RESEARCH LETTER

Phosphatidylcholine levels of peanut-nodulating *Bradyrhizobium* sp. SEMIA 6144 affect cell size and motility

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Abstract

Phosphatidylcholine, the major phospholipid in eukaryotes, is found in rhizobia and in many other bacteria interacting with eukaryotic hosts. Phosphatidylcholine has been shown to be required for a successful interaction of *Bradyrhizobium japonicum* USDA 110 with soybean roots. Our aim was to study the role of bacterial phosphatidylcholine in the *Bradyrhizobium*–peanut (*Arachis hypogaea*) symbiosis. Phospholipid *N*-methyltransferase (Pmt) and minor phosphatidylcholine synthase (Pcs) activities were detected in crude extracts of the peanut-nodulating strain *Bradyrhizobium* sp. SEMIA 6144. Our results suggest that phosphatidylcholine formation in *Bradyrhizobium* sp. SEMIA 6144 is mainly due to the phospholipid methylation pathway. Southern blot analysis using *pmt*- and *pcs*-probes of *B. japonicum* USDA 110 revealed a *pcs* and multiple *pmt* homologues in *Bradyrhizobium* sp. SEMIA 6144. A *pmtA* knockout mutant was constructed in *Bradyrhizobium* sp. SEMIA 6144 that showed a 50% decrease in the phosphatidylcholine content in comparison with the wild-type strain. The mutant was severely affected in motility and cell size, but formed wild-type-like nodules on its host plant. However, in coinoculation experiments, the *pmtA*-deficient mutant was less competitive than the wild type, suggesting that wild-type levels of phosphatidylcholine are required for full competitivity of *Bradyrhizobium* in symbiosis with peanut plants.

Introduction

Peanut (*Arachis hypogaea* L.) is an agriculturally valuable plant originally coming from South America and later disseminated to the rest of the world. China leads in the production of peanuts, having a share of about 37.5% of the overall world production, followed by India (19%) and Nigeria (11%). The United States of America, Argentina, Brazil, Mexico and Nicaragua are the major producers in the Americas (FAOSTAT, 2009). In symbiotic association with *Bradyrhizobium* sp. (Urtz & Elkan, 1996), peanut plants can fix atmospheric nitrogen, reducing the need for expensive and environmentally damaging nitrogen fertilizers. During symbiosis, rhizobia are hosted intracellularly and a molecular dialogue between the two partners is required to coordinate the events leading to the symbiosis and to avoid host defence responses (Kistner & Parniske, 2002). The relevance of rhizobial cell surface components in the symbiotic interaction has been described in several studies (Perret *et al.*, 2000; Fraysse *et al.*, 2003). However, few studies have focused on the importance of membrane lipids of rhizobia (Minder *et al.*, 2001; López-Lara *et al.*, 2005; Vences-Guzmán *et al.*, 2008).

There is general agreement that phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and cardiolipin are the major phospholipids in rhizobia (Wilkinson, 1988). While phosphatidylethanolamine, phosphatidylglycerol and cardiolipin are common phospholipids in many bacteria, phosphatidylcholine is restricted to a limited number of genera, and seems to be more common in those that establish close interactions with eukaryotes (Sohlenkamp *et al.*, 2003). It was speculated that phosphatidylcholine might serve some special function during host–pathogen/symbiont interactions. The importance of

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phosphatidylcholine for the establishment of successful interactions with eukaryotic hosts is exemplified by the fact that *Agrobacterium tumefaciens* mutants lacking phosphatidylcholine are unable to form tumours in susceptible plants (Wessel *et al.*, 2006), that the human pathogen *Brucella abortus* requires phosphatidylcholine for full virulence (Comerci *et al.*, 2006) and that phosphatidylcholine synthesis is required for optimal function of virulence determinants in *Legionella pneumophila* (Conover *et al.*, 2008). In *Sinorhizobium meliloti*, which can form nitrogen-fixing nodules on its host plant alfalfa, phosphatidylcholine can be synthesized by two entirely different biosynthetic pathways. In the methylation pathway, the enzyme phospholipid *N*-methyltransferase (PmtA) forms phosphatidylcholine by three successive methylations of phosphatidylethanolamine (de Rudder *et al.*, 2000). The second pathway is dependent on the supply of choline and consists of the direct condensation of choline and CDP-diacylglycerol in a reaction catalysed by phosphatidylcholine synthase (Pcs) (Sohlenkamp *et al.*, 2000). *Sinorhizobium meliloti* mutants deficient in either pathway show wild-type-like phosphatidylcholine levels when grown on complex medium while a mutant defective in both pathways does not form phosphatidylcholine and shows a severe reduction of the growth rate with respect to the wild-type (de Rudder *et al.*, 2000). Furthermore, the *S. meliloti* mutant lacking phosphatidylcholine is unable to form nodules on alfalfa (Sohlenkamp *et al.*, 2003). In contrast to *S. meliloti*, in a *pmtA*-deficient *Bradyrhizobium japonicum* mutant, the phosphatidylcholine content is reduced from 52% to 6%. This reduction in the phosphatidylcholine content did not prevent nodule formation, but drastically reduced nodule occupancy and nitrogen-fixation ability (Minder *et al.*, 2001). Recently, Hacker *et al.* (2008) have reported the presence of multiple functional phospholipid *N*-methyltransferases (Pmts) exhibiting different substrate specificities in *B. japonicum* and proposed a model in which phosphatidylcholine biosynthesis is achieved mainly by the concerted action of PmtA and PmtX1. Although it has been reported that *B. japonicum* is unable to take up choline (Boncompagni *et al.*, 1999), its genome contains a functional Pcs (Hacker *et al.*, 2008) and some Pcs activity can be detected in cell extracts of *B. japonicum* (Martínez-Morales *et al.*, 2003).

Little is known about the participation of phosphatidylcholine in the physiological response of rhizobia-nodulating peanut roots. This feature is especially interesting because the infection process in peanut is different from other legumes because the rhizobia spread intercellularly by cortical cells at the middle lamellae (crack entry mechanism) and structures resembling infection threads have never been observed (Boogerd & van Rossum, 1997).

Bradyrhizobium sp. SEMIA 6144 (called SEMIA 6144 in the rest of the article) is a slow-growing strain recommended for inoculating peanut roots (Gomes-Germano *et al.*, 2006). In this strain, 44% of the total phospholipids corresponded to phosphatidylcholine, followed by phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, and it was suggested that phosphatidylcholine may be involved in the bacterial response to environmental conditions (Medeot *et al.*, 2007). The present work was designed to identify the genes involved in phosphatidylcholine biosynthesis as well as to isolate and mutate the homologous *pmtA* gene in SEMIA 6144 in order to elucidate the role of phosphatidylcholine in free-living bacteria and during symbiosis with peanut plants.

Materials and methods

Bacteria and growth conditions

Table 1 lists the bacterial strains and plasmids used in this work. *Escherichia coli* was grown on Luria–Bertani medium (Miller, 1972) at 37 °C. *Sinorhizobium meliloti* 1021 was grown on tryptone yeast medium (Beringer, 1974). *Bradyrhizobium japonicum* USDA 110spc4 and SEMIA 6144 were grown on B^{-} medium (van Brussel *et al.*, 1977) or on yeast extract mannitol (YEM) medium (Somasegaran & Hoben, 1994), all at 28 °C. Antibiotics were added at the following concentrations ($\mu g\,mL^{-1}$) when required: carbenicillin 100, tetracycline 10, spectinomycin 200, kanamycin 50, gentamicin 10 for *E. coli*, and nalidixic acid 40, spectinomycin 100 and gentamicin 40 for SEMIA 6144.

Plasmids pBBR1MCS-5, pK18*mobsacB* and their derivatives were mobilized from *E. coli* S17-1 into the receptor strain SEMIA 6144 (Simon *et al.*, 1983).

Determination of Pmt and Pcs activities

To obtain cell-free protein crude extracts, cells were ruptured using a French press as described previously (Martínez-Morales *et al.*, 2003). Pmt activity was determined according to de Rudder *et al.* (1997). To detect minor Pcs activities, the assay developed originally for *S. meliloti* (Sohlenkamp *et al.*, 2000) was performed according to the modifications described by Martínez-Morales *et al.* (2003).

DNA manipulations

DNA manipulations were performed according to standard procedures (Sambrook & Russell, 2001). DNA and derived protein sequences were analysed using the NCBI BLAST network server (Altschul *et al.*, 1997). Probes for *pmt* or *pcs* genes were obtained from plasmids indicated in Table 1. DNA probes were labelled with the DIG-DNA labelling kit (Roche Molecular Biochemicals, Germany).

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strain		
DH5α	recA1, Φw80 lacZΔM15	Hanahan (1983)
S17-1	RP4-2 (Tc::Mu) (Km::Tn7) integrated in the chromosome	Simon *et al*. (1983)
Rhizobial strain		
B. japonicum USDA 110spc4	Sp^R wild type	Regensburger & Hennecke (1983)
Bradyrhizobium sp. SEMIA 6144	Wild type	FEPAGRO-MIRCEN, Brazil
Bradyrhizobium sp. DBM13	SEMIA 6144 derivative; Sp^R *pmtA*::Sp integrated in the chromosome	This study
Sinorhizobium meliloti 1021	Sm^R of wild-type SU47	Meade *et al*. (1982)
Plasmid		
pUC18	Cloning vector, Cb^R	Yanisch-Perron *et al*. (1985)
pBluescript II SK (+)	Cloning vector, Cb^R *lacZ*	Stratagene
pK18*mobsacB*	Suicide vector, Km^R	Schäfer *et al*. (1994)
pBBR1MCS-5	Broad-host-range vector, Gm^R	Kovach *et al*. (1995)
pHY109	Broad-host-range vector containing the Sp^R cassette	Østeras *et al*. (1998)
pTB2559	pcs_{Sm} in pET9a	Sohlenkamp *et al*. (2000)
pTB2084	$pmtA_{Sm}$ in pET3a	de Rudder *et al*. (2000)
pTB2117	$pmtA_{Bj}$ in pET3a	Minder *et al*. (2001)
pCCS24	pcs_{Bj} in pET9a	Hacker *et al*. (2008)
pCCS20	$pmtX1_{Bj}$ in pET9a	Hacker *et al*. (2008)
pCCS36	$pmtX2_{Bj}$ in pET9a	Hacker *et al*. (2008)
pCCS37	$pmtX3_{Bj}$ in pET9a	Hacker *et al*. (2008)
pDBM01	2.5-kb fragment containing *pmtA* of SEMIA 6144 as HindIII insert in pUC18, Cb^R	This study
pDBM07	2.5-kb fragment containing *pmtA* of SEMIA 6144 as HindIII insert in pBBR1MCS-5, Gm^R	This study
pDBM11	2-kb XhoI–EcoRV fragment containing *pmtA* of SEMIA 6144 as the XhoI/SmaI insert in pBluescript II SK (+), Cb^R	This study
pDBM12	4-kb fragment containing *pmtA* of SEMIA 6144 disrupted by an Sp cassette in pDBM11, Cb^R, Sp^R	This study
pDBM14	4-kb fragment containing *pmtA* of SEMIA 6144 disrupted by an Sp cassette in pK18*mobsacB*, Km^R, Sp^R	This study

Sm^R, Km^R, Cb^R, Gm^R, Sp^R, Tc^R: streptomycin, kanamycin, carbenicillin, gentamicin, spectinomycin and tetracycline resistance, respectively. *Sm* and *Bj* denote genes from *Sinorhizobium meliloti* 1021 and *Bradyrhizobium japonicum* USDA 110, respectively.

Chemiluminescent detection of the DIG label was performed using CSPD (Roche Molecular Biochemicals).

Construction of a *Bradyrhizobium* sp. SEMIA 6144 *pmtA*-deficient mutant

To obtain a DNA fragment containing the *pmtA* gene, a size-selected genomic library of SEMIA 6144 was constructed. SEMIA 6144 genomic DNA was digested with HindIII, and DNA fragments with sizes of around 2.5 kb were cloned into pUC18. Clones carrying the gene of interest were selected by colony hybridization using *pmtA* of *B. japonicum* USDA 110 (Minder *et al*., 2001) as a probe. A positive clone (pDBM01) was selected and its insert was sequenced. After XhoI digestion of pDBM01, a partial EcoRV digestion was performed and the 2-kb XhoI–EcoRV fragment was isolated from an agarose gel and ligated into XhoI/SmaI-digested pBluescript II SK (+), resulting in plasmid pDBM11. The internal EcoRV site present in *pmtA* was used for mutagenesis. A spectinomycin-resistance cassette obtained as a SmaI fragment from pHY109 was inserted into EcoRV-digested pDBM11, resulting in pDBM12. Finally, the 4-kb XhoI–XbaI fragment from pDBM12 was ligated into SalI/XbaI-digested pK18*mobsacB*, resulting in plasmid pDBM14. The pDBM14 construct contains the interrupted *pmtA* gene flanked on both sides by 1 kb of DNA from SEMIA 6144. Plasmid pDBM14 was introduced by biparental mating into SEMIA 6144. After mating for 2 days at 28 °C, the bacterial mix was plated on YEM medium with nalidixic acid, spectinomycin and kanamycin to select against *E. coli* donor cells and for SEMIA 6144 recipient cells harbouring the suicide plasmid integrated into its chromosome. Resistant SEMIA 6144 colonies were grown in liquid YEM medium for 24 h before being streaked out on YEM medium containing spectinomycin and 10% w/v saccharose to select for the loss of the vector backbone. Double-crossover events were

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confirmed by PCR and Southern blot. The *Bradyrhizobium* sp. SEMIA 6144 *pmtA*-deficient mutant was called DBM13.

To complement the mutant, the HindIII fragment of pDBM01 was cloned into the broad-host-range vector pBBR1MCS-5 that had been digested with HindIII, resulting in pDBM07.

In vivo labelling of *Bradyrhizobium* sp. strains with [^{14}C]acetate

The lipid compositions of SEMIA 6144 wild type, DBM13, DBM13 complemented with pDBM07 and DBM13 harbouring the vector pBBR1MCS-5 were determined after labelling with 37 kBq mL^{-1} [1-^{14}C]acetate sodium salt (New England Nuclear, 2.26 GBq mmol^{-1}) for 72 h. Lipids were extracted according to Bligh and Dyer (1959). The chloroform phase was used for lipid analysis on thin layer chromatography (TLC) plates and the individual lipids were quantified as described previously (Medeot *et al.*, 2007).

Bacterial cell size determination

Bacterial cultures in YEM medium were grown for 72 h until the mid-exponential phase was reached. Cells were observed with a Zeiss microscope (Axiophot Carl Zeiss) equipped with a Canon PC1089 Powershot G6 7.1-megapixel digital camera (Canon Inc., Japan). Photographs were processed and sizes were determined using software AXIOVISION 4.1 (Carl Zeiss).

Motility assays

The protocols were adapted from those of Dèziel *et al.* (2001). Swim plates (YEM medium with 0.3% agar) were point-inoculated with a toothpick and incubated for 48 h at 28 °C. Swimming was assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

Plant infection test

Seeds of *A. hypogaea* L. cv. Blanco Manfredi M68, obtained from INTA Manfredi (Córdoba, Argentina), were surface-sterilized, grown in sand and inoculated according to Dardanelli *et al.* (2009). Uninoculated plants did not develop nodules.

For the competition assay, surface-sterilized seedlings were coinoculated with parental strain SEMIA 6144 and DBM13 in a 1 : 1 ratio. Bacteria were reisolated from surface-sterilized nodules and identified based on the spectinomycin resistance marker. Controls included uninoculated peanut plants and plants inoculated with each individual strain.

Nucleotide sequence accession number

The nucleotide sequence of the *pmtA* gene from SEMIA 6144 has been deposited in the GenBank database under accession number FJ820331.

Results and discussion

Phosphatidylcholine metabolism in the peanut-nodulating strain *Bradyrhizobium* sp. SEMIA 6144

Pmt and Pcs activities were determined *in vitro* using cell-free protein crude extracts and radiolabelled substrates. A major Pmt activity was found in SEMIA 6144 (Fig. 1a), while only a minor Pcs activity was detected (Fig. 1b). This is similar to the situation found for cell extracts of *B. japonicum* USDA 110 (Martínez-Morales *et al.*, 2003). Although minor Pcs activity was detected, it was found that SEMIA 6144 is incapable of incorporating [^{14}C]choline from the medium (data not shown). This is consistent with a previous study that had shown that all the rhizobial strains tested, except *B. japonicum*, possess a choline uptake activity and can use choline as a carbon, nitrogen and energy source for growth (Boncompagni *et al.*, 1999).

Identification of genes for phosphatidylcholine biosynthesis

We have used the two *S. meliloti* genes involved in phosphatidylcholine biosynthesis (*pmtA* and *pcs*) and the *B. japonicum* phosphatidylcholine biosynthesis genes *pmtA*, *pmtX1*, *pmtX2*, *pmtX3* and *pcs* as probes against SEMIA 6144 genomic DNA. Hybridizations performed under low-stringency conditions (5 × SSC, 58 °C) showed that only *pcs*, *pmtA*, *pmtX1* and *pmtX2* probes from *B. japonicum* hybridized with SEMIA 6144 genomic DNA, while no hybridization was observed when *S. meliloti* probes were used (data not shown).

This is in agreement with the genetic and physiological similarities between *B. japonicum* USDA 110 and SEMIA 6144 (Gomes-Germano *et al.*, 2006).

Isolation and cloning of the *Bradyrhizobium* sp. SEMIA 6144 *pmtA* homologue

All previous data indicate that phosphatidylcholine biosynthesis in strain SEMIA 6144 resembles that described for *B. japonicum* where *pmtA* is a critical gene for phosphatidylcholine biosynthesis (Minder *et al.*, 2001; Hacker *et al.*, 2008). Therefore, we proceeded to clone the *pmtA* gene from SEMIA 6144 and to create a *pmtA*-deficient mutant. Two $pmtA_{Bj}$-hybridizing bands were observed in the EcoRV digestion of SEMIA 6144 genomic DNA (data not shown), indicating the presence of an internal EcoRV restriction site that was later used for interruption of the *pmtA* gene. The

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2.5-kb HindIII fragment hybridizing with $pmtA_{Bj}$ (data not shown) was cloned into pUC18, resulting in plasmid pDBM01. The DNA sequence of SEMIA 6144 *pmtA* showed high identity (92%) with the *pmtA* sequence of *B. japonicum* USDA 110 (Y09633). The SEMIA 6144 *pmtA* gene is located downstream of the heat shock-controlled *dnaKJ* chaperone operon (data not shown), which is the same gene organization as in *B. japonicum* (Minder *et al*., 2001). Comparison of the predicted amino acid sequence of SEMIA 6144 PmtA with other rhizobial PmtA sequences (data not shown) revealed the presence of the motif VVEXGXGXG, which is the same consensus motif found in PmtA of *B. japonicum* for the *S*-adenosylmethionine (SAM)-binding site present in SAM-dependent methyltransferases (Minder *et al*., 2001; Sohlenkamp *et al*., 2003).

The *Bradyrhizobium* sp. SEMIA 6144 *pmtA*-deficient mutant forms less phosphatidylcholine than the wild-type strain

To understand the biological role of phosphatidylcholine in the peanut-nodulating strain SEMIA 6144, a *pmtA*-deficient mutant (DBM13) was constructed as described in Materials and methods. Table 2 shows that the wild-type strain possesses phosphatidylcholine ($47.6 \pm 3.9\%$ of total phospholipids) and phosphatidylethanolamine ($27.5 \pm 6.5\%$) as major phospholipids. In contrast, DBM13 showed a marked decrease of phosphatidylcholine and a concomitant increase of phosphatidylethanolamine ($24.8 \pm 3.8\%$ and $57.6 \pm 5.2\%$, respectively), indicating that *pmtA* plays a major role in phosphatidylcholine biosynthesis in SEMIA 6144. Probably, the significant amounts of phosphatidylcholine still remaining in DBM13 are due to activities encoded by other functional *pmt* genes. In a similar way, the biosynthesis of phosphatidylcholine in *B. japonicum* USDA 110 is achieved through the action of different Pmt activities (Hacker *et al*., 2008).

The reduction in phosphatidylcholine and the increase in phosphatidylethanolamine in the mutant DBM13 were accompanied by a decrease in the cardiolipin level (Table 2). A slight reduction in cardiolipin had also been observed in the *pmtA* mutant of *B. japonicum* (Minder *et al*., 2001). When DBM13 was complemented with pDBM07, carrying the wild-type *pmtA* gene, the phospholipid levels were restored to those of the wild type, while phospholipid levels in DBM13 containing the empty vector pBBR1MCS-5 were similar to those of *pmtA*-deficient cells (Table 2).

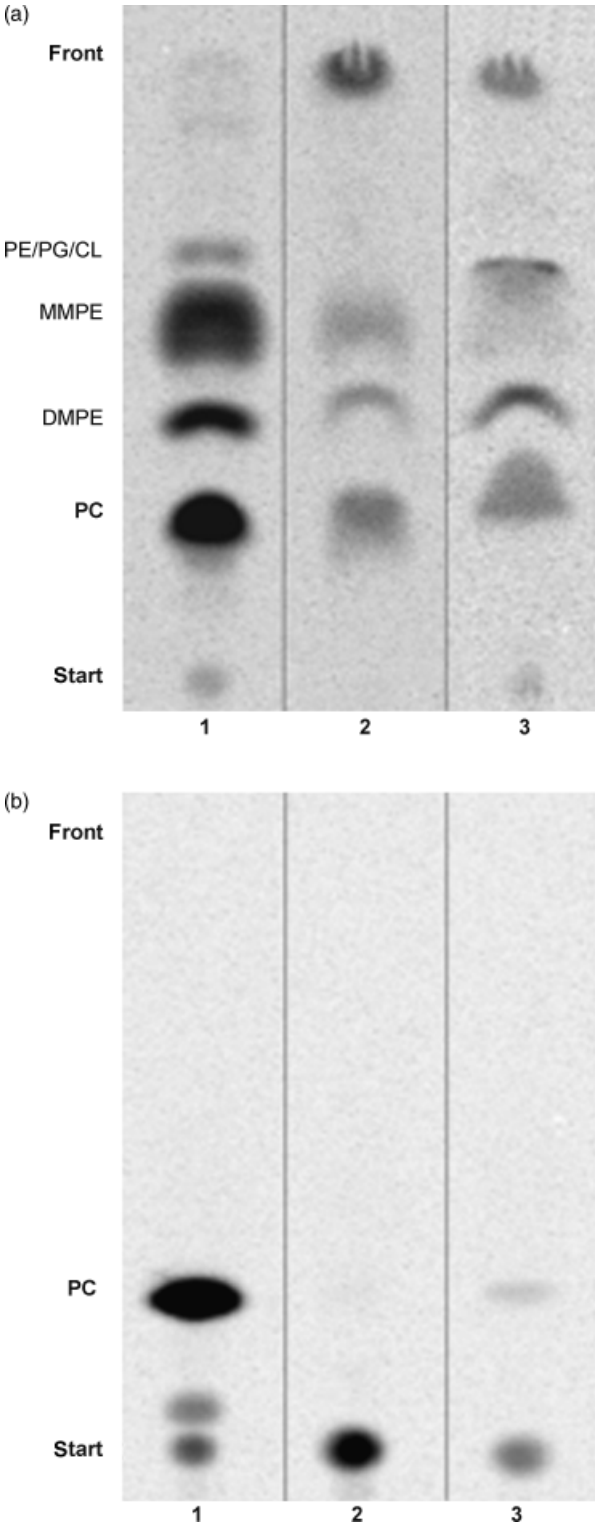

Fig. 1. Determination of Pmt and Pcs activities. The Pmt activity (a) was performed according to de Rudder *et al*. (1997) using *S*-adenosyl-L-[methyl-^{14}C]methionine (SAM) as a radiolabelled substrate. The Pcs activity (b) was determined according to Martínez-Morales *et al*. (2003) using the substrates [methyl-^{14}C]choline and CDP-diacylglycerol. Bacterial lipids formed *in vitro* were extracted according to Bligh & Dyer (1959) and analysed by TLC. Each lane in the figure is derived from different TLCs developed in parallel. (1) *Sinorhizobium meliloti* 1021, (2) *Bradyrhizobium japonicum* USDA 110spc4, (3) *Bradyrhizobium* sp. SEMIA 6144. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; MMPE, monomethylphosphatidylethanolamine; DMPE, dimethylphosphatidylethanolamine; PC, phosphatidylcholine.

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Table 2. Membrane lipid composition of *Bradyrhizobium* sp. SEMIA 6144 wild type, *pmtA*-deficient mutant (DBM13), DBM13 complemented with plasmid pDBM07 and DBM13 harbouring the vector pBBR1MCS-5

Lipid labelled (% of total $^{14}C \pm SD$)				
	SEMIA 6144 wild type	*pmtA*-deficient mutant	*pmtA*-deficient mutant × pDBM07	*pmtA*-deficient mutant × pBBR1 MCS-5
PA	2.4 ± 1.0	1.3 ± 0.4	3.3 ± 1.3	2.0 ± 0.6
PC	47.6 ± 3.9	24.8 ± 3.8	56.6 ± 2.5	25.0 ± 3.9
PG	7.0 ± 2.3	7.2 ± 1.1	6.4 ± 0.1	7.2 ± 2.2
PE	27.5 ± 6.5	57.6 ± 5.2	20.1 ± 1.4	56.4 ± 8.0
CL	13.4 ± 3.3	5.3 ± 0.7	9.6 ± 2.2	5.8 ± 0.7
UL	4.1 ± 0.7	3.0 ± 0.6	3.9 ± 0.5	3.6 ± 0.5

Bradyrhizobium sp. SEMIA 6144 strains were grown in B^- medium. [1-^{14}C]acetate, sodium salt (37 kBq mL^{-1}) was added. Aliquots of the total lipid extracts were analysed by TLC and the fractions were quantified by radioactivity measured in a liquid scintillation counter (Beckman LS 60001 C). Values represent means ± SD of three independent experiments. PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; UL, unidentified lipid(s) migrating with the solvent front.

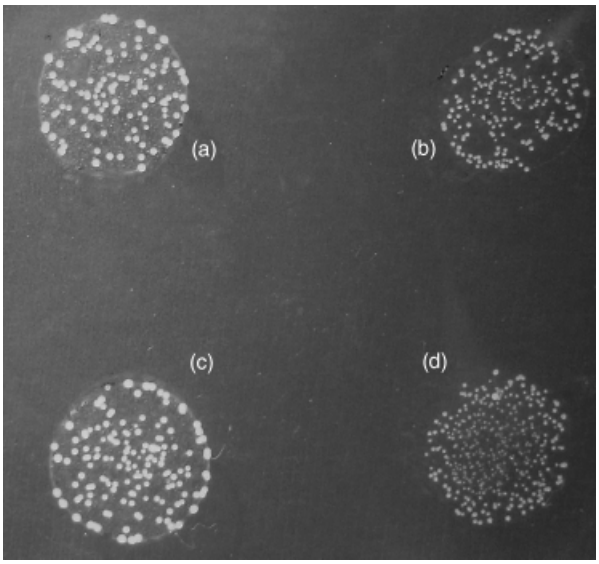

Fig. 2. Size of *Bradyrhizobium* sp. SEMIA 6144 wild-type and DBM13 colonies. The strains were grown on YEM medium for 72 h. (a) SEMIA 6144 wild type, (b) DBM13, (c) DBM13 × pDBM07 and (d) DBM13 × pBBR1MCS-5.

Bradyrhizobium sp. SEMIA 6144 *pmtA*-deficient cells are affected in cell and colony size

In order to characterize the phenotype of SEMIA 6144 *pmtA*-deficient mutant, their growth behaviour was monitored under aerobic growth conditions in rich YEM medium (Somasegaran and Hoben, 1994) and in B^- minimal medium (van Brussel *et al.*, 1977). Although the viability of the parental and its isogenic mutant strain determined as CFU mL^{-1} was similar in all culture media tested (data not shown), we found that the $OD_{620\,nm}$ of the DBM13 cultures was always lower than that in its parental strain. Furthermore, we noticed that wild-type colonies were larger than colonies of the mutant strain (Fig. 2). Determination of cell size under the light microscope showed that wild-type cells were longer than DBM13 cells (Table 3). Both phenotypes, colony and cell size were recovered when plasmid pDBM07 was introduced into DBM13. The recovery in cell and colony size of the complemented mutant correlates with the recovery of its phosphatidylcholine levels (Table 2).

The formation of cardiolipin domains at the cell pole and the division site plays an important role in selection and recognition of the division site by cell cycle and cell division proteins in *E. coli* (Mileykovskaya *et al.*, 2009). Because the level of cardiolipin was reduced to more than half in DBM13 with respect to wild-type cells (Table 2), it is possible that the decrease in cell size is due to the reduction of cardiolipin. Bernal *et al.* (2007) found a similar decrease in the cell length in a cardiolipin synthase-deficient mutant of *Pseudomonas putida* and concluded that changing the amount of anionic phospholipids led to cell division before the cell had reached the average size of wild-type cells.

Table 3. Average size of *Bradyrhizobium* sp. SEMIA 6144 wild type and DBM13 cells

Strain	Size (μm)
SEMIA 6144 wild type	2.31 ± 0.38
SEMIA 6144 *pmtA*-deficient mutant (DBM13)	1.86 ± 0.25
DBM13 × pDBM07	2.25 ± 0.37
DBM13 × pBBR1MCS-5	1.48 ± 0.16

Cells were examined under the Axiophot (Carl Zeiss) light microscope. The images were acquired with a Canon PC1089 Powershot G6 7.1-megapixel digital camera (Canon Inc., Japan) and processed with the AXIOVISION 4.1 software (Carl Zeiss).

A *Bradyrhizobium* sp. SEMIA 6144 *pmtA* mutant is deficient in flagellar-mediated motility

Comparing the motility of the wild type and DBM13 on soft plates (0.3% agar), the zones of swimming of the mutant were smaller than that of the wild type (Fig. 3). Complementation experiments confirmed the correlation between defective motility and the mutation in the *pmtA* gene (Fig. 3c). The reduced diameter of *pmtA*-deficient mutant colonies suggests that they were impaired in motility and/or chemotaxis. Shi *et al.* (1993) showed a possible relation between zwitterionic membrane phospholipids and motility by observing that the *E. coli* flagellar master operon was repressed by the loss of phosphatidylethanolamine in the *pssA* null and *psd-2* mutants. The defects in motility observed in our work are in agreement with data reported by Conover *et al.* (2008) and by Klüsener *et al.* (2009) in

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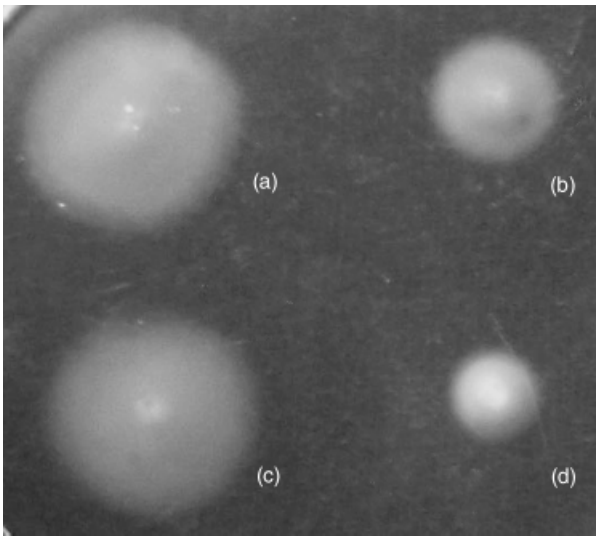

Fig. 3. Swimming motility of *Bradyrhizobium* sp. SEMIA 6144 strains. The assay was performed at 28 °C on YEM swim plates (0.3% agar). (a) SEMIA 6144 wild type, (b) DBM13, (c) DBM13 × pDBM07 and (d) DBM13 × pBBR1MCS-5. Similar results were obtained in three independent experiments.

other bacteria. Mutants of *L. pneumophila* lacking phosphatidylcholine are unable to transit to a motile state and have low levels of flagellin protein (Conover *et al.*, 2008). Also in *A. tumefaciens*, the loss of phosphatidylcholine resulted in reduced motility (Klüsener *et al.*, 2009).

Symbiotic performance of the *Bradyrhizobium* sp. SEMIA 6144 *pmtA*-deficient mutant

All peanut plants infected with DBM13 developed normal nodules, with the red colour indicative of leghaemoglobin and also showed wild-type parameters with respect to the levels of nitrogen-fixation activity and the amount of dry matter produced per plant (data not shown). Therefore, the phosphatidylcholine level encountered in DBM13 (Table 2) was sufficient to develop functional nitrogen-fixing nodules. Hacker *et al.* (2008) reported wild-type-like symbiotic characteristics for soybean plants infected with *B. japonicum pmtX2*, *pmtX3*, *pmtX4* or *pcs* mutants, but all of which showed wild-type levels of phosphatidylcholine. On the other hand, soybean plants inoculated with *pmtA* mutants of *B. japonicum*, which were severely affected in phosphatidylcholine biosynthesis, showed drastic nitrogen-fixation defects (Minder *et al.*, 2001).

When peanut roots were coinoculated with the wild-type and DBM13 strains in a 1 : 1 inoculum ratio, DBM13 was detected in only 27.8 ± 6.5% of the total nodules, indicating a defect in their nodulation competitiveness. We related this defect in competitiveness of DBM13 to its lack of motility and/or chemotaxis because many earlier reports indicate their importance for competitive nodulation (Caetano-Anollés *et al.*, 1988; Barbour *et al.*, 1991; Alexandre *et al.*, 2004; Miller *et al.*, 2007). Therefore, wild-type levels of phosphatidylcholine could be important for the competitive abilities of SEMIA 6144 in the rhizosphere.

Two major changes occur in the membrane lipid composition in the mutant with respect to the wild type: firstly, the fact that in the *pmtA*-deficient mutant phosphatidylethanolamine is the most abundant phospholipid instead of phosphatidylcholine should cause major changes in the membrane properties. Phosphatidylethanolamine has a smaller headgroup, can hydrogen bond through its ionizable amine and has the unique property of undergoing a bilayer-to-nonbilayer physical transition influenced by its fatty acid content and the temperature (Dowhan, 1997). These changes could lead to modifications in the structure of transmembrane α-helices of membrane proteins, altering the packing of these helices (Dowhan, 1997). As a consequence, membrane-associated functions of DBM13, such as motility, might be affected. Secondly, the amount of cardiolipin is strongly reduced in the *pmtA*-deficient mutant. This reduction might be a direct effect of the decrease in phosphatidylcholine and the increase in phosphatidylethanolamine. Possibly, by decrease of cardiolipin, the cell size might be affected. Finally, the change in the proportion between anionic and zwitterionic lipids could be important in seemingly diverse membrane-associated processes.

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