

# The phosphatidylcholine synthase of *Pseudomonas putida* A ATCC 12633 is responsible for the synthesis of phosphatidylcholine, which acts as a temporary reservoir for Al<sup>3+</sup>

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In *Pseudomonas putida* A ATCC 12633 cells grown with tetradecyltrimethylammonium bromide and exposed to Al<sub>3</sub>Cl, phosphatidylcholine (PC) levels increased, which alleviated stress caused by the Al<sup>3+</sup>. Here we cloned and sequenced a gene from this strain that encodes a phosphatidylcholine synthase (PCS) and characterized a *pcs*-deficient mutant. In the *pcs*-deficient mutant, PC could not be detected, whereas the mutant could be successfully complemented and expressed the enzyme, indicating that PC synthesis occurs exclusively via the PCS pathway in this organism. Although under non-stressing growth conditions the *pcs*-deficient mutant showed growth like that of the wild-type strain, the mutant was much more sensitive when challenged with Al<sup>3+</sup>, which strongly supports the supposition that PC is involved in the response of *P. putida* to Al<sup>3+</sup> and acts as a temporary reservoir of available ions through the formation of Al<sup>3+</sup>:PC complexes.

## INTRODUCTION

Phospholipid metabolism plays an important role in cellular responses to environmental changes. Most bacteria contain phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) as their major membrane-forming lipids (Rock *et al.*, 1996). Phosphatidylcholine (PC) has also been found in an increasing number of prokaryotes, mainly in symbionts, pathogens and photosynthetic bacteria (Sohlenkamp *et al.*, 2003). In bacteria, PC plays a particularly important role, including participating in successful interactions with eukaryotic hosts (Minder *et al.*, 2001; Daiyasu *et al.*, 2005; Comerci *et al.*, 2006; Wessel *et al.*, 2006; Conover *et al.*, 2008) and functioning in the response to environmental stress, such as that produced by high temperature, high salt concentration, low oxygen tension and the presence of acetic acid (Russell, 1992; Tang & Hollingsworth, 1998; Hanada *et al.*, 2001; Medeot *et al.*, 2007). The phospholipid (PL) composition of *Pseudomonas putida* A ATCC 12633

cultivated under unstressed culture conditions is similar to that described for other fluorescent *Pseudomonas* species (Ramos *et al.*, 1997; Albelo & Domenech 1997; Pinkart & White, 1997). However, in the presence of the quaternary ammonium compound tetradecyltrimethylammonium bromide (TTAB), *P. putida* A ATCC 12633 responds through quantitative changes in its PL composition, with specific variations in the content of phosphatidic acid (PA), PG and CL (Boeris *et al.*, 2007). In addition, in *P. putida* A ATCC 12633 cells grown with TTAB and exposed to Al<sub>3</sub>Cl, an increase in PC was detected, which led us to propose a physiological role for PC as a temporary reservoir for available Al<sup>3+</sup> through the formation of Al<sup>3+</sup>:PC complexes (Boeris *et al.*, 2009). These complexes are utilized as an Al<sup>3+</sup> reservoir in the membrane. When *P. putida* ATCC 12633 cells are grown in the presence of TTAB and Al<sup>3+</sup>, the bacteria can sequester the ion, permitting the total oxidation of TTAB as a source of carbon, nitrogen and energy (Lucchesi *et al.*, 2010).

Two pathways for PC biosynthesis are known in bacteria: the methylation pathway, in which PE undergoes three methylation steps catalysed by the enzyme phospholipid *N*-methyltransferase (PMT), and the phosphatidylcholine synthase (PCS) pathway, in which choline is directly condensed with CDP-diacylglycerol (CDP-DAG) by the enzyme PCS (Sohlenkamp *et al.*, 2003; Martínez-Morales *et al.*, 2003 and citations therein). Evidence for the existence of a PCS pathway has been reported in Gram-negative bacteria, such as *Sinorhizobium meliloti*, *Rhizobium leguminosarum* and

**Abbreviations:** CDP-DAG, CDP-diacylglycerol; CL, cardiolipin; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PCS, phosphatidylcholine synthase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; PMT, phospholipid *N*-methyltransferase; PSS, phosphatidylserine synthase; TTAB, tetradecyltrimethylammonium bromide.

The GenBank/EMBL/DDBJ accession numbers for the *pcs* gene sequences of *P. putida* KT2440 and *P. putida* A ATCC 12633 are JN368426 and JN680354, respectively.

*Pseudomonas aeruginosa* (López-Lara & Geiger, 2001; Wilderman *et al.*, 2002; Martínez-Morales *et al.*, 2003). We have shown an increase in PCS activity in cell-free extracts of *P. putida* A ATCC 12633 grown in the presence of TTAB and Al<sub>3</sub>Cl, and that Al<sup>3+</sup> is an activator of the enzyme (Boeris *et al.*, 2009). However, the mechanism and genes involved in PC biosynthesis in this bacterium have not been elucidated. In this report we cloned the *pcs* gene from *P. putida* KT2440. The *P. putida* A ATCC 12633 *pcs* homologue was also disrupted to generate the corresponding mutant. We found that the *pcs*-deficient mutant did not have detectable levels of PC and was much more sensitive than the wild-type strain when challenged with Al<sup>3+</sup>, supporting the involvement of PC in the response to Al<sup>3+</sup> in *P. putida* A ATCC 12633.

## 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. *P. putida* KT2440 and *P. putida* A ATCC 12633 were grown aerobically at 30 °C while shaking in LB medium, or in a basal salt liquid medium (HPi-BSM) (Lucchesi *et al.*, 1989) with 20 mM glucose and 18.7 mM NH<sub>4</sub>Cl or 0.15 mM (50 mg l<sup>-1</sup>) TTAB as the carbon and nitrogen sources, respectively. *Escherichia coli* strains were grown on LB medium at 37 °C. Growth was measured as OD<sub>660</sub> using a spectrophotometer (Beckman DU 640). When appropriate, media were supplemented with the following antibiotics at the indicated concentrations: for *E. coli*, 20 µg chloramphenicol ml<sup>-1</sup>, 100 µg gentamicin ml<sup>-1</sup>, 50 µg kanamycin ml<sup>-1</sup> and 25 µg streptomycin ml<sup>-1</sup>; for *P. putida*, 120 µg

gentamicin ml<sup>-1</sup>, 100 µg streptomycin ml<sup>-1</sup> and 75 µg kanamycin ml<sup>-1</sup>.

To determine survival rates, cells were grown in HPi-BSM with glucose and NH<sub>4</sub>Cl until the culture reached an OD<sub>660</sub> of approximately 0.8–1.0. At this point, the culture was divided into three parts; one part was kept as a control and either 50 mg TTAB l<sup>-1</sup> or 0.1 mM AlCl<sub>3</sub> was added to the other two parts. The number of viable cells (c.f.u. ml<sup>-1</sup>) was determined by plating serially diluted cell suspensions on LB plates both before TTAB or AlCl<sub>3</sub> was added and 15 min after their addition (Duque *et al.*, 2004). The experiments were performed three times. Errors in experiments were less than 2% of each value.

**Cloning and expression of an ORF potentially encoding a protein with putative PCS activity.** To manipulate DNA, recombinant DNA techniques were performed according to standard protocols (Sambrook & Russell, 2001). A 618 bp DNA fragment containing the ORF *pp0731* was amplified from *P. putida* KT2440 genomic DNA using the primers oLop731F and oLop731R (Table 2). The PCR product was cloned into pCR2.1-TOPO (Promega) to generate plasmid pPB03 (Table 1), which was sent to Eurofins (Germany) for sequencing. pPB03 was digested with *Nde*I and *Bam*HI, and the fragments corresponding to *pp0731* were cloned into vector pET9a (Studier *et al.*, 1990) to obtain the expression plasmid pPB03A (Table 1) in which the potential *pcs* gene can be overexpressed under the control of the T7 promoter. *E. coli* strain BL21(DE3)/pLysS, which expresses the T7 polymerase under the control of the *lac* promoter, was transformed with the respective expression plasmids. For the expression assay, *E. coli* BL21(DE3)/pLysS harbouring the *pcs* gene from *S. meliloti* (pTB2559) (Sohlenkamp *et al.*, 2000), *pp0731* from *P. putida* KT2440 (pPB03A) or pET9a alone was grown in LB medium with kanamycin (50 µg ml<sup>-1</sup>) and chloramphenicol (20 µg ml<sup>-1</sup>) to OD<sub>660</sub> ~0.3. Then, 200 µM IPTG and 1 µCi [1-<sup>14</sup>C]acetate (60 mCi mmol<sup>-1</sup>; 2.26 GBq mmol<sup>-1</sup>, New England Nuclear) was added to 1 ml aliquots

**Table 1.** Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<b><i>P. putida</i> strains</b>		
A ATCC 12633	Wild-type	Palleroni (1992)
KT2440	Wild-type	Nelson <i>et al.</i> (2002)
PB01	Gentamicin-resistant, <i>pcs::aacC</i> , insertional mutant of <i>P. putida</i> A ATCC 12633	This study
<b><i>E. coli</i> strains</b>		
DH5α	Host for plasmid amplification	Hanahan (1983)
BL21(DE3)/pLysS	Host for expression	Studier <i>et al.</i> (1990)
<b>Plasmids</b>		
pCR2.1-TOPO	Cloning vector, ampicillin- and kanamycin-resistant	Invitrogen
pK18mob	Mobilizable vector, Km <sup>r</sup> oriColE1 Mob <sup>+</sup> lacZ <sup>+</sup>	Schäfer <i>et al.</i> (1994)
pBBR1MCS-5	Broad-host-range cloning vector, gentamicin-resistant Tra <sup>-</sup> Mob <sup>-</sup>	Kovach <i>et al.</i> (1995)
pBBR1MCS-2	Broad-host-range cloning vector, kanamycin-resistant Tra <sup>-</sup> Mob <sup>-</sup>	Kovach <i>et al.</i> (1995)
pKNG101	Cloning vector for chromosomal insertion, streptomycin-resistant Sac <sup>s</sup> Tra <sup>-</sup> Mob <sup>+</sup> oriR6K	Kaniga <i>et al.</i> (1991)
pET9a	Expression vector, kanamycin-resistant	Studier <i>et al.</i> (1990)
pTB2559	<i>pcs</i> gene of <i>S. meliloti</i> in pET9a	Sohlenkamp <i>et al.</i> (2000)
pPB03A	<i>pcs</i> -like ORF ( <i>pp0731</i> ) from <i>P. putida</i> KT2440 (618 bp) as <i>Nde</i> I/ <i>Bam</i> HI insert in pET9a	This study
pPB03	<i>pcs</i> -like ORF ( <i>pp0731</i> ) from <i>P. putida</i> KT2440 (618 bp) insert in pCR2.1-TOPO	This study
pPB13	<i>pcs</i> -like ORF ( <i>pp0731</i> ) from <i>P. putida</i> KT2440 (618 bp) as <i>Bam</i> HI/ <i>Sal</i> I insert in pK18mob	This study
pPB15	1.2 kb <i>Bam</i> HI/ <i>Sal</i> I fragment containing <i>pp0731::aacC1</i> in pK18mob	This study
pPB21	1.2 kb <i>Bam</i> HI/ <i>Sal</i> I fragment containing <i>pp0731::aacC1</i> in pKNG101	This study
pPB24	<i>pcs</i> -like ORF from <i>P. putida</i> A ATCC 12633 (618 bp) as <i>Bam</i> HI/ <i>Bam</i> HI insert in pBBR1MCS-2	This study

**Table 2.** Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')*	Relevant characteristics
oLop731F	AGGAATAC <u>ATAT</u> GCCCACATTGGCGCGTGTG	<i>Nde</i> I restriction site overlapping the start codon of <i>pp0731</i>
oLop731R	AAAGGATCCTTACCAGGGATGAAAGTAAATAGTG	<i>Bam</i> HI restriction site after the stop codon of <i>pp0731</i>
oLPB19-0731	CGCGGATCCATGCCACATTGGCGCGTGTG	Located at the start of the <i>pp0731</i> gene in the genome sequence of <i>P. putida</i> KT2440
oLPB20-0731	ACGCGTGCAGCTTACCAGGGATGAAAGTAAATAGTG	Located after the stop codon of the <i>pp0731</i> gene in the genome sequence of <i>P. putida</i> KT2440
UpGm:	GGGT <u>TCTAG</u> ACGATGTTTGTATGT	Flanking the <i>aacC1</i> gene from pBBR1MCS-5
DownGm:	ATCTAT <u>CTAG</u> ACGGCTTGAACGAA	Flanking the <i>aacC1</i> gene from pBBR1MCS-5

\*Incorporated restriction sites are underlined.

of the cultures. Two hours after induction, cells were harvested by centrifugation and lipids were extracted.

Also, for the expression assay, *E. coli* strains were grown in HPI-BSM with 20 mM glucose and 18.7 mM NH<sub>4</sub>Cl supplemented with kanamycin (50 µg ml<sup>-1</sup>) and chloramphenicol (20 µg ml<sup>-1</sup>) to OD<sub>660</sub> ~0.3. At this point, 200 µM IPTG and 100 µM choline were added. Five hours after induction, cells were harvested and lipids were extracted and analysed by 2D-TLC.

#### Inactivation of a putative *pcs* gene from *P. putida* A ATCC 12633.

To inactivate the *pp0731* gene homologue in *P. putida* A ATCC 12633, the gene was mutated by the insertion of a gentamicin-resistance cassette. The oligonucleotide primers oLPB19-0731 and oLPB20-0731 (Table 2) were used to amplify approximately 600 bp of genomic DNA containing the *P. putida* KT2440 *pp0731* gene, which introduced *Bam*HI and *Sal*I sites (underlined in the table) into the PCR product. After digestion with the enzymes, the PCR product was cloned at the *Bam*HI/*Sal*I sites into pK18mob (Schäfer *et al.*, 1994) to yield plasmid pPB13 (Table 1). Next, a 600 bp *Xba*I fragment containing the *aacC1* gene, which encodes gentamicin resistance, was PCR-amplified from pBBR1MCS-5 (Kovach *et al.*, 1995) using the oligonucleotide primers shown in Table 2. This fragment was ligated to pPB13, which had been previously digested with the same enzymes, producing pPB15 (Table 1). The 1.2 kb *Bam*HI/*Sal*I fragment containing the *pp0731::aacC1* allele was subcloned into the suicide vector pKNG101 (Kaniga *et al.*, 1991) to obtain pPB21 (Table 1). Finally pPB21 was used for allelic exchange, in which it was electroporated into *P. putida* A ATCC 12633. Merodiploids were selected from cells grown in LB medium plus streptomycin (100 µg ml<sup>-1</sup>) and gentamicin (120 µg ml<sup>-1</sup>) (first crossover) and streaked onto LB plates containing 10% sucrose and gentamicin (120 µg ml<sup>-1</sup>) to score for loss of the pPB21 suicide vector, which contains a *sacB* gene. Gentamicin-resistant colonies that were able to grow in the presence of sucrose were purified (Kaniga *et al.*, 1991). The correct insertion of the mutant allele of *pp0731* into the chromosome was verified by PCR analysis (results not shown). The *pp0731*-deficient *P. putida* A ATCC 12633 strain was named *P. putida* PB01.

For complementation, the *pcs* gene was amplified from *P. putida* A ATCC 12633 genomic DNA using the oligonucleotides oLPB19-0731 and oLop731R (Table 2), which introduced *Bam*HI sites (underlined in the table) into the PCR product. After digestion with the enzyme, the PCR product was cloned into the *Bam*HI site of vector pBBR1MCS-2 (Kovach *et al.*, 1995) to yield plasmid pPB24 (Table 1). Finally, pPB24 was transformed into *P. putida* PB01 using electroporation. The strains containing pPB24 were selected in LB medium with kanamycin (75 µg ml<sup>-1</sup>).

#### Incorporation of [methyl-<sup>14</sup>C]choline into *P. putida* A ATCC 12633.

*P. putida* A ATCC 12633 was grown in HPI-BSM with 50 mg TTAB l<sup>-1</sup> or 50 mg TTAB l<sup>-1</sup> plus 0.1 mM choline until the culture reached an OD<sub>660</sub> of approximately 0.2. At this point, 1 µCi ml<sup>-1</sup> [methyl-<sup>14</sup>C]choline (55 mCi mmol<sup>-1</sup>; 2.07 GBq mmol<sup>-1</sup>, New England Nuclear) was added. When necessary, after 30 min of incubation with radiolabeled choline, both cultures (with or without the addition of exogenous choline) were divided, and growth and choline incorporation were continued in the presence or absence of 0.1 mmol AlCl<sub>3</sub> l<sup>-1</sup>. After the time indicated in each experiment, 100 µl samples were removed and the cells were collected by centrifugation at 12 000 g for 10 min. Aliquots of supernatant and cells were quantified based on radioactivity measured in a liquid scintillation counter (Beckman LS 60001 C).

#### Phospholipid radiolabelling and extraction.

The lipid compositions of *P. putida* A ATCC 12633 wild-type and mutant strains were determined following labelling with [1-<sup>14</sup>C]acetate (60 mCi mmol<sup>-1</sup>; 2.26 GBq mmol<sup>-1</sup>, New England Nuclear) or [methyl-<sup>14</sup>C]choline (55 mCi mmol<sup>-1</sup>; 2.07 GBq mmol<sup>-1</sup>, New England Nuclear) (de Rudder *et al.*, 1997; Sohlenkamp *et al.*, 2000). Briefly, cultures of wild-type and mutant strains were grown in LB medium or HPI-BSM with 50 mg TTAB l<sup>-1</sup> or 50 mg TTAB l<sup>-1</sup> plus 0.10 mmol choline l<sup>-1</sup> to OD<sub>660</sub> ~0.2. After the addition of 1 µCi ml<sup>-1</sup> [1-<sup>14</sup>C]acetate or [methyl-<sup>14</sup>C]choline to each culture, the cultures were incubated for 2 or 12 h, according to whether they were previously grown in LB medium or HPI-BSM, respectively. Then, the cells were collected and washed twice with centrifugation at 8000 g for 10 min to eliminate the unincorporated [1-<sup>14</sup>C]acetate or [methyl-<sup>14</sup>C]choline, and the lipids were extracted. In experiments where the cells were exposed to AlCl<sub>3</sub>, after the addition of [methyl-<sup>14</sup>C]choline to each culture, the cultures were divided into two parts: one part was kept as a control, and to the other 10 mmol AlCl<sub>3</sub> l<sup>-1</sup> was added. At 15 or 180 min after AlCl<sub>3</sub> addition, the cells were collected and washed, and lipids were extracted. The same procedures and conditions were used for the unlabelled samples. Lipids were extracted from the cells according to the method of Bligh & Dyer (1959) and separated using 1D or 2D TLC plates (silica gel HLF, 250 microns, Analtech) according to Boeris *et al.* (2007). Separated lipids were detected with iodine vapour and identified based on comparison with purified standards purchased from Sigma. The positions of radiolabelled lipids were determined by autoradiography on Agfa-Gevaert film. Spots were quantified using a Phosphorimager (Storm 820, Molecular Dynamics). For non-radioactive samples, phospholipid classes were quantified by phosphorus determination as described by Fiske & Subbarow (1925). The experiments were performed four times.

**Al<sup>3+</sup> detection in cells.** *P. putida* A ATCC 12633, *P. putida* PB01 and *P. putida* PB01 containing pPB24 or pBBR1MCS-2 alone were grown in LB medium until the culture reached an OD<sub>660</sub> of approximately 0.80. At this point the cells were harvested by centrifugation at 8000 g for 10 min, washed twice with 0.9% (w/v) NaCl and diluted in HPi-BSM to OD<sub>660</sub> ~0.4. Cells were immobilized on microscope slides by the addition of cold 1:1 (v/v) methanol/acetone and incubated for 20 min at -20 °C. A subset of immobilized cells were treated with 10 µM AlCl<sub>3</sub> for 10 s (to allow incorporation of the ion into the membrane), washed with 0.9% (w/v) NaCl and stained by the addition of 10 µM morin for 30 s (Browne *et al.*, 1990). The slides were washed with 0.9% (w/v) NaCl to eliminate free fluorochrome. Samples were observed with a Zeiss microscope (Axiovert 135) equipped with an AxioCam camera.

**Determination of PCS and PMT activities.** Cell suspensions of *P. putida* A ATCC 12633 or *P. putida* PB01 grown to the beginning of the stationary phase were harvested by centrifugation at 10 000 g for 10 min at 4 °C, washed with 0.9% (w/v) NaCl, and resuspended in 20 mM Tris-HCl buffer, pH 8. Washed cells were disrupted by sonication in an ice bath at 20 000 Hz using a Vibra-Cell ultrasonic processor 10 times for 10 s each time. Whole cells and cell debris were removed by centrifugation, and the supernatant of the cellular extracts was used for PCS and PMT determination in a fashion similar to that described by Sohlenkamp *et al.* (2000) and de Rudder *et al.* (1997), respectively. The products of PCS and PMT activity were identified by TLC, developed with *n*-propanol/propionic acid/chloroform/water (3:2:2:1, v/v) (Higgins, 1987). Protein concentration was determined using the method of Bradford (1976) with BSA as the standard.

## RESULTS

### Synthesis of PC in *P. putida* A ATCC 12633

We have demonstrated that an increase in PCS activity can be detected in cell-free extracts of *P. putida* A ATCC 12633 grown in the presence of TTAB and Al<sub>3</sub>Cl, and that Al<sup>3+</sup> is an activator of the enzyme (Boeris *et al.*, 2009). We investigated the presence of PMT activity using cell-free extracts of *P. putida* A ATCC 12633 grown under the above culture conditions and none of the three methylated PE derivatives products usually formed by PMT [phosphatidylmonomethylethanolamine (MMPE), phosphatidyl dimethylethanol amine (DMPE) and PC] was detected (not shown). Taken together, these data indicate that PCS of *P. putida* A ATCC 12633 is, under the culture conditions used in this study, solely responsible for the ability of this organism to synthesize PC.

In order to determine whether the synthesis of PC in *P. putida* A ATCC 12633 by PCS is dependent on an exogenous supply of choline (Sohlenkamp *et al.*, 2000, 2003; Comerci *et al.*, 2006), the PL composition of *P. putida* A ATCC 12633 was analysed when grown in a basal medium in the presence of TTAB, with or without the addition of choline. Previously we determined that after 30 min, 75% of the [methyl-<sup>14</sup>C]choline was incorporated into *P. putida* A ATCC 12633 grown with TTAB with or without the addition of exogenous choline (results not shown). The radiolabelled choline was incorporated into the cellular membranes and the PL composition was

qualitatively the same under all culture conditions. PE was the most abundant PL, while the proportions of the other components, PG, CL, PC and lysophosphatidylethanolamine (LPE), ranged from 2 to 6%, and PA was around 0.5%. These results indicate that the synthesis of PC in *P. putida* A ATCC 12633 does not depend on the presence of choline in the culture medium. When both cultures (with or without the addition of exogenous choline) were treated with [methyl-<sup>14</sup>C]choline and after 30 min exposed to Al<sup>3+</sup>, the incorporation of [methyl-<sup>14</sup>C]choline was similar to results obtained in the absence of the ion (after 30 min, 73% of the [methyl-<sup>14</sup>C]choline was incorporated under both culture conditions). Based on the incorporation of radiolabelled choline into lipid fractions after 15 or 180 min in contact with AlCl<sub>3</sub>, the most significant change associated with the presence of Al<sup>3+</sup> under both culture conditions was a sustained increase in the total amount of PC: from 1.86 ± 0.01 (without Al<sup>3+</sup>) to 4.00 ± 0.31 (15 min with Al<sup>3+</sup>) or to 3.79 ± 0.05 (180 min with Al<sup>3+</sup>) (*n*=3) arbitrary units measured by the Phosphorimager.

### Identification of homologues of *P. putida* pcs

In order to identify the *P. putida* pcs gene, we subjected the *S. meliloti* PCS (AAL53937) protein sequence (Sohlenkamp *et al.*, 2000) to BLAST analysis against the *P. putida* KT2440 genome, a strain closely related to *P. putida* A ATCC 12633 that has been completely sequenced (Nelson *et al.*, 2002).

The *P. putida* KT2440 genome contains an ORF (*pp0731*) predicted to encode a protein with significant homology to the PCS from *S. meliloti* and *P. aeruginosa* (26 and 35% amino acid identity, respectively). Based on sequence homology, in *P. putida* KT2440 the ORF *pp0731* has been annotated as a putative phosphatidylserine synthase (PSS) (NP\_7428929). In addition, another ORF (*pp4677*) is also annotated in *P. putida* KT2440 as a putative PSS (NP\_742892) (Nelson *et al.*, 2002). The potential gene product of the ORF *pp4677* encodes a protein that shows the typical motif DG(X)<sub>2</sub>AR(X)<sub>8</sub>G(X)<sub>3</sub>D(X)<sub>3</sub>D that is characteristic of CDP-alcohol phosphatidyltransferases, including subclass II PSSs (Williams & McMaster, 1998; Sohlenkamp *et al.*, 2003); however, the motif highly predictive for PCSs, DG(X)<sub>2</sub>AR(X)<sub>12</sub>G(X)<sub>3</sub>D(X)<sub>3</sub>D (Sohlenkamp *et al.*, 2003), is absent (Fig. 1). While our search using the BLASTP algorithm also revealed significant similarities of the ORF *pp0731* of *P. putida* KT2440 to some subclass II PSSs (32, 34 and 41% amino acid identity with PSSs from *Bacillus subtilis*, *S. meliloti* and *Agrobacterium* sp. 317459, respectively) (Fig. 1), the ORF *pp0731* from *P. putida* KT2440 showed 12 amino acid residues between the conserved Arg and the second conserved Gly, which is described as motif characteristic for PCSs from *S. meliloti*, *P. aeruginosa* and *Borrelia burgdorferi* (Martínez-Morales *et al.*, 2003). For this reason, in order to identify a *P. putida* pcs gene, this ORF was selected.

PSS-Smel	MEEP RHEQSAGIPEAAEHVGATGTHDKARGPRLREIPLR--LMVPMITVLAICAGLSGVRLAFENR-----	65
PSS-Agsp	-----MARSDSGRGPRLREIPFR--LLVPLNITVLAICAGLSGVRLAIEGR-----	44
PSS-Bsub	-----MNYIPCMITIGNFICGLLAHSLLYHN-----	27
PP4677	-MSE RPEEPNKP SDAESLLPVDEHVEEGHDAEGRKVRHGGIYLLPNLFTANLFAFYSIISMSAQALSAGDPREASK	79
PCS-Paer	-----MPVNL SMTPI NKAKAWG-----V-HAVTASGVILALLALLALVDNK-----	40
PCS-Smel	-----MKFFNYRRVPEYAEIRAFS-----V-HILTASGSFLAFLGVVAAAEHR-----	41
PCS-Bbur	-----MKNINILILAW-----LVHIFTASGLIVGLYSIISIVNGN-----	34
PP0731	-----MPTLARVFPFVRLLDLANILTMFNIGLAFGTVYFALQRR-----	39
<p style="text-align: center;">* * * * *</p>		
PSS-Smel	-FELAVAMVLLAAFLDGDIDGRIARLLKATSSF---GQOMDSLADIINFGVAPALVLYVFLDQARS LGWIAALIYAIYAV	140
PSS-Agsp	-FELAVGMVLLAAFLDGDVGRVARMMKATSKF---GEQOMDSLADIVNFGVAPALVYIYLLDQARSIGWIGALIYVIAA	119
PSS-Bsub	-IHSALVLI FTGMFLDFD GMAARKLNAVSDM---GRELDSFADLVTFGVAP SMLAYSVALYTLPIGILCALTYSICG	102
PP4677	YFAFAAIAIFVAMVLDGLDGRVARM TNTQSAF---GAEYDSLSDMVAFGVAPALLAFGWALGDMGKVGWVAFIYVAGA	155
PCS-Paer	-PQACLLWLGLALLVDGLDGLT LARKYEVKEMLP HFDG SVLDLVIDYLYVFP AIF IYR YIPLPEH-FEL LAVGVILVSS	118
PCS-Smel	-FVDMFWLGLLALLVDGIDGPIARKVQVKEVLPNWSGDTLDNVIDYVTVYVLLPAFALYQSGMIGEP-WSFVAAGAIYVSS	119
PCS-Bbur	-YSLLLKLTVIGLIIDGIDGTMARKLKVKE LPEIDGTLDDNITDYIN YTFIPVIFVYLGFEIEEK-YKVAICIGILSS	112
PP0731	-FVLAATMICLAAMLDFLDGHIARRYLAA NSAQRAF GKHLDSFADLLNFSITPALVILLDQSLSA---TLAAVTLVFTA	115
<p style="text-align: center;">* * * * *</p>		
PSS-Smel	GLRLARFNVAERQVKASWQSEYFVGVPAPAGAMLVLLPVYLG LGLAPERTFALIASVYTVLIAFLMVSRLPVWSGKSE	220
PSS-Agsp	GLRLARFNVMIERPVKAPWQSEFFVGV PAMP GAMLVLLPVYLGFLGVEPKPFAYVAAAYTVLIGYLLISRLPVWSGKSS	199
PSS-Bsub	MLRLSKFNIEQ-----SKLP TFI GMP I P F A G---MCLVILSFTYN-----PILLAIGTCGLSYLMVSKIKFPHFKKH	166
PP4677	ALRLARFN TQVG-----TADKRYF IGLASPAAGV VAGT V WAF S D Y G I Q G S K L S F L V A L L V A A G M L M V S N I K Y N S F K E L	230
PCS-Paer	LFCFCNVNMKS-----TDNYFVGFPAAWNVA VYFYVLDLHPWV-----NLATV L V L A A L T L T R M K F L H P F R V R Q F	184
PCS-Smel	A I Y Y A D M G M K T-----D E Y F F S G F P V V W N M V V F T L F V I Q A S E V T-----A S I V V L S V I L T F L P I N F L H P V R V K R L	185
PCS-Bbur	A Y Q F S R T D A K T-----N D N Y F R G F P S L W N L F V I A L I F K M E Q I T-----N L I T M S I C I I T S F I P K I F I Y P S K T K E L	178
PP0731	I L R L S L F G M P G-----T S A G G Y Q G L P T T Y S G F V F A L N I F A L M G L S A G H Y G-----A G V I V G L M A L V G I I Q V T S L R I P K Y G A P	185
<p style="text-align: center;">* * * * *</p>		
PSS-Smel	NRVRRDLVLPVILVVVYVATLMTYT WETMV---VTAVAYLVSLPFGARSWQRKYG-----DWPGGHNDGLPGDSE	289
PSS-Agsp	TRIRRDVLVPMILVVVLYVAMLSFTWEVLV---LTVAAYLVFIPISARLWHRRYGTLTIEEDAHDDANGNGLD RGI-	274
PSS-Bsub	AAEN-----LESGRWN-----	177
PP4677	DLKGRVPFV-AILAVVLVFAVVS D P P R I L L---L I F L A Y A A S G P I Q F L L K A R R R K A-----	283
PCS-Paer	MPLNIAVTFVWLISG L L I V Q P A D L P I L L G---L W F A A S A Y F V G I C L W R S A R E W F G-----	238
PCS-Smel	RPLNLGIFLVWSVLGMYALLLHFETPPWVVV---G V V A T G L Y L V I G F I L Q I F P K L G R A-----	241
PCS-Bbur	RK I T I P I T I I S C L I F V V S I F S E L S T T A L K M A K T V L I L Y F A Y L T L A S I Y L T Y K T R N R-----	234
PP0731	STVA-----CFAMLFG-----LS-----T Y F H P W-----	205

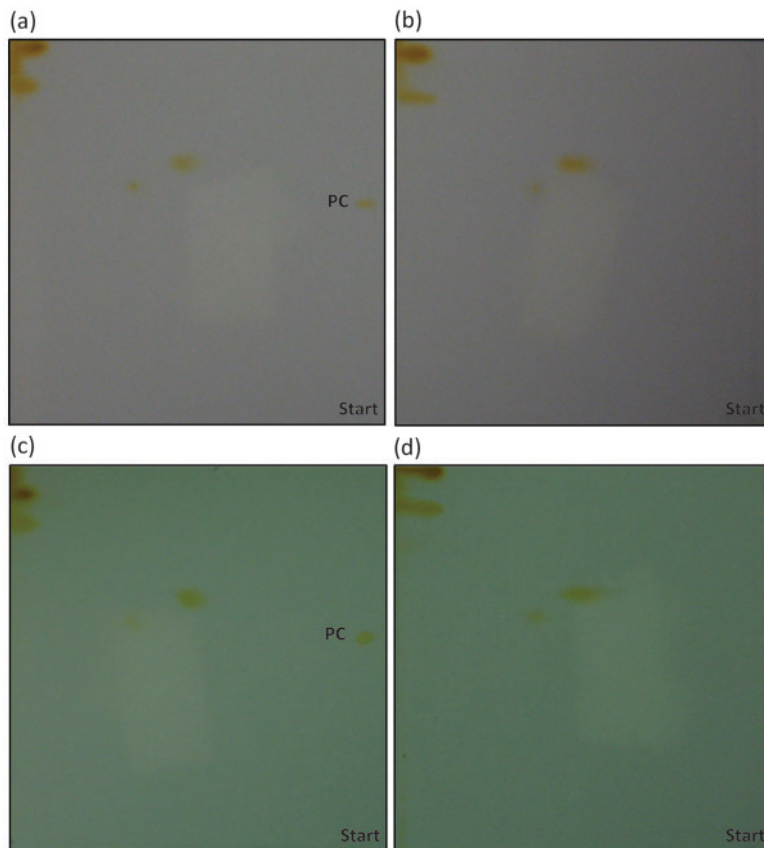
**Fig. 1.** Comparison of the *P. putida* KT2440 PCS homologue with protein sequences derived from CDP-alcohol phosphatidyltransferase. The *P. putida* KT2440 PCS homologue sequence (PP0731) was aligned with PCS from *S. meliloti* (PCS-Smel, AAL53937), PCS from *P. aeruginosa* (PCS-Paer, NP002516), PCS from *B. burgdorferi* (PCS-Bbur, NP\_212383), PSS from *S. meliloti* (PSS-Smel, AF247564), PSS from *Agrobacterium* sp. 317459 (PSS-Agsp, AAL01116), PSS from *Bacillus subtilis* (PSS-Bsub, D38022) and ORF PP4677 (putative PSS, NP\_746786) from *P. putida* KT2440. Identical residues are marked with asterisks. Residues thought to be characteristic of the CDP-alcohol phosphatidyltransferase motif are indicated by black circles. Full stops represent semi-conserved residues and colons represent conserved residues. Alignment was performed using the program CLUSTAL X.

The selected ORF *pp0731* was cloned into vector pET9a and expressed in *E. coli* BL21(DE3)/pLysS, a PC-deficient organism. It has been shown that *E. coli* does not synthesize PC (Ames, 1968; Sohlenkamp *et al.*, 2003); however, *E. coli* does synthesize CDP-DAG, which can be utilized by PCS to form PC. We compared the membrane phospholipid content of *E. coli* transformed by pTB2559 (harbouring the *pcs* gene from *S. meliloti*) or pPB03A [harbouring the ORF (*pp0731*) from *P. putida* KT2440] with that of *E. coli* transformed with vector alone. When the cells were grown in LB medium, after induction with IPTG, PC was detected only in *E. coli* transformed with pTB2559 or pPB03A (results not shown). When the cells were grown in saline medium, after induction with IPTG, PC was present only in transformed *E. coli* when choline was supplied in the culture medium (Fig. 2a, c). Without supplementation by additional choline in the medium, PC was undetectable even in *E. coli* transformed with pTB2559 or pPB03A (Fig. 2b, d). As predicted, *E. coli* transformed with vector alone did not produce PC,

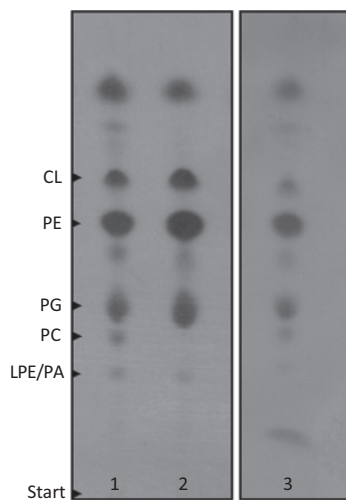
irrespective of the addition of choline to the medium (results not shown).

### Biochemical and physiological analysis in an insertional *pcs* mutant of *P. putida* A ATCC 12633

The *P. putida* A ATCC 12633 *pcs* homologue was disrupted; the corresponding mutant was generated and analysed for PC production. As expected, wild-type *P. putida* A ATCC 12633 contains PC after growth in LB medium (Fig. 3, lane 1). In contrast, PC was not detected in the *pcs*-deficient mutant (Fig. 3, lane 2). Also, compared with the wild-type, mutants had a 130 % increase in the relative amount of CL, whereas the relative amounts of the other anionic (PG, PA) or zwitterionic (PE, LPE) PLs remained relatively constant (results not shown). To validate the function of the *pcs* gene, we measured PCS activity in cell-free extracts of *P. putida* A ATCC 12633 wild-type and mutant strains. PCS activity was detected in the cell-free extracts from the wild-type strain only [ $35.8 \pm 0.13$  nmol PC min<sup>-1</sup> ( $\mu$ g protein)<sup>-1</sup> ( $n=3$ )].



**Fig. 2.** Expression of the *pcs* homologue gene (*pp0731*) from *P. putida* KT2440. *E. coli* BL21(DE3)/pLysS cells containing pPB03A (a, b) or pTB2559 (c, d) were grown in HPI-BSM with 20 mM glucose and 18.7 mM NH<sub>4</sub>Cl as the carbon and nitrogen sources, respectively, to OD<sub>660</sub> ~0.3. At this point, 200 μM IPTG and 0.1 mM choline (a, c) were added. Five hours after induction, lipids were extracted and analysed by 2D-TLC.



**Fig. 3.** TLC of the total PL extracted from *P. putida* A ATCC 12633 (lane 1), *P. putida* PB01 (lane 2) and *P. putida* PB01 containing pPB24 (lane 3). Cells were grown in LB medium until the culture reached OD<sub>660</sub> ~0.2. At this point, 1 μCi [1-<sup>14</sup>C]acetate was added. Two hours after induction, lipids were extracted and analysed.

The mutant strain was complemented with plasmids containing the *pcs* gene and then analysed for PC production. When the *pcs*-deficient mutant was transformed with pPB24 (Table 1), PC production was restored (Fig. 3, lane 3), indicating that the mutation was successfully complemented.

In order to characterize the phenotype of the *pcs*-deficient *P. putida* mutant, its growth behaviour was monitored. The mutant strain *P. putida* PB01 grew at a rate similar to that of the wild-type in rich medium (LB) or in basal salt medium with glucose and NH<sub>4</sub>Cl as the carbon and nitrogen sources, respectively, indicating that PC is not essential for the viability of *P. putida* cells. To assess whether mutations in PC biosynthesis influence the response of *P. putida* A ATCC 12633 to TTAB and Al<sup>3+</sup>, cells were grown on HPI-BSM with glucose and NH<sub>4</sub>Cl to exponential phase (OD<sub>660</sub> 0.8). Then, 50 mg TTAB l<sup>-1</sup> or 0.1 mM Al<sup>3+</sup> was added, and cellular viability was determined. We found that the *P. putida* PB01 mutant strain was more sensitive to TTAB stress than the wild-type strain. The mutant cells reached a cell density of 10<sup>9</sup> c.f.u. ml<sup>-1</sup> 15 min after TTAB addition, which was approximately 10<sup>3</sup>-fold less than the cell density of the wild-type cells exposed to TTAB. Interestingly, the *pcs* mutant was much more sensitive than the wild-type strain when challenged with Al<sup>3+</sup>, and showed a significant loss of viability (from 10<sup>12</sup> to 10<sup>6</sup> c.f.u. ml<sup>-1</sup> 15 min after Al<sup>3+</sup> addition). Considering that we previously demonstrated that at the concentrations used, Al<sup>3+</sup> does not produce

changes in the intracellular and extracellular pH values (Liffourrena *et al.*, 2008), this negative effect cannot be attributed to the acidification of the medium by the addition of  $Al^{3+}$ . Also,  $Al^{3+}$  in the membrane of wild-type and mutant strains grown in LB medium was visualized using the fluorochrome 2',3,4',5,7-pentahydroxyflavone (morin reagent). Green fluorescence was homogeneously distributed along the cell membrane. In the mutant strain, the distribution of green fluorescence was quite similar to that of wild-type cells, but the mutant had a lower level of fluorescence intensity in the cell membrane, consistent with a reduced presence of  $Al^{3+}$  (Fig. 4b and c, respectively). As expected, the distribution and intensity of green fluorescence was restored in complemented cells (Fig. 4d).

## DISCUSSION

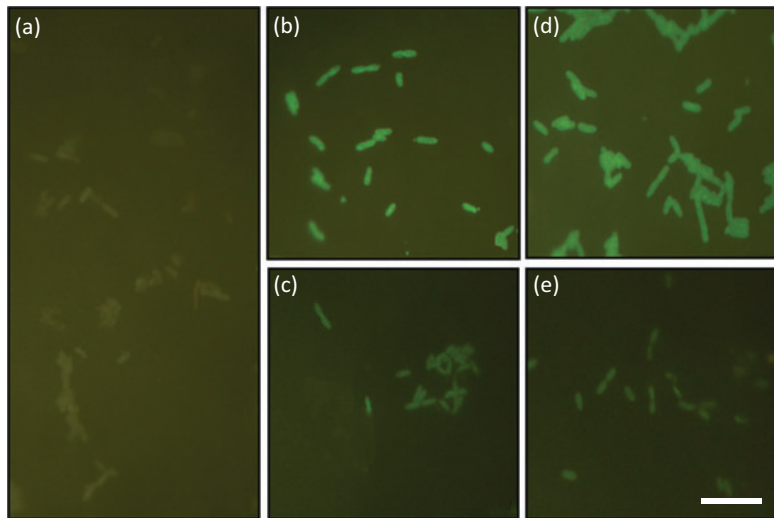
*P. putida* A ATCC 12633 is able to modify the composition of PL in its membranes in response to stress produced by the presence of the quaternary ammonium compound TTAB, and thus compensates for changes induced by this environmental contaminant (Boeris *et al.*, 2007). Our first interesting observation was that in *P. putida* A ATCC 12633 cells grown with TTAB and exposed to  $Al_3Cl$ , the PC content increased through the activation of PCS, suggesting that PC biosynthesis is dependent on the PCS pathway (Boeris *et al.*, 2009). The genomes of *Pseudomonas* species harbour two genes encoding homologues of the *pmtA* and *pcs* genes of *Rhodobacter sphaeroides* and *S. meliloti*, respectively, which suggests that the methylation of PE and/or the condensation of choline with CDP-DAG could account for PC biosynthesis in this genus (Wilderman *et al.*, 2002; Sohlenkamp *et al.*, 2003). Based on sequence similarity to the *R. sphaeroides* gene for *pmtA*, we did not detect a *P. putida* KT2440 orthologue that methylated PE and therefore was involved in the biosynthesis of PC. Also, PMT activity could not be detected in cellular extracts from *P. putida* grown in minimal medium with glucose and ammonium as carbon

and nitrogen sources, confirming that the methylation of PE could not account for PC synthesis in this strain. A similar situation has been described in *P. aeruginosa*, where the synthesis of PC occurs only through the PCS pathway, because the methylation pathway is not functional (Wilderman *et al.*, 2002).

In this work, the ORF (PP0731) from *P. putida* KT2440, which shows a motif characteristic of PCSs from *S. meliloti*, *P. aeruginosa* and *B. burgdorferi* (Martínez-Morales *et al.*, 2003) (Fig. 1), was successfully amplified from genomic DNA, cloned into the pET9a vector and expressed in *E. coli* BL21(DE3)/pLysS, a PC-deficient organism. Introduction of *pp0731* into *E. coli* resulted in the production of PC which was dependent on the supplementation of choline in the medium. This clearly demonstrates that this ORF encodes a functional PCS enzyme capable of condensing choline directly with CDP-DAG to yield PC. Additionally, the data from the generation of a *pcs* gene mutant that does not contain PC combined with the results from the transformation of the *pcs*-deficient mutant with an expression vector containing the *P. putida pcs* homologue (Fig. 3) indicate that the PCS pathway is the sole mechanism for PC synthesis in *P. putida*.

Investigation of the PL composition of *P. putida* A ATCC 12633 revealed that the synthesis of PC does not depend on the presence of choline in the culture medium, although *P. putida* A ATCC 12633 is able to take up choline and we were able to detect the incorporation of radiolabelled choline into PL of *P. putida* A ATCC 12633 cells. This result strongly indicates that the PCS pathway for PC formation in *P. putida* A ATCC 12633 uses endogenous free choline as the substrate, and suggests that in this organism a *de novo* pathway for choline biosynthesis might exist.

Several reports have suggested that PC is required for the survival of *S. meliloti* (de Rudder *et al.*, 2000), symbiosis in *Bradyrhizobium japonicum* (Minder *et al.*, 2001), and for virulence and adhesion in plant, human and animal



**Fig. 4.**  $Al^{3+}$  detection in cells. Cells were grown in LB medium to early exponential phase, immobilized on microscope slides, treated with  $AlCl_3$ , and stained by addition of morin reagent as described in Methods. *P. putida* A ATCC 12633 (b), *P. putida* PB01 (c), *P. putida* PB01 harbouring pPB24 (d) and *P. putida* PB01 harbouring pBBR1MCS-2 alone (e). (a) Cells of *P. putida* A ATCC 12633 stained with morin but without any added aluminium (negative control). Bar, 4.5  $\mu m$ .

pathogens (Wessel *et al.*, 2006; Comerci *et al.*, 2006; Conde-Alvarez *et al.*, 2006; Conover *et al.*, 2008). From our observation that PC is absent in the *pcs*-deficient mutant but that this mutant strain grew at a rate similar to that of wild-type in rich medium or in basal salt medium with glucose and  $\text{NH}_4\text{Cl}$  as the carbon and nitrogen sources, we reached a tentative conclusion that this PL is not essential for the viability of *P. putida* cells. In the *pcs*-deficient mutant, the amount of CL increased 1.5-fold; it is possible that this increase might be an efficient way to counteract any possible damage resulting from the lack of PC. It is known that in bacteria, CL has a distribution that reflects its participation in cell division and in the response to different stress conditions (Xia & Dowhan, 1995; Bernal *et al.*, 2007).

In comparison with wild-type cells, the most relevant phenotype of the PC-deficient mutant strain was its increased sensitivity to exposure to  $\text{Al}^{3+}$ , indicating that PC plays an important role in the initial response to this ion. This hypothesis is in agreement with the presence of less  $\text{Al}^{3+}$  in the cell membranes in the *pcs*-deficient mutant (Fig. 4) and with earlier reports demonstrating that in multilamellar vesicles,  $\text{Al}^{3+}$  binds to the phosphate group of PC and should be viewed as a temporary reservoir of biologically available aluminium (MacKinnon *et al.*, 2004, 2006). Previous studies in our laboratory have demonstrated that when *P. putida* A ATCC 12633 cells grow on TTAB, an accumulation of intracellular trimethylamine is found; however, after the addition of 0.1 mM  $\text{AlCl}_3$ , the internal concentration of intracellular trimethylamine decreases through the formation of a soluble  $\text{Al}^{3+}$ -trimethylamine complex, and TTAB is fully consumed without the accumulation of undesirable compounds (Liffourrena *et al.*, 2008). We also demonstrated that in *P. putida* A ATCC 12633, PCS activity is activated by  $\text{Al}^{3+}$ , and this ability might allow the bacterium to rapidly increase the relative amount of PC in its membrane and therefore adjust its biochemical properties more rapidly (Lucchesi *et al.*, 2010). Thus, the results obtained in this study, showing the sensitivity of the PC-deficient mutant strain to  $\text{Al}^{3+}$ , strongly support our conclusion that the PC localized in the membrane acts as a temporary reservoir for available  $\text{Al}^{3+}$  through the formation of  $\text{Al}^{3+}$ :PC complexes. These complexes reduce the amount of free toxic  $\text{Al}^{3+}$  and allow *P. putida* A ATCC 12633, in the presence of TTAB and  $\text{Al}^{3+}$ , to obtain the ions necessary to achieve the total oxidation of the physiologically relevant quaternary ammonium compound TTAB as a source of carbon, nitrogen and energy.

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