Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Content of cardiolipin of the membrane and sensitivity to cationic surfactants in *Pseudomonas putida*

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Keywords
adaptation, cardiolipin, cardiolipin synthase genes, cationic surfactants, *Pseudomonas putida*.

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2015/2071: received 14 October 2015, revised 27 June 2016 and accepted 13 July 2016

doi:10.1111/jam.13238

Abstract

Aims: To establish the role of cardiolipin (CL) of the membrane in response to the presence of tetradecyltrimethylammonium in *Pseudomonas putida* A (ATCC 12633).
Methods and Results: Two ORFs of *Ps. putida* A (ATCC 12633), which in *Ps. putida* KT2440 encode the putative CL synthase genes *cls* and *cls2*, were cloned, sequenced and mutated. Only the double mutant lacking *cls* and *cls2* showed a reduction of the CL content, 83% lower than the amount produced by the wild-type. Accompanying this change was a 40% decrease in the content of unsaturated fatty acid. Consequently, the membrane of the mutant was more rigid than the one of the parental strain, as observed using fluorescence polarization techniques. The mutant strain showed reduced viability in the presence of tetradecyltrimethylammonium. The incorporation of exogenous CL into its membrane relieved sensitivity to the cationic detergent.
Conclusions: *Pseudomonas Putida* cells with low levels of CL die in the presence of tetradecyltrimethylammonium, because they cannot counter the fluidizing effect of the cationic surfactant.
Significance and Impact of the Study: The modification in the membrane phospholipids composition allows knowing the adaptation strategy of *Ps. putida* when these bacteria are exposed to cationic surfactant.

Introduction

Bacteria can adapt to different environmental stress conditions by modifying their membrane and, therefore, its flexibility and adaptation capability determine the survival ability of the bacteria. Among the main responses of most bacteria to environmental perturbations are the alteration of saturated/unsaturated ratio of fatty acids (Ramos *et al.* 2002; Mrozik *et al.* 2004) and the isomerization of *cis* to *trans* double bonds (Diefenbach *et al.* 1992; Heipieper *et al.* 2003; Bernal *et al.* 2007a). Modifications in the polar heads of anionic phospholipids (PL) have also been associated with the response to certain types of stress such as solvents, cationic surfactants, changes in pH and high salinity (Von Wallbrunn *et al.* 2002; López *et al.* 2006; Bernal *et al.* 2007b; Boeris *et al.* 2007; MacGilvray *et al.* 2012; Heredia *et al.* 2014).

Pseudomonas putida A (ATCC 12633) was described as a pollutant degrader that can utilize the cationic surfactant tetradecyltrimethylammonium bromide (TTAB) as a sole carbon, nitrogen and energy source (Liffourrena *et al.* 2008). In this bacterial strain, the response to the stress by TTAB involves quantitative changes in its membrane PL, with increases in the phosphatidic acid (PA), phosphatidylglycerol (PG) contents and decrease of cardiolipin (CL) (Boeris *et al.* 2007, 2009). Also, in TTAB-exposed cells, the unsaturated fatty acid (UFA) content decreases as TTAB concentration in the medium increases. Analysis of fatty acid composition of *Ps. putida* showed that PG contains the major proportion of saturated fatty acid (SFA) (89%) while CL contains an elevated proportion of UFA (18%). Thus, in the presence of TTAB, the increase in PG and, consequently in SFA, together with the decrease in CL, enhances membrane

resistance, reversing the fluidizing effect of TTAB (Heredia *et al.* 2014).

Taking into account both that, in *Ps. putida*, CL constitutes a reservoir of UFA and the need of the decrease in the UFA content as adaptation mechanism to tolerate cationic surfactants (Heredia *et al.* 2014), we hypothesized that a strain deficient in CL could be able to tolerate high TTAB concentration. It has been described that, in *Ps. putida*, CL is synthetized by cardiolipin synthase (Cls) that catalyses the condensation of two PG molecules to yield CL and glycerol (Von Wallbrunn *et al.* 2002; Bernal *et al.* 2007b). Here, we report the isolation, characterization and generation of mutants in the coding gene(s) for the synthesis of CL in *Ps. putida* A (ATCC 12633). We tested the influence of *cls*-mutants on the response of cells exposed to TTAB and demonstrated that the reduction in the levels of CL in mutant cells was accompanied by reduced viability in the presence of the cationic surfactant. The tolerance to TTAB could be restored by incorporating exogenous CL. Thus, we conclude that the presence of CL in regular amounts—that is, levels found in wild-type cells—is necessary to counteract the biocide effect of TTAB.

Materials and methods

Bacterial strains, plasmids and culture media

The strains and plasmids used in this study are described in Table 1. Luria-Bertani (LB) medium was used for strain maintenance. *Pseudomonas putida* A (ATCC 12633) was grown aerobically at 30°C at late exponential phase (OD660 0·8) in LB medium or in a basal salt liquid medium (HPi-BSM) (Lucchesi *et al.* 1989) with 20 mmol l^{-1} glucose and 18·7 mmol l^{-1} NH_4Cl. *Escherichia coli* strains were grown in LB media at 37°C. Growth was measured at an absorbance of 660 nm (OD_{660}) using a spectrophotometer (Beckman DU 640, Fullerton, CA). When appropriate, media were supplemented with the following antibiotics at the indicated concentrations: gentamicin (Gm) 20 μg ml^{-1}, kanamycin (Km) 50 μg ml^{-1},tetracycline (Tc) 10 μg ml^{-1} and streptomycin (Sm) 25 μg ml^{-1} for *E. coli*; Gm 60 μg ml^{-1}, Km 75 μg ml^{-1}, Tc 120 μg ml^{-1} and Sm 100 μg ml^{-1} for *Ps. putida*.

For evaluating the survival of strains in the presence of different stressors, the wild-type and double cls/cls2 mutant strain were cultured overnight in LB medium. The cultures were harvested by centrifugation at 8000 ***g*** for 10 min and, after serial dilutions, aliquots were plated on LB medium or HPi-BSM with glucose and NH_4Cl and different stressors: temperature (37°C), pH (5–10), saline stress (NaCl 0·15 to 0·5 mol l^{-1}) and TTAB (50–150 mg l^{-1}). When necessary, the number of viable cells (CFU ml^{-1}) was determined by plating the serially diluted cell suspensions on LB plates before TTAB was added and 10, 30, 60, and 120 min after the addition of TTAB.

For the growth of bacteria in the presence of exogenously added CL, 50 μg ml^{-1} of bovine CL (Sigma Chemical Co., St. Louis, MO) was added to overnight cultures grown in LB medium. The cultures were harvested by centrifugation at 8000 ***g*** for 10 min and, after serial dilutions, aliquots were plated on HPi-BSM plates containing 50 μg ml^{-1} of bovine CL, glucose 20 mmol l^{-1}, NH_4Cl 18·7 mmol l^{-1}, and the addition of variable concentrations of TTAB (from 0 to 150 mg l^{-1}). Some bacteria were grown in the presence of exogenously added TopFluor-CL, 1,1′,2,2′-tetraoleoyl cardiolipin[4-(dipyrrometheneborondifluoride)butanoyl] (Avanti Polar Lipids, Inc., Alabaster, AL). In this case, cells were grown in HPi-BSM with 0·1 μmol l^{-1} of TopFluor-CL at late exponential phase (OD_{660} 0·8) and were harvested by centrifugation at 8000 ***g*** for 10 min. Pellets were washed and the cells were immobilized on microscope slides and observed with a Zeiss microscope (Axiovert 135, Jena, Germany) equipped with an AxioCam camera.

The cell morphology of the strains was examined by Transmission electron microscopy (TEM). The protocol for TEM sample preparation was previously described (Cristofolini *et al.* 2012). The cells were examined in an Elmiskop 101 transmission electron microscope (Siemens, Germany) and the acquisition of images was performed with DIGITAL MICROGRAPH software (Gatan, Japan).

Cloning of *cls* ORFs and generation of mutants

Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell 2001). The ORFs *pp5364* and *pp3264* of *Ps. putida* KT2440 annotated as *cls* and *cls2*, respectively, served as the basis for designing oligonucleotides for the amplification of *cls* and *cls2* genes of *Ps. putida* A (ATCC 12633). The oligonucleotides oLUpCls4 and oLDownCls4 (Table 2) were used to amplify 1800 bp of *cls* gene from *Ps. putida* A (ATCC 12633) genomic DNA, introducing *Bam*HI and *Sal*I sites. The PCR product was cloned into vector pUC18 to yield plasmid pPB07 (Table 1). For inactivation of the *cls* gene, pPB07 was cut with *Stu*I and *Nco*I and the 600 bp StuI/NcoI fragment containing the *aacC1*, which encodes Gm resistance, was inserted into the *cls* gene, giving rise to pPB08 (Table 1). The 1·8 kb *Bam*HI/*Sal*I fragment containing the *cls::aacC1* allele was subcloned into the suicide vector pKNG101 (Kaniga *et al.* 1991) to yield plasmid pPB09 (Table 1), which was then introduced into *Ps. putida* A (ATCC 12633) by electroporation.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	References
Pseudomonas putida strains		
A (ATCC 12633)	Wild-type	Palleroni (1992)
cls mutant	Gentamicin-resistant, *Δcls::aacC1* deletion mutant of *Ps. putida* A (ATCC 12633)	This study
cls2 mutant	Kanamicin-resistant, *cls2::aph(3′)-IIa* insertion mutant of *Ps. putida* A (ATCC 12633)	This study
cls/cls2 mutant	Gentamicin and Kanamicin-resistant, *Δcls::aacC1* and *cls2::aph(3′)-IIa* mutant of *Ps. putida* A (ATCC 12633)	This study
cls/cls2/pGL08	*cls/cls2* mutant transformed with a pGL08 containing *cls* gene of *Ps. putida* A (ATCC 12633)	This study
cls/cls2/pGL09	*cls/cls2* mutant transformed with a pGL09 containing *cls2* gene of *Ps. putida* A (ATCC 12633)	This study
Plasmids		
pGem®T Easy	Cloning vector, ampicillin-resistant	Promega
pKNG101	Cloning vector for chromosomal insertion streptomycin-resistant Sac^{s} Tra^{-} Mob^{+} oriR6K	Kaniga *et al.*(1991)
pBBR1MSC-3	Broad-host-range cloning vector, Tetracycline-resistant Tra^{-} Mob^{-}	Kovach *et al.* (1995)
pUC18	Cloning vector, ampicillin-resistant	Yanisch-Perron *et al.* (1985)
pPB07	*cls*-like ORF from *Ps. putida* A (ATCC 12633) (1·8 Kb) as BamHI/*Sal*I insert in pUC18	This study
pPB08	1·8 kb *Bam*HI/*Sal*I fragment containing *Δcls::aacC1* in pUC18	This study
pPB09	1·8 kb *Bam*HI/*Sal*I fragment containing *Δcls::aacC1* in pKNG101	This study
pRH01	*cls2*-like ORF from *Ps. putida* A (ATCC 12633) (1·5Kb) as BamHI/*Sal*I insert in pGem®T Easy	This study
pRH02	2·5 kb BamHI/SalI fragment containing *cls2:: aph(3′)-IIa*- in pGem®T Easy	This study
pRH03	2·5 kb BamHI/SalI fragment containing *cls2:: aph(3′)-IIa*- in pKNG101	This study
pGL08	*cls-like* ORF from *Ps. putida* A (ATCC 12633) (1400 bp) as *Sal*I/*Bam*HI insert in pBBR1MCS-3	This study
pGL09	*cls2-like* ORF from *Ps. putida* A (ATCC 12633) (1200 bp) as *Sal*I/*Bam*HI insert in pBBR1MCS-3	This study

Table 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)*	Relevant characteristics
UpCls4	CGCGGATCCGTTGCAGCATCTGGGCTTGTACCAGTGG	*Bam*HI restriction site located at the 202 bp upstream of start codon of the *cls* gene
DownCls4	ACGCGTCGACTGAGAAGACCCTGCGGATCTGGCGTTTGT	*Sal*I restriction site located at the 237 bp downstream of stop codon of the of the *cls2* gene
oLPB23-3264	CGCGGATCCGATCAATGGCGAGCAGTATT	*Bam*HI restriction site overlapping the start codon of *cls2*
oLPB24-3264	ACGCGTCGACAGGTTAAAGGCGTAGCACAC	*Sal*I restriction site located 267 bp downstream of stop codon of the of the *cls2* gene
oLGL01	ACGCGTCGACGTGTACTCGGCAAGCAGGAT,	*Sal*I restriction site overlapping the start codon of *cls*
oLGL02	CGCGGATCCGGGAAGATTTGTACCGCTGA	*Bam*HI restriction site after the stop codon of *cls*
oLGL12	CCGCTCGAGGTGAACCGACCTTGGGTAGA,	*Xho*I restriction site overlapping the start codon of *cls2*
oLGL13	ACGCGTCGACGCGGCTTGAACGAA	*Sac*I restriction site after the stop codon of *cls2*

*Restriction sites are underlined.

Similarly, using the oligonucleotides oLPB23 and oLPB24 (Table 2), a 1500 bp fragment of *cls2* gene was amplified from genomic DNA of *Ps. putida* A (ATCC 12633), introducing *Bam*HI and *Sal*I sites. The PCR product was cloned into pGem®-T Easy (Promega, Madison, WI), producing plasmid pRH01 (Table 1). For inactivation of the *cls2* gene, pRH01 was digested with *Stu*I, and the *Stu*I-restricted Km cassette (*aph(3′)lla*) was inserted into the gene to obtain plasmid pRH02 (Table 1). The 2·5 kb *Bam*HI/*Sal*I fragment containing the *cls2::aph(3′)lla* was subcloned into pKNG101, producing pRH03 (Table 1).

To obtain the double-mutant *cls/cls2*, pRH03 (Table 1) was introduced into the single *Ps. putida cls* mutant by electroporation. Merodiploids of *Ps. putida cls, cls2* and *cls/cls2* mutant were selected as described Kaniga *et al.* (1991). In all cases, the correct insertion of the mutant allele of *cls* and/or *cls2* into the *Ps. putida* chromosome was confirmed by PCR.

For complementation assays, the *cls* gene was amplified using genomic DNA from *Ps. putida* A (ATCC 12633) as template and the oligonucleotides oLGL01 and oLGL02 (Table 2), which introduced *Sal*I and *Bam*HI sites into the PCR product. After digestion with the enzymes, the PCR product was cloned into pBBR1MCS-3 vector (Kovach *et al.* 1995) to generate pGL08 (Table 1). Finally, pGL08 was transformed into *Ps. putida cls/cls2* mutant by

electroporation. For complementation of *cls2* gene, the oligonucleotides used were oLGL12 and oLGL13 (Table 2), which introduced *XhoI* and *SacI* sites into the PCR product. The product was cloned in the pBBR1MCS-3 vector to yield the plasmid pGL09 (Table 1), which was introduced by electroporation to *Ps. putida cls/cls2* mutant. Strains containing pGL08 and pGL09 were selected in LB medium with tetracycline (120 μg ml^{-1}).

Lipids radiolabelling, extraction and transesterification

The lipid composition of wild-type *Ps. putida* A (ATCC 12633) and its isogenic mutants were determined following labelling with [1-^{14}C]-acetate (60 mCi mmol^{-1}, 2·26 Gbq mmol^{-1}; New England Nuclear, Boston, MA) (de Rudder *et al.* 1997). Lipids were extracted from the cells following the Bligh & Dyer method (1959) and they were later separated by thin-layer-chromatography (TLC, silica gel HLF, 250 μm, from Analtech, Newark, DE) according to Boeris *et al.* (2007), visualized with iodine vapours and identified by comparison with purified standards (Sigma Chemical Co., SL). The position of radiolabelled lipids was determined by autoradiography on Agfa-Gevaert film. Spots were scrapped off the plates and the fractions were quantified by radioactivity measurement in a liquid scintillation counter (Beckman LS 60001 C).

Fatty acid methyl esters (FAMEs) were prepared from total PL extracts using BF_3 10% in methanol (Morrison and Smith 1964), they were then extracted with hexane and finally concentrated under stream of N_2 gas. The FAMEs were analysed by using a Hewlett Packard 5890 II gas chromatograph (GC) equipped with a methyl silicone column (length 50 m; inner diameter 0·2 mm; film thickness 0·33 μm) and 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^{-1} to 250°C (Heredia *et al.* 2014). Fatty acids were identified by comparison of retention times of purified standards (Sigma Chemical Co., SL).

Determination of membrane fluidity

Membrane fluidity was determined by measuring the fluorescence polarization of the 1,6-diphenyl-1,3,5-hexatriene (DPH) probe inserted into cytoplasmic membranes (Mykytczuk *et al.* 2007). The relationship between the probe polarization ratio and cytoplasmic membrane fluidity is inversely proportional (Litman and Barenholz 1982). Following the procedures described by Heredia *et al.* (2014), cells of *Ps. putida* A (ATCC 12633), wild-type and its isogenic mutants, grown in HPi-BSM medium with glucose and NH_4Cl to OD_{660} 0·8–1·00, were incubated with DPH and 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 width for the excitation and emission beams was 12 and 10 nm respectively. The degree of polarization was calculated from the polarization ratio (P) using the expression: $P = (I_{VV} - I_{VH}G)/(I_{VV} + I_{VH}G)$. I_{VV} and I_{VH} are the vertically and horizontally polarized components of light intensities emitted after excitation by vertically polarized light, and G is the sensitivity factor of the detection system (Lakowicz 1999).

NAO staining

Pseudomonas putida A (ATCC 12633) and its isogenic mutants were grown in LB medium up to an OD_{660} 0·8, harvested by centrifugation at 8000 ***g*** for 10 min, washed twice with NaCl 0·9% (w/v) and diluted to an OD_{660} 0·4. Cells were stained by the addition of the fluorescent dye 10-*N*-Nonyl acridine orange (NAO) at a final concentration of 200 nmol l^{-1}. After 1 h of incubation at room temperature in the dark, the cells were immobilized on microscope slides by the addition of cold methanol/acetone (1 : 1, v/v) and incubated for 20 min at −20°C. Samples were analysed with a Zeiss microscope (Axiovert 135) equipped with an AxioCam camera.

Determination of Cls activity

Cells of *Ps. putida* A (ATCC 12633) and its isogenic mutants were grown to late exponential phase ($D_{660\ nm}$ of about 0·8), harvested by centrifugation at 10 000 ***g*** for 10 min at 4°C, washed and resuspended in phosphate buffer 10 mmol l^{-1} (pH 7·4). Cells were finally lysed by sonication in an ice bath at 20 000 Hz using a Vibra cell ultrasonic processor for 10 cycles (10 s per cycle). After centrifuging at 20 000 ***g*** for 30 min, the clear supernatant was removed and used for Cls determination in a similar fashion as described by Guo and Tropp (2000) using the fluorescent lipid NBD-PG (1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-il)amino] dodecanoyl}-sn-glycero-3-[phospho-rac-(1-glycerol)]; Avanti Polar Lipids, Inc.). The activity was measured at 30°C in a 100 μl reaction mixture containing potassium 320 mmol l^{-1} phosphate buffer, pH 7·0, 10 mmol l^{-1} β-mercaptoethanol, 0·03% Triton X-100, 1 μmol l^{-1} NBD-PG and 0·5 mg ml^{-1} of protein. Cell extracts boiled during 5 min were used as a blank. After 60 min of incubation at 30°C, the reaction was stopped by the addition of 350 μl of methanol/chloroform (2 : 1, v/v) and the lipids were extracted by Bligh and Dyer method (1959). Lipids were separated by TLC

with chloroform/methanol/acetic acid 65 : 25 : 8 (by vol) as the solvent. TLC plates were scanned and the spots were quantified using a Phosphorimager (Image Station 4000MM PRO; Carestream, Molecular Imaging, Rochester, NY). Relative amounts of each lipid were determined by fluorescence imaging with excitation at 460 nm and emission at 535 nm.

Cls assays using ^{14}C-PE and PG as substrate were performed as described by Tan *et al.* (2012). ^{14}C-PE (specific activity, 9 nCi nmol^{-1}) was prepared through growth of *Ps. putida* A (ATCC 12633) in a medium with 1 μCi ml^{-1} [1-^{14}C]-acetate and was used as substrate for Cls assays. The extraction and isolation of the ^{14}C-PE was performed as described above for PL. The reaction mixture of 100 μl consisted of PBS 1×, 0·1% Triton X-100, 10 mmol l^{-1} β-mercaptoethanol, 5 μmol l^{-1} ^{14}C-PE, 5 μmol l^{-1} PG and 1 mg ml^{-1} of protein. After 60 min of incubation at 30°C, the reaction was stopped by the addition of 350 μl of methanol/chloroform (2 : 1, v/v) and the lipids were extracted according to the Bligh and Dyer (1959). The products were separated by TLC plates using chloroform/methanol/acetic acid (65 : 25 : 8, by vol) as solvents.

Protein concentrations were measured by the Bradford method (1976) with bovine serum albumin (BSA; Sigma Chemical Co., SL) as a standard.

Nucleotide sequence accession number

The nucleotide sequences of *cls* and *cls2* genes from *Ps. putida* A (ATCC 12633) were deposited in the GenBank database, under accession numbers KF835427 and KF835428 respectively.

Results

Identification of *Pseudomonas putida* A (ATCC 12633) *cls* homologues

The number of CL synthase genes (*cls*) varies among bacterial species. In *Ps. putida* KT2440, the ORFs *pp5364* and *pp3264* were annotated as *cls* and *cls2* respectively. Using oligonucleotides based on these sequences, we amplified and cloned the putative *cls* and *cls2* genes of *Ps. putida* A (ATCC 12633) in pUC18 and pGem®-TEasy vectors (Table 1) respectively. A BLAST search at the National Center for Biotechnology Information (NCBI) indicated that *cls* and *cls2* are 88 and 99% identical to the genes *cls* and *cls2* of *Ps. putida* KT2440 respectively. The deduced gene product of the *cls* gene shares 68, 70, 27 and 23% of the amino acid sequence identity with Cls of *Ps. putida* DOT-T1E, *Ps. putida* P8, *Bacillus subtilis* and *Staphylococcus aureus* respectively (Von Wallbrunn *et al.* 2002; López *et al.* 2006; Bernal *et al.* 2007b; Koprivnjak *et al.* 2011). Moreover, the deduced gene product of the *cls2* showed significant homology to Cls2 from *E. coli* and *Staph. aureus* (55 and 25% amino acid identity respectively) (Guo and Tropp 2000; Koprivnjak *et al.* 2011).

Lipids composition of *Pseudomonas putida* strains

To test the role of *cls* and/or *cls2* in the synthesis of CL by *Ps. putida* A (ATCC 12633), we constructed isogenic mutants in which *cls (Δcls::aacC1)* and/or *cls2 (cls2:: aph (3′)-IIa-)* were disrupted. Successful disruption of these genes was performed as described in the Materials and Methods section and confirmed by PCR (data not shown). After the growth of the strains in saline medium with glucose and ammonium chloride to late exponential phase ($OD_{660\ nm}$ of about 0·8), bacterial lipids were extracted and analysed (Table 3). No significant differences were observed in CL levels between the wild-type and the single mutants. In contrast, a significant decrease in the amount of CL was detected in the *cls/cls2* mutant (6-fold decrease). We also observed that the double mutant had a twofold increase in the relative amount of PG and PA, whereas the relative amounts of zwitterionic PL (PE, LPE, PC) remained relatively constant with respect to values obtained in the wild-type (Table 3). The CL present in the membrane of the wild-type and of the double mutant was visualized using the fluorescent dye 10-*N*-nonyl acridine orange (NAO). In the wild-type, green fluorescence spots were distributed at the poles of the cells and near septa during cell division (Fig. 1a). In the double-mutant strain, a lower intensity fluorescent dye was homogeneously distributed among the cell membrane with no apparent intense regions (Fig. 1b). This result was consistent with a reduced amount of CL. No changes were detected in the CL distribution between the wild-type strain and the single mutants (not shown). In addition, the morphology of the *cls/cls2* mutant is significantly different to that of the wild-type strain, as judged by TEM examination (Fig. 1d,e).

Based on GC–MS analyses, no substantial differences were detected between the *Ps. putida* wild-type and *cls/cls2* mutant in terms of the presence of acyl chain length profiles of FA. In the wild-type and *cls/cls2* mutant, the most abundant SFA was stearic acid (18 : 0), representing up to 60 and 40% of the total FA, respectively, followed by palmitic acid (16 : 0) which represented 25 and 40% of the total FA respectively. Palmitoleic acid (16 : 1 Δ9) and vaccenic acid (18 : 1 Δ11) were dominant within the UFA. However, we observed that the content of UFA in *cls/cls2* mutant cells was approx. 40% lower than the one found in wild-type cells (6·63 ± 0·62 and 11·20 ± 5·04 respectively),

Table 3 Phospholipidic composition of *Pseudomonas putida* A (ATCC 12633) and its isogenic mutants

Strain	Wild-type	*cls* mutant	*cls2* mutant	*cls/cls2* mutant
PL	% Total of PL			
CL	11·38 ± 0·70	12·10 ± 0·77	13·18 ± 0·81	1·90 ± 0·58
PG	9·60 ± 0·42	10·29 ± 1·03	10·36 ± 0·32	19·94 ± 0·98
PE	67·58 ± 2·91	65·01 ± 2·66	60·45 ± 2·75	62·09 ± 1·32
PC	6·13 ± 0·92	5·76 ± 1·55	6·16 ± 2·11	5·66 ± 0·93
LPE	3·52 ± 0·12	3·57 ± 0·71	4·63 ± 1·03	4·81 ± 0·27
PA	0·60 ± 0·21	0·91 ± 0·03	0·72 ± 0·35	1·55 ± 0·77
NI	1·19 ± 0·15	2·36 ± 0·23	3·70 ± 1·23	4·05 ± 1·55

PL, phospholipids; CL, cardiolipin; PG, phosphatidylglicerol; PE, Phosphatidylethanolamine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, Phosphatidic acid; NI, no identified.

Pseudomonas putida A (ATCC 12633) and its isogenic mutants were grown in HPi-BSM with 20 mmol l^{-1} glucose, 18·7 mmol l^{-1} NH_4Cl and 1 μCi ml^{-1} [1-^{14}C]-acetate up to late exponential phase (OD_{660} 0·8). At this point the cells were collected and the PL were extracted and resolved by TLC. The distribution of labels was determined as described in the Materials and Methods section. Results are expressed as mean values ± SD (n = 4).

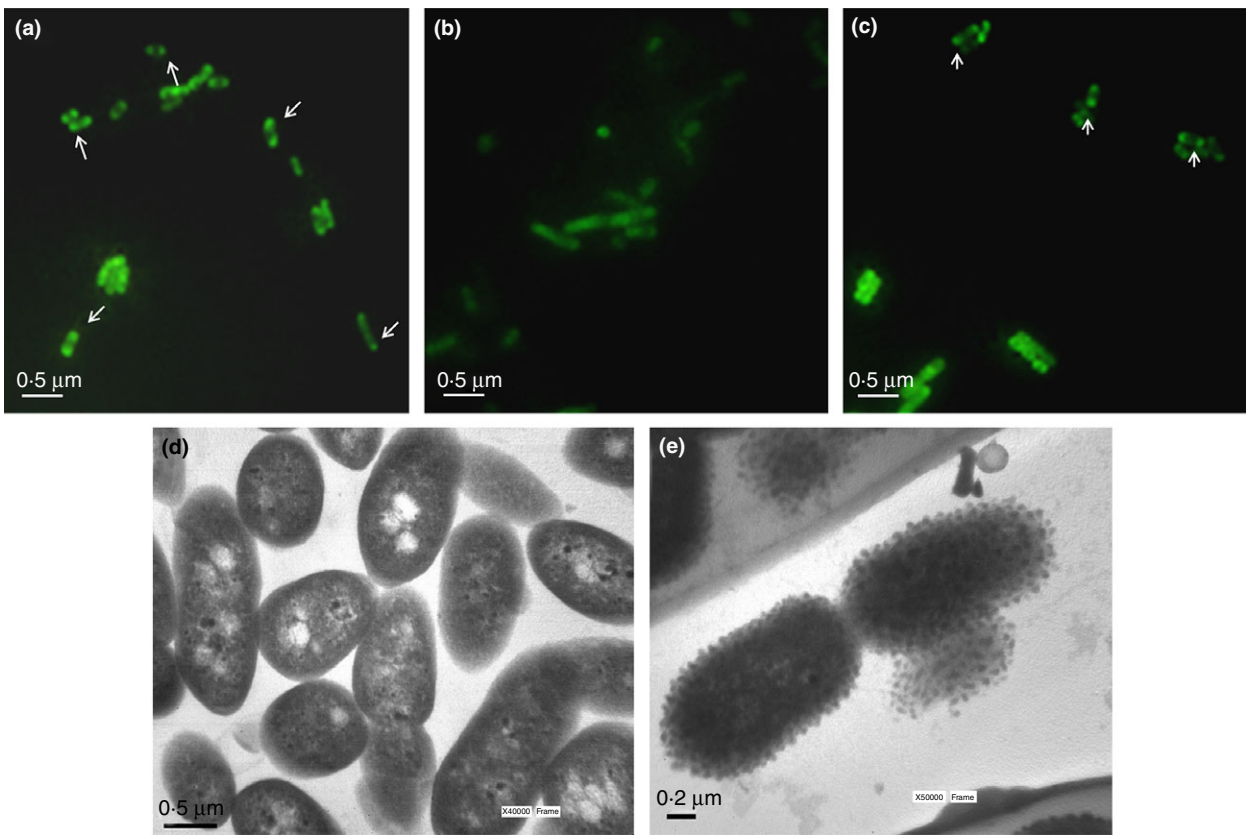

Figure 1 Visualization of cardiolipin (CL) in membranes and transmission electron microscopy (TEM) of *Pseudomonas putida* A (ATCC 12633) and its isogenic *cls/cls2* mutant. Cells grown in Luria-Bertani (LB) medium to the late log phase (OD_{660} 0·8) were stained with NAO: (a) *Ps. putida* A (ATCC 12633), (b) *cls/cls2* mutant. (c) *cls/cls2* mutant was grown in HPi-BSM with 20 mmol l^{-1} glucose, 18·7 mmol l^{-1} NH_4Cl and 0·1 μmol l^{-1} of TopFluor-CL at OD_{660} 0·8. (d) *Ps. putida* A (ATCC 12633) and (e) *cls/cls2* mutant cells were grown in LB medium and after reaching early stationary phase were analysed by TEM. The arrows indicate CL domains in poles of the cells.

increasing the SFA/UFA ratio up to 13·71, while in wild-type cells this value was 7·92. When evaluating fluidity of the membranes by DPH fluorescence polarization, we found a P value of 0·12 ± 0·02 and of 0·22 ± 0·01 for wild-type and *cls/cls2* mutant cells respectively. These results coupled with the reduced content of UFA in the

cls/cls2 mutant indicate a higher rigidity of the membrane in the cls/cls2 mutant.

cls and *cls2* gene products

To validate the function of the *cls* genes, we measured Cls activity. Cell-free extracts of *Ps. putida* A (ATCC 12633) wild-type and its isogenic single mutants could convert NBD-PG to CL, indicating that Cls activity was present in these extracts. However, the Cls activity assayed in the extracts of *cls/cls2* double mutant was practically undetectable (Fig. 2a, lane 4). When the *cls/cls2* mutant strain was transformed with pGL08 or pGL09 (Table 1), Cls activity was restored (Fig. 2b, lane 2 and 3), indicating that the mutations were successfully complemented. In addition, when we evaluated Cls activity using PE as the phosphatidyl donor to PG (Tan *et al.* 2012) in cell-free extracts of *cls/cls2* mutant strains, Cls activity was detected (Fig. 2c, lane 3).

Relationship between cardiolipin content and survival of *Pseudomonas putida* exposed to TTAB

We investigated the role of the content of CL in the adaptive mechanisms of *Ps. putida* to TTAB. Given that CL in other micro-organisms has been suggested to participate in different stress responses, we also determined the survival of wild-type and mutant strain in the presence of other stressors agents. The wild-type and double *cls/cls2* mutant strain were cultured in LB medium overnight. The cultures were then serially diluted and cell survival was evaluated based on the cell growth after exposure to different stressors: temperature, pH, saline stress and the presence of the cationic surfactant TTAB. Our results indicated that a temperature shift from 30 to 37°C and pH shift from 7·0 to 10 did not affect survival (data not shown). *Pseudomonas putida* and its isogenic *cls/cls2* mutant were equally sensitive to acid pH; neither was able to grow at pH 5. In LB medium supplemented with different NaCl concentrations, (ranging from 0·15 to 0·5 mol l^{-1} of NaCl), the *cls/cls2* double-mutant survival was reduced only at 0·5 mol l^{-1} of NaCl (not shown). However, we found that the decrease in CL content markedly influences the tolerance to cationic surfactants. We observed a nearly 6-fold reduction in survival of the cls/cls2 double mutant after 30-min exposure to 50 mg l TTAB (from 3×10^9 CFU ml^{-1} to 5×10^3 CFU ml^{-1}), whereas there was no decrease in survival of the wild-type under the same condition. Considering that double-mutant strain had a decreased content of CL (Table 2,

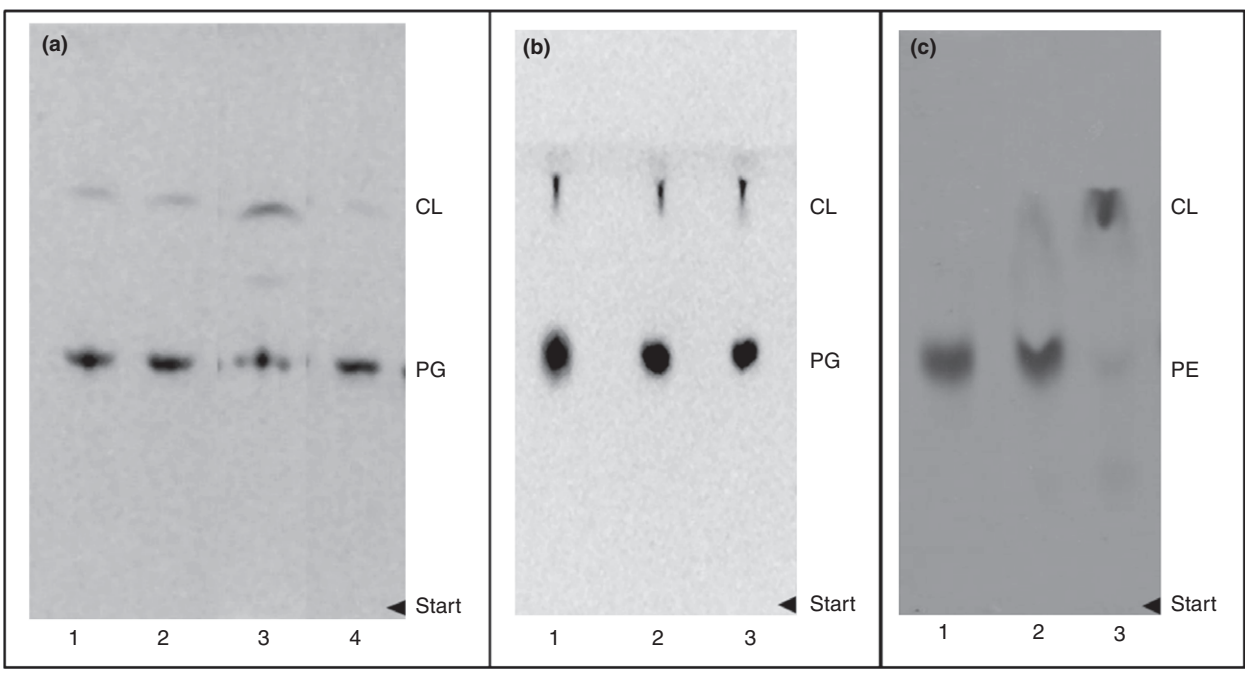

Figure 2 Cls activity of *Pseudomonas putida* wild-type, cardiolipin isogenic mutants and complementary strains using NBD-PG (a, b) or ^{14}C-PE and PG (c) as substrate. Enzyme assay and identification of phospholipids was performed as described in the Materials and Methods section. (a): Lane 1: *Ps. putida* A (ATCC 12633), lane 2: *cls* mutant, lane 3: *cls2* mutant, lane 4: *cls/cls2* mutant. (b): Lane 1: *Ps. putida* A (ATCC 12633), lane 2: *cls/cls2*/pGL08 (complementary strain), lane 3 *cls/cls2*/pGL09 (complementary strain). (c): Lane 1: Standard ^{14}C-PE, lane 2: *Ps. putida* A (ATCC 12633), lane 3: *cls/cls2* mutant.

Fig. 1b) and that this characteristic resulted in a TTAB-sensitive phenotype, we evaluated if exogenous CL could be incorporated from the medium by the mutant to restore the tolerance to the detergent. As shown in Fig. 1c, exogenous TopFluor-CL was incorporated by the double-mutant strain and the green fluorescence spots were distributed at the poles of the cells and near septa during cell division. Similar to what we found in wild-type cells (Fig. 3a), the mutant strain exhibited improved tolerance at the highest concentrations of TTAB when exogenous CL was incorporated (Fig. 3b). This shows a correlation between the survival of the double-mutant cells and the CL content in their membrane. When evaluating fluidity of the membranes in the cls/cls2 mutant, we found, in the absence of TTAB, a *P* value of 0·22 ± 0·01. After 1 min of the addition of TTAB 50 mg l^{-1}, the *P* value decreased to 0·15 ± 0·02 (Fig. 4). This response to TTAB treatment was also observed in wild-type cells, where the *P* value decreased from 0·12 ± 0·02 in the absence of TTAB to 0·08 ± 0·01 in its presence, indicating that the surfactant has a fluidizing effect on the hydrophobic core region of the cell membrane. However, differently to what occurred in wild-type cells, within 15 min of exposure to TTAB, the double-mutant cells did not return to a similar *P* value as the one observed in the absence of the surfactant (Fig. 4). These results clearly indicate that the double-mutant cells were unable to counteract the fluidizing effect of the detergent.

Discussion

Different studies show that bacteria with deficiency in the content of CL are more susceptible to different stress situations. This is explained by the multiple functions exerted by CL in for example, cell division and DNA replication (Mileykovskaya and Dowhan 2009; Maloney *et al.* 2011; Renner and Weibel 2011), functionality and stability of certain membrane proteins (Romantsov *et al.* 2007; Schlame 2008), performance of efflux pump (Bernal *et al.* 2007b) and membrane bioenergetic processes (Arias-Cartin *et al.* 2012 and citations therein). In the present study, we analysed the role of the CL levels in response to the presence of TTAB in *Ps. putida* A (ATCC 12633) cell mutant strains, where the *cls* genes have been

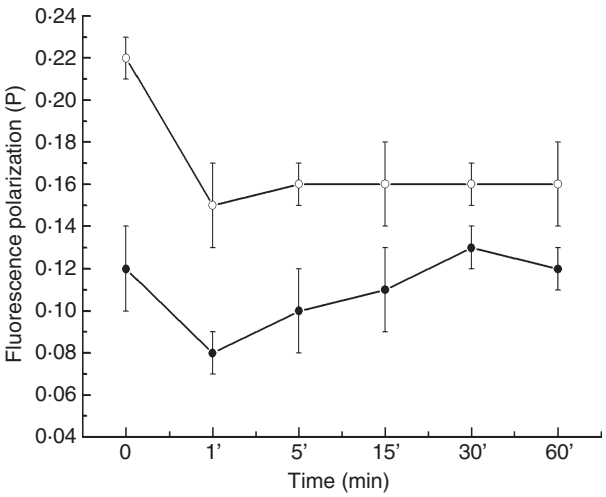

Figure 4 Fluorescence polarization of DPH in *Pseudomonas putida* A (ATCC 12633) wild-type (●) and in the *cls/cls2* mutant (○) exposed to tetradecyltrimethylammonium bromide (TTAB). Cells cultivated in Luria-Bertani medium at late exponential phase were harvested, washed in sterile buffer and resuspended in same buffer to obtain a turbidity of 0·2 at 660 nm. The fluorescent membrane probe was added to the resuspended culture and incubated to allow incorporation into the cytoplasmic membranes. Aliquots of cells exposed to TTAB 50 mg l^{-1} were collected at different times and fluorescence polarization was measured. Results are means of three independent experiments ± SD.

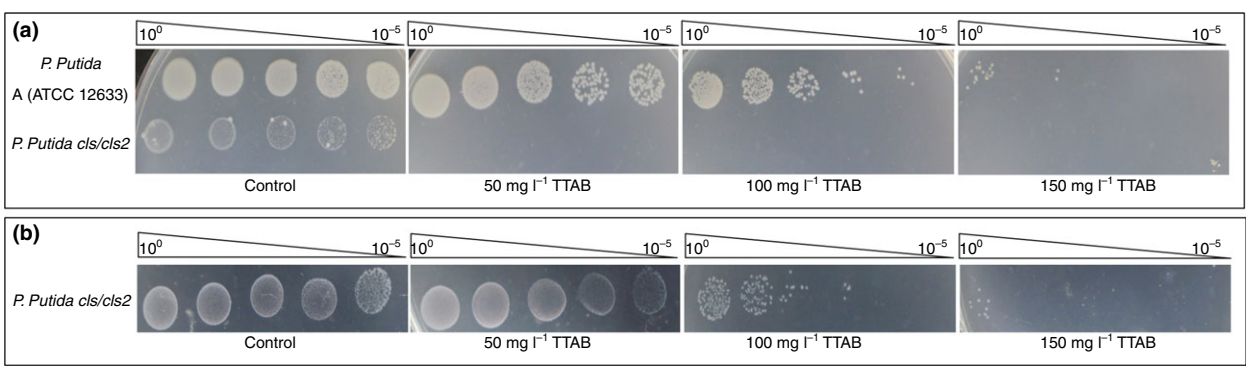

Figure 3 Survival of *Pseudomonas putida* A (ATCC 12633) and *Ps. putida cls/cls2* mutant in the presence of tetradecyltrimethylammonium bromide (TTAB). The wild-type strain and *cls/cls2* mutant were grown in Luria-Bertani overnight, serially diluted and then plated on HPi-BSM with glucose and NH_4Cl; without or with 50, 100 and 150 mg l^{-1} of TTAB (a) and 50 μg ml^{-1} cardiolipin (b).

disrupted. The knowledge of the sequences of putative *cls* and *cls2* encoding genes from *Ps. putida* KT2440 strain (Nelson *et al.* 2002) facilitated the amplification of two fragments from *Ps. putida* A (ATCC 12633) and led to the sequencing of 1800 and 1500 bp DNA fragments containing the *cls* and *cls2* respectively. Sequence comparisons and alignments revealed that the *Ps. putida cls* and *cls2* are related to members of the PLD superfamily which shows two $HXK(X)_4D(X)_6G(X)_2N$ motifs, a characteristic of numerous prokaryotic cardiolipin synthases (Tropp 1997). The fact that only the *cls/cls2* double mutant presented a marked reduction in the CL content (a decrease of about 83%), together with results obtained from the catalytic conversion of 2 PG to CL plus glycerol with extracts of each single mutant and complemented double mutant (Fig. 2a,b), pointed out that both ORFs were involved in the CL synthesis in *Ps. putida* A (ATCC 12633). In other micro-organisms, such as *Staph. aureus*, disruption of two *cls* genes resulted in lower levels of CL in the membranes (Koprivnjak *et al.* 2011; Tsai *et al.* 2011). In contrast, in *Ps. putida* DOT T1, *B. subtilis* and *Xantomonas campestris*, which harbour 2, 3 and 6 putative cls genes respectively, the disruption of only one cls gene resulted in a significant deficiency in CL levels of the membrane (López *et al.* 2006; Bernal *et al.* 2007b; Moser *et al.* 2014). In the double *Ps. putida* A (ATCC 12633) mutant strain, a reduced CL accumulation during logarithmic phase of growth was detected (1·9% of total PL), indicating that other enzyme activity responsible for the formation of CL was still present. In bacteria, it has been described that the final step in CL biosynthesis is the phosphatidyl transfer from one PG molecule to a second PG molecule to form CL and glycerol (Von Wallbrunn *et al.* 2002; Bernal *et al.* 2007b). However, in *E. coli*, the CL synthesis has also been described using PE as the phosphatidyl donor to PG instead of a second PG (Tan *et al.* 2012). Results presented in Fig. 2c show the production of CL after incubation of cell-free extracts of the double mutant with ^{14}C-labelled PE and PG, indicating the presence of Cls activity. Therefore, this Cls activity that used PG and PE as substrates would be responsible for the synthesis of the low levels of CL detected in the *Ps. putida cls/cls2* mutant.

The results of the present work showed that the PL composition required for the viability of *Ps. putida* cells under different stress conditions is flexible. The observation that temperature shift and pH shift or high salinity conditions had little effect on the growth of *cls/cls2* mutant strain suggests that CL alone is not responsible for bacterial survival under these conditions. However, results from the physiological characterization of the double-mutant strains showed that the disruption of both CL biosynthesis genes is lethal when cells are exposed to TTAB. In the *cls/cls2* mutant, the levels of CL were low (1·9% of total PL) while PG level remained high (18% of total PL) (Table 3). Thus, it is clear that, in the double-mutant cells, the decreases in the content of CL were compensated by an increase in the percentage of PG, and, as a result, it is likely that the density of negative surface charges in the membrane remains nearly constant. However, the increase of PG is not enough to compensate the fluidizing effect of TTAB and, consequently, cell death occurs. Previously, we have found that, in *Ps. putida,* CL is a reservoir for UFA and that a decrease in these UFA is important for counteracting the fluidization effect induced by TTAB (Heredia *et al.* 2014). We detected that the content of UFA in *cls/cls2* mutant cells was approx. 40% lower than in wild-type cells, and when we evaluated fluidity of membranes by means of DPH fluorescence polarization, the *P* value was $0{\cdot}22 \pm 0{\cdot}01$, indicating a higher rigidity. However, when the double mutant was exposed to TTAB, the *P* value decreased from $0{\cdot}22 \pm 0{\cdot}01$ to $0{\cdot}15 \pm 0{\cdot}02$ after 1 min exposure. This fluidizing effect of the detergent on the membranes could not be reversed over time and it was accompanied by cell death (Figs 3a and 4). These results clearly indicate that the double-mutant cells cannot counteract the fluidizing effect of the detergent provide support for the idea that CL is needed for the stability and functionality of cell membranes. It is known that CL stimulates changes in the physical properties of the membrane and that it decreases the lateral interaction within the monolayer leaflet, which favours the creation of membrane folds (Nichols-Smith *et al.* 2004). This is the reason why CL is concentrated in polar and septal regions of the bacterial cell (Mileykovskaya and Dowhan 2009). The low amount of CL detected in the double mutant was homogeneously distributed along the cell membrane with no apparent microdomains in regions of high curvature, such as the poles of the bacterial cells (Fig. 1b). Apparently, this uniform distribution of CL is critical to the topology of the membrane and this is reflected in the formation of structures of membrane, such as blebs or vesicles, which cause that the morphology of the cells lacking CL is very different to the wild- type strain (Fig. 1d). The changes produced by the low levels of CL in the membrane of *Ps. putida* allows the TTAB to pass through the membrane, reach its site of action and destroy the cell, as bacteria cannot counteract the fluidizing effect of the detergent. Supporting this behaviour, we observed that the double-mutant strain was able to assimilate exogenous CL into the membrane and that this incorporation promoted tolerance to the detergent (Fig. 3b). Thus, we conclude that regular amounts of CL in the membrane of *Ps. putida* are needed to counteract the fluidizing effect produced by the cationic surfactant TTAB.

Acknowledgements

GIL and PSB are Career Members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). GAL and RMH, are grateful for fellowships from CONICET. This work was supported by grants from SECYT–UNRC and CONICET of Argentina.

Conflict of Interest

No conflict of interest declared.

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