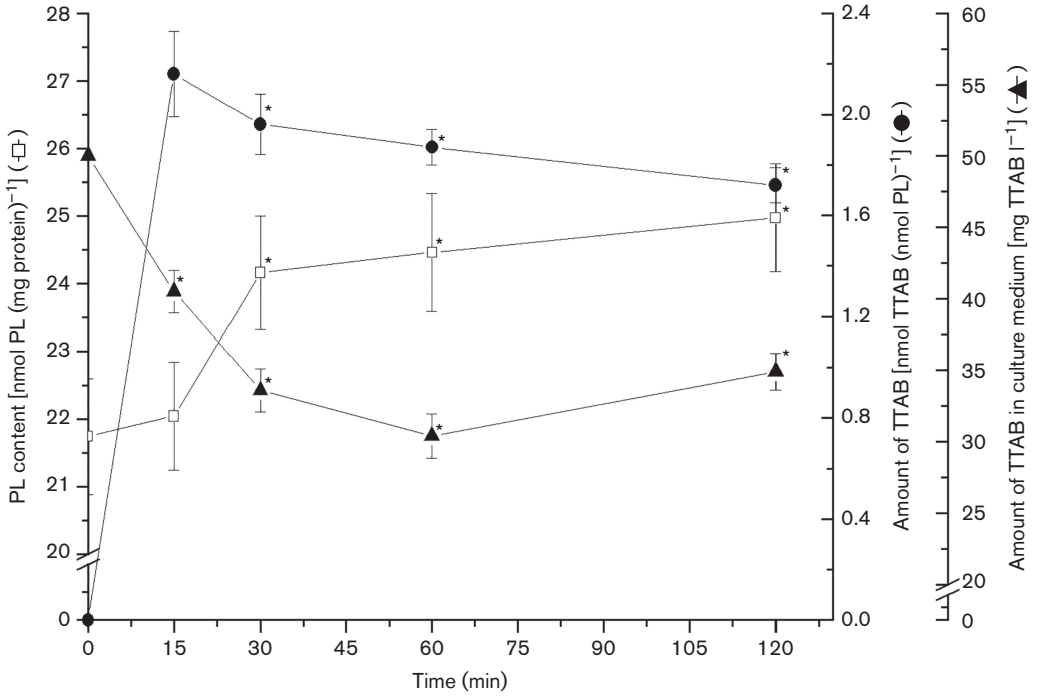

Fig. 3. Relationship between the formation of OMVs and the elimination of TTAB. *P. putida* cells were grown in HPi-BSM with glucose and NH_4Cl until late-exponential phase (OD_{660} 0.8). At this point, the culture was divided in two parts: one was kept as a control and the other was treated with 50 mg TTAB l^{-1}. At different times, the PL content (□) and the amount of TTAB adherent to the cells (●) or free in the culture medium (▲) were determined. Error bars show means ± SDs (n=3). Values with asterisks are significantly different ($P \leq 0.05$) according to the ANOVA test.

Factors contributing to OMV biogenesis in the presence of TTAB

The generation of space between the outer membrane cross-links and the peptidoglycan seems to be a general prerequisite for the creation of OMVs (Deatherage *et al.*, 2009; Schwechheimer *et al.*, 2014). An abundant lipoprotein (Lpp) in *Escherichia coli*, or its counterpart in other Gram-negative bacteria, covalently links the outer membrane and the peptidoglycan (OM-peptidoglycan), providing structural envelope integrity (Braun, 1975; Braun & Wolff, 1975; Deatherage *et al.*, 2009 and citations therein).

It is possible to distinguish between two types the Lpp: the peptidoglycan cross-linked form of (bound form) and the outer membrane lipid-anchored but uncross-linked form of (free form). Hoekstra *et al.* (1976) reported that the peptidoglycan-binding Lpp plays a crucial role in the production of OMVs in *E. coli*. They showed that OMV formation starts with an outwardly bulging event towards the outer membrane, occurring mostly where there is less peptidoglycan-binding Lpp, which can cause a weak linkage between the peptidoglycan and the outer membrane of the bacterium. In our study, the Lpp of *P. putida* A (ATCC 12633) was obtained after cleaving of the peptidoglycan–Lpp complex, as described in Methods. When the soluble fraction obtained after this treatment was resolved by 15 % SDS-PAGE, a protein with a molecular mass of approximately 9 kDa was revealed (Fig. 4). This band was excised and the MALDI-TOF analysis of the spots showed that the 9 kDa polypeptide shared 98 % sequence identity with PP_5226 (GenBank accession no. NP_747327.1) of *P. putida* KT 2440, a Lppl family lipoprotein (Pseudomonas Genome Database). Digestion of the peptidoglycan–Lpp complex with lysozyme showed that the amount of the bound form of Lpp (Lpp covalently cross-linked to the peptidoglycan), detected after growth of *P. putida* with TTAB, decreased twofold with respect to the values found after growth without TTAB [16 ± 2 and 28 ± 3 μg (mg cell envelope protein)$^{-1}$ (n=3), respectively]. Given the inverse relationship between OMV production and Lpp-cross-linking, regardless of the overall amount of Lpp present in the cells (Schwechheimer *et al.*, 2014 and citations therein), we suggest that this decrease in the content of Lpp covalently cross-linked to the peptidoglycan in the presence of the QAC could be related to areas of loss of contact between the outer membrane and the peptidoglycan, facilitating the formation of OMVs. Additionally, TTAB, due to its amphiphilic nature, may contribute to OMV biogenesis when added exogenously to the culture medium, by inducing curvature of the

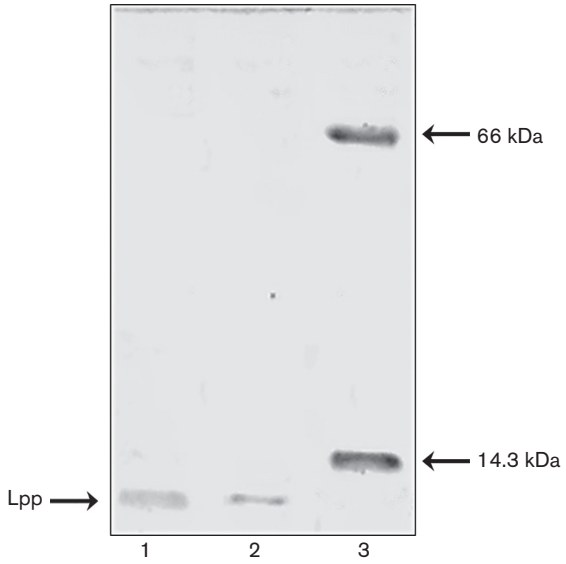

Fig. 4. Isolation of Lpp cross-linked to the peptidoglycan of *P. putida* A (ATCC 12633). SDS-PAGE (15 %) of Lpp cross-linked to peptidoglycan extracted by treatment with lysozyme and stained with Coomassie blue R-250. Cells were grown with glucose/NH_4Cl (lane 1) and with TTAB (lane 2). Molecular mass standards (Bio-Rad): bovine albumin (66 kDa) and lysozyme (14.3 kDa) (lane 3).

membrane. As shown in Fig. 1(e), the cells of *P. putida* A (ATCC 12633) exposed to 50 mg TTAB l^{-1} possess intact cell membranes but with outward membrane protrusions that probably result in the formation of membrane blebs. Accordingly, TTAB could contribute to the formation of these protrusions by interacting directly with the outer membrane and, thus, stimulating OMV formation. To analyse the induction of the curvature of the membrane by TTAB, we used a RBC model and analysed the crenation of the membrane produced by the asymmetrical expansion of the outer leaflet relative to the inner. This model has also been applied to demonstrate the formation of OMVs in *P. aeruginosa* (Schertzer & Whiteley, 2012 and citations therein). As shown in Fig. 5(a, b), 5 μmol TTAB l^{-1} induced RBC crenation to form echinocytes (RBCs with outward membrane protrusions; Fig. 5d). A higher proportion of RBCs were crenated after treatment with 10 μmol TTAB l^{-1} (Fig. 5a, c), suggesting a concentration-dependent response.

The crenation of RBCs can be antagonized by compounds, such as chlorpromazine, that expand the inner leaflet of the membrane and induce RBC cup formation (Schertzer & Whiteley, 2012). We showed that increasing amounts of chlorpromazine in samples of TTAB-treated RBCs resulted in a concentration-dependent antagonistic effect, with crenation completely abolished when TTAB was present at a

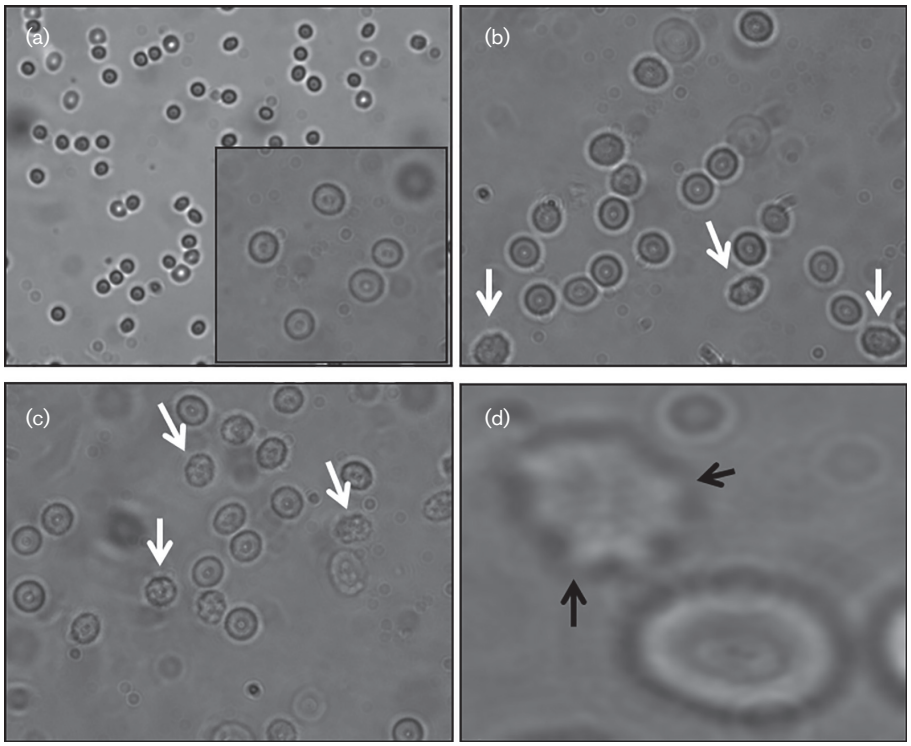

Fig. 5. Effect of TTAB on membranes of RBCs. TTAB was added to washed RBCs and observed using light microscopy. (a) RBCs in PBS; (b, c) RBCs treated with TTAB 5 μmol l^{-1} and 10 μmol l^{-1}, respectively. White arrows indicate the echinocytes. (d) An enlargement of part of the image in (b) showing the echinocytes and normal RBCs. Black arrows indicate spicule-containing cells. (a) × 100 magnification; (b, c) × 400 magnification.

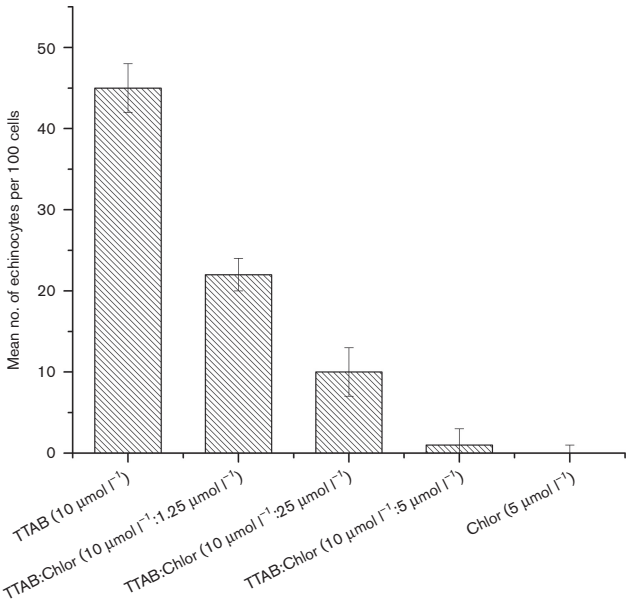

Fig. 6. Antagonization of the TTAB effect by chlorpromazine. Washed RBCs were exposed to 10 μmol TTAB l^{-1}, to 10 μmol TTAB l^{-1} plus 1.25–5 μmol chlorpromazine l^{-1} or to 5 μmol chlorpromazine l^{-1} alone. Bars represent mean numbers of echinocytes per 100 cells. Means were calculated from at least three independent determinations. Error bars show the SEs. Chlor, Chlorpromazine.

ratio of 2 : 1 with chlorpromazine (Fig. 6). The fact that the effect of TTAB was successfully antagonized by the addition of chlorpromazine confirms the proposed mechanism. Therefore, the results obtained with the RBC model, transposed to what occurs in cells of *P. putida* A (ATCC 12633) exposed to TTAB, lead us to propose that the addition of TTAB contributes to OMV formation, through a physical mechanism that induces curvature of the membrane. Although the mechanism of OMV biogenesis is not completely known, taken together, our results suggest that the physical process associated with decrease in the levels Lpp covalently cross-linked to the peptidoglycan, detected in *P. putida* cells grown with TTAB, markedly contributes to the formation of these vesicles.

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