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Multiple Phospholipid *N*-Methyltransferases with Distinct Substrate Specificities Are Encoded in *Bradyrhizobium japonicum*▽

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Phosphatidylcholine (PC) is the major phospholipid in eukaryotic membranes. In contrast, it is found in only a few prokaryotes including members of the family *Rhizobiaceae*. In these bacteria, PC is required for pathogenic and symbiotic plant-microbe interactions, as shown for *Agrobacterium tumefaciens* and *Bradyrhizobium japonicum*. At least two different phospholipid *N*-methyltransferases (PmtA and PmtX) have been postulated to convert phosphatidylethanolamine (PE) to PC in *B. japonicum* by three consecutive methylation reactions. However, apart from the known PmtA enzyme, we identified and characterized three additional *pmt* genes (*pmtX1*, *pmtX3*, and *pmtX4*), which can be functionally expressed in *Escherichia coli*, showing different substrate specificities. *B. japonicum* expressed only two of these *pmt* genes (*pmtA* and *pmtX1*) under all conditions tested. PmtA predominantly converts PE to monomethyl PE, whereas PmtX1 carries out both subsequent methylation steps. *B. japonicum* is the first bacterium known to use two functionally different Pmts. It also expresses a PC synthase, which produces PC via condensation of CDP-diacylglycerol and choline. Our study shows that PC biosynthesis in bacteria can be much more complex than previously anticipated.

Phosphatidylcholine (PC) or lecithin is one of the major structural constituents of eukaryotic membranes (10). In contrast, only ca. 10% of all bacterial species are predicted to produce PC (39). Eukaryotes synthesize PC by two alternative pathways: the CDP-choline pathway or the methylation pathway (22). In the CDP-choline pathway, also known as the Kennedy pathway, free choline is converted to PC via choline-phosphate and CDP-choline as intermediates (21). In the methylation pathway, PC is formed by three successive methylations of phosphatidylethanolamine (PE) via the intermediates monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE) using the methyl donor *S*-adenosyl-L-methionine (SAM). In higher eukaryotes, such as human or rats, a single phospholipid *N*-methyltransferase (Pmt) performs all three methylation reactions (36). Lower eukaryotes, such as yeast or *Neurospora*, possess two different Pmts with distinct substrate specificities (6, 24). One Pmt predominantly catalyzes the first methylation step, converting PE to MMPE, and is therefore referred to as phosphatidylethanolamine *N*-methyltransferase. A second Pmt mainly catalyzes the second and third methylation steps from MMPE via DMPE to PC.

Prokaryotes were originally believed to use only the methylation pathway for PC biosynthesis (43). Indeed, mutants of *Rhodobacter sphaeroides* and *Zymomonas mobilis* deficient in Pmt were unable to form PC (1a, 41). However, an alternative pathway in which choline is directly condensed with CDP-diacylglycerol (CDP-DAG) by the PC synthase (Pcs) was later discovered in the legume symbiont *Sinorhizobium meliloti* (38). Similar activities have been demonstrated in *Pseudomonas aeruginosa*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, *Mesorhizobium loti*, *Legionella pneumophila*, *Agrobacterium tumefaciens*, and *Brucella abortus* (4, 26, 45, 46). Most intriguingly, evidence for a eukaryotic-like CDP-choline pathway was found in *Treponema denticola* (23), indicating that prokaryotic PC biosynthesis is very diverse.

The lipid composition of biological membranes defines their stability and integrity (34). It undergoes adaptational changes in response to environmental conditions, such as oxygen tension, temperature, salinity, or medium composition (27, 42). Recently, a fundamental role of PC during infection of eukaryotic hosts has been recognized. A *B. japonicum* mutant with decreased PC levels was unable to establish an efficient symbiosis with its soybean host, although vegetative growth was largely unaffected (30). An *S. meliloti* mutant unable to form PC failed to establish a proper symbiosis with its host (8, 39). PC is also required for virulence of the plant pathogen *A. tumefaciens* (45). Important human pathogens such as *P. aeruginosa*, *L. pneumophila*, *Borrelia burgdorferi*, and *Brucella melitensis* are also known to possess PC (26, 44, 46). Interestingly, it has been reported recently that PC is necessary for the full virulence of *Brucella abortus* (4, 5). This clearly supports the earlier hypothesis that PC might play an important role in many host-microbe interactions (14, 39).

Since a *B. japonicum pmtA* mutant was still able to produce reduced amounts of MMPE, DMPE, and PC, at least one additional Pmt (PmtX) was predicted in this organism (30). In the present study, we set out to determine the complete PC biosynthesis inventory of *B. japonicum*. We describe a Pcs and multiple Pmt enzymes, that have distinct substrate specificities.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* cells were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
DH5α	Host for plasmid amplification	16b
S17-1	RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome	37a
BL21(DE3)/pLysS	Host for expression	40
B. japonicum		
USDA110 spc4	Sp^r (wild type)	35
H1	Sp^r Km^r *nifH*::Tn*5*	16a
110-48	Sp^r Tc^r *nifH′-′lacZ* chromosomally integrated	15a
5569	Sp^r Km^r *pmtA*::[Km>] deletion mutant of USDA110 spc4	30
5570	Sp^r Km^r *pmtA*::[<Km] deletion mutant of USDA110 spc4	30
5702	Sp^r Sm^r *pcs*::[ΩSm/Sp] deletion mutant of USDA110 spc4	This study
BO203	Sp^r Km^r *pmtX3*::[Km>] deletion mutant of USDA110 spc4	This study
BO204	Sp^r Km^r *pmtX3*::[<Km] deletion mutant of USDA110 spc4	This study
BO213	Sp^r Tc^r *pmtA′-′lacZ* chromosomally integrated	This study
BO214	Sp^r Tc^r *pmtX1′-′lacZ* chromosomally integrated	This study
BO216	Sp^r Tc^r *pmtX2′-′lacZ* chromosomally integrated	This study
BO222	Sp^r Tc^r *trmU′-′lacZ* chromosomally integrated	This study
BO223	Sp^r Tc^r *bll6635′-′lacZ* chromosomally integrated	This study
BO224	Sp^r Tc^r *pmtX3′-′lacZ* chromosomally integrated	This study
BO230	Sp^r Km^r *pmtX2*::[Km>] deletion mutant of USDA110 spc4	This study
BO231	Sp^r Km^r *pmtX2*::[<Km] deletion mutant of USDA110 spc4	This study
BO248	Sp^r Tc^r *pmtX4′-′lacZ* chromosomally integrated	This study
BO254	Sp^r Km^r *pmtX4*::[Km>] deletion mutant of USDA110 spc4	This study
BO255	Sp^r Km^r *pmtX4*::[<Km] deletion mutant of USDA110 spc4	This study
BO261	Sp^r Tc^r *pcs′-′lacZ* chromosomally integrated	This study
BO265	Sp^r Tc^r bll8165′-′*lacZ* chromosomally integrated	This study
Plasmids		
pBCSK(+)	High-copy cloning vector; Cm	Stratagene, Amsterdam, The Netherlands
pBSL86	Km cassette flanked by polylinker; Ap	1
pET9a	High-copy T7 tag expression vector; Km	40
pET24b(+)	High-copy His tag expression vector; Ap	Novagen, Darmstadt, Germany
pHP45::Ω	ΩSm/Sp cassette flanked by polylinker; Km	33a
pRK404	Broad-host-range vector	9
pSUP202	Mobilizable narrow-host-range vector; Ap Cm Tc; *oriT* from RP4	37a
pSUP202pol4	pSUP202 derivative with part of polylinker from pBluescript-II KS1 between EcoRI and PstI; Tc	13
pSUP482	Mobilizable *lacZ* fusion narrow-host-range vector; Tc	30a
pUC18	High-copy cloning vector; Ap	43a
pBO203	pSUP202pol4 derivative carrying Δ*pmtX3*::[Km>]	This study
pBO204	pSUP202pol4 derivative carrying Δ*pmtX3*::[<Km]	This study
pBO213	pSUP482 derivative carrying a *pmtA′-′lacZ* translational fusion	This study
pBO214	pSUP482 derivative carrying a *pmtX1′-′lacZ* translational fusion	This study
pBO216	pSUP482 derivative carrying a *pmtX2′-′lacZ* translational fusion	This study
pBO222	pSUP482 derivative carrying a *trmU′-′lacZ* translational fusion	This study
pBO223	pSUP482 derivative carrying a bll6635′-′*lacZ* translational fusion	This study
pBO224	pSUP482 derivative carrying a *pmtX3′-′lacZ* translational fusion	This study
pBO230	pSUP202pol4 derivative carrying Δ*pmtX2*::[Km>]	This study
pBO231	pSUP202pol4 derivative carrying Δ*pmtX2*::[<Km]	This study
pBO234	pET24b derivative for expression of *B. japonicum pmtX4*	This study
pBO248	pSUP482 derivative carrying a *pmtX4′-′lacZ* translational fusion	This study
pBO254	pSUP202pol4 derivative carrying Δ*pmtX4*::[Km>]	This study
pBO255	pSUP202pol4 derivative carrying Δ*pmtX4*::[<Km]	This study
pBO265	pSUP482 derivative carrying a bll8165′-′*lacZ* translational fusion	This study
pBO267	pSUP482 derivative carrying a *pcs′-′lacZ* translational fusion	This study
pCCS20	pET9a derivative for expression of *B. japonicum pmtX1*	This study
pCCS27	pRK404 derivative for expression of *B. japonicum pmtA*	This study
pCCS36	pET9a derivative for expression of *B. japonicum pmtX2*	This study
pCCS37	pET9a derivative for expression of *B. japonicum pmtX3*	This study
pCCS67	pCCS27 derivative for coexpression of *B. japonicum pmtA* and *pmtX3*	This study
pCCS115	pBO234 derivative for coexpression of *B. japonicum pmtX1* and *pmtX4*	This study
pRJ5295	pSUP202pol4 derivative carrying a 440-bp gene internal fragment of *pmtX1*	This study
pRJ5702	pSUP202pol4 derivative carrying Δ*pcs*::[ΩSm/Sp]	This study
pTB2117	pET3a derivative for expression of *B. japonicum pmtA*	30

[a] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; Sp, spectinomycin.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
pmtX3UP-EcoRI (O1)	AAAGAATTCCGAAAGCCAGGACCCATAACCAC
pmtX3DWN (O2)	GGACGCCGCGACACCGAACTGAAG
pmtA_lacZ_UP (O32)	CGAGGTCCTGAAGGACAAGGACAA
pmtA_lacZ_DWN (O33)	AAAACTGCAGCATGTCTGGACCGGACGGGAC
pmtX1_lacZ_DWN (O35)	AAAACTGCAGCATGCCCCCTGCCCCGGAAA
pmtX2_lacZ_UP_long (O37)	AAAAGAATTCCGTTGCCCAGCACGTAATAG
pmtX2_lacZ_DWN (O38)	AAAACTGCAGCATGATCTTAGCCATCGGTGA
pmtX3_lacZ_UP (O39)	AAAAGAATTCAAGCCTCCCCAACGTCGTCCT
pmtX3_lacZ_DWN (O40)	AAAACTGCAGCATGCGAGATCTCCCTCGCGAT
USX2_up (O45)	AAAAGCGGCCGCGTGTTGCGGTGGAGAAAAAT
USX2_dwn (O46)	AAAACTGCAGCCGAACACGAGATCGTAGA
DSX2alt_Up (O53)	AAAACTGCAGCCGAGCGCCGCCTGATTCC
DSX2alt_Dwn (O54)	AAAAGAATTCGTGCGTCGCCTGGTTGATGTGGT
trmU_lacZ_UP-EcoRI (O57)	AAAAGAATTCGAGGACTGGTCGGTCGA
trmU-lacZ_DWN_PstI (O58)	AAAACTGCAGCATGGGTTCTTAAGGTGTGACG
bll6635_lacZ_UP_EcoRI (O59)	AAAAGAATTCTTCTTGGTCACCGACTGGAC
bll6635_lacZ_DWN_PstI (O60)	AAAACTGCAGCATCAACCACGGATATTTCAG
X4_NdeI_UP (O61)	AAAAAAAACATATGCCCAACGATTTCCTCTCCTTC
X4_HindIII_DWN (O62)	AAAAAAGCTTTCAGCGCGCGGCGGAGTAGTCG
X4_LZ_UP (O63)	AAAAGAATTCCGCGCTGACCAACCTCGTG
X4_LZ_DWN (O64)	AAAACTGCAGCATGCGCACTCCGGCATC
pcs_LZ_2_DWN (O73)	AAAACTGCAGCATGGCTTCTGCTATCAG
Bll8165_LZ_DWN2 (O78)	AAAACCCGGGACATTGCGATCGTGCTCGA
pmtX4_DS_UP (O82)	AAAACTGCAGAGATGAAGCTCGTCACGG
pmtX4_DS_DWN (O83)	AAAATCTAGAGGCCCGACCAAGGAGCCC
Sig238	GGTGCGCGGCTAGAAAAT
Sig239	GTAGCGGATCAGCGAAAAGT
Sig241	GCCCGAATTCGTGTGGAACTCGGTCGAGACAT
Sig242	GCCCGGATCCACAATTCGCCACAAAATCA
Sig243	GCCCGGATCCATTGATCCGCTTCGCAAGAT
Sig244	AAGGCTGCAGAATTGGAATCCATCCGCAAC
PmtX1_5	ACGTCATATGGCAGCAGACATCTCGCGGGCCGGGGTC
PmtX1_3	ACGTGGATCCTCACGATTTGCGGTAGCGGATCAGCGAAAAG
PmtX2_5	ACGTCATATGGCTAAGATCATGAACCTTGACGGCACCCAG
PmtX2_3	ACGTGGATCCTCACGCGGCCTTGGCGACGTCGACCTTGCGG
PmtX3_5	ACGTCATATGTTGTCTGCTGACATCCTGCCGTTCTTCC
PmtX3_3	ACGTGGATCCTCACGCGCAGGGGGGACGGCGGCTGATCCGGTAC

[a] Incorporated restriction sites are underlined.

routinely grown at 37°C in Luria-Bertani (LB) medium (29) supplemented with ampicillin (200 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), or tetracycline (10 μg ml^{-1}) if required. *B. japonicum* strains were propagated aerobically at 30°C in PSY complex medium (35) supplemented with 0.1% (wt/vol) L-arabinose. Anaerobic cultures were grown in yeast extract-mannitol (YEM) medium supplemented with 10 mM KNO_3 (7) under nitrogen atmosphere. If appropriate, antibiotics were added at the following concentrations) to *B. japonicum* cultures: chloramphenicol, 20 μg ml^{-1} (for counterselection against *E. coli* donor strains); kanamycin, 100 μg ml^{-1} (solid media) and 50 μg ml^{-1} (liquid media); spectinomycin, 100 μg ml^{-1}; streptomycin, 50 μg ml^{-1}; and tetracycline, 50 μg ml^{-1} (solid media) and 30 μg ml^{-1} (liquid media).

Routine DNA work and sequence analysis. Recombinant DNA work and Southern blotting were performed according to standard protocols (37). Probes for Southern blot hybridizations were generated by PCR and labeled with digoxigenin according to manufacturer's instructions (Roche, Mannheim, Germany). The isolation of chromosomal DNA from *B. japonicum* was carried out as described previously (15).

Homology searches were performed by using the National Center for Biotechnology Information's BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of *pmt* and *pcs* mutants. Construction of the *pmtA* deletion mutant has been described (30). In order to construct a *pmtX1* mutant by a gene disruption strategy, an internal fragment comprising nucleotides 38 to 477 was PCR amplified using the oligonucleotides Sig238 and Sig239 (for oligonucleotide sequences, see Table 2). The resulting 440-bp StuI-Ecl136II fragment was directly cloned into the suicide vector pSUP202pol4 linearized with SmaI to yield plasmid pRJ5295 (see Fig. 3B).

For construction of the *pmtX2* deletion mutant, the *pmtX2* up- and downstream regions (645 and 593 bp, respectively) were amplified by using the oligonucleotide pairs O45-O46 and O53-O54, respectively. After cloning both fragments into pBCSK(+), the 1.2-kb PstI fragment of pBSL86 containing the neomycin phosphotransferase II cassette (Km^r) was placed between them in both possible orientations. The 2.4-kb EcoRI-NotI inserts of the resulting plasmids were ligated into the 6.7-kb EcoRI-NotI vector fragment of pSUP202pol4, yielding plasmids pBO230 and pBO231.

In order to delete *pmtX3*, a 1.8-kb DNA fragment carrying the gene was PCR amplified by using primer pair O1-O2. A 1.7-kb EcoRI-NotI fragment from the amplification product was cloned into vector plasmid pBCSK(+). Subsequently, a 249-bp PstI fragment of the *pmtX3* coding region was replaced by a 1.2-kb PstI kanamycin cartridge from pBSL86 (see Fig. 3D). The 2.6-kb EcoRI-NotI inserts of the resulting plasmids were ligated into the 6.7-kb EcoRI-NotI vector fragment of pSUP202pol4, yielding plasmids pBO203 and pBO204.

The *pmtX4* deletion constructs were cloned as described for *pmtX2*. The up- and downstream regions (418 and 520 bp, respectively) of *pmtX4* were amplified with the primer pairs O63/O64 and O82/O83.

To delete the *pcs* gene, up- and downstream regions (704 and 662 bp, respectively) were PCR amplified with the primer pairs Sig241-Sig242 and Sig243-Sig244. After PCR products were cloned in pUC18, the 2-kb BamHI fragment of pHP45::Ω containing a Sm/Sp resistance cassette was inserted in the same orientation as the *pcs* gene. Finally, the 3.4-kb EcoRI-PstI insert was cloned into pSUP202pol4, yielding pRJ5702.

Finally, the suicide plasmids were mobilized from *E. coli* S17-1 into *B. japonicum* 110spc4 for marker replacement mutagenesis as described previously (16). The correct genomic structure of all mutant strains was confirmed by Southern blot analysis of genomic DNA digested with appropriate restriction enzymes.

Construction of chromosomally integrated, translational *'lacZ* fusions. For the construction of *'lacZ* fusion strains, suitable translational *lacZ* fusions present on pSUP202pol4 derivates were integrated into the chromosome of

wild-type *B. japonicum* via homologous recombination (see Fig. 3). The correct genomic structure of all mutant strains was confirmed by Southern blot hybridization with appropriate digoxigenin-11-dUTP-labeled DNA probes.

β-Galactosidase assays. The β-galactosidase activity of *B. japonicum* cells grown aerobically in PSY complex medium or anaerobically in YEM medium was measured according to standard protocols (29).

In vivo labeling of *B. japonicum* with [^{14}C]acetate and quantitative analysis of lipid extracts. The lipid compositions of *B. japonicum* wild type and mutant strains were determined after labeling with [1-^{14}C]acetate. Cultures (1 ml) in PSY were inoculated from precultures grown in the same medium. After the addition of 0.5 μCi of [1-^{14}C]acetate (56.0 mCi/mmol) to each culture, the cultures were incubated for 48 h. Cells were harvested by centrifugation, washed with 500 μl of water, and resuspended in 100 μl of water. Lipid extraction was done according to the method of Bligh and Dyer (2). The chloroform phase was used for lipid analysis on HPTLC silica gel 60 plates (Merck, Darmstadt, Germany). After one-dimensional separation with *n*-propanol–propionic acid–chloroform–water (3:2:2:1) as the running solvent the individual lipids were quantified by using a Bio-Imager (Fujifilm Europe GmbH, Düsseldorf, Germany) and the Aida Image Analyzer v. 4.03 software (Raytest GmbH, Straubenhardt, Germany).

Cloning and expression of the putative *pmt* genes of *B. japonicum* in *E. coli*. Candidate open reading frames (ORFs) *pmtX1*, *pmtX2*, and *pmtX3* were PCR amplified by using oligonucleotide primers that introduced NdeI/BamHI restriction sites (Table 2) and were cloned into the expression plasmid pET9a (40), resulting in plasmids pCCS20, pCCS36, and pCCS37, respectively. Candidate ORF *pmtX4* was PCR amplified by using oligonucleotide primers that introduced NdeI/HindIII restriction sites and cloned into pET24b (40), resulting in plasmid pBO234. Expression plasmids were transformed into the *E. coli* expression strain BL21(DE3)/pLysS. Cultures were grown in LB medium containing the appropriate antibiotics. At a density of 5×10^8 cells/ml, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 200 μM. Aliquots of 1 ml were transferred to sterile culture tubes and after 1 h of induction 0.5 μCi of [1-^{14}C]acetate (56.0 mCi/mmol; Amersham) or [^{14}C-*methyl*]methionine (40 to 60 mCi/mmol; Amersham) were added to each culture. One hour later the cells were harvested and extracted as described above.

To coexpress *pmtA* from *B. japonicum* with the candidate ORFs in *E. coli*, the expression cassette containing the T7 promoter and the *pmtA* gene was subcloned from pTB2117 (30) as a BamHI/BglII fragment into the broad-host-range plasmid pRK404 (9), resulting in plasmid pCCS27. *E. coli* BL21(DE3)/pLysS was transformed with pCCS27 and one of the plasmids pCCS20, pCCS36, pCCS37, or pBO234. Growth of the cells, induction, labeling, and extraction of the lipids was done as described above.

To coexpress *pmtA* from *B. japonicum* with two or three candidate ORFs in *E. coli*, new constructs had to be made: the *pmtX3* gene was subcloned as a BamHI/BglII fragment from pCCS37 into the BamHI site of pCCS27, resulting in plasmid pCCS67, containing *pmtA* and *pmtX3* both under control of the T7 promoter. The *pmtX1* gene was subcloned as a BamHI/SphI fragment from pCCS20 into the BglII/SphI-digested plasmid pBO234, resulting in plasmid pCCS115, containing *pmtX1* and *pmtX4* under the control of the T7 promoter. Growth of the cells, induction, labeling, and lipid extraction were done as described above.

Plant infection tests. Surface sterilization of soybean seeds (*G. max* L.Merr cv. Williams 82), inoculation, plant cultivation, and acetylene reduction assays were done as described previously (15, 16). For determination of specific acetylene reduction activity (expressed as μmol of ethylene formed/h/g [dry weight] of nodule) all nodules from each plant were collected, dried overnight at 80°C, and weighed.

RESULTS

Multiple genes coding for putative Pmts in the genome of *B. japonicum*. Earlier work had suggested that at least two Pmts are present in *B. japonicum* (30). However, previous attempts to identify a *pmtX* gene in the *B. japonicum* genome via low-stringency Southern blot hybridization had failed (30). More recently, the complete genome sequence of *B. japonicum* USDA110 (19) made it possible to search for *pmtX* candidates using bioinformatics tools. Two different families of prokaryotic Pmts have been described (1a, 8). We used as a query the sequences of *S. meliloti* PmtA and *R. sphaeroides* PmtA, which were the first members to be described in each family (25) to identify candidate genes coding for additional Pmts. Using the amino acid sequence of PmtA from *S. meliloti* as a query revealed three close homologs. As described earlier, *B. japonicum* PmtA shows homology to PmtA from *S. meliloti* (30) (Fig. 1). Both proteins share 35% identical and 56% similar amino acids (30, 39). Two additional candidates were identified that we named PmtX3 (Bll8166; identity on amino acid level, 33%; similarity, 53%) and PmtX4 (Blr4804; identity, 32%; similarity, 50%).

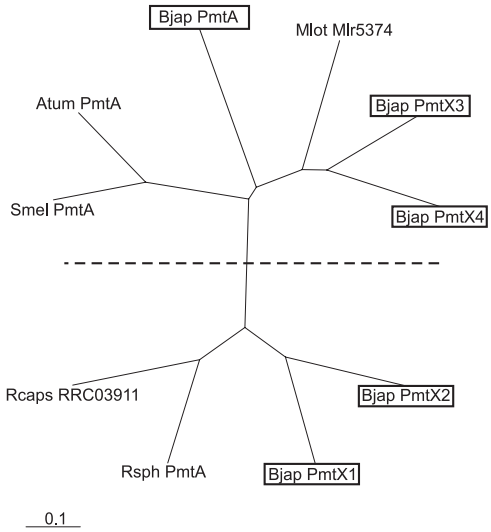

FIG. 1. Unrooted phylogenetic tree of selected bacterial Pmt enzymes. The sequences used for the construction of the tree include the *B. japonicum* Pmt proteins (Bjap_PmtA, Blr0681; Bjap_PmtX1, Bll6994; Bjap_PmtX2, Bll6634; Bjap_PmtX3, Bll8166; Bjap_PmtX4, Blr4804) (19), the PmtA proteins from *S. meliloti* (Smel_PmtA; accession number AF201699) and *R. sphaeroides* (Rsph_PmtA, L07247), PmtA from *A. tumefaciens* (Atum PmtA, AE009001), a Pmt-like ORF from the genome of *Rhodobacter capsulatus* (Rcaps RRC03911; Ergo-light database; http://www.ergo-light.com/), and one from the genome of *M. loti* (Mlot Mlr5374, BA000012). The bradyrhizobial Pmt enzymes are highlighted by surrounding black boxes. The dashed line indicates the separation of the different types of Pmt enzymes. The tree was constructed by using the program CLUSTAL X (ftp://ftp-igbmc.u-strasbg.fr/pub/). Distances between sequences are expressed as 0.1 changes per amino acid residue.

Using the amino acid sequence of PmtA from *R. sphaeroides* as a query, two close homologs showed up, which we named PmtX1 (Bll6994; identity, 39%; similarity, 59%) and PmtX2 (Bll6634; identity, 41%; similarity, 61%). All predicted Pmt-like proteins contain the consensus motif for *S*-adenosylmethionine (SAM)-utilizing methyltransferases (17) thought to be involved in SAM binding (18) (data not shown).

To summarize, our bioinformatic analysis revealed that in addition to the already-known *pmtA* gene the *B. japonicum* genome contains four ORFs coding for putative Pmts. To make PC biosynthesis in this organism even more complex, its genome also contains a *pcs* gene encoding a Pcs (26).

Expression of the individual *pmt* genes in *E. coli*. Analysis of *B. japonicum* mutants deficient in the *pmtA* gene and heterologous expression of the *B. japonicum pmtA* in *E. coli* indicated that PmtA appears to be responsible mainly for the first methylation step in PC biosynthesis (30). Additional Pmts should

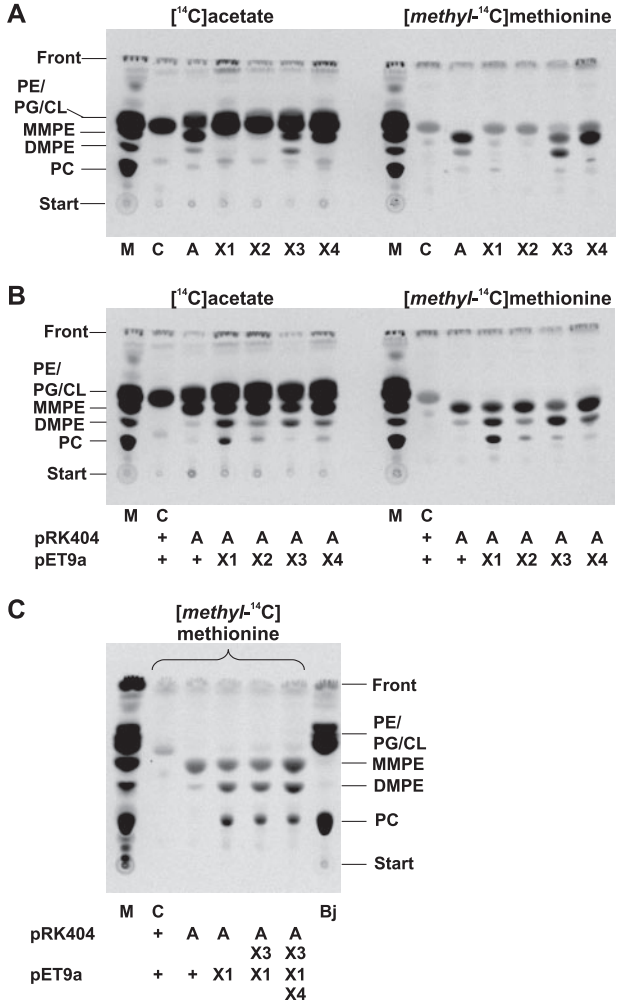

FIG. 2. Lipid formation after heterologous expression of *pmtA* from *B. japonicum* and four genes encoding putative Pmt enzymes from *B. japonicum* in *E. coli*. *E. coli* BL21(DE3)/pLysS cells expressing the indicated enzymes were labeled with [^{14}C]acetate or [*methyl*-^{14}C]methionine during growth on LB medium. Panel A shows the resulting phospholipid profile after single expression of (putative) Pmts in *E. coli*: panels B and C show the resulting phospholipid profile after coexpression of the phospholipid methyltransferase PmtA from *B. japonicum* with ORFs encoding putative Pmts in *E. coli*. After extraction, lipids were separated by one-dimensional TLC. Lane M, marker (*S. meliloti* lipids labeled with [^{14}C]acetate); lane C, control with BL21(DE3)/pLysS/pET9a (+pRK404 for panels B and C); lane A, *pmtA*; lane X1, *pmtX1*; lane X2, *pmtX2*; lane X3, *pmtX3*; and lane X4, *pmtX4* (genes encoded in the indicated vector backbone [see also Materials and Methods and Table 1]); lane Bj, *B. japonicum* lipids labeled with [^{14}C]acetate. The lipids PG, cardiolipin (CL), PE, MMPE, DMPE, and PC are indicated.

therefore carry out the subsequent methylation steps converting MMPE into DMPE and PC. To detect this activity, we expressed PmtA, PmtX1, PmtX2, PmtX3, and PmtX4 in *E. coli* BL21(DE3)/pLysS. Lipids were radiolabeled by [^{14}C]acetate or [*methyl*-^{14}C]methionine, and the lipid composition of the strains was analyzed by using thin-layer chromatography (TLC) (Fig. 2). The amount of [^{14}C]methionine-labeled lipids was quantified (Table 3). *E. coli* does not have Pmts and therefore only produces the membrane lipids PE, phosphatidylglycerol (PG), and cardiolipin. Expression of *pmtA* from *B. japonicum* led to the formation of mainly MMPE and minor amounts of DMPE (Fig. 2A and Table 3) as described earlier (30). If at all, only minute amounts of methylated PE derivatives were formed when PmtX1 or PmtX2 were expressed in *E. coli*. When PmtX3 was expressed, MMPE and DMPE were produced in almost equal amounts. Expression of PmtX4 led to the formation of large quantities of MMPE and only small amounts of DMPE.

Coexpression of multiple *pmt* genes in *E. coli*. Since PmtA catalyzes very efficiently the formation of MMPE, one of the *pmtX* genes might encode a Pmt responsible for the second and/or third methylation step of PC biosynthesis. We therefore expressed PmtA together with each of the candidate ORFs in *E. coli*. Lipids were radiolabeled by [^{14}C]acetate or [*methyl*-^{14}C]methionine, and the lipid composition of the strains was analyzed by using TLC (Fig. 2B). The amount of [^{14}C]methionine incorporated into each lipid was quantified (Table 3). As described above, mainly MMPE was detected when only PmtA was expressed. Coexpression of PmtA with PmtX1 led to the formation of significant amounts of DMPE and PC. When PmtA and PmtX2 were expressed together, mainly MMPE and possibly minor amounts of DMPE and PC were detected, indicating that, if at all, PmtX2 is an inefficient Pmt enzyme under these conditions. Coexpression of PmtA and PmtX3 caused a lipid spectrum similar to that caused by the expression of PmtX3 alone, with slightly increased levels of DMPE. Since both PmtA and PmtX4 preferentially catalyze the first methylation step, their coexpression also led mainly to the formation of MMPE.

Wild-type *B. japonicum* grown in complex PSY medium produces mainly PC and only small amounts of MMPE and DMPE (30). In an attempt to simulate this lipid composition heterologously, we coexpressed two, three, or four different Pmts from *B. japonicum* in *E. coli*. However, the expression of PmtA and PmtX1 caused the maximal formation of DMPE and PC methylation products in *E. coli* hosts. The relative

TABLE 3. Formation of methylated derivatives of PE upon expression of genes encoding putative Pmts from *B. japonicum* in *E. coli*[a]

Derivative	Avg incorporation (Bq) ± SD										
	No *pmt*	Single expression					Double expression				
		pmtA	*pmtX1*	*pmtX2*	*pmtX3*	*pmtX4*	*pmtA*/-	*pmtA*/*pmtX1*	*pmtA*/*pmtX2*	*pmtA*/*pmtX3*	*pmtA*/*pmtX4*
PE	7.4 ± 2.4	1.1 ± 0.7	6.8 ± 2.9	7.6 ± 1.7	2.9 ± 1.4	12.0 ± 2.6	1.3 ± 0.9	1.1 ± 0.6	1.4 ± 1.3	0.9 ± 0.9	2.1 ± 1.9
MMPE		28.1 ± 9.7	1.5 ± 0.9	1.1 ± 1.0	15.3 ± 3.6	164.0 ± 14.0	23.8 ± 6.5	21.3 ± 4.7	38.0 ± 10.7	15.7 ± 2.4	70.0 ± 26.4
DMPE		5.3 ± 1.4	0.8 ± 0.5	0.8 ± 0.8	17.4 ± 4.5	2.3 ± 1.4	2.0 ± 1.2	21.1 ± 5.1	4.6 ± 1.6	29.8 ± 5.1	8.4 ± 3.7
PC		1.1 ± 1.1	1.4 ± 0.9	0.8 ± 0.8	1.4 ± 0.9	1.6 ± 0.9	0.8 ± 0.9	24.7 ± 7.4	2.3 ± 1.7	1.6 ± 1.1	1.2 ± 1.2

[a] *E. coli* BL21(DE3)/pLysS cells expressing the indicated *pmt* genes were labeled with [*methyl*-^{14}C]methionine during growth in LB medium. A total of 18,500 Bq were used per culture. After extraction, lipids were separated by one-dimensional TLC, and the incorporated radioactivity was determined. Numbers in the table give the average incorporation of three independent experiments.

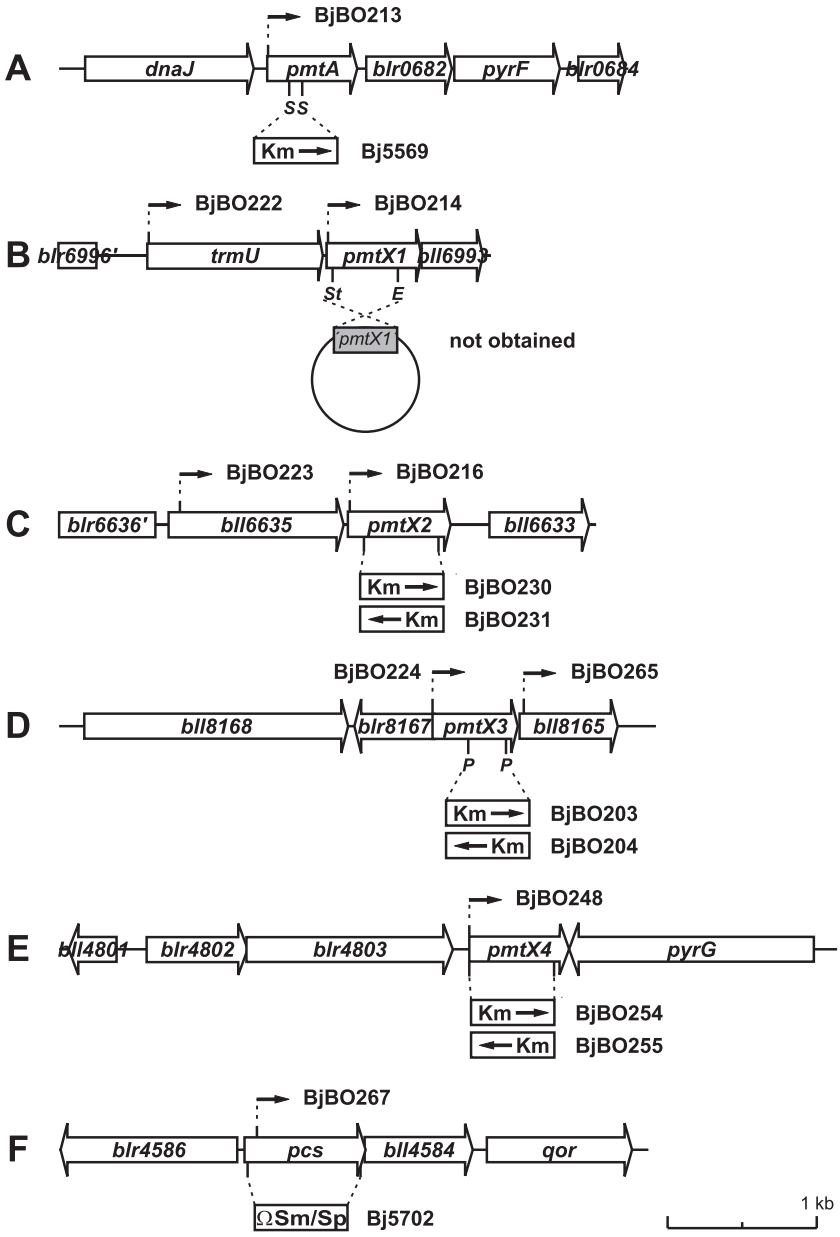

FIG. 3. Physical and genetic maps of the *B. japonicum pmtA* (A), *pmtX1-4* (B to E), and *pcs* (F) gene regions. Physical maps are given for SalI, StuI, Ecl136II, and PstI (indicated by S, St, E, and P, respectively). Black arrows indicate the position of chromosomally integrated translational *lacZ* fusions and point to the corresponding strain designation. Below each map, the insertion sites for antibiotic resistance cassettes (Km in the case of *pmtA* and *pmtX2-4*; ΩSm/Sp in the case of *pcs*) or the plasmid integration site are depicted. Antibiotic resistance cassettes are not drawn to scale. Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; *dnaJ*, chaperone protein DnaJ; *pmtA*, Pmt; blr0682, putative oxidoreductase; *pyrF*, orotidine-5′-monophosphat-decarboxylase; blr0684, blr6996, bll6993, bll6633, blr8167, bll4801, and bll4584, hypothetical ORFs; *trmU*, tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase; *pmtX1*, phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17); blr6636, ATP synthase subunit; *pmtX2*, phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17); bll6635, probable glycosyl transferase; bll8168, Na^+/H^+ antiporter; *pmtX3*, Pmt; bll8165, putative alkaline phosphatase; blr4802, two-component response regulator; blr4803, two-component hybrid sensor and regulator; *pmtX4*, Pmt; *pyrG*, CTP synthetase; blr4586, putative oxidoreductase protein; *pcs*, Pcs; *qor*, quinone oxidoreductase.

amounts of DMPE and PC could not be increased by expressing additionally PmtX3 or PmtX3 and PmtX4 (Fig. 2C), indicating that PmtX3 and PmtX4 have no important role for overall PC formation.

Expression of *pmt* genes and the *pcs* gene in *B. japonicum*. In order to understand the complexity of PC biosynthesis in *B. japonicum*, we studied the expression of all potential PC biosynthesis enzymes using the corresponding reporter gene fusions to these and some flanking genes (Fig. 3). We first measured β-galactosidase activity during aerobic growth in complex medium. The reporter strains harboring chromosomally integrated, translational *lacZ* fusions were assayed 24, 48, and 72 h after inoculation to an optical density at 600 nm (OD_{600}) of 0.02, which corresponds to early-exponential

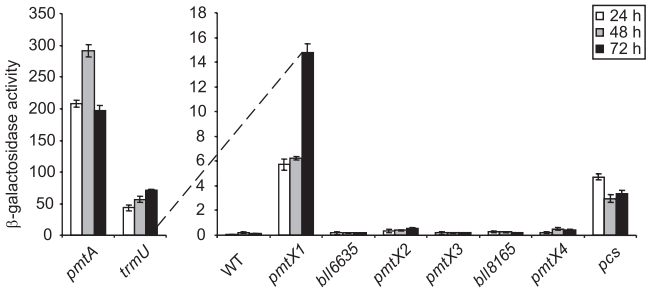

FIG. 4. Expression of *B. japonicum* PC biosynthesis genes. The expression of chromosomally integrated, translational *lacZ* reporter gene fusions (see Fig. 3) was measured as described previously (see Materials and Methods). Samples were collected after 24, 48, and 72 h. The results of a typical experiment, in which two parallel cultures of each strain were grown and assayed in duplicate, are shown. Four independent experiments were performed with individual strains.

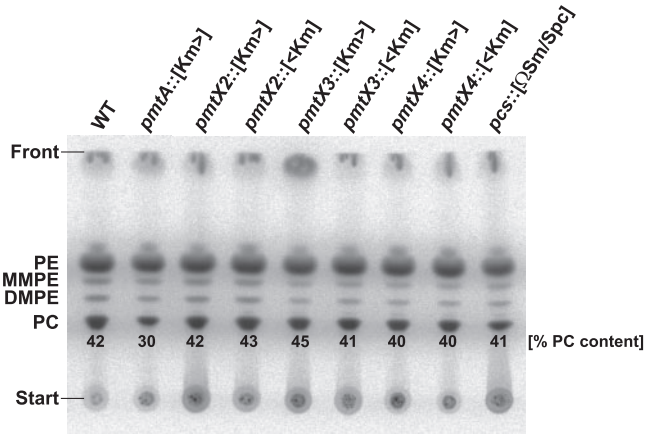

FIG. 5. Phospholipid profile of PC biosynthesis mutants after in vivo labeling with [^{14}C]acetic acid. *B. japonicum* strains were labeled with [^{14}C]acetate during growth in PSY medium. After extraction, lipids were separated by one-dimensional TLC. The lipids PC, DMPE, MMPE, and PE are indicated. The relative PC content (as the percentage of total phospholipids) is given below each PC spot.

(OD_{600} = 0.3), late-exponential (OD_{600} = 1.0), and stationary-phase (OD_{600} = 1.2) cultures, respectively. *B. japonicum* wild type, which does not encode an endogenous β-galactosidase, served as a negative control.

Neither *pmtX2*, *pmtX3*, and *pmtX4* nor the genes potentially forming an operon with *pmtX2* and *pmtX3* (*bll6635* and *bll8165*, respectively) were expressed in *B. japonicum* (Fig. 4). This finding was confirmed by primer extension analysis (data not shown) and by recently deposited microarray data in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE8478). Consistent with our previous primer extension analysis (30), significant expression between 200 and 300 MU was detected for the *pmtA'-'lacZ* fusion. The only other expressed Pmt gene was *pmtX1*, with approximately 10 MU throughout the growth curve. The *trmU* gene, a potential tRNA methyltransferase that is located upstream of *pmtX1* (Fig. 3B) shows about 50 MU (Fig. 4). The *pcs* gene is only weakly (3 to 4 MU) expressed.

The *pmtA* gene is symbiotically important (30), and a main physiological signal during nodulation is a reduction in the cellular oxygen level (11, 12). Hence, we tested whether the expression of PC biosynthesis genes was altered under anaerobic conditions. In order to control the establishment of anaerobic conditions, a *nifH'-'lacZ* fusion was measured in parallel. Again, *pmtA*, *trmU*, and *pmtX1* were the only significantly expressed enzymes. An overall reduced expression of the *pmtA'-'lacZ* fusion under anaerobic growth (aerobically, 200 to 350 MU; anaerobically, 100 to 150 MU) might be due to some differences in the medium for aerobic and anaerobic cultivation (see Materials and Methods). Aerobic cultivation in YEM medium normally used for anaerobic cultivation resulted in similar reduction of β-galactosidase activity (data not shown). Similar to aerobic conditions, *pmtX2* and *pmtX3* were not expressed during oxygen deprivation (data not shown). The *pmtX4* and *pcs* fusions were not tested. At the time these fusions were obtained, recent microarray studies had shown that expression of these genes did not change under microaerobic conditions (NCBI GEO database; http://www.ncbi.nlm.nih.gov/geo/; accession number GSE8478).

Construction and phospholipid profiling of *B. japonicum* PC biosynthesis mutants. To examine the phenotypic importance of each putative PC biosynthesis gene, we constructed a series of individual deletion mutants (Fig. 3). Despite numerous attempts, the *pmtX1* gene could not be inactivated by a simple plasmid insertion strategy.

The lipid composition of *B. japonicum* wild type and all obtained PC biosynthesis mutants was examined after in vivo labeling with [1-^{14}C]acetate. The wild-type strain possesses PE (52.2% of total phospholipids) and PC (42.4%) as major phospholipids and contains only small amounts of MMPE (2.3%) and DMPE (1.8%) in the membrane (Fig. 5). Except for the *pmtA* mutant, all other strains showed a wild-type-like membrane composition, as one might expect from the expression data described above. The *pmtA* mutant, on the other hand, showed a marked decrease of PC and a concomitant increase of PE in the membrane (30.4 and 63.7% of total phospholipids, respectively), The MMPE and DMPE levels remained unchanged (2.5 and 1.8%).

Since the *pmtA* mutant still produced significant amounts of PC, we tested whether the alternative Pcs pathway or another Pmt enzyme might be upregulated in this strain. Since expression of *pmtX4* was strongly induced (Fig. 6), it appears that PmtX4 partially compensates for the loss of *pmtA* under these conditions.

Symbiotic performance of *B. japonicum pmt* and *pcs* mutants. Since disruption of the *pmtA* gene resulted in a severe nitrogen fixation defect (30), we tested whether any of the other mutants also showed reduced symbiotic properties. Plants infected with *B. japonicum* wild type showed nitrogen fixation activity of about 93.7 μmol of C_2H_4/h/g (dry weight) of nodule, whereas plants infected with a *nifH* mutant showed only 5% of the wild-type level (Fig. 7). All plants infected with *B. japonicum pmtX2*, *pmtX3*, *pmtX4*, or *pcs* mutants developed normal nodules with the typical red color indicative of the presence of leghemoglobin (data not shown) and showed wild-type levels of nitrogen fixation activity. Consistent with previous results, the *pmtA* mutant elicited defective nodules capable of only reduced nitrogen fixation activity. This indicates that

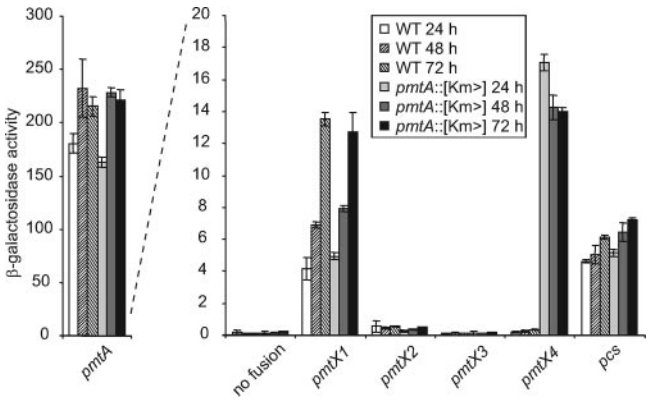

FIG. 6. Expression of the PC biosynthesis genes in the *pmtA* mutant compared to the wild type. The expression of chromosomally integrated, translational *lacZ* reporter gene fusions (see Fig. 3) was measured as described previously (see Materials and Methods). Samples were collected after 24, 48, and 72 h. The results of a typical experiment, in which two parallel cultures of each strain were grown and assayed in duplicate, are shown. Four independent experiments were performed with individual strains.

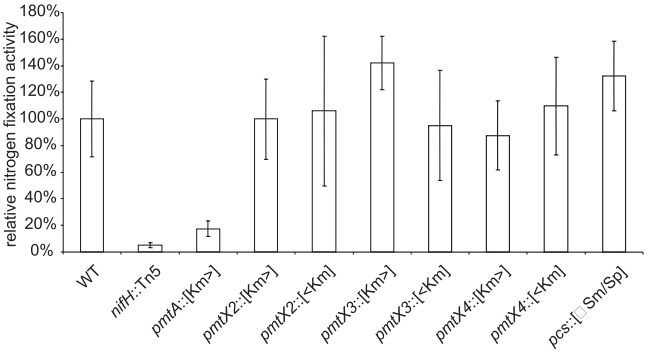

FIG. 7. Symbiotic phenotype of *B. japonicum pmt* and *pcs* mutants. The nitrogen fixation activity was measured as the amount of C_2H_2 reduced per hour per gram (dry weight) of nodule. A 100% wild-type (WT) activity corresponds to 93.7 μmol of C_2H_2/h/g. Mean values and standard deviations derive from at least eight individual plants, except for the *pcs* mutant with six individual plants.

the PmtA activity and/or the presence of sufficient PC are of importance for the symbiotic performance of *B. japonicum*.

DISCUSSION

All PC-synthesizing prokaryotes with an active methylation pathway that have been characterized thus far, such as *S. meliloti*, *R. sphaeroides*, or *A. tumefaciens*, possess only one Pmt enzyme capable of performing all three methylation steps (1a, 8, 45). Here, we report the presence of four functional Pmt enzymes with different substrate specificities in *B. japonicum*. The presence of more than one Pmt might be rare but is probably not exclusive to *B. japonicum*. In a bioinformatic survey, we identified four good Pmt candidates in the genome of *Rhodopseudomonas palustris*, which is closely related to *Bradyrhizobium*. Three putative Pmt-encoding genes are present in the genomes of *Rhizobium etli*, *R. leguminosarum*, and *Nitrobacter winogradsky*. Two putative Pmt enzymes are encoded in *Granulibacter bethesdensis*, *M. loti* MAFF303099, *Fulvimarina pelagi*, and *Aurantimonas* sp. (data not shown; genome sequences were accessed via the TIGR database; http://www.tigr.org/). The presence of two Pmts seems to be typical in lower eukaryotes, such as *Saccharomyces cerevisiae* or *Neurospora crassa*, which possess two different Pmt enzymes with distinct substrate specificities (6, 24). The presence of multiple *pmt* genes possibly reflects a need to adjust the membrane composition in response to environmental changes.

Why are there so many *pmt* genes in some organisms but not in others? We studied the complexity of prokaryotic PC biosynthesis using *B. japonicum* as a model organism. Apart from PmtA, at least one additional enzyme is required since PmtA alone produces predominantly MMPE. PmtX1 was shown to be responsible for converting MMPE via the DMPE intermediate to PC. Only *pmtA* and *pmtX1* genes were significantly expressed in *B. japonicum* under all conditions tested. However, expression experiments with *pmtX2*, *pmtX3*, or *pmtX4* in *E. coli* show that at least PmtX3 and PmtX4 function as Pmt enzymes. The fact that *B. japonicum* retains these Pmts intact suggests that there is some physiological demand for them. Otherwise, cryptic genes tend to accumulate missense mutations, as was shown for a member of the small heat shock gene family in *Bradyrhizobium* sp. (*Parasponia*) (33). *B. japonicum* is notorious for encoding multigene families such as the sigma factors RpoH1 to RpoH3 (32), the *groESL*-like chaperonin family (13), small heat shock proteins (31), and the transcription activators FixK1 and FixK2 (28). Members of these families often have overlapping functions but differ in the way they are regulated.

Little is known about regulation of PC biosynthesis in prokaryotes. It is unclear under which conditions, if at all, *pmtX2*, *pmtX3*, and *pmtX4* are expressed in *B. japonicum*. In contrast to the *pmtA* mutant, deletion of these genes did not confer a symbiotic defect, indicating that they do not play an essential role in plant-microbe interaction under the conditions tested. Accordingly, anaerobic conditions did not induce expression of the *pmtX* genes. Expression of *pmtA* was affected by the medium composition rather than the oxygen supply. This seems to contradict results from a previous study showing a decrease in PC levels under anaerobic conditions and suggesting a possible oxygen-dependent regulation of PC biosynthesis gene expression (42). However, both the medium ingredients and the cultivation conditions were different in both studies. There is more evidence that external conditions and the nutrient supply influence PC biosynthesis in *B. japonicum*. The amount of *pmtA* transcripts was reduced three- to fourfold under heat shock conditions (30). Recent experiments with *Bradyrhizobium* SEMIA 6144 showed that PC amounts were decreased by heat stress and increased with salinity (27). Finally, a microarray analysis of an *irr* mutant lacking the iron-responsive regulator Irr showed that *pmtX1* (and its upstream *trmU* gene) was downregulated approximately threefold under iron-limited conditions compared to the parental strain (47). This result was reproduced with our *pmtX1'-'lacZ* fusion (data not shown). Interestingly, transcription of the gene for the phosphatidylglycerophosphate synthase (*pgsA*), catalyzing the first reaction from CDP-DAG to PG, was decreased 2.6-fold under the same conditions (47). These findings suggest a possible

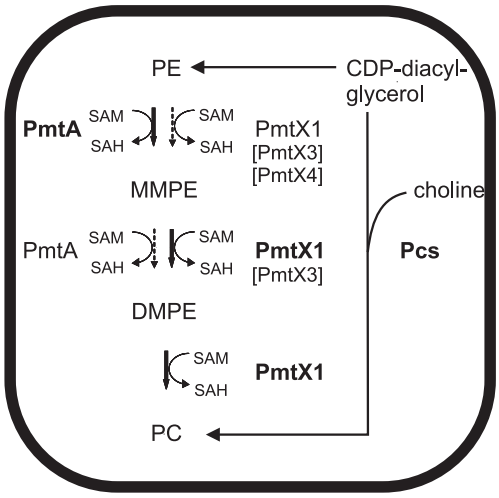

FIG. 8. Model of PC biosynthesis in *B. japonicum*. The model is based on the phospholipid profiles after expression of each enzyme in *E. coli* and integrates the corresponding gene expression data from *B. japonicum* grown aerobically in complex culture medium. Thick arrows and boldface letters indicate the predominant reaction(s) performed by each enzyme. Enzymes in brackets are not expressed in B. *japonicum* wild type but are functional when expressed in *E. coli*. SAH, *S*-adenosylhomocysteine.

regulatory connection between iron availability and phospholipid biosynthesis in *B. japonicum*.

Although it has been reported that *B. japonicum* is unable to take up choline (3), it expresses a functional Pcs, which requires choline as precursor. A previous study already showed Pcs activity in cell extracts of *B. japonicum* (26). Expression of the *pcs* gene was low but significant under all conditions tested. As speculated for *A. tumefaciens* and *B. abortus* (5, 20), *B. japonicum* might be able to synthesize choline.

On the basis of our analysis we propose an updated model for PC biosynthesis in *B. japonicum* (Fig. 8). The previously postulated Pmt activity catalyzing the second and third methylation step was shown to derive from PmtX1. PC biosynthesis is achieved by the concerted action of PmtA and PmtX1. Although PmtX4 and PmtX3 are able to perform the initial one or two methylation reactions when expressed in *E. coli*, their role in *B. japonicum* is not clear. The conditions under which they come into play remain to be elucidated. In an alternative route, PC can be synthesized in a one-step reaction by the Pcs pathway. One interesting avenue for future studies will be to study the mechanistic differences between the various Pmts biochemically, especially in cases in which closely related sequences produce different product spectra.

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REFERENCES

1. **Alexeyev, M. F.** 1995. Three kanamycin resistance gene cassettes with different polylinkers. BioTechniques **18:**52–56.
1a. **Arondel, V., C. Benning, and C. R. Somerville.** 1993. Isolation and functional expression in *Escherichia coli* of a gene encoding phosphatidylethanolamine methyltransferase (EC 2.1.1.17) from *Rhodobacter sphaeroides*. J. Biol. Chem. **268:**16002–16008.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. **37:**911–917.
3. **Boncompagni, E., M. Osterås, M. C. Poggi, and D. le Rudulier.** 1999. Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection. Appl. Environ. Microbiol. **65:**2072–2077.
4. **Comerci, D. J., S. Altabe, D. de Mendoza, and R. A. Ugalde.** 2006. *Brucella abortus* synthesizes phosphatidylcholine from choline provided by the host. J. Bacteriol. **188:**1929–1934.
5. **Conde-Alvarez, R., M. J. Grilló, S. P. Salcedo, M. J. de Miguel, E. Fugier, J. P. Gorvel, I. Moriyón, and M. Iriarte.** 2006. Synthesis of phosphatidylcholine, a typical eukaryotic phospholipid, is necessary for full virulence of the intracellular bacterial parasite *Brucella abortus*. Cell. Microbiol. **8:**1322–1335.
6. **Crocken, B. J., and J. F. Nyc.** 1964. Phospholipid variations in mutant strains of *Neurospora crassa*. J. Biol. Chem. **239:**1727–1730.
7. **Daniel, R. M., and C. A. Appleby.** 1972. Anaerobic-nitrate, symbiotic and aerobic growth of *Rhizobium japonicum*: effects on cytochrome P 450, other haemoproteins, nitrate and nitrite reductases. Biochim. Biophys. Acta **275:**347–354.
8. **de Rudder, K. E. E., I. M. López-Lara, and O. Geiger.** 2000. Inactivation of the gene for phospholipid *N*-methyltransferase in *Sinorhizobium meliloti*: phosphatidylcholine is required for normal growth. Mol. Microbiol. **37:**763–772.
9. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
10. **Exton, J. H.** 1994. Phosphatidylcholine breakdown and signal transduction. Biochim. Biophys. Acta **1212:**26–42.
11. **Fischer, H. M.** 1996. Environmental regulation of rhizobial symbiotic nitrogen fixation genes. Trends Microbiol. **4:**317–320.
12. **Fischer, H. M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
13. **Fischer, H. M., M. Babst, T. Kaspar, G. Acuña, F. Arigoni, and H. Hennecke.** 1993. One member of a *groESL*-like chaperonin multigene family in *Bradyrhizobium japonicum* is coregulated with symbiotic nitrogen fixation genes. EMBO J. **12:**2901–2912.
14. **Goldfine, H.** 1984. Bacterial membranes and lipid packing theory. J. Lipid Res. **25:**1501–1507.
15. **Göttfert, M., S. Hitz, and H. Hennecke.** 1990. Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. Mol. Plant-Microbe Interact. **3:**308–316.
15a. **Gubler, M., and H. Hennecke.** 1988. Regulation of the *fixA* gene and *fixBC* operon in *Bradyrhizobium japonicum*. J. Bacteriol. **170:**1205–1214.
16. **Hahn, M., and H. Hennecke.** 1984. Localized mutagenesis in *Rhizobium japonicum*. Mol. Gen. Genet. **193:**46–52.
16a. **Hahn, M., L. Meyer, D. Studer, B. Regensburger, and H. Hennecke.** 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. **3:**159–168.
16b. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
17. **Haydock, S. F., J. A. Dowson, N. Dhillon, G. A. Roberts, J. Cortes, and P. F. Leadlay.** 1991. Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*: sequence similarities between EryG and a family of *S*-adenosylmethionine-dependent methyltransferases. Mol. Gen. Genet. **230:**120–128.
18. **Ingrosso, D., A. V. Fowler, J. Bleibaum, and S. Clarke.** 1989. Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. Common sequence motifs for protein, DNA, RNA, and small molecule *S*-adenosylmethionine-dependent methyltransferases. J. Biol. Chem. **264:**20131–20139.
19. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**189–197.
20. **Karnezis, T., H. C. Fisher, G. M. Neumann, B. A. Stone, and V. A. Stanisich.** 2002. Cloning and characterization of the phosphatidylserine synthase gene of *Agrobacterium* sp. strain ATCC 31749 and effect of its inactivation on production of high-molecular-mass (1→3)-β-D-glucan (curdlan). J. Bacteriol. **184:**4114–4123.
21. **Kennedy, E. P.** 1989. Discovery of the pathways for the biosynthesis of

phosphatidylcholine, p. 1–9. *In* D. E. Vance (ed.), Phosphatidylcholine metabolism, vol. 249. CRC Press, Boca Raton, FL.
22. **Kent, C.** 1995. Eukaryotic phospholipid biosynthesis. Annu. Rev. Biochem. **64:**315–343.
23. **Kent, C., P. Gee, S. Y. Lee, X. Bian, and J. C. Fenno.** 2004. A CDP-choline pathway for phosphatidylcholine biosynthesis in *Treponema denticola*. Mol. Microbiol. **51:**471–481.
24. **Kodaki, T., and S. Yamashita.** 1987. Yeast phosphatidylethanolamine methylation pathway: cloning and characterization of two distinct methyltransferase genes. J. Biol. Chem. **262:**15428–15435.
25. **López-Lara, I. M., C. Sohlenkamp, and O. Geiger.** 2003. Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. Mol. Plant-Microbe Interact. **16:**567–579.
26. **Martínez-Morales, F., M. Schobert, I. M. López-Lara, and O. Geiger.** 2003. Pathways for phosphatidylcholine biosynthesis in bacteria. Microbiology **149:** 3461–3471.
27. **Medeot, D. B., M. A. Bueno, M. S. Dardanelli, and M. G. de Lema.** 2007. Adaptational changes in lipids of *Bradyrhizobium* SEMIA 6144 nodulating peanut as a response to growth temperature and salinity. Curr. Microbiol. **54:**31–35.
28. **Mesa, S., H. Hennecke, and H. M. Fischer.** 2006. A multitude of CRP/FNR-like transcription proteins in *Bradyrhizobium japonicum*. Biochem. Soc. Trans. **34:**156–159.
29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. **Minder, A. C., K. E. de Rudder, F. Narberhaus, H. M. Fischer, H. Hennecke, and O. Geiger.** 2001. Phosphatidylcholine levels in *Bradyrhizobium japonicum* membranes are critical for an efficient symbiosis with the soybean host plant. Mol. Microbiol. **39:**1186–1198.
30a. **Minder, A. C., H. M. Fischer, H. Hennecke, and F. Narberhaus.** 2000. Role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum*. J. Bacteriol. **182:**14–22.
31. **Münchbach, M., A. Nocker, and F. Narberhaus.** 1999. Multiple small heat shock proteins in rhizobia. J. Bacteriol. **181:**83–90.
32. **Narberhaus, F., M. Kowarik, C. Beck, and H. Hennecke.** 1998. Promoter selectivity of the *Bradyrhizobium japonicum* RpoH transcription factors in vivo and in vitro. J. Bacteriol. **180:**2395–2401.
33. **Nocker, A., N. P. Krstulovic, X. Perret, and F. Narberhaus.** 2001. ROSE elements occur in disparate rhizobia and are functionally interchangeable between species. Arch. Microbiol. **176:**44–51.
33a. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
34. **Raetz, C. R. H., and W. Dowhan.** 1990. Biosynthesis and function of phospholipids in *Escherichia coli*. J. Biol. Chem. **265:**1235–1238.
35. **Regensburger, B., and H. Hennecke.** 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. **135:**103–109.
36. **Ridgway, N. D., and D. E. Vance.** 1987. Purification of phosphatidylethanolamine N-methyltransferase from rat liver. J. Biol. Chem. **262:**17231–17239.
37. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37a. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
38. **Sohlenkamp, C., K. E. E. de Rudder, V. Röhrs, I. M. López-Lara, and O. Geiger.** 2000. Cloning and characterization of the gene for phosphatidylcholine synthase. J. Biol. Chem. **275:**18919–18925.
39. **Sohlenkamp, C., I. M. López-Lara, and O. Geiger.** 2003. Biosynthesis of phosphatidylcholine in bacteria. Prog. Lipid Res. **42:**115–162.
40. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
41. **Tahara, Y., T. Yamashita, A. Sogabe, and Y. Ogawa.** 1994. Isolation and characterization of *Zymomonas mobilis* mutant defective in phosphatidylethanolamine *N*-methyltransferase. J. Gen. Appl. Microbiol. **40:**389–396.
42. **Tang, Y., and R. I. Hollingsworth.** 1998. Regulation of lipid synthesis in *Bradyrhizobium japonicum*: low oxygen concentrations trigger phosphatidylinositol biosynthesis. Appl. Environ. Microbiol. **64:**1963–1966.
43. **Vance, D. E., and N. D. Ridgway.** 1988. The methylation of phosphatidylethanolamine. Prog. Lipid Res. **27:**61–79.
43a. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
44. **Wang, X. G., J. P. Scagliotti, and L. T. Hu.** 2004. Phospholipid synthesis in *Borrelia burgdorferi*: BB0249 and BB0721 encode functional phosphatidylcholine synthase and phosphatidylglycerolphosphate synthase proteins. Microbiology **150:**391–397.
45. **Wessel, M., S. Klüsener, J. Gödeke, C. Fritz, S. Hacker, and F. Narberhaus.** 2006. Virulence of *Agrobacterium tumefaciens* requires phosphatidylcholine in the bacterial membrane. Mol. Microbiol. **62:**906–915.
46. **Wilderman, P. J., A. I. Vasil, W. E. Martin, R. C. Murphy, and M. L. Vasil.** 2002. *Pseudomonas aeruginosa* synthesizes phosphatidylcholine by use of the phosphatidylcholine synthase pathway. J. Bacteriol. **184:**4792–4799.
47. **Yang, J., I. Sangwan, A. Lindemann, F. Hauser, H. Hennecke, H. M. Fischer, and M. R. O'Brian.** 2006. *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. Mol. Microbiol. **60:**427–437.