Pathways for phosphatidylcholine biosynthesis in bacteria

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Phosphatidylcholine (PC) or lecithin is the major phospholipid in eukaryotic cells. In addition to being the major structural component of cellular membranes, PC plays an important role in signal transduction as it is a major source of lipid second messengers (Exton, 1994). In eukaryotic organisms, PC can be synthesized by two alternative biosynthetic pathways, the CDP-choline pathway or the methylation pathway (Kent, 1995). In the CDP-choline pathway, also known as the Kennedy pathway, free choline is converted to PC via the intermediates choline phosphate and CDP-choline through the sequential actions of choline kinase, CTP:phosphocholine cytidylyltransferase and CDP-choline : 1,2-diacylglycerol cholinephosphotransferase. In the methylation pathway, PC is formed by three successive methylations of phosphatidylethanolamine (PE) via the intermediates monomethyolphosphatidylethanolamine (MMPE) and dimethyolphosphatidylethanolamine (DMPE) using the methyl donor S-adenosylmethionine and the enzyme phospholipid N-methyltransferase (Pmt) (Fig. 1). Although in mammals the three methylation activities are due to a single gene, in yeast, two different genes encoding class I and class II PMTs are involved (Sohlenkamp et al., 2003). Class II PMTs (PEM1/CHO2 in Saccharomyces cerevisiae) catalyse the first methylation step from PE to MMPE, whereas class I PMTs (PEM2/OPI3 in S. cerevisiae) catalyse the second and third methylation steps from MMPE via DMPE to PC.

The Gram-negative model bacterium Escherichia coli or the Gram-positive model bacterium Bacillus subtilis both contain only PE, phosphatidylglycerol and cardiolipin as major membrane phospholipids and therefore it was thought that this holds true for most bacteria. However, a recent estimate suggests that probably more than 10% of all bacteria contain PC as a membrane phospholipid (Sohlenkamp et al., 2003). Only the methylation pathway of PC biosynthesis was thought to occur in prokaryotes...
(Rock et al., 1996); however, a second pathway for PC biosynthesis exists, the phosphatidylcholine synthase (PCS) pathway (Fig. 1), which is distinct from the CDP-choline pathway and seems to be exclusive for bacteria (de Rudder et al., 1997, 1999; Sohlenkamp et al., 2000). In this second pathway, choline is condensed directly with CDP-diacylglycerol to form PC and CMP in a reaction catalysed by PCS. The existence of a PCS pathway has been proposed for a number of bacteria (Sohlenkamp et al., 2000, 2003; López-Lara & Geiger, 2001; López-Lara et al., 2003) and now there is evidence that this pathway exists in Pseudomonas aeruginosa (Wilderman et al., 2002) and at least indirect evidence that it occurs in Agrobacterium (Karnezis et al., 2002).

Bacterial genes encoding Pmt proteins are quite dissimilar and the two families presently known, with members resembling either the Rhodobacter PmtA (Arondel et al., 1993) or the Sinorhizobium PmtA (de Rudder et al., 2000), are more similar to methyltransferases with other substrate specificities than to each other (López-Lara & Geiger, 2001; Sohlenkamp et al., 2003). Recently, the gene encoding Pmt in Acetobacter aceti has been identified (Hanada et al., 2001) and its product belongs to the rhodobacterial PmtA family. A Pmt protein (PmtA) from Bradyrhizobium japonicum resembles the sinorhizobial PmtA but surprisingly the bradyrhizobial PmtA shows a peculiar substrate specificity as it seems to perform only the first methylation step efficiently, converting PE to MMPE (Minder et al., 2001).

In this study, we investigated the PMT and PCS pathways of PC biosynthesis in different bacteria. We identified various genes encoding Pmt or Pcs. Based on our studies, we suggest that if bacteria are able to form PC, they often can do so via either of the pathways.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study and their relevant characteristics are shown in Table 1. *Escherichia coli* (37°C), Agrobacterium tumefaciens (29°C), Brucella melitensis (37°C), *P. aeruginosa* (37°C) and Burkholderia strains (29°C) were grown on Luria–Bertani (LB) medium (Miller, 1972) at the growth temperatures indicated. Sinorhizobium (Rhizobium) meliloti strains were grown on LB/MC medium (Glazebrook & Walker, 1991), Mesorhizobium loti was grown on YEM medium (Vincent, 1970), Rhodobacter sphaeroides was grown on Sistrom’s medium (Sistrom, 1962) and Bradyrhizobium japonicum was grown on PSY complex medium supplemented with 0.1% (w/v) L-arabinose (Regensburger & Hennecke, 1983), all at 29°C. *Legionella pneumophila* was grown on AYE medium at 37°C (Feeley et al., 1979) and *Borrelia burgdorferi* was grown on BSK-H medium (Barbour–Stoenner–Kelley; Sigma) at 34°C.

Antibiotics were added to media at the following concentrations (μg ml⁻¹) when required: gentamicin 150, in the case of *P. aeruginosa*; carbenicillin 100, tetracycline 10, gentamicin 20, kanamycin 50, in the case of *E. coli*.

**Inactivation of the P. aeruginosa pcs gene and a pmtA-homologous ORF.** For inactivation of the pcs gene (PA3857) and of a pmtA homologue (PA0798), both potentially involved in PC
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>recA1, φ80 lacZΔM15</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>DH5α</td>
<td>Expression strain</td>
<td>Studier et al. (1990)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Sm′ Sp′ hsdR RP4-2 kan::Tn7 tet::Mu, integrated in the chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Wild-type, biovar I, ATCC 33970</td>
<td>Hamilton &amp; Fall (1971)</td>
</tr>
<tr>
<td>S17-1</td>
<td>Isolated from a tick</td>
<td>ATCC 35210^T</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector, carbenicillin-resistant</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pET9a</td>
<td>Expression vector, kanamycin-resistant</td>
<td>Studier et al. (1990)</td>
</tr>
<tr>
<td>pLysS</td>
<td>Production of lysozyme for repression of T7 polymerase, chloramphenicol-resistant</td>
<td>Studier et al. (1990)</td>
</tr>
<tr>
<td>pEX18Tc</td>
<td>Gene replacement vector, tetracycline-resistant</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pPS58</td>
<td>aacC1–GFP, ampicillin- and gentamicin-resistant</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pMSB05</td>
<td>Pseudomonal pcs-containing Taq-amplified 1 kb SpHl–EcoRI insert in pEX18Tc</td>
<td>This study</td>
</tr>
<tr>
<td>pMSB07</td>
<td>pcs (PA3857) : : aacC1–GFP in pEX18Tc</td>
<td>This study</td>
</tr>
<tr>
<td>pMSB06</td>
<td>Pseudomonal pmtA homologue containing Taq-amplified 0.8 kb SpHl–EcoRI insert in pEX18Tc</td>
<td>This study</td>
</tr>
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</table>
biosynthesis in *P. aeruginosa*, the respective genes were mutated by insertion of a gentamicin-resistance cassette following the strategy described by Hoang et al. (1998). Using PCR, specific oligonucleotides (CAGTGCATGCAACTGAGGATCTT and GCCGGAATTCGGTTCCTTGCGATGATAG) and Taq polymerase, a 1 kb fragment was amplified from genomic DNA containing the pseudomonal *pcs* gene. Suitable restriction sites (underlined) for cloning the fragment were introduced by PCR with the oligonucleotides. After restriction with *Sph*I and *EcoRI*, the PCR-amplified DNA fragment was cloned into the suicide vector pEX18Tc, to obtain plasmid pMSB05. For inactivation of the *pcs* gene, pMSB05 was cut with *SalI* and the *Sal*-restricted gentamicin-resistance cassette of pPS858 was inserted into the *pcs* gene giving rise to pMSB07. Similarly, using the oligonucleotides CAGTGCATGCAACTGAGGATCTT and GCCGGAATTCGGTTCCTTGCGATGATAG, a 0-8 kb fragment was amplified from genomic DNA containing a pseudomonal gene homologous to *pmtA* from *Rhodobacter sphaeroides*. After restriction with *Sph*I and *EcoRI*, the fragment was cloned into pEX18Tc, to obtain plasmid pMSB06. For inactivation of the *pmtA*-homologous gene, pMSB06 was cut with Xhol and the *Sal*-restricted gentamicin-resistance cassette of pPS858 was inserted into the *pmtA* homologue giving rise to pMSB08. The suicide vectors were transferred into *P. aeruginosa* PAO1 by diparental mating using *E. coli* S17-1 as donor. Gentamicin-resistant *P. aeruginosa* strains were isolated on minimal medium M9 (Miller, 1972) containing succinate (40 mM) and gentamicin and streaked onto LB plates containing 0.5% sucrose and gentamicin to score for loss of the pEX18Tc suicide vector, which contains a *sacB* gene. Gentamicin-resistant colonies able to grow in the presence of sucrose were purified. The absence of the wild-type genes and the presence of the disrupted mutant genes were verified by PCR. The *pcs*-deficient *P. aeruginosa* strain was named PAOPGS and that deficient in the *pmtA* homologue was designated PAOPM.T.

**DNA manipulations.** Recombinant DNA techniques were performed according to standard protocols (Sambrook et al., 1989). DNA was sequenced by the chain termination method (Sanger et al., 1977) using PET9a derivatives. The DNA region sequenced and the deduced proteins were analysed using the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) BLAST network server (Altschul et al., 1997).

**In vivo labelling of bacterial strains with [1-14C]acetate or [methyl-14C]methionine and analysis of lipid extracts.** The lipid compositions of different bacterial strains were determined following labelling with [1-14C]acetate. The incorporation of methyl groups into lipids was evaluated following labelling with [methyl-14C]methionine. Cultures (1 ml) of bacterial strains in the corresponding medium were inoculated from pre-cultures grown in the same medium. After addition of 0.4 μCi [1-14C]acetate (60 mCi mmol⁻¹; 2.22 GBq mmol⁻¹) or 1 μCi [methyl-14C]methionine (55 mCi mmol⁻¹; 2.03 GBq mmol⁻¹) to each culture, the cultures were incubated for either 4 or 16 h. Cells were harvested by centrifugation, washed with 500 μl of water and resuspended in 100 μl of water. The lipids were extracted according to Bligh & Dyer (1959). The chloroform phase was used for lipid analysis on TLC plates and, after one-dimensional separation (de Rudder et al., 1997), visualization of labelled compounds was obtained by autoradiography.

**Preparation of cell-free crude extracts.** Cell-free crude extracts were made from cells obtained from 0-5 l of exponentially growing cultures. After harvesting by low-speed centrifugation at 4 °C, each cell pellet was resuspended in 20 mM Tris/Cl buffer, pH 8.5. The cell suspension was passed twice through a French pressure cell at 20 000 p.s.i. (138 MPa). Unbroken cells and cell debris were removed by centrifugation at 7000 g for 20 min to obtain the cell-free extract. Protein concentrations were determined by the method of Dulley & Grieve (1975).
**Determination of PMT activity.** This was done essentially as described previously (de Rudder et al., 1997).

**Determination of PCS activity.** To detect minor PCS activities, the assay optimized originally for the enzyme from *S. meliloti* (Sohlenkamp et al., 2000) was modified in a way that higher concentrations of both substrates were used. The assay to determine PCS activity contained, in a total volume of 50 μl in Eppendorf tubes, 50 μg protein, 50 mM Tris/HCl, pH 8-0, 10 mM MnCl2, 343 μM CDP-diacylglycerol, 0-2% (v/v) Triton X-100 and 100 μM [methyl-14C]choline (55 mCi mmol-1). In some cases, MnCl2 was replaced by equimolar concentrations of CoCl2. The mixtures were incubated for 15 min in a 30 ºC water bath and stopped by mixing with 188 μl of methanol/chloroform (2:1, v/v). Addition of 63 μl chloroform and 63 μl water led to phase separation; after washing the chloroform phase once with another 100 μl of water, it was dried, re-dissolved in 10 μl of methanol/chloroform (1:1, v/v) and subjected to one-dimensional TLC.

**Cloning and expression of ORFs potentially encoding proteins with Pmt or Pcs activities.** Using specific oligonucleotides (Table 2), suspected pmtA or pcs genes were amplified from genomic DNA with Pfu polymerase and, after restriction with NdeI and BamHI, were cloned into pET9a (Studier, 1990), which expresses the T7 polymerase under the control of the lac promoter. Correct in-frame cloning and the correct sequence were demonstrated by DNA sequencing (data not shown). *E. coli* BL21(DE3)(pLysS) (Studier et al., 1990), which expresses the T7 polymerase under the control of the lac promoter, was transformed with the respective expression plasmids. At a cell density of 5 x 10⁸ cells ml⁻¹, IPTG was added to a final concentration of 0-1 mM. After 4 h induction, cells were harvested, resuspended in 20 mM Tris/HCl, pH 8-0, and stored at −20 ºC. Cells were lysed by thawing and two additional freeze–thaw cycles. Lysates were treated for 20 min with DNase I (Roche) from 200 units ml⁻¹ and subjected to one-dimensional TLC.

**RESULTS**

**PMT activities in cell-free bacterial extracts**

PMT activities were determined in cell-free extracts obtained from different bacteria and the potential products were separated by TLC (Fig. 2). None of the potential products (MMPE, DMPE, PC) usually formed by Pmt was formed from cell-free extracts of *E. coli* (lane 3), *Brucella melitensis* (lane 8) and *Klebsiella pneumoniae* (lane 13) or by *P. aeruginosa* and its two mutant derivatives (lanes 14, 15, 16). In *Burkholderia cepacia* extracts (lane 11), methyl transfer to the lipid fraction and maybe to MMPE does occur; however, labelled DMPE or PC are not formed in this case. Instead, in *Burkholderia cepacia*, major label incorporation had occurred into faster-migrating, unidentified lipids. All three products of PMT activity are clearly formed by *S. meliloti* (lane 4), *A. tumefaciens* (lane 5), *Rhizobium leguminosarum* (lane 6), *Bradyrhizobium japonicum* (lane 7), *M. loti* (lane 9), *Rhodobacter sphaeroides* (lane 10) and *L. pneumophila* (lane 12).

**Methylation of lipids in Burkholderia strains**

Using a standard cell-free assay for Pmt, *Burkholderia cepacia* ATCC 29352 failed to form DMPE and PC (Fig. 2, lane 11 and Fig. 3, lane 6). We therefore investigated whether multiple methylation of PE could occur in vivo by labelling cell suspensions of *S. meliloti* and different *Burkholderia* strains with [methyl-14C]methionine. In *S. meliloti*, four lipid compounds were formed, three that migrated at the positions expected for MMPE, DMPE or

**Table 2. PCR primers used in this study**

The forward primers incorporated an Ndel restriction site (underlined) overlapping the start codon of the respective genes. The reverse primers encoded a BamHI restriction site (underlined) after the stop codon. Names are explained in legends of Figs 7 and 8.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
</tr>
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<tbody>
<tr>
<td>Lpne Pcs forward</td>
<td>TCTAGGATAACATGGAATCCATAAAGCC</td>
</tr>
<tr>
<td>Lpne Pcs reverse</td>
<td>AAATTACGGATCCCTAATTTTATTAT</td>
</tr>
<tr>
<td>Lpne PmtA forward</td>
<td>AGGATAACATATGCTGTCCTTCAGTA</td>
</tr>
<tr>
<td>Lpne PmtA reverse</td>
<td>ATTCAGGGATCTCAATTATTTTACGTG</td>
</tr>
<tr>
<td>Paer Pcs forward</td>
<td>AGGAAATCATATGCATCCCAACAGGAC</td>
</tr>
<tr>
<td>Paer Pcs reverse</td>
<td>AAGATGTCGCTTCGGAACACCGTCG</td>
</tr>
<tr>
<td>Paer PmtA-like ORF long version forward</td>
<td>AGGAATGATCGGTGCCGGCCCG</td>
</tr>
<tr>
<td>Paer PmtA-like ORF short version forward</td>
<td>AGGAAATCATATGCATCAATTTTATTTAG</td>
</tr>
<tr>
<td>Paer PmtA-like ORF reverse</td>
<td>TTATACAGGATCTCAGGCCG</td>
</tr>
<tr>
<td>Bbur Pcs forward</td>
<td>AGGAATGATCGGTGCCGGCCCG</td>
</tr>
<tr>
<td>Bbur Pcs reverse</td>
<td>AAAAGATGTCGCTTCGGAACAGGAA</td>
</tr>
<tr>
<td>Mlot Pcs forward</td>
<td>AGGATAACATATGCGCCGACGGAA</td>
</tr>
<tr>
<td>Mlot Pcs reverse</td>
<td>TAAAGTGGATCCTCAGGCCCTTTTGG</td>
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<tr>
<td>Mlot PmtA-like ORF forward</td>
<td>AGGAAATCATATGCGCCGACGGAA</td>
</tr>
<tr>
<td>Mlot PmtA-like ORF reverse</td>
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<tr>
<td>Mlot PmtA forward</td>
<td>AGGAAATCATATGCGCCGACGGAA</td>
</tr>
<tr>
<td>Mlot PmtA reverse</td>
<td>TTATACAGGATCTCAGGCCG</td>
</tr>
</tbody>
</table>
PC and one that migrated at the position of PE (Fig. 3, lane 1). In addition to the methylation of the ethanolamine head group of PE, many bacteria are able to methylate cis-unsaturated fatty acyl residues of phospholipids, thereby causing the formation of fatty acyl residues containing a cyclopropane ring (Grogan & Cronan, 1997). In this latter reaction, labelled methyl groups can be incorporated into phospholipids, especially into PE and to some extent into PC (de Rudder et al., 1997). However, for formation of MMPE and DMPE, methylation of the ethanolamine head group of PE is required, as this constitutes the only pathway of MMPE and DMPE formation, at least in S. meliloti (de Rudder et al., 1997). Therefore, the formation of radiolabelled MMPE and DMPE is indicative of a functional PMT pathway. In the case of Burkholderia cepacia ATCC 29352, three lipid compounds were formed that migrate at the positions expected for MMPE, DMPE or PC (Fig. 3, lane 2), demonstrating that all three methylations of the PE head group occurred in vivo. Burkholderia cepacia ATCC 29352 therefore possesses a PMT pathway for PC formation; however, using our standard assay, the Pmt enzyme activity could not be clearly detected in cell-free extracts of this organism (Fig. 2, lane 11; Fig. 3, lane 6). In Burkholderia cepacia ATCC 25602, only one compound was formed that migrated like MMPE (Fig. 3, lane 3), whereas in Burkholderia tropicalis and Burkholderia caryophylli four compounds were formed, two of which migrated like MMPE and DMPE (Fig. 3, lanes 4 and 5), while the nature of the two faster-migrating substances is unknown at this point. Burkholderia species therefore seem to vary significantly in their ability to form methylated derivatives of PE. Similar in vivo studies performed with P. aeruginosa PAO1 also failed to detect MMPE, DMPE or PC (data not shown), strongly suggesting that no methylation pathway for PC biosynthesis exists in P. aeruginosa PAO1.

PCS activities in cell-free bacterial extracts

PCS activities were determined in the presence of MnCl₂ in cell-free extracts obtained from different bacteria and the products were identified by TLC (Fig. 4). Cell-free extracts of E. coli (lane 3), Rhodobacter sphaeroides (lane 10), Burkholderia cepacia (lane 11) or K. pneumoniae (lane 13) did not form any PC and therefore were devoid of PCS activity. A reduced amount of PC was formed by Bradyrhizobium japonicum extracts (lane 7). PC was formed by extracts of S. meliloti (lane 4), A. tumefaciens (lane 5), Rhizobium leguminosarum (lane 6), Brucella melitensis (lane 8), M. loti (lane 9), L. pneumophila (lane 12), Borrelia burgdorferi (data not shown) and P. aeruginosa PAO1 (lane 14). The pcs mutant of P. aeruginosa (PAOPCS) was devoid of PCS activity (lane 15), whereas the putative pmtA mutant PAOPMT retained PCS activity (lane 16). The PCS activity was also determined in the presence of CoCl₂ instead of MnCl₂ since Co²⁺ has been reported to fulfill the cation requirement of some CDP-alcohol phosphatidyltransferases (Taniguchi et al., 1986). Under these conditions, the Pcs from L. pneumophila displayed the same level of activity as in the presence of MnCl₂ (data not shown), whereas the Pcs from S. meliloti, A. tumefaciens, Rhizobium leguminosarum or Brucella melitensis had
reduced activity and that from *Bradyrhizobium japonicum, M. loti* or *P. aeruginosa* was not functional (data not shown).

**Identification of bacterial ORFs encoding Pmt or Pcs enzymes**

Based on sequence comparison with known Pmt or Pcs enzymes, ORFs from numerous organisms were suggested to encode Pmt or Pcs activities (López-Lara & Geiger, 2001; Sohlenkamp et al., 2003; López-Lara et al., 2003). To evaluate some of these predictions, selected ORFs were cloned and expressed in *E. coli*, and cell-free extracts were assayed for Pmt or Pcs activity. Expression of PmtA from *S. meliloti* from plasmid pTB2084 (Fig. 5, lane 4) leads to the formation of MMPE, DMPE and PC, as demonstrated previously (de Rudder et al., 2000). Putative homologues were compared with the enzyme activity caused by PmtA from *S. meliloti*. In *M. loti*, two homologues of the sinorhizobial PmtA had been identified, one (mlr4753) with a high similarity and the other (mlr5374) with a lower similarity to the sinorhizobial PmtA. Expression of the ORF with the higher similarity to the sinorhizobial PmtA (mlr4753), present in pFM20, caused the formation of MMPE, DMPE and PC (lane 5); therefore, this ORF encodes a Pmt (Mlot PmtA). Expression of the ORF with the lower similarity to the sinorhizobial PmtA (mlr5374), cloned in pFM18, might cause the formation of MMPE and possibly DMPE (lane 6) and, like the PmtA of *Bradyrhizobium japonicum* (Minder et al., 2001), the expressed ORF mlr5374 seems unable to form PC (lane 6). Expression of an ORF from *L. pneumophila* from pFM14 which encodes a homologue to the rhodobacterial PmtA leads to the formation of MMPE, DMPE and PC (lane 7), demonstrating that this ORF encodes a functional Pmt. In contrast, expression of an ORF (PA0798) from *P. aeruginosa* (in pFM16) which shows homology to the ORF encoding the rhodobacterial PmtA did not cause the formation of any of the methylated derivatives of PE (data not shown). We also tried to express a longer version of PA0798 (from pFM17) using as the starting methionine one located 114 aa upstream of the predicted start. Similarly, also with the longer version expressed, we could not observe the formation of any of the methylated derivatives of PE (data not shown).

Expression of *pcs* from *S. meliloti* from pTB2559 in *E. coli* leads to the formation of PC (Fig. 6, lane 2) in a Pcs enzyme assay as demonstrated previously (Sohlenkamp et al., 2000). Expression of ORFs homologous to the one encoding sinorhizobial Pcs shows that the respective ORF (BAB48080) from *M. loti* harboured in pFM15 (lane 3), an ORF deduced from a fragment of the unfinished genome of *L. pneumophila* present in pFM3 (lane 4), the ORF (PA3857) from *P. aeruginosa* in pTB2906 (lane 5) and an ORF (BB0249) from *Borrelia burgdorferi* in pTB2902 (lane 6) produce PC in the Pcs reaction and therefore encode functional Pcs enzymes.
**DISCUSSION**

PC, a major membrane lipid in eukaryotes, is also encountered in diverse eubacterial groups. Studies with *Sinorhizobium meliloti* demonstrated that PC can be synthesized via two different routes, the PMT (de Rudder et al., 2000) pathway, represented by phospholipid N-methyltransferase (PmtA) enzymes, or the PCS (de Rudder et al., 1999; Sohlenkamp et al., 2000) pathway. In recent years, numerous homologues of PmtA or PCS have been described, but only a few have been evaluated experimentally (Hanada et al., 2001; Minder et al., 2001; Wilderman et al., 2002).

In this work, we have shown that PmtA homologues encode PMT activities responsible for PMT pathways in *L. pneumophila* (Lpne PmtA) or *M. loti* (Mlot PmtA). PMT activities were detected in cell-free extracts of *A. tumefaciens* as well as of *Rhizobium leguminosarum*. Also, a homologue of the sinorhizobial PmtA (Smel PmtA) can be detected in the genome of *A. tumefaciens* (Atum ORF1, 66 % identity on amino acid level) and a homologue of the mesorhizobial PmtA (Mlot PmtA) can be detected in the genome of *Rhizobium leguminosarum* (Rleg ORF1, 66 % identity on amino acid level) (see Fig. 7); therefore, we suggest that Atum ORF1 as well as Rleg ORF1 encode functional Pmt enzymes. In cell-free extracts of *Brucella melitensis* no PMT activity was detected. This was surprising as the *Brucella melitensis* genome contains a homologue (Bmel ORF1, 65 % identity on amino acid level) with high similarity to the sinorhizobial PmtA (Smel PmtA). One possibility is that the expression or activity of Bmel ORF1 is down-regulated under the conditions for *Brucella melitensis* growth. Expression of the PmtA homologue (Paer ORF) of *P. aeruginosa* in *E. coli* did not cause PMT activity nor was such activity detected by *in vitro* or *in vivo* assays of *P. aeruginosa*, suggesting that *P. aeruginosa* has no methylation pathway for PC biosynthesis. A similar conclusion was drawn from recent studies performed by Wilderman et al. (2002). Earlier work on the methylation pathway of PC biosynthesis in *Bradyrhizobium japonicum* genome (Minder et al., 2001) suggested that besides a phospholipid N-methytransferase (PmtA) catalysing efficiently the first methylation, and therefore the formation of MMPE, another phospholipid methyltransferase (PmtX) activity must exist in this organism which efficiently performs the second and third methylation required for PC formation via the methylation pathway. In the *Bradyrhizobium japonicum* genome sequence (Kaneko et al., 2002), two homologues (Bjap ORF1 and Bjap ORF2) of the rhodobacterial PmtA exist that could qualify as candidates for PmtX.

In the work presented here, we have shown that *pcs*-homologous ORFs encode PCS activities and must constitute the enzymes responsible for a PCS pathway in *M. loti* (Mlot Pcs), *P. aeruginosa* (Paer Pcs), *L. pneumophila* (Lpne Pcs) and *Borrelia burgdorferi* (Bbur Pcs). Based on the PCS activities detected in cell-free extracts of *A. tumefaciens* as well as in *Rhizobium leguminosarum* and based on the fact that homologues of the sinorhizobial Pcs (Smel Pcs) can be detected in the genomes of *A. tumefaciens* (Atum ORF2, 80 % identity on amino acid level) and *Rhizobium leguminosarum* (Rleg ORF2, 78 % identity on amino acid level) (see Fig. 8), we suggest that Atum ORF2 as well as Rleg ORF2 encode functional Pcs enzymes. Although we were unable to detect PCS activity in cell-free extracts of *Bradyrhizobium japonicum* with our previous enzyme assay (Minder et al., 2001), with the enzyme assay used in the present study, which employs higher substrate concentrations, we were able to detect a minor PCS activity and based on the fact that a homologue (44 % identity on amino acid level) of the sinorhizobial Pcs (Smel Pcs) can be detected in the genome of *Bradyrhizobium japonicum* (Bjap ORF3) (see Fig. 8), we suggest that Bjap ORF3 encodes a functional Pcs. The PCS activity detected in cell-free extracts of *Brucella melitensis* and the fact that a homologue (68 % identity on amino acid level) of the mesorhizobial Pcs (Mlot Pcs) can be detected in the genome of *Brucella melitensis* (Rleg ORF2) (see Fig. 8) suggest that Bmel ORF2 encodes a functional Pcs. *Pseudomonas fluorescens* and *Pseudomonas syringae* both possess homologues to Pcs from *P. aeruginosa* (Pflu ORF 54 % identity and Psyr ORF 56 % identity on amino acid level, respectively) suggesting that these two
pseudomonads also possess a PCS pathway. The fact that no PCS activity could be detected in cell-free extracts of *Rhodobacter sphaeroides* is in agreement with earlier studies (Arondel *et al.*, 1993). Surprisingly, a Pcs homologue (Rsph ORF) exists in *Rhodobacter sphaeroides* and more studies will be required in order to establish whether Rsph ORF encodes a Pcs activity and why *Rhodobacter sphaeroides* seems devoid of a functional PCS pathway. So far, all known PCSs form a group clearly distinct from other CDP-alcohol phosphatidyltransferases (Sohlenkamp *et al.*, 2003), such as, for example, the phosphatidylserine synthases (Pss) (Fig. 7) (Sohlenkamp *et al.*, 2000, 2003) is encountered in all enzymes showing that activity and therefore this motif is highly predictive for PCSs.

In a recent estimate, we proposed that more than 10% of the eubacteria contain PC as a membrane lipid (Sohlenkamp *et al.*, 2003). Based on the studies presented here, we propose that *S. meliloti*, *A. tumefaciens*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, *M. loti* and *L. pneumophila* possess both bacterial pathways for PC biosynthesis, i.e. the PMT and PCS pathways. Therefore, if bacteria possess PC, they are usually able to form it via both pathways. Some bacteria such as *Zymomonas mobilis* (Tahara *et al.*, 1994), *Rhodobacter sphaeroides* (Arondel *et al.*, 1993) or some *Burkholderia* strains might possess only the PMT pathway for PC formation. Notably, some important pathogens (*Brucella melitensis*, *P. aeruginosa* and *Borrelia burgdorferi*) seem to possess only the PCS pathway for PC formation and therefore presumably depend on choline supplies from their hosts for the ability to synthesize PC. *P. aeruginosa* is an opportunistic pathogen and poses a serious threat to cystic fibrosis patients. *Brucella melitensis*, frequently found in dairy products, can cause brucellosis, whereas the tick-transmitted spirochaete *Borrelia burgdorferi* is the causative agent of Lyme disease. After initial infection, all three aforementioned pathogens tend to persist in their hosts and when established as persistent infections are difficult to eliminate from the host with presently available treatments.

**Fig. 7.** Unrooted phylogenetic tree of Pmt enzymes and Pmt-like ORFs. The tree was constructed using the program CLUSTAL W at [http://www.expasy.ch/](http://www.expasy.ch/) (Thompson *et al.*, 1994). Distances between sequences are expressed as 0-1 changes per amino acid residue. Sequences used for the tree were *Sinorhizobium meliloti* PmtA (Smel PmtA; GenBank accession no. AF201699), PmtA from *M. loti* (Mlot PmtA; mll4753), PmtA from *Bradyrhizobium japonicum* (Bjap PmtA; Y09633), PmtA from *Rhodobacter sphaeroides* (Rsph PmtA; L07247), PmtA from *Acetobacter acetii* (Aace PmtA; AB019196), PmtA from *Legionella pneumophila* (Lpne PmtA; ORF deduced from fragment of unfinished genome), an ORF deduced from the genome of *Agrobacterium tumefaciens* (Atum ORF1; accession no. AE009001), an ORF from the genome of *Rhizobium leguminosarum* (Rleg ORF1; rhiz775c03.q1k), an ORF from the genome of *Brucella melitensis* (Bmel ORF1; AE009632), two ORFs deduced from the genome of *Bradyrhizobium japonicum* (Bjap ORF1 and Bjap ORF2; accession nos AP005960 and AP005959, respectively), an ORF deduced from the genome of *M. loti* (Mlot ORF; mlt5374), an ORF deduced from the genome of *Yersinia pestis* (Ypes ORF; AJ414156), an ORF deduced from the genome of *P. aeruginosa* (Paer ORF; AE004515), a sinorhizobial ORF (Smel ORF; AE007209), the 16S rRNA methyltransferase KgsA from *E. coli* (Eco KgsA; M11054), an ORF from the genome of *Pyrococcus furiosus* (Pfur ORF; AE007209), and an ORF from contig 2543 of the unfinished genome of *Silicibacter pomeroyi* (Spom ORF1).
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**Fig. 8.** Unrooted phylogenetic tree of phosphatidylserine synthases (Pss), Pcs enzymes and Pcs-like ORFs. The tree was constructed using the program CLUSTAL W at http://www.expasy.ch/ (Thompson et al., 1994). Distances between sequences are expressed as 0–1 changes per amino acid residue. Sequences used for the tree are Pss from *Agrobacterium tumefaciens* (Atum Pss; GenBank accession no. AF410774), Pss from *Bacillus subtilis* (Bsub Pss; D38022), Pss from *Helicobacter pylori* (Hpyl Pss; AAC45587), Pcs from *Sinorhizobium meliloti* (Smel Pcs; AAF27310), Pcs from *P. aeruginosa* (Paer Pcs; ORF PA3857), Pcs from *M. loti* (Mlot Pcs; BAB48080), Pcs from *Legionella pneumophila* (Lpne Pcs; ORF deduced from fragment of unfinished genome), Pcs from *Borrelia burgdorferi* (Bbur Pcs; BB0249), the sequences of an ORF from the genome of *A. tumefaciens* (Atum ORF2; AAK87563), an ORF from contig rhiz659e06.q1n of the unfinished genome of *Rhizobium leguminosarum* bv. viciae (Rleg ORF2), an ORF from the genome of *Brucella melitensis* (Bmel ORF2; AAL53937), an ORF from the genome of *Rhodobacter sphaeroides* (Rsph ORF; contig 110, gene 289), an ORF from the unfinished genome of *Rhodobacter capsulatus* (Rcap ORF; RRC00355), an ORF from contig 68 of the unfinished genome of *Silicibacter pomeroyi* (Spom ORF2), an ORF from the genome of *Rhodopseudomonas palustris* (Rpal ORF; contig 58, gene 1464), an ORF from the genome of *Bradyrhizobium japonicum* (Bjap ORF3; AP005951), an ORF from the genome of *P. fluorescens* (Pflu ORF; contig 309, gene 76) and an ORF from the genome of *P. syringae* (Psyr ORF; contig 5668).
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