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Phosphatidylethanolamine Is Not Essential for Growth of *Sinorhizobium meliloti* on Complex Culture Media

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In addition to phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylethanolamine (PE), *Sinorhizobium meliloti* also possesses phosphatidylcholine (PC) as a major membrane lipid. The biosynthesis of PC in *S. meliloti* can occur via two different routes, either via the phospholipid N-methylation pathway, in which PE is methylated three times in order to obtain PC, or via the phosphatidylcholine synthase (Pcs) pathway, in which choline is condensed with CDP-diacylglycerol to obtain PC directly. Therefore, for *S. meliloti*, PC biosynthesis can occur via PE as an intermediate or via a pathway that is independent of PE, offering the opportunity to uncouple PC biosynthesis from PE biosynthesis. In this study, we investigated the first step of PE biosynthesis in *S. meliloti* catalyzed by phosphatidylserine synthase (PssA). A sinorhizobial mutant lacking PE was complemented with an *S. meliloti* gene bank, and the complementing DNA was sequenced. The gene coding for the sinorhizobial phosphatidylserine synthase was identified, and it belongs to the type II phosphatidylserine synthases. Inactivation of the sinorhizobial *pssA* gene leads to the inability to form PE, and such a mutant shows a greater requirement for bivalent cations than the wild type. A sinorhizobial PssA-deficient mutant possesses only PG, CL, and PC as major membrane lipids after growth on complex medium, but it grows nearly as well as the wild type under such conditions. On minimal medium, however, the PE-deficient mutant shows a drastic growth phenotype that can only partly be rescued by choline supplementation. Therefore, although choline permits Pcs-dependent PC formation in the mutant, it does not restore wild-type-like growth in minimal medium, suggesting that it is not only the lack of PC that leads to this drastic growth phenotype.

Rhizobia are soil bacteria that are able to form a symbiosis with legume plants that leads to the formation of nitrogen-fixing root nodules. The formation and functioning of this symbiosis are based on specific recognition of signal molecules, which are produced by both the bacterium and the plant partner. Recognition factors of the bacterial endosymbiont include lipochitin oligosaccharides or nodulation (Nod) factors, extracellular polysaccharides, lipopolysaccharides, K-antigens, and cyclic glucans (55), and these factors are required for nodule formation, the infection process, and the colonization of the root nodule. Recently it was demonstrated that adequate levels of certain bacterial membrane lipids, i.e., phosphatidylcholine (PC), also are required in order to allow the formation of a fully functional symbiosis between *Bradyrhizobium japonicum* and its host plant soybean (38). Under conditions of phosphate limitation, phosphorus-free membrane lipids (sulfolipids, ornithine-containing lipids, and diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine lipids) are formed in rhizobia (22). Rhizobial mutants lacking the ability to form any one of these phosphorus-free membrane lipids form effective nitrogen-fixing root nodules (31), demonstrating that not all major bacterial membrane lipids are required for the onset of a successful symbiosis.

Escherichia coli is the prokaryote with the best-studied membrane lipid biosynthesis. For *E. coli* three major membrane phospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL), are present. Certain functions have been defined for specific membrane phospholipids in *E. coli*. Anionic phospholipids (PG and CL) are involved in the initiation of DNA replication (64) and in the translocation of outer membrane precursor proteins (28). The zwitterionic PE is essential for proper functioning of the electron transfer chain (36), for the assembly and function of lactose permease (4, 6), and for motility and chemotaxis (51).

The membrane lipid composition in *Sinorhizobium meliloti* is more complex. In addition to PE, PG, and CL, *S. meliloti* membranes possess PC as a major membrane lipid, as well as monomethyl PE (MMPE) and dimethyl PE (DMPE) as minor membrane lipids. Two pathways for PC biosynthesis exist for *S. meliloti*. PC can be synthesized by the phospholipid N-methylation (Pmt) pathway from PE (12), or it can be synthesized by the PC synthase (Pcs) pathway directly from free choline and CDP-diacylglycerol (13, 52). Therefore, PC biosynthesis in this organism can happen via PE as an intermediate or in the alternative Pcs pathway in a PE-independent manner, offering the opportunity to uncouple PC biosynthesis from PE biosynthesis. Previously we described *S. meliloti* mutants deficient in the Pmt pathway as well as the Pcs pathway and therefore completely lacking PC (12). These mutants were not only strongly affected in their vegetative functions but also lacked the ability to form nitrogen-fixing root nodules on their host plant alfalfa (12, 53).

The first committed step in the biosynthesis of PE is cata-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
S. meliloti 1021	SU47 *str-21*	34
Sm 1021 derivatives		
KDR309	PmtA-deficient chemical mutant	14
KDR310	PE-deficient chemical mutant	14; this work
CS111	*pssA* gene replaced with a gentamicin resistance cassette	This work
E. coli		
DH5α	*recA*1 ϕ80 *lacZΔM15*	24
BL21(DE3)/pLysS	Expression strain	57
Plasmids		
pLAFR3	Cosmid vector, tetracycline resistant	56
pRK2013	Helper plasmid, kanamycin resistant	20
pRK404	Broad-host-range vector, tetracycline resistant	15
pBBR1MCS-3	Broad-host-range vector, tetracycline resistant	27
pACΩ-Gm	Broad-host-range vector containing gentamicin resistance cassette	49
pET3a	Expression vector, carbenicillin resistant	57
pET9a	Expression vector, kanamycin resistant	57
pK18*mobsacB*	Suicide vector, kanamycin resistant	47
pMP3510	Broad-host-range vector, tetracycline resistant	54
pUC18/pUC19	Cloning vectors, carbenicillin resistant	65
pCOS9.4	*pssA*-containing DNA of *S. meliloti* in pLAFR3	This work
pTB2009	*pssA*-containing 6.6-kb *Bam*HI/*Bam*HI insert in pRK404	This work
pTB2036	*pssA*-containing 2.1-kb *Bam*HI/*Hin*dIII insert in pRK404	This work
pTB2086	*psd*-containing *Hin*dIII/*Bcl*I insert in pRK404	This work
pTB2104	*pssA*-containing *Eco*RI/*Bam*HI-insert in pMP3510	This work
pTB2112	*pssA* gene of *S. meliloti* in pMP3510	This work
pTB2559	*pcs* gene of *S. meliloti* in pET9a	52
pTB2919	*pssA* gene of *S. meliloti* in pET9a	This work

lyzed by phosphatidylserine synthase (Pss). A gene coding for the Pss enzyme (*pssA*) has been found and cloned in prokaryotes (10, 21, 41), lower eukaryotes like *Saccharomyces cerevisiae* (29, 40), and plants (11). Pss is responsible for the formation of phosphatidylserine from CDP-diacylglycerol and serine (EC 2.7.8.8). In a subsequent step, phosphatidylserine is decarboxylated by phosphatidylserine decarboxylase (Psd) to yield PE.

Genetic and biochemical studies have revealed the existence of two different types of Pss. Subclass I Pss, the prototype of which is the Pss from *E. coli*, was thought to occur primarily in gram-negative bacteria (32). Members of the type I subclass are soluble enzymes that seem to be tightly associated with the ribosomal fraction (44). Subclass I Pss are part of a protein superfamily that furthermore includes CL synthases, poxvirus envelope proteins, phospholipases D, and nucleases (26). In contrast, subclass II Pss, like the *Bacillus subtilis* Pss or the *S. cerevisiae* Pss, are not related on a sequence level to type I Pss and are predicted to be integral membrane proteins. Subclass II Pss are related to phosphatidylinositol synthases, phosphatidylglycerolphosphate synthases, and Pcs (53). So far, *pssA* genes have been cloned or characterized from *E. coli*, *B. subtilis*, *S. cerevisiae*, *Helicobacter pylori*, *Agrobacterium* sp. strain ATCC 31749, and *Tritium aestivum* (wheat). However, a detailed mutant characterization with respect to lipid pattern and growth has been performed only with the mutant of type I Pss from *E. coli*.

Here we describe the functional complementation of a PE-deficient chemical mutant of *S. meliloti* with a sinorhizobial gene bank and the isolation of the sinorhizobial gene (*pssA*) coding for a type II Pss. A mutant in which the sinorhizobial *pssA* gene is replaced with an antibiotic marker was constructed, and we show that PE is not essential for growth of *S. meliloti* in complex medium. However, on minimal media, PE-deficient mutants of *S. meliloti* show severe growth defects that can only partially be rescued by a functional PC biosynthesis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used and their relevant characteristics are shown in Table 1. *S. meliloti* strains were grown at 30°C either in complex tryptone yeast extract (TY) medium containing 4.5 mM $CaCl_2$ (2) or in morpholinepropanesulfonic acid (MOPS) minimal medium using glucose (15 mM) as a carbon source (1). Choline chloride (Sigma) was added to the minimal medium in the amounts indicated. For determination of growth rates, strains were first cultivated on TY medium containing 4.5 mM $CaCl_2$ before being resuspended at cell densities of 9×10^7 cells ml^{-1} in TY medium containing $CaCl_2$ concentrations varying between 0 and 50 mM and grown for more than three generations per subcultivation. The growth rates of strains were determined during the third subcultivation in TY medium with the respective calcium concentration.

E. coli strains were cultured on Luria-Bertani medium at 37°C. Antibiotics were added to the media at the following final concentrations when required (in micrograms per milliliter): 500 for streptomycin, 200 for neomycin, 70 for gentamicin, 20 for piperacillin, 4 for tetracycline for *S. meliloti*, and 100 for carbenicillin, 10 for gentamicin, and 20 for tetracycline for *E. coli*.

Cosmids, pRK404, pMP3510, and pK18*mobsacB* derivatives were mobilized into *S. meliloti* strains by triparental mating using the mobilizing plasmid pRK2013 as described previously (45).

DNA manipulations. Recombinant DNA techniques were performed according to standard protocols (46). DNA was sequenced by the dideoxy-mediated chain termination method using an SQ3 sequencer (Hoefer) and pUC19 derivatives.

Functional complementation of PE-deficient mutant *S. meliloti* KDR310. Cosmids of a sinorhizobial gene bank (52) were mobilized into mutant KDR310 (14), and transconjugants were selected for wild-type-like growth on minimal medium. From colonies able to grow like the wild type on minimal medium, cosmids were isolated and transformed in *E. coli* DH5α, and the respective DNA inserts were analyzed.

In vivo labeling of *S. meliloti* with [^{14}C]acetate and quantitative analysis of lipid extracts. The lipid compositions of *S. meliloti* 1021 wild-type and mutant strains were determined following labeling with [1-^{14}C]acetate (Amersham). Cultures (1 ml) of wild-type and mutant strains in TY medium were inoculated from precultures grown in the same medium. After addition of 0.4 μCi of [^{14}C]acetate (60 nCi/nmol) to each culture, the cultures were incubated for either 4 or 16 h. The cells were harvested by centrifugation, washed with 500 μl of water, and resuspended in 100 μl of water. The lipids were extracted according to the method of Bligh and Dyer (3). The chloroform phase was used for lipid analysis on thin-layer chromatography (TLC) plates (high-performance TLC aluminum sheets, silica gel 60; Merck), and after one-dimensional or two-dimensional separation using the solvent systems described previously (14), the individual lipids were quantified as described previously (14) or by using a PhosphorImager (Storm 820; Molecular Dynamics).

Cloning of the Pss gene (*pssA*) of *S. meliloti* for complementation and expression studies. The oligonucleotide primers oTBL235 (5′-gAg gAA TTC ATA Tgg AAg AgC CCC g-3′) and oTBL236 (5′-AgA AgC TTg gAT CCT ATT CgC TAT CAC CCg-3′), introducing *Eco*RI/*Nde*I sites and *Hin*dIII/*Bam*HI sites (underlined), respectively, were used in the PCR to amplify the sinorhizobial *pssA* gene using the plasmid pTB2036 as a template. After digestion with *Eco*RI and *Hin*dIII, the PCR product was cloned into pUC19 to yield the plasmid pTB2071. The latter was digested with *Nde*I and *Bam*HI, and the *pssA*-containing fragment was recloned into pET3a to yield pTB2081. The plasmid pTB2081 was digested with *Xba*I and *Bam*HI, and the *pssA* gene was recloned into the *Xba*I/*Bam*HI sites of pMP3510 to yield pTB2112, a plasmid used for complementation studies with *S. meliloti*. Alternatively, pTB2081 was digested with *Nde*I and *Bam*HI, and the *pssA* gene was recloned into the *Nde*I/*Bam*HI sites of pET9a to obtain pTB2919, a plasmid used for expression of sinorhizobial PssA in *E. coli*.

Exchange of the sinorhizobial *pssA* gene with a gentamicin resistance cassette. The oligonucleotide primers opss5 (5′-ACg TTC TAg ATg gAg gCg AAg CgC TTC gAC AAg TCg ATg gAg C-3′) and opss6 (5′-ACg TgC CCg ggC CTT gTC gTg CgT gCC ggT ggC g-3′) were used in the PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.8 kb of genomic DNA upstream of the putative *pssA* gene from *S. meliloti*, introducing *Xba*I and *Sma*I sites (underlined) into the PCR product. Similarly, the primers opss7 (5′-ACg TCC Cgg gAg ACg ATg gTC gTg ACT gCg gTg gCC TAT C-3′) and opss8 (5′-ACg TCT gCA ggA AAg Cgg CAT CCC CTT CgA gCT Cgg CAT C-3′) were used to amplify about 1.8 kb of genomic DNA downstream of the *pssA* gene, introducing *Sma*I and *Pst*I sites (underlined) into this PCR product. After digestion with the respective enzymes, the PCR products were cloned as *Xba*I/*Sma*I or *Sma*I/*Pst*I fragments into pUC18 to yield the plasmids pCCS01 and pCCS02, respectively. Then, the *Xba*I/*Sma*I fragment from pCCS01 was recloned into pBBR1MCS-3 (27) to yield pCCS03. The plasmid pCCS02, linearized with *Sma*I, was cloned into the *Sma*I site of pCCS03. The plasmid containing the two sinorhizobial genomic fragments adjacent was named pCCS04. pCCS04 was digested with *Pst*I, the pUC18 backbone was cut out, and the rest of pCCS04 was religated to yield pCCS04a. The plasmid pCCS04a was digested with *Sma*I, and the gentamicin resistance cassette derived from pACΩ-Gm (49) was cloned as a *Sma*I fragment into pCCS04a to yield pCCS04b. The plasmid pCCS04b was digested with *Pst*I and *Xba*I to reclone the regions usually flanking the sinorhizobial *pssA* gene and the gentamicin resistance gene located in between those regions as a *Pst*I/*Xba*I fragment into the suicide vector pK18*mobsacB* (47) to yield pCCS05. Via triparental mating using pRK2013 as a helper plasmid, pCCS05 was introduced into the wild-type strain *S. meliloti* 1021. Transconjugants were selected on TY medium containing neomycin and gentamicin to select for single recombinants in a first step. pK18*mobsacB* contains the *sacB* gene (50), which confers sucrose sensitivity to many bacteria. Growth of the single recombinants on high levels of sucrose will therefore select for a further recombination and the loss of the vector backbone of pK18*mobsacB* from the bacterial genome. Single recombinants were grown under nonselective conditions in complex medium for 1 day before being plated on TY medium containing 10% (wt/vol) sucrose and gentamicin. Several large and small colonies grew after 5 days, and the membrane lipids of 16 candidates were analyzed by in vivo labeling during growth on complex medium with [^{14}C]acetate and subsequent TLC (data not shown). Six clones lacking PE were identified. Southern blot analysis confirmed that the PE-deficient strains were indeed double recombinants in which the *pssA* gene was replaced with a gentamicin resistance cassette (data not shown). Using PCR, the orientation of the gentamicin resistance cassette was determined, and in CS111 the cassette has the same orientation as the replaced *pssA* gene.

Determination of phospholipid methyltransferase activity in the presence or absence of added PE. Cultures (30 ml) of mutant or wild-type strains were grown to an optical density at 620 nm of 0.6. Cells were harvested by centrifugation for 10 min at 5,000 × *g*, and the pellets obtained were resuspended in 3 ml (total volume) of ice-cold reaction buffer (100 mM Tris-HCl [pH 9.5]). The cell suspension was passed three times through a French pressure cell at 20,000 lb/in^2. Unbroken cells and cell debris were removed by centrifugation at 4,000 × *g* for 4 min to obtain the cell extract. The protein concentration was determined by the method of Dulley and Grieve (17). The phospholipid methyltransferase activity was determined by quantifying the transfer of labeled methyl groups from *S*-adenosyl-L-methionine to chloroform-soluble material. Under the conditions chosen, methylated derivatives of PE were the only chloroform-soluble substances formed (14). When the effect of added PE was studied, first, per reaction tube, 100 μg of PE (L-α-phosphatidylethanolamine, dipalmitoyl; Sigma) in 10 μl of chloroform-methanol (1:1 [vol/vol]) was mixed with 3.6 μl of 1% Triton X-100 (wt/vol) and dried under vacuum before other components were added. Therefore, in a total of 180 μl in Eppendorf reaction tubes, the reaction mixture contained 100 μg of PE or no PE, 0.02% Triton X-100, 200 μg of sinorhizobial protein, 100 μg of bovine serum albumin, and 125 nCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (100 nCi/nmol) in 100 mM Tris-HCl (pH 9.5). The mixtures were incubated for 15 min in a 30°C water bath. The reactions were stopped by addition of 180 μl of ice-cold 20% (wt/vol) trichloroacetic acid, and precipitations were completed by a 10-min incubation on ice. The precipitates were pelleted by centrifugation (5 min, 14,000 × *g*), dissolved by the addition of 60 μl of 1 M Tris base, and neutralized by adding 40 μl of 20% (wt/vol) trichloroacetic acid, respectively. Lipids were extracted according to the method of Bligh and Dyer (3). The chloroform phases were dried, and aliquots of the redissolved lipids were counted in scintillation vials.

Determination of Pcs and Pss activities. *E. coli* BL21(DE3)/pLysS strains harboring one of the plasmids pTB2919, pTB2559, or pET9a were grown in Luria-Bertani medium containing the appropriate antibiotics. At a cell density of 5×10^8 cells/ml, isopropyl-β-D-thiogalactoside was added to a final concentration of 100 μM. After 4 h of induction, cells were harvested and stored at −20°C. The cell pellets were resuspended in 50 mM Tris-HCl, pH 8.0, and cell extracts were prepared as described above.

The optimized standard assay to determine Pcs activity (13) contained, in a total volume of 50 μl in Eppendorf tubes, 50 μg of protein, 50 mM Tris-HCl, pH 8, 10 mM $MnCl_2$, 20 μM CDP-DAG (cytidine 5′-diphospho-*sn*-glycerol, 1,2-dipalmitoyl; Sigma), 0.2% (wt/vol) Triton X-100, and 50 μM [*methyl*-^{14}C]choline (55 mCi/mmol; Amersham Biosciences). The mixture was incubated for 30 min at 30°C in a water bath, and the reaction was stopped by mixing with 188 μl of methanol-chloroform (2:1 [vol/vol]). Addition of 63 μl of chloroform and 63 μl of water led to phase separation, and after washing of the chloroform phase with another 100 μl of water, it was dried and an aliquot was analyzed using one-dimensional TLC. The Pss assay was performed in an identical way, except that [^{14}C]choline was replaced with equimolar amounts of [U-^{14}C]serine (151 mCi/mmol; Amersham Biosciences). In some Pss assays, 10 mM hydroxylamine, an inhibitor of Psd (7, 18, 61), was present in the reaction mixture.

Nucleotide sequence accession number. The nucleotide sequence (2,183 bp) of the *Hin*dIII-*Bam*HI fragment complementing the PE-deficient mutant KDR310 has been deposited in GenBank under accession number AF247564.

RESULTS

Identification of a PE-deficient mutant of *S. meliloti*. During our search for phospholipid *N*-methyltransferase-deficient mutants of *S. meliloti* using a colony autoradiography method (42), we had identified six chemically generated mutants that showed no phospholipid *N*-methyltransferase (Pmt) activity when the respective cell-free crude extracts were assayed (14). One-dimensional TLC of mutant lipid extracts demonstrated that none of the mutants was able to form MMPE or DMPE after growth on complex medium (data not shown). However, in one of the mutant extracts (KDR310), even another lipid seemed to be absent. To further compare the phospholipid patterns of the wild type and mutant KDR310, two-dimensional TLC analysis of the lipids was performed (Fig. 1). The *S.*

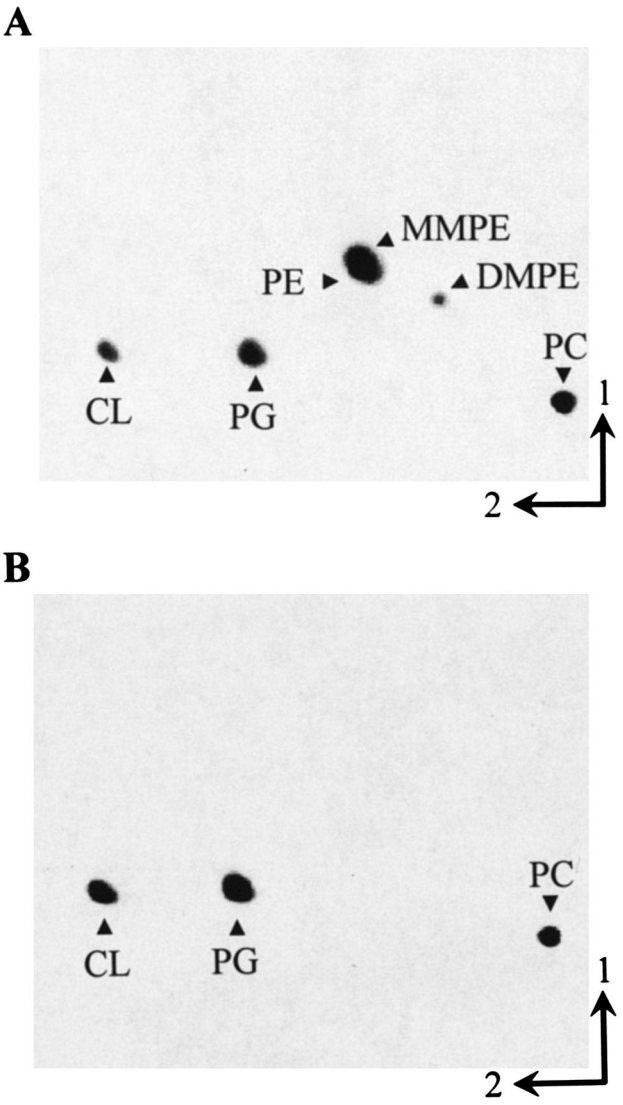


FIG. 1. Separation of [^{14}C]acetate-labeled lipids from *S. meliloti* 1021 wild type (A) and PE-deficient mutant KDR310 (B) by two-dimensional TLC. Phospholipids are indicated.

meliloti wild type possesses PG, CL, PE, MMPE, DMPE, and PC as membrane phospholipids (Fig. 1A). Mutant KDR310, however, has only PG, CL, and PC and lacks MMPE, DMPE, and PE (Fig. 1B). Staining of TLC plates with ninhydrin confirmed that in the case of the mutant KDR310, no ninhydrin-positive compound could be detected at the position where PE was expected (data not shown). The relative amounts of the individual phospholipids from the *S. meliloti* 1021 wild type and mutant KDR310 are shown in Table 2. In mutant KDR310, the relative amounts of all three remaining phospholipids are elevated, with a large increase in the relative quantities of the anionic phospholipids PG and CL (from 19 to 60%) and a smaller increase in the relative amount of PC (from 27 to 36%).

PE restores phospholipid *N*-methyltransferase activity in mutant KDR310. The absence of PE in membranes of mutant KDR310 and therefore the lack of one substrate could explain why we did not detect any Pmt activity in our usual assay that did not contain externally added PE (14). The effect of PE on Pmt activities of different sinorhizobial cell extracts was studied in the presence of the detergent Triton X-100 to ensure equal accessibility of added lipid (Table 3). When PE was added to *S. meliloti* wild-type extracts, the methyl groups transferred to lipid increased by a factor of 33.7. Addition of PE to the Pmt-deficient mutant KDR309 increased the amount of radiolabeled methyl groups incorporated into the lipid phase by a factor of 6.0. As no Pmt activity is present in KDR309, the stimulation of methyl group incorporation into the lipid phase might be due to cyclopropane synthases acting on the fatty acyl groups of PE (23). In the case of mutant KDR310, however, the stimulation of Pmt activity by PE was 27.1-fold (Table 3), suggesting that, as in the wild type, stimulation of methyl group incorporation into lipids by KDR310 extracts was much higher than by KDR309 extracts and that Pmt activity was present in KDR310. However, in KDR310, Pmt activity can only be detected when external PE is added.

TABLE 2. Phospholipid composition of *S. meliloti* 1021 (wild type) and PE-deficient mutant strain KDR310 after growth in complex medium

Lipid	Lipid composition (% of total ^{14}C)	
	Wild type	KDR310
PE + MMPE	44.6	0.3
DMPE	3.1	0.0
PC	26.9	35.8
PG	13.4	36.7
CL	5.5	23.0
Other	6.5	4.2

PE-deficient mutant KDR310 is defective in Pss. Formation of phosphatidylserine (PS) and PE by cell extracts of *S. meliloti* wild type and PE-deficient mutant KDR310 was studied by incorporation of [^{14}C]serine into lipid products (Fig. 2). With wild-type extracts, only PS was formed when hydroxylamine, an inhibitor of Psd activity, was present in the reaction mixture, demonstrating the presence of Pss activity in the *S. meliloti* wild type (lane 1). When hydroxylamine was omitted in the Pss assay, both PS and PE were formed in the crude cell extracts of the wild type (lane 3). For mutant KDR310, neither PS nor PE was formed under such conditions, demonstrating that Pss activity was absent in this mutant (lanes 2 and 4). As a control to verify that other activities were not impaired, we investi-

TABLE 3. Stimulation of in vitro phospholipid *N*-methyltransferase activity by added PE[a]

Strain	Incorporation of methyl groups (pmol/mg of protein/min)		
	Without PE	With PE	Stimulation (*x*-fold)
Wild type	0.71	23.91	33.7
KDR309	0.13	0.78	6.0
KDR310	0.23	6.24	27.1

[a] The assay for phospholipid *N*-methyltransferase was done with cell-free extracts of *S. meliloti* wild-type 1021, Pmt-deficient mutant KDR309, and PE-deficient mutant KDR310 after growth in complex medium.

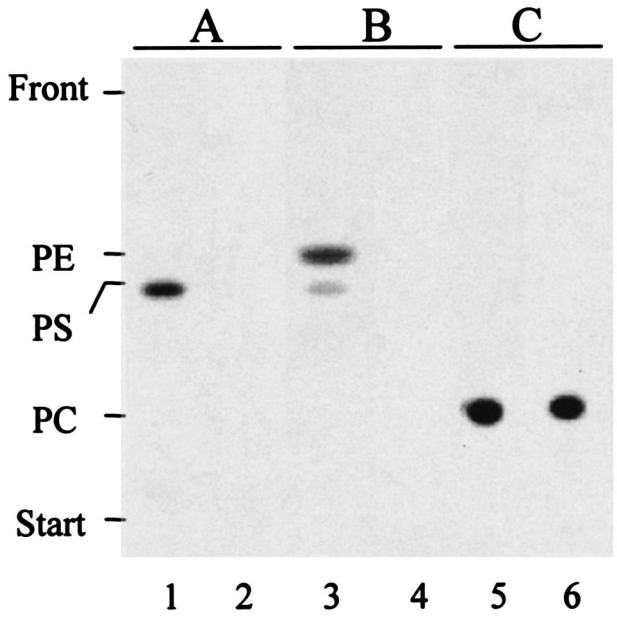


FIG. 2. Mutant KDR310 is defective in Pss activity. Lipid products obtained by in vitro activity tests for Pss in the presence of hydroxylamine (A) or without hydroxylamine (B) and for Pcs (C) were separated by one-dimensional TLC. Activity tests were performed with cell extracts from the *S. meliloti* wild-type strain (lanes 1, 3, and 5) and PE-deficient mutant strain, KDR310 (lanes 2, 4, and 6), that had been grown in complex medium.

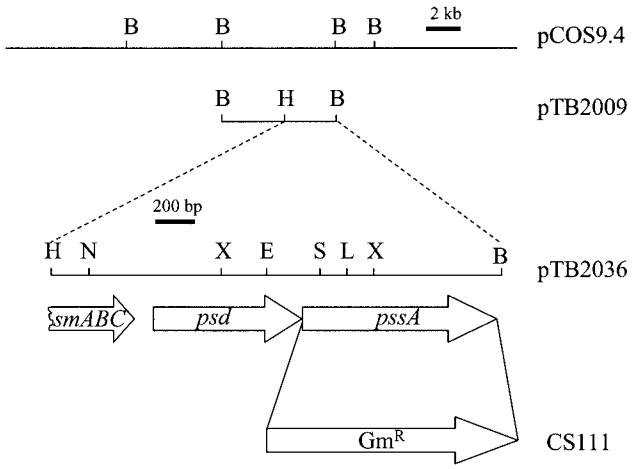


FIG. 3. Genomic region of *S. meliloti* 1021 complementing the PE-deficient mutant KDR310. Schematic representation of the genomic DNA insert of cosmid pCOS9.4 complementing PE deficiency. The subcloning strategy is shown with the 6.6-kb DNA region (pTB2009) and the 2.1-kb *Hin*dIII-*Bam*HI fragment that was sequenced (pTB2036). The location and direction of one partial ORF, encoding an ABC transporter-like protein (*smABC*), and two complete ORFs, encoding Psd (*psd*) and Pss (*pssA*), are shown by arrows. The replacement of the *pssA* gene by a gentamicin resistance cassette (Gm^R) in mutant CS111 is indicated. Restriction sites (B, *Bam*HI; L, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; S, *Sma*I; X, *Xho*I) used are shown.

gated the recently discovered Pcs activity (13, 52) and could show that both wild-type (lane 5) and mutant KDR310 (lane 6) extracts were able to incorporate radiolabeled choline into PC.

Complementation of *S. meliloti* mutants deficient in Pmt. During our search for Pmt-deficient mutants of *S. meliloti*, we had identified nine chemically generated mutants that showed no or reduced Pmt activity when the respective cell extracts were assayed (14). A cosmid, pCOS24.1, that was able to complement mutant KDR309 (12) also complemented most other mutants (KDR296, KDR304, KDR339, KDR345, KDR365) that were devoid of Pmt activity (data not shown). However, the PE-deficient mutant KDR310 was not complemented by pCOS24.1, indicating that genes complementing KDR310 would be of another complementation group. *E. coli* HB101, carrying a genomic library of *S. meliloti* that had been constructed in the cosmid vector pLAFR3 as described previously (52), was conjugated with *S. meliloti* KDR310. In contrast to the PE-deficient mutant KDR310, which hardly grew on minimal medium, complemented mutants should be able to do so, and we selected for transconjugant colonies able to grow like the wild type on minimal medium. From growth-complemented mutants, the cosmids were isolated and transformed into DH5α for further analysis. Three types of overlapping cosmids were able to complement the PE-deficient phenotype of mutant KDR310. One representative, cosmid pCOS9.4, was used for further studies (Fig. 3). Subcloning of restriction fragments, comprising regions of overlapping DNA, in the broad-host-range vector pRK404 and subsequent analysis for complementation of the PE-deficient mutant KDR310 shows that a plasmid (pTB2009) containing a 6.6-kb *Bam*HI-*Bam*HI DNA fragment is able to restore formation of PE, MMPE, and DMPE (data not shown). A subclone of the complementing DNA containing a 2.1-kb *Bam*HI-*Hin*dIII fragment in pRK404 (pTB2036) (Fig. 3) is also able to complement mutant KDR310 (Fig. 4, lane 4). The DNA sequence of the 2.1-kb *Bam*HI-*Hin*dIII fragment was determined, and analysis of the sequence (2,183 bp) revealed one partial open reading frame (ORF) and two complete ORFs (Fig. 3). The complementing 2.1-kb fragment was further subcloned to analyze which of the complete ORFs was able to complement the mutant KDR310. Plasmid pTB2104, containing a 1.1-kb *Eco*RI-*Bam*HI fragment including the second complete ORF, was able to complement

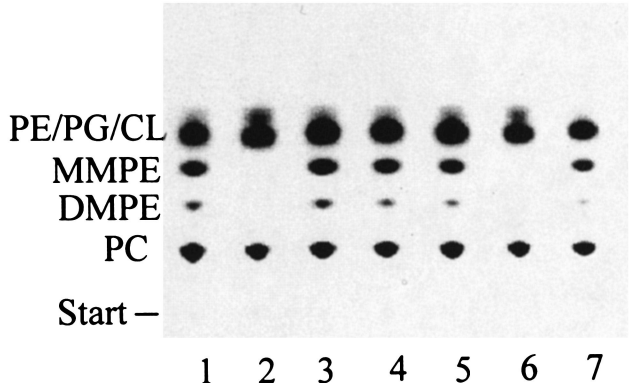


FIG. 4. Complementation of PE-deficient mutant KDR310. Lipids of *S. meliloti* strains containing different plasmids were radiolabeled with [^{14}C]acetate during growth in complex medium and separated by one-dimensional TLC. The following strains were analyzed: *S. meliloti* wild type 1021 (lane 1), PE-deficient mutant KDR310 (lane 2), and KDR310 containing either pCOS9.4 (lane 3), pTB2036 (lane 4), pTB2104 (lane 5), pTB2086 (lane 6), or pTB2112 (lane 7).

the PE-deficient mutant (Fig. 4, lane 5), whereas plasmid pTB2086, containing a 1.4-kb *Hin*dIII-*Bcl*I DNA fragment with the first complete ORF, was not able to do so (Fig. 4, lane 6). In order to confirm these results, the second complete ORF was amplified by PCR and cloned into pMP3510 (pTB2112). Expression of this second ORF complemented PE biosynthesis in KDR310 (Fig. 4, lane 7).

Gene bank searches with the NCBI BLAST program showed that the gene product of the partial ORF (positions 1 to 387) shows homology to members of the drug peptide and lipid export family (DPL) of ATP binding cassette (ABC) transporters (9). A relative of this partial ORF, the MsbA lipid flippase of *E. coli*, is involved in lipid export and presumably in flipping lipids from the inner layer to the outer layer of the cytoplasmic membrane (16), raising the question whether this sinorhizobial gene might be involved in lipid transport. The first complete ORF (positions 497 to 1192), encoding a protein of 232 amino acids, was found to be preceded by a putative ribosome binding site. The predicted protein sequence contains a conserved domain typical for Psd (COG0688), but it is only distantly related to the Psd proteins from *E. coli* (30), *B. subtilis* (33), and *S. cerevisiae* (8, 58, 59, 60) for which functions have been shown. Psd enzymes of the latter group contain a conserved amino acid pattern (L/G)GST for *E. coli* and *S. cerevisiae* that has been described as a motif where probably the proteolytic cleavage of Psd into α- and β-fragment and the formation of the pyruvoyl group occur (62). ORFs containing the conserved domain COG0688 fall into two quite different families, and whereas functions have been shown for several representatives of the above-mentioned family, for no member of the second family, to which the putative *S. meliloti* Psd belongs, has a function been demonstrated. In the *S. meliloti* Psd, the conserved amino acid pattern of the putative cleavage site is reduced to GS.

The second complete ORF follows immediately downstream of *psd*, suggesting that the two ORFs might form an operon. The potential gene product of the latter ORF (positions 1205 to 2071) encodes a protein of 289 amino acids that shows the typical motif [DG(X)2AR(X)8G(X)3D(X)3D] characteristic for CDP-alcohol phosphatidyltransferases (53, 63), like phosphatidylglycerolphosphate synthases, phosphatidylinositol synthases, amino alcohol phosphotransferases, and type II Pss. The best homologues to the latter ORF for which a function has been shown are the type II Pss from *Agrobacterium* sp. ATCC 31749 (60% identity, 70% similarity on amino acid level; accession number AAL01116) and *Helicobacter pylori* (31% identity, 47% similarity on amino acid level; G64653). Therefore, this second complete ORF probably encodes a type II Pss.

The identified *pssA* gene codes for a protein that has Pss activity but no Pcs activity. The sinorhizobial PssA protein is of the type II subgroup. Type II Pss share a conserved domain with Pcs, phosphatidylinositol synthases, and phosphatidylglycerolphosphate synthases (53). Due to this similarity, we wanted to investigate whether the sinorhizobial Pss enzyme would show Pcs activity in addition to the Pss activity and whether the Pcs enzyme would show Pss activity in addition to Pcs activity. Pss activity and Pcs activity were studied in cell-free crude extracts from *E. coli* BL21(DE3)/pLysS strains that had expressed either the *pssA* gene from *S. meliloti* or the *pcs* gene from *S. meliloti* or that were harboring the pET9a plasmid only. Neither Pcs activity nor Pss activity could be detected in the crude extract not expressing any sinorhizobial gene (Fig. 5), meaning that the type I *E. coli* Pss does not interfere with either the Pss assay or the Pcs assay (lanes 1 and 2). In the crude extract containing the Pcs of *S. meliloti*, only Pcs activity (lane 3) and no Pss activity (lane 4) could be detected, whereas in the crude extract containing the sinorhizobial Pss, no Pcs activity (lane 5) but only Pss activity (lane 6) was detected.

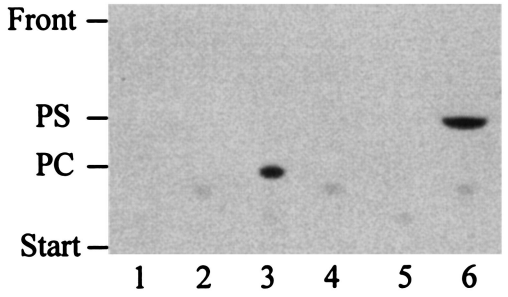


FIG. 5. Pss and Pcs do not overlap in their substrate specificities. Lipids formed during in vitro activity tests for Pcs and Pss were separated by one-dimensional TLC. Pcs (lanes 1, 3, and 5) or Pss (lanes 2, 4, and 6) activities were assayed with cell-free crude extracts from *E. coli* BL21(DE3)/pLysS strains expressing either no rhizobial gene when harboring pET9a only (lanes 1 and 2), *pcs* of *S. meliloti* from pTB2559 (lanes 3 and 4), or *pssA* from *S. meliloti* from pTB2919 (lanes 5 and 6).

Replacement of the sinorhizobial *pssA* gene with a gentamicin resistance cassette. When we tried to characterize the PE-deficient mutant KDR310 in more detail, we noticed that this chemical mutant reverted easily, thereby impeding a rigorous characterization. In order to be able to tackle the role(s) that PE plays in *S. meliloti*, we decided to substitute the whole *pssA* gene with a cassette conferring resistance to the antibiotic gentamicin. The genomic regions flanking the *pssA* gene from *S. meliloti* were amplified by PCR, and a gentamicin cassette was cloned between them. This whole construct was introduced into the suicide vector pK18*mobsacB*, and the resulting plasmid was conjugated into *S. meliloti* 1021, leading to the exchange of the entire *pssA* gene with the gentamicin cassette (Fig. 3). Mutant candidates were analyzed by in vivo labeling with [^{14}C]acetate and subsequent separation of the radiolabeled lipids by one-dimensional TLC. Six mutants lacking PE and its methylated derivatives were identified (data not shown). Substitution of the *pssA* gene with the gentamicin resistance cassette was confirmed by Southern blotting (data not shown). One of the six allelic mutants (CS111) was selected for further analysis. The lipid pattern of the mutant CS111 was identical to the lipid pattern shown above for the PE-deficient chemical mutant KDR310 (Fig. 1B). In order to find out if there might be an additional activity leading to PE synthesis, we performed in vivo labeling of CS111 cells grown in complex TY medium with [^{32}P]phosphate. When the lipid extracts were analyzed by two-dimensional TLC, no radioactivity above background levels could be detected where the PE spot was to be expected (data not shown).

Growth of the PE-deficient knockout mutant CS111. Mutants of *E. coli* that are deficient in PE are conditionally lethal

TABLE 4. Correlation between Ca^{2+} concentration and generation times of different rhizobial strains when grown in complex medium[a]

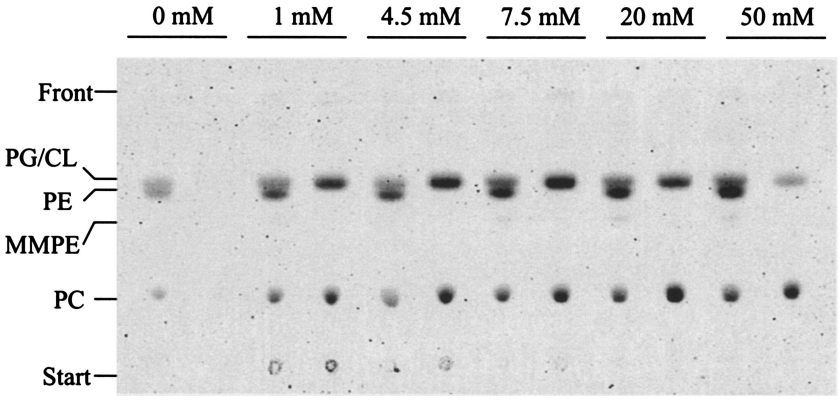
Ca^{2+} concn (mM)	Avg generation time (min)			
	1021	CS111	CS111/pTB2112	CS111/pMP3510
1	190 ± 10	253 ± 31	197 ± 15	680 ± 35
4.5	181 ± 14	217 ± 24	200 ± 25	624 ± 128
7.5	167 ± 21	205 ± 26	170 ± 20	373 ± 64
20	170 ± 8	180 ± 26	171 ± 18	303 ± 86
50	155 ± 15	165 ± 10	157 ± 6	227 ± 50

[a] *S. meliloti* 1021 (wild type), PE-deficient mutant CS111, CS111/pTB2112 (complemented mutant), and CS111/pMP3510 (mutant harboring the empty vector only) were grown in complex TY medium containing between 1 and 50 mM $CaCl_2$. Numbers in the table give the average generation time of at least three independent growth experiments ± standard deviation.

and require a millimolar concentration of bivalent cations like Ca^{2+} or Mg^{2+} to grow (10). We therefore expected a drastic growth phenotype for the PE-deficient *S. meliloti* mutant CS111 as well. Growth of CS111, wild-type *S. meliloti* 1021, and CS111 harboring either the complementing plasmid pTB2112 or vector pMP3510 only was compared on TY medium supplemented with different concentrations of $CaCl_2$ (0 to 50 mM). On TY medium lacking calcium, mutant CS111 was not able to grow, whereas the wild type was able to grow for one passage but then stopped growing as well (data not shown). This result suggests that CS111 needs more calcium than the wild type to sustain growth. When additional calcium was present in the TY growth medium, a clear difference in growth between mutant CS111 and the wild type could be observed at calcium concentrations up to 7.5 mM, but this difference was getting smaller with increasing calcium concentrations (Table 4). The difference in growth is much more striking when CS111 carrying the complementing plasmid (pTB2112) is compared to CS111 harboring the empty vector (pMP3510). At 1 mM and 4.5 mM $CaCl_2$, the generation time of the strain lacking PE was more than three times longer than the generation time of the complemented mutant CS111/pTB2112 (Table 4). With increasing calcium concentrations, the difference in generation time between CS111/pTB2112 and CS111/pMP3510 decreased, but it did remain significant. A possible explanation for this drastic difference might be that CS111/pMP3510, due to the absence of PE from its membranes, has a greater sensitivity to tetracycline than the complemented mutant CS111/pTB2112, although both strains carry a tetracycline resistance gene. Resistance to tetracycline is conferred by TetA, a transporter of the major facilitator superfamily (19). The increased sensitivity towards tetracycline of CS111/pMP3510 compared to that of CS111/pTB2112 might be due to reduced transporter activity caused by the lack of PE in the membrane, in analogy to what has been described for the major facilitator superfamily lactose transporter (LacY) from *E. coli* (4, 5, 6). By adding increasing amounts of calcium, which has been shown to stimulate tetracycline transport, the tetracycline hypersensitivity might be masked partially (48). Antibiotic hypersensitivity of a mutant deficient in PE has been described before for *E. coli* (43). Using antibiotic susceptibility disks (Oxoid), we compared the susceptibilities of CS111 and 1021 towards 18 different antibiotics on TY medium containing 0.5, 4.5, or 20 mM $CaCl_2$. Mutant CS111 seemed to be more sensitive towards carbenicillin, neomycin, and tetracycline (data not shown), and the increased sensitivity of CS111 appeared to be more pronounced when less calcium was present in the medium. In the case of tetracycline, the explanation for the higher resistance at higher calcium concentrations again might be that calcium is stimulating tetracycline export from the cells (48). The hypersensitivity towards these antibiotics was confirmed by determining the MICs of these antibiotics for both strains when grown in TY medium containing 4.5 mM $CaCl_2$. The PE-deficient mutant CS111 was two- to fourfold more sensitive towards these antibiotics (data not shown).

The lack of the non-bilayer-forming lipid PE leads to filamentous growth of PE-deficient mutants of *E. coli* (35). This growth phenotype might be a direct effect of the lack of the non-bilayer-forming lipid PE, as no other non-bilayer-forming lipid seems to be part of the *S. meliloti* membrane, but there is also evidence that the high negative charge density on the cell surfaces of *E. coli* cells lacking PE is partially responsible for these defects in cell division (37). When grown in complex medium, mutant cells should lack PE, MMPE, and DMPE, but when grown in choline-free minimal medium, they should lack PE, MMPE, DMPE, and PC, which should lead to an excess of negative charge at the membrane. Mutant cells and wild-type cells were cultivated on complex medium and obtained from the exponential growth phase before being analyzed by light microscopy for their morphologies. In addition, mutant and wild-type cells were cultivated on MOPS minimal medium with or without supplementation of 100 μM choline. Under all growth conditions mentioned, no significant difference in cell morphology could be observed when comparing the wild type, 1021, and the PE-deficient mutant CS111 (data not shown).

Lipid composition of PE-deficient mutant CS111 during growth on complex medium. The difference in growth between the PE-deficient mutant CS111 and the wild type, 1021, at low calcium concentrations prompted us to study the membrane lipid composition of the two strains after growth in TY medium containing different concentrations of calcium (Fig. 6). Lipid composition of wild-type strain 1021 and the PE-deficient mutant CS111 was analyzed by in vivo labeling with [^{14}C]acetate and subsequent lipid analysis by TLC. In the wild type, PG and CL comprised 33 to 36%, PE comprised 34 to 39%, and PC comprised between 28 and 32%, and notably, the wild type exhibited a similar lipid composition over the whole range of calcium concentrations used. Without added calcium in the growth medium, hardly any radioactivity was incorporated into membrane lipids of mutant CS111, which is consistent with the earlier observation that the mutant does not grow on medium lacking Ca^{2+}. At calcium concentrations up to 7.5 mM, PG and CL comprise 63 to 66% of the membrane lipids whereas the residual 34 to 37% are PC, which is in agreement with the results obtained for lipid composition of the PE-deficient chemical mutant KDR310 (Table 2). Surprisingly, at calcium concentrations of 20 and 50 mM, PC becomes the major membrane lipid in mutant CS111 (relative amount of PC increases to 55%) and the proportion of anionic membrane lipids (PG and CL) decreases to 45% (Fig. 6). Therefore, in contrast to that of the wild type, the lipid profile of the PE-deficient mutant CS111 changes depending on the calcium concentration in the medium, and whereas at low concentrations of calcium, PE is largely replaced by the anionic phospholipids PG and



%	Wt	M	Wt	M	Wt	M	Wt	M	Wt	M	Wt	M
CL/PG	33	---	35	63	35	63	34	66	33	45	33	45
PE	35	---	34	---	35	---	38	---	38	---	39	---
PC	32	---	31	37	30	37	28	34	29	55	28	55

FIG. 6. Lipid analysis of wild-type *S. meliloti* 1021 and Pss-deficient mutant CS111 after in vivo labeling with [^{14}C]acetate in complex TY medium containing different concentrations of $CaCl_2$. *S. meliloti* wild-type 1021 (Wt) and mutant CS111 (M) were grown in complex TY medium containing between 0 and 50 mM $CaCl_2$. [^{14}C]acetate-labeled lipids were extracted, separated by one-dimensional TLC, and quantified using a PhosphorImager. Relative amounts (percent) of individual lipids are given in the table at bottom.

CL, at concentrations of 20 mM calcium and above, the zwitterionic PC functions as a major replacement lipid for PE.

Choline supplementation partly rescues the growth defect of PE-deficient *S. meliloti* mutant CS111 on minimal medium. The PE-deficient sinorhizobial mutant CS111 grows surprisingly well on complex medium even at relatively low concentrations of calcium ions. Unlike *E. coli*, *S. meliloti* possesses with PC a second major zwitterionic lipid in its membranes. Since wild-type *S. meliloti* can synthesize PC via the Pmt pathway or via the Pcs pathway, even a mutant deficient in Pss will be able to synthesize PC via the Pcs pathway when grown in medium containing choline. We speculated that the presence of PC might make up for the lack of PE. Cultivation of *S. meliloti* in defined choline-free medium would completely prevent the synthesis of PC. Strains were pregrown for one passage on TY medium containing 4.5 mM $CaCl_2$. Then, cells were washed twice with MOPS minimal medium before being cultured in the same medium in the presence of different amounts of choline. *S. meliloti* 1021 wild-type cells grew well in MOPS medium lacking or containing up to 10 μM choline (Fig. 7). When higher choline concentrations (100 μM or 1 mM) were present in the medium, growth of the wild type was inhibited. In contrast, growth of PE-deficient mutants was much worse on MOPS minimal medium. Choline concentrations up to 1 μM did not improve growth of the PE-deficient mutant. A partial restoration of wild-type-like growth was observed when the medium was supplemented with 10 μM choline or more. Interestingly, in minimal medium supplemented with 1 mM choline, the mutant grew even better than the wild type (Fig. 7). The addition of 0.01% (wt/vol) Casamino Acids (Difco) restored growth of the PE-deficient mutant almost to the growth rate observed on complex TY medium (data not shown). Since growth of the PE-deficient mutant CS111 was much worse than that of the wild type on MOPS minimal medium when supplemented with choline but was nearly as good as that of the wild type on complex medium and on minimal medium supplemented with small amounts of Casamino Acids, we suggest that some compound(s) other than choline and present in complex medium or in the Casamino Acids preparation is needed for optimal growth of a PE-deficient sinorhizobial mutant.

DISCUSSION

During a screening for mutants of *S. meliloti* deficient in Pmt activity, we identified a number of chemical mutants that showed reduced or no Pmt activity (14). Mutant KDR309, deficient in the sinorhizobial gene (*pmtA*) for Pmt, was complemented by a cosmid (pCOS24.1) containing sinorhizobial

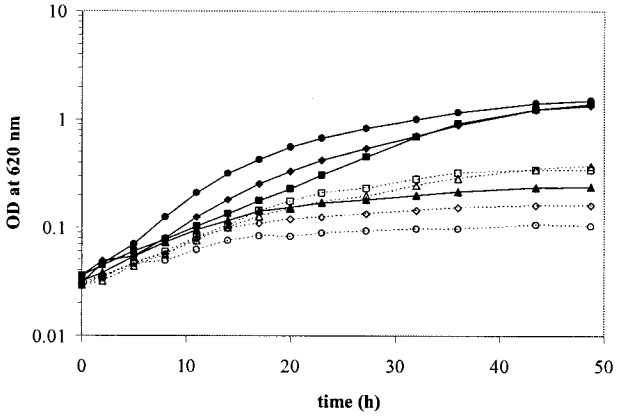


FIG. 7. The growth phenotype of the PE-deficient mutant CS111 is partly rescued by choline supplementation to the medium. *S. meliloti* 1021 (solid lines, closed symbols) and the Pss-deficient mutant CS111 (dashed lines, open symbols) were grown in MOPS minimal medium without choline supplementation (circles) or in the presence of 10 μM choline (diamonds), 100 μM choline (squares), or 1 mM choline (triangles).

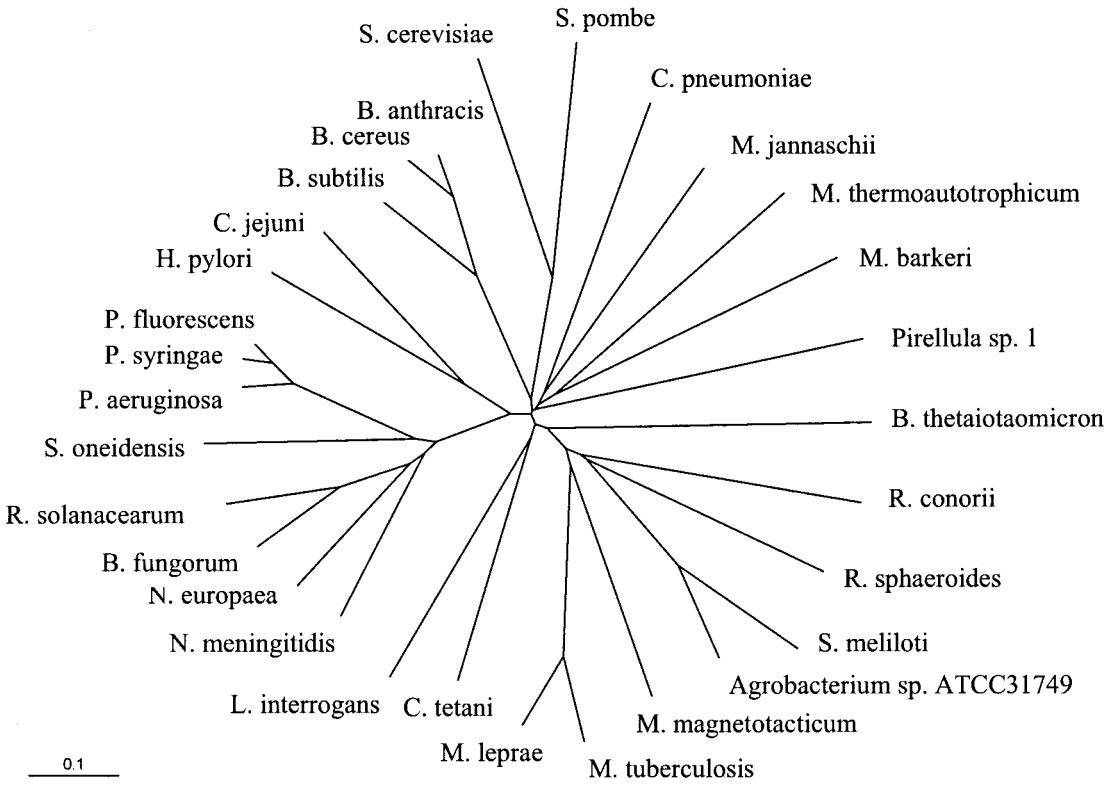


FIG. 8. Unrooted phylogenetic tree of subclass II Pss and Pss-like ORFs. The tree was constructed using the program ClustalW (http://www.expasy.ch/). Distances between sequences are expressed as 0.1 changes per amino acid residue. Accession numbers are as follows: *Saccharomyces cerevisiae* Cho1p, NP_010943;, *Schizosaccharomyces pombe* Pss, NP_588326; *Chlamydophila pneumoniae* ORF, NP_225177; *Methanococcus jannaschii* ORF, NP_248207; *Methanobacterium thermoautotrophicum* Pss, A69004; *Methanosarcina barkeri* ORF, ZP_00079280; *Pirellula* sp. ORF, NP_865741; *Bacteroides thetaiotaomicron* ORF, NP_811145; *Rickettsia conorii* ORF, NP_359963; *Rhodobacter sphaeroides* ORF, ZP_00008123; *Sinorhizobium meliloti* Pss, AAG00422; *Agrobacterium* sp. ATCC31749 Pss, AAL01116; *Magnetospirillum magnetotacticum* ORF, ZP_00056392; *Mycobacterium tuberculosis* Pss, P96282; *Mycobacterium leprae* ORF, NP_301340; *Clostridium tetani* ORF, NP_781048; *Leptospira interrogans* ORF, NP_711632; *Neisseria meningitidis* ORF, NP_274337; *Nitrosomonas europaea* ORF, NP_841370; *Burkholderia fungorum* ORF, ZP_00029658; *Ralstonia solanacearum* ORF, NP_520194; *Shewanella oneidensis* ORF, NP_719120; *Pseudomonas aeruginosa* ORF, NP_253381; *Pseudomonas syringae* ORF, NP_790823; *Pseudomonas fluorescens* ORF, ZP_00083027; *Helicobacter pylori* Pss, AAB66379; *Campylobacter jejuni* ORF, NP_282262; *Bacillus subtilis* Pss, NP_388109; *Bacillus cereus* ORF, NP_830762; *Bacillus anthracis* ORF, NP_843464.

DNA (12). Most of the other Pmt-deficient chemical mutants were complemented by the same cosmid or *pmtA*-containing subclones of it, suggesting that the original defect of these mutants was within the sinorhizobial *pmtA* gene. One mutant isolated in the original screening (KDR310) was complemented by another cosmid containing sinorhizobial DNA (pCOS9.4) or by subclones containing an intact sinorhizobial *pssA* gene encoding for Pss. Originally we did not detect Pmt activity in KDR310, because this mutant cannot form the substrate PE needed for this reaction. When external PE is added to cell extracts of KDR310, Pmt activity can be demonstrated.

Whereas eukaryotes can form PE via the CDP-ethanolamine pathway, prokaryotes usually form PE from CDP-diacylglyceride, employing Pss and the Psd reaction. Replacement of the *pssA* gene with a gentamicin resistance-conferring cassette in *S. meliloti* eliminates the formation of PE and of methylated PE derivatives, demonstrating that the knockout of the *pssA* gene eliminates Pss activity and that no other pathway for PE biosynthesis exists in *S. meliloti.* The complementation of *pssA*-deficient mutants with broad-host-range plasmids containing the sinorhizobial 2.1 kb with the *pssA* gene (pTB2036) or only the PCR-amplified *pssA* gene (pTB2112) demonstrates that this single gene restores Pss activity in *S. meliloti*.

Sequence analysis of the sinorhizobial *pssA* gene revealed that *S. meliloti* Pss is a type II Pss. Type II Pss were originally thought to be specific for gram-positive bacteria (32), but recent experimental data (21, 25, 39) and the wealth of information provided by the large number of genome projects indicate that type II Pss occur in all three kingdoms of life (archaea, eubacteria, eukaryotes) (Fig. 8). They are therefore not restricted to gram-positive bacteria but can be found in such diverse organisms as *Methanococcus jannaschii* (archaebacteria), *Leptospira interrogans* (spirochetes), *Pirellula* sp. (*Planctomycetales*), *Pseudomonas fluorescens* (γ-proteobacteria), *Neisseria meningitidis* (β-proteobacteria), *Helicobacter pylori* (ε-proteobacteria), and *Rhodobacter sphaeroides* (α-proteobacteria). In contrast, the type I subclass of Pss seems to be restricted to certain families of the γ-proteobacteria. Homologues to the type I *E. coli* Pss can be found, for example, in *Yersinia pestis* (*Enterobacteriaceae*), *Vibrio cholerae* (*Vibrionaceae*), *Haemophilus influenzae* (*Pasteurellaceae*), *Shewanella oneidensis* (*Alteromonaceae*), and *Pseudomonas putida*

(*Pseudomonadaceae*). In the family of the pseudomonads some strains seem to have the subclass I Pss (*P. putida*), others seem to have the subclass II Pss (*Pseudomonas aeruginosa*), and surprisingly, *P. fluorescens*, *Pseudomonas syringae*, and *S. oneidensis* seem to have good homologues for both subclasses of Pss.

For the characterization of a sinorhizobial mutant lacking Pss activity, the mutant CS111 was created in which the *pssA* gene was replaced with a gentamicin resistance cassette. When grown in complex medium, *S. meliloti* forms PG, CL, PE, MMPE, DMPE, and PC as major membrane lipids. Mutant CS111, deficient in Pss activity, lacks PE, MMPE, and DMPE. On complex medium or other media containing choline, CS111 is able to form PC via the Pcs pathway (13, 52). No additional membrane lipid is synthesized to make up for the deficiency of PE, but the relative amounts of both the anionic lipids PG and CL and the zwitterionic lipid PC increase, with the anionic lipids presenting 63 to 66% of the lipids. Interestingly, at calcium concentrations of 20 mM or higher the relative amounts of membrane lipids are different, and membranes contain more PC than anionic lipids. The conditionally lethal phenotype of *E. coli* mutants deficient in PE can be rescued by millimolar concentrations of certain bivalent cations, such as Ca^{2+}, Mg^{2+}, and Sr^{2+}, with calcium being the most efficient ion. It has been suggested that the presence of high concentrations of these ions enables the bilayer-forming lipid CL to change into the reverse-hexagonal-phase configuration and thereby substitute for PE, which prefers the reverse-hexagonal-phase configuration (5). *S. meliloti* needs calcium supplementation for growth, and TY medium complemented with 4.5 mM $CaCl_2$ is a standard growth medium for *S. meliloti*. Therefore, we had to use Ca^{2+} in our studies instead of Mg^{2+}, which was used in the studies with *E. coli.* Similarly as observed in the case of *Agrobacterium* (25), *S. meliloti* seems to be much less affected by the lack of PE than *E. coli*, and there might be several reasons for this. First, PE forms about 70% of the membrane lipids in *E. coli* but only about 30% in *S. meliloti.* Furthermore, *S. meliloti* contains significant amounts of MMPE and DMPE, which are less likely to form a non-bilayer phase than PE. When PE is lost in a PssA-deficient mutant, MMPE and DMPE are lost as well; thus, the loss of PE might be less severe and could be compensated by an increase in CL. Another reason why the lack of PE is affecting *S. meliloti* less than *E. coli* might have been the presence of PC, another zwitterionic lipid, in the membranes of *S. meliloti.* To test this hypothesis, we studied growth of *S. meliloti* in minimal medium with the supplementation of choline. The supplementation with 10 μM choline or more partly complemented the growth phenotype of PE-deficient *S. meliloti* mutants under these conditions. Such elevated choline concentrations were impairing growth of the wild type, 1021, for an unknown reason. The observation that no drastic growth difference between the mutant and the wild type can be observed when both strains are grown in complex medium but that growth of the mutant is much worse than that of the wild type in minimal medium supplemented with choline indicates that some other factor from the complex medium is needed for optimal growth.

Interestingly, PE seems to be essential for some microorganisms, whereas it is not for others. *S. cerevisiae* cannot grow without it, and similarly, Ge and Taylor (21) were not able to construct an *H. pylori* mutant deficient in Pss, indicating that PE might be essential for this organism. In contrast, an *Agrobacterium* mutant deficient in Pss seemed to be unaffected in growth (25). The requirement of PE for growth of some bacteria and not in the case of some others parallels the requirement of PC for some bacteria but not for others. No specific phenotype was detected for PC-deficient mutants of *R. sphaeroides* and *Zymomonas mobilis*, whereas relatively drastic phenotypes for the PC-deficient mutants were described for *S. meliloti* and *B. japonicum*. In order to explain these observations, far more has to be learned about the roles which different membrane lipids play for membrane function, i.e., in what form they are responsible for protein activity or stability.

An interesting aspect for future studies to address is how the PE-deficient *S. meliloti* strain behaves during symbiosis. Mutants of *B. japonicum* that were deficient in Pmt and had a reduced amount of PC in their membranes were impaired in their symbiotic performance (38), and double mutants of *S. meliloti* that were deficient in Pcs and PmtA activity and therefore completely lacking PC were not able to form nodules on their host plant, *Medicago sativa* (alfalfa) (53). If the lack of PC has such a drastic effect on symbiotic performance, one might expect that the symbiotic performance of mutants deficient in the other major zwitterionic membrane lipid, PE, might be seriously affected. Such studies are currently under way.

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