Sinorhizobium meliloti phospholipase C required for lipid remodeling during phosphorus limitation

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Rhizobia are Gram-negative soil bacteria able to establish nitrogen-fixing root nodules with their respective legume host plants. Besides phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine, rhizobial membranes contain phosphatidylcholine (PC) as a major membrane lipid. Under phosphate-limiting conditions of growth, some bacteria replace their membrane phospholipids with lipids lacking phosphorus. In *Sinorhizobium meliloti,* these phosphorus-free lipids are sulfoquinovosyl diacylglycerol, ornithine-containing lipid, and diacylglyceryl trimethylhomoserine (DGTS). Pulse–chase experiments suggest that the zwitterionic phospholipids phosphatidylethanolamine and PC act as biosynthetic precursors of DGTS under phosphorus-limiting conditions. A *S. meliloti* mutant, deficient in the predicted phosphatase SMc00171 was unable to degrade PC or to form DGTS in a similar way as the wild type. Cell-free extracts of *Escherichia coli*, in which SMc00171 had been expressed, convert PC to phosphocholine and diacylglycerol, showing that SMc00171 functions as a phospholipase C. Diacylglycerol , in turn, is the lipid anchor from which biosynthesis is initiated during the formation of the phosphorus-free membrane lipid DGTS. Inorganic phosphate can be liberated from phosphocholine. These data suggest that, in *S. meliloti* under phosphate-limiting conditions, membrane phospholipids provide a pool for metabolizable inorganic phosphate, which can be used for the synthesis of other essential phosphorus-containing biomolecules. This is an example of an intracellular phospholipase C in a bacterial system; however, the ability to degrade endogenous preexisting membrane phospholipids as a source of phosphorus may be a general property of Gram-negative soil bacteria.

bacterial cell envelope | diacylglycerol | diacylglyceryl trimethylhomoserine | phospholipid turnover | phosphatidylcholine

Animal cells have access to relatively abundant sources of phosphorus for the formation of biomolecules such as membrane phospholipids and nucleic acids. The characteristic lipid composition for a particular animal cell membrane is thought to result from a steady state between formation and turnover of the lipids. In contrast, plants and many environmental microbes often live in environments where available phosphorus is a growth-limiting factor. The strategies employed by organisms to deal with phosphorus limitation include: (*i*) increased solubilization of phosphorus-containing compounds; (*ii*) more efficient uptake into cells; and (*iii*) less phosphorus use when synthesizing their biomolecules (1). The replacement of phospholipids by galacto- and sulfolipids in plant membranes constitutes an important adaptive process for growth on phosphate-limited soils. In *Arabidopsis thaliana*, several phospholipases D and C (2–5) are induced under phosphate-limiting conditions, and they degrade membrane phospholipids to phosphatidic acid or diacylglycerol (DAG), respectively. DAG then serves as the initial substrate for the formation of galacto- and sulfolipids, which lack phosphorus.

In some bacteria, the membrane phospholipids are partially replaced during phosphate limitation by phosphorus-free lipids, including *Bacillus subtilis* (6), *Pseudomonas diminuta* (7), *Pseudomonas fluorescens* (8), and *Rhodobacter sphaeroides* (9). *Sinorhizobium meliloti* is a soil bacterium that can form a symbiosis with legumes such as alfalfa. In the symbiotic state, *S. meliloti* fixes molecular nitrogen in nodules on the roots of the legume plant. *S. meliloti* usually synthesizes phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylethanolamine (PE), monomethyl-PE (MMPE), and phosphatidylcholine (PC) as its major membrane lipids (10). However, under phosphorus-limiting conditions, *S. meliloti* replaces most of its phospholipids with membrane-forming lipids that do not contain phosphorus, such as the sulfolipid sulfoquinovosyl diacylglycerol (SL), an ornithine-containing lipid (OL), or diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) (11). These phosphorus-free membrane lipids are not important for the symbiotic lifestyle of *S. meliloti*, but they are required for optimal growth under phosphorus-limiting conditions (12).

The expression of many genes of the phosphorus-limitation stress response in *S. meliloti* is regulated at the transcriptional level by the two-component PhoR-PhoB signal transduction system in which phosphorylated PhoB modulates gene expression by binding to specific Pho boxes (13, 14). The main membrane lipid formed under phosphorus limitation in *S. meliloti* is DGTS. Two structural genes (*btaAB*) required for DGTS biosynthesis were first described in *Rhodobacter sphaeroides* (15) and later in *S. meliloti* (12). BtaA converts DAG into diacylglyceryl-homoserine (DGHS), and BtaB catalyzes the 3-fold methylation of DGHS to yield DGTS (15, 16). Expression of BtaA and BtaB requires induction by the PhoB regulator. Another minor DAG-containing, phosphorus-free membrane lipid in *S. meliloti* is SL, and *sqdB* is required for its formation (17). At least four structural genes (*sqdA*, *sqdB*, *sqdC*, and *sqdD*) are involved in SL biosynthesis in *R. sphaeroides*, but the detailed overall mechanism for sulfolipid assembly in bacteria still needs to be defined (18).

Before the present study, it was not clear how the DAG needed for DGTS biosynthesis was formed. Here, we show that preexisting phospholipids are metabolic precursors of DAG-containing, phosphorus-free membrane lipids in *S. meliloti*. The PhoB-controlled gene *smc00171* encodes a phospholipase C that degrades zwitterionic phospholipids to DAG and the respective phosphoalcohol. DAG is then used for the formation of the phosphorus-free membrane lipids.

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Results

Membrane Phospholipids of *S. meliloti* Are Degraded Under Phosphorus-Limiting Conditions. Under phosphate-limiting conditions of growth, *S. meliloti* replaces its membrane phospholipids by lipids not containing phosphorus (11) such as SL, OL, and DGTS. To understand whether or not sinorhizobial phospholipids might act as biosynthetic precursors for membrane lipids lacking phosphorus, we performed pulse–chase experiments, labeling with radioactive acetate in high-phosphate conditions during the pulse period. When the chase was performed at high-medium concentrations of inorganic phosphate (Pi), the radioactivity incorporated into the individual sinorhizobial phospholipids was maintained over time, suggesting that under such conditions, phospholipids are metabolically stable in this organism. In contrast, when the chase was performed at low concentrations of Pi, some phospholipids continuously lost radiolabel; in particular, the relative amounts of the zwitterionic PC, PE, and MMPE (Fig. 1 and Table S1) were continuously reduced (PC from 60% to 12% and PE + MMPE from 25% to 3%). Little or no degradation was observed for the anionic phospholipids PG and CL (Fig. 1 and Table S1) at low concentrations of Pi. As noted previously (11) and observed again here (Fig. 1 and Table S1), the amount of SL increased slightly under conditions of phosphorus limitation, whereas the amount of OL strongly increased. DGTS becomes the predominant membrane lipid in *S. meliloti* (11) (Fig. 1 and Table S1), and its relative amount increases from 0% to 63%. Remarkably, most of the radiolabel lost from the zwitterionic phospholipids PC, PE, and MMPE appeared in the DGTS (Fig. 1 and Table S1), suggesting that PC, PE, and MMPE are metabolic precursors of DGTS.

To understand whether or not DGTS formation was required for the degradation of zwitterionic phospholipids, similar pulse–chase experiments were performed with the *btaA*-deficient mutant DGTS1 that is unable to catalyze the first step of DGTS biosynthesis (12). Under phosphorus-limiting conditions, the *btaA*-deficient mutant degraded PC, PE, and MMPE to a similar degree as the wild-type, which shows that biosynthesis of DGTS is not a requirement for the degradation of zwitterionic phospholipids. Many processes induced under phosphorus-limiting conditions are controlled by the regulator PhoB. In pulse–chase experiments, we, therefore, analyzed whether or not the *phoB*-deficient mutant H838 (11) degraded phospholipids in a similar way as the wild-type. Under phosphorus-limiting conditions, the *phoB*-deficient mutant was unable to degrade PC, PE, and MMPE, suggesting that the hypothetical structural genes encoding the phospholipid-degrading activity should require active PhoB for their induction.

***smc00171*-Deficient Mutant of *S. meliloti* Is Unable to Degrade Phosphatidylcholine Under Phosphate Limitation.** If membrane phospholipids are biosynthetic precursors of DGTS, we expected that they should be degraded by a phospholipase D to phosphatidic acid and subsequently, by a phosphatase to DAG or alternatively, by a phospholipase C directly to DAG. We, therefore, hypothesized that potential phospholipases C or D or phosphatases controlled by PhoB might be responsible for the degradation of zwitterionic phospholipids under phosphorus-limiting conditions. *S. meliloti* has two genes that encode for putative phospholipases D, *smb20094*, and *smc04448*. Also, four genes (*smc00620*, *smc02634*, *smc01907*, and *smc00171*) encoding putative phosphatases are strongly induced under Pi limitation in a PhoB-dependent manner (13, 14). Mutants deficient in each of the six potential phospholipase or phosphatase genes were constructed and analyzed in pulse–chase experiments. Mutants PLD20094, PLD04448, PHO00620, or PHO02634 (deficient in *smb20094*, *smc04448*, *smc00620*, or *smc02634,* respectively) degraded zwitterionic phospholipids similarly to the wild-type and formed DGTS (Fig. 2, lanes 1–3, 5, 6). SMc02634 was recently shown to be a PhoX alkaline phosphatase (19), which is found in many bacteria but is distinct from the well-characterized PhoA family. Mutant PHO01907, deficient in *smc01907*, also degraded zwitterionic phospholipids and formed DGTS (Fig. 2, lane 4); however, the rate of PC degradation in this mutant was slower than in the wild-type. Remarkably, mutant PHO00171, deficient in *smc00171*, did not degrade PC or PE + MMPE (Fig. 2, lane 7) under phosphorus-limiting conditions. The formation of DGTS was much reduced (Fig. 2, lane 7) compared with the wild-type (Fig. 2, lane 1), suggesting that the gene encoded by *smc00171* is required for the degradation of the zwitterionic membrane phospholipids.

SMc00171 Restores Phosphatidylcholine and Phosphatidylethanolamine Degradation in a *smc00171*-Deficient Mutant. A quantitative comparison of the membrane lipid compositions of mutant PHO00171 and wild-type under phosphorus-limiting conditions (Fig. S1 and Table S2) confirms that the reduced amounts of PC and PE + MMPE, as well as the increased amounts of DGTS detected in the wild-type depend on an intact *smc00171* gene. When the gene encoded by *smc00171* and its upstream regulatory region was introduced in a

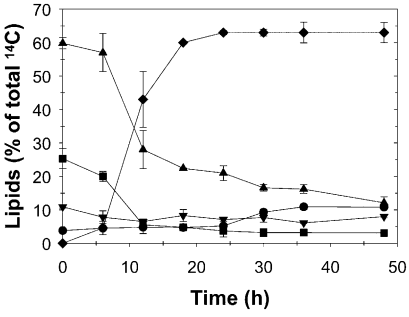

Fig. 1. Pulse–chase experiment suggesting that zwitterionic phospholipids function as biosynthetic precursors of phosphorus-free membrane lipids in *S. meliloti*. After labeling *S. meliloti* with [^{14}C]-labeled acetate during growth on minimal medium in the presence of 1.3 mM Pi, cells were washed and resuspended in minimal medium containing 0.02 mM Pi. During the chase period, aliquots were taken at different time points and lipids were quantified. Percentages of individual lipid classes are shown for the different time points of chase: ▲ PC, phosphatidylcholine; ■ PE, phosphatidylethanolamine; + MMPE, monomethyl-PE; ◆ DGTS, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine; ▼ PG, phosphatidylglycerol; + CL, cardiolipin; ● OL, ornithine-containing lipid; and + SL, sulfolipid. The data represent mean values ± SD of three independent experiments.

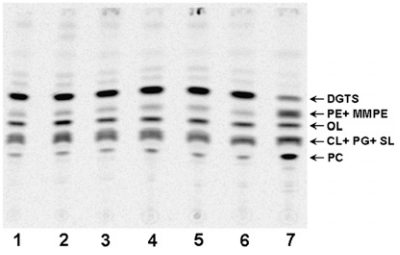

Fig. 2. Mutant deficiency in *smc00171* degrades less zwitterionic phospholipids (PC, PE, and MMPE) and forms less DGTS under phosphorus limitation. Different strains were pulse-labeled on minimal medium and chased at low phosphate concentrations as described in *Materials and Methods*. After 24 h of chase, [^{14}C]acetate-labeled lipids were extracted and separated by 1D-TLC. Lipids included *S. meliloti* wild type (lane 1), the *smb20094*-deficient knock-out mutant PLD20094 (lane 2), the *smc04448*-deficient knock-out mutant PLD04448 (lane 3), the *smc01907*-deficient knock-out mutant PHO01907 (lane 4), the *smc02634*-deficient knock-out mutant PHO02634 (lane 5), the *smc00620*-deficient knock-out mutant PHO00620 (lane 6), and the *smc00171*-deficient knock-out mutant PHO00171 (lane 7). The lipids PC, PE, MMPE, PG, CL, OL, SL, and DGTS are indicated.

broad host-range vector (pCP00171) into the PHO00171 mutant background, PHO00171 × pCP00171 again showed reduced amounts of PC and PE + MMPE as well as increased amounts of DGTS. Therefore, in this complemented mutant, the membrane lipid composition is similar to that displayed by wild-type (Figs. S1 and S2 and Table S2). In mutant PHO00171 containing an empty broad host-range plasmid (PHO00171 × pRK404), the membrane lipid composition remained mutant-like (Figs. S1 and S2 and Table S2).

SMc00171 Is a Phospholipase C. To prove our hypothesis that SMc00171 has phospholipase activity, we developed an enzymatic assay for the SMc00171 activity. Different *Escherichia coli* cell-free extracts were incubated with phosphatidylcholine, and potential products were analyzed. When [^{14}C]PC was incubated with a cell-free extract of *E. coli* harboring SMc00171-expressing pC00171, the formation of two compounds was observed (Fig. 3*A*, lane 1), which migrated like the two DAG isomers 1,2-DAG and 1,3-DAG. When [^{14}C]PC was incubated with a cell-free extract of *E. coli* harboring the empty pET9a plasmid, only a small amount of a DAG-like compound was formed (Fig. 3*A*, lane 2), whereas incubation with buffer only did not cause degradation of PC (Fig. 3*A*, lane 3). Mass spectrometry (see *SI Materials and Methods*) of lipid extracts showed that a cell-free extract of *E. coli* that expressed SMc00171 converted the added artificial deuterated substrate 1,2-dipalmitoyl-D62-*sn*-glycero-3-phosphocholine to the corresponding DAG 1,2-dipalmitoyl-D62-*sn*-glycerol (Fig. 3*B*), whereas such a conversion did not occur in *E. coli* extracts lacking SMc00171. Incubation of [^{32}P]-labeled PC with a known phospholipase C from *Clostridium perfringens* leads to the formation of phosphocholine and a small amount of Pi (Fig. 3*C*). Incubation of [^{32}P]PC with a cell-free extract of *E. coli* expressing SMc00171 led to the formation of several water-soluble products, among them phosphocholine as the major product and some Pi (Fig. 3*C*). Although incubation with an extract from *E. coli* lacking SMc00171 also caused the formation of several water-soluble products, the main product was Pi; no phosphocholine formation was observed (Fig. 3*C*). The formation of the products DAG and phosphocholine is linear with time, and extracts of *E. coli* expressing SMc00171 showed a specific activity of phosphocholine formation of 1.36 ± 0.30 pmol/min per mg protein.

Discussion

S. meliloti assembles its membranes mainly with the phospholipids PG, CL, PE, MMPE, and PC when grown in phosphate-rich culture media. On growth of *S. meliloti* under phosphorus-limiting conditions, most of the phospholipids are replaced by membrane lipids that do not contain phosphorus (i.e., OL, SL, and DGTS) (11). Our pulse–chase experiments suggest that PC, PE, and MMPE are remodeled to be the biosynthetic precursors of DGTS. A *btaA*-deficient mutant of *S. meliloti*, unable to perform the first step in DGTS biosynthesis (Fig. 4), is still able to degrade zwitterionic phospholipids under phosphorus limitation, showing that the phospholipid disappearance does not require formation of DGTS. In contrast, phospholipids are not degraded during phosphorus limitation in a *phoB*-deficient mutant, suggesting that the gene required for phospholipid degradation is controlled by the PhoB regulator.

Analysis of different mutants deficient in distinct predicted phospholipases or in PhoB-controlled predicted phosphatases showed that a mutant lacking the predicted phosphatase SMc00171 was unable to degrade PC, PE, or MMPE or synthesize DGTS during phosphate limitation. Complementation of the *smc00171*-deficient mutant with the intact gene *in trans* restored the ability to degrade PC, PE, and MMPE and synthesize DGTS. Therefore, *smc00171* seemed to encode a function that was responsible for the degradation of zwitterionic membrane phospholipids under phosphorus-limiting conditions.

Cell-free extracts of *E. coli* expressing SMc00171 specifically cleave PC into DAG and phosphocholine, whereas vector-control extracts do not. Therefore, SMc00171 seems to be a sinorhizobial phospholipase C that degrades endogenous zwitterionic phospholipids when appropriate, liberating the phosphoalcohol and DAG (Fig. 4). Because no other phospholipase C is known to date from *S. meliloti,* we have renamed this gene *plcP* to highlight its

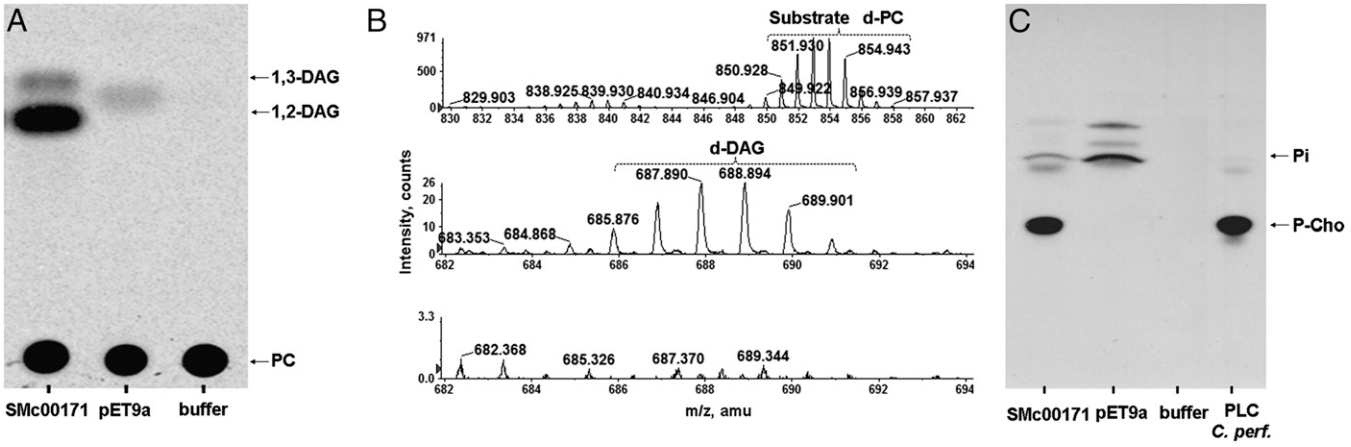

Fig. 3. Cell-free extracts of SMc00171-expressing *E. coli* convert PC to DAG and phosphocholine. Cell-free extracts of *E. coli* BL21 (DE3) × pLysS × pC00171 expressing SMc00171 or of BL21 (DE3) × pLysS containing the empty pET9a vector were incubated with 89.3 nmol [^{14}C]PC (112 mCi/mmol) for 2 h (*A*) or with 1,2-dipalmitoyl-D62-*sn*-glycero-3-phosphocholine (d-PC) for 16 h (*B*). After extraction, radiolabeled lipids were analyzed by TLC using n-hexane:diethyl ether:acetic acid (30:70:1, vol/vol) as a solvent system and detected by autoradiography (*A*), whereas deuterated lipids were analyzed by mass spectrometry (*B*). Mass spectrometric analysis (*B*) of d-PC substrate (*Top*), lipids formed by incubation with cell-free extracts of *E. coli* BL21 (DE3) × pLysS × pC00171 expressing SMc00171 (*Middle*), and BL21 (DE3) × pLysS containing the empty pET9a vector (*Bottom*) are shown. Both d-PC and d-DAG were detected as their acetate adduct $[M+Ac]^-$ ions in the negative ion mode. 1,2-DAG, 1,2-diacylglycerol; 1,3-DAG, 1,3-diacylglycerol; d-DAG, 1,2-dipalmitoyl-D62-*sn*-glycerol. Cell-free extracts of SMc00171-expressing *E. coli* convert PC to phosphocholine (*C*). Cell-free extracts of *E. coli* BL21 (DE3) × pLysS × pC00171 expressing SMc00171 (lane 1) and of BL21 (DE3) × pLysS containing the empty pET9a vector (lane 2), buffer (lane 3), or phospholipase C from *C. perfringens* (lane 4) were incubated with 75 pmol [^{32}P] PC (100 000 cpm/75 pmol), respectively. After lipid extraction, the aqueous phase was concentrated and separated by TLC using water:ethanol:ammonium hydroxide (91:95:6, vol/vol) as a solvent system. Phosphocholine (P-Cho) and Pi are indicated.

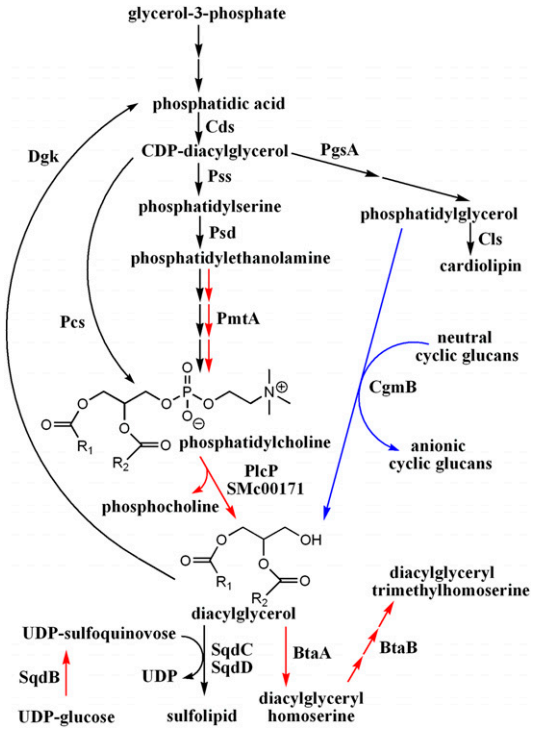

Fig. 4. Membrane lipid formation, turnover, and recycling in *Sinorhizobium meliloti* are shown. The major membrane phospholipids of *S. meliloti*, PE, PC, PG, and CL, are formed by well-known pathways. Under phosphorus-limiting conditions, zwitterionic phospholipids are degraded to DAG, whereas during low osmolarity conditions, phosphoglycerol head groups of PG are transferred to form anionic cyclic glucans and DAG. DAG can be recycled to phosphatidic acid or serve as lipid anchor during the formation of DAG-based phosphorus-free membrane lipids such as sulfolipid or DGTS. Cds, CDP-DAG synthase (SMc02096); Pcs, phosphatidylcholine synthase (SMc00247); Pss, phosphatidylserine synthase (SMc00552); Psd, phosphatidylserine decarboxylase (SMc00551); PmtA, phospholipid *N*-methytransferase (SMc00414); PgsA, PG–phosphate synthase (SMc00601); Cls, cardiolipin synthase (SMc02076); Dgk, DAG kinase (SMc04213); SqdB, UDP-sulfoquinovose synthase (SMc03961); BtaA, *S*-adenosylmethionine: DAG 3-amino-3-carboxypropyl transferase (SMc01848); BtaB, diacylglyceryl homoserine *N*-methyltransferase (SMc01849); CgmB, cyclic glucan-modifying phosphoglycerol transferase (SMc04438); PlcP, phospholipase C (SMc00171). Steps increased under phosphorus limitation (red) or at low osmolarity (blue) are highlighted.

induction during phosphorus starvation. Phospholipases D or C that degrade phospholipids under phosphorus limitation are known from plants (2–5), but none of them show homology to PlcP (SMc00171). Although numerous extracellular bacterial phospholipases C have been reported (20), PlcP (SMc00171) is an example of a bacterial phospholipase C that degrades endogenous phospholipids. PlcP (SMc00171) belongs to the PfamPF00149 protein family of phosphoesterases (21), the members of which hydrolyze a wide variety of protein and nucleotide substrates. The four motifs (DxH, GD, GNHD, and GHxH) of the calcineurin-like phosphoesterase superfamily (22) are present in PlcP (SMc00171) (Fig. S3). PlcP also shows weak similarities to LpxH of *E. coli*, which hydrolyzes uridine diphosphate-2,3-diacylglucosamine to uridine monophosphate and lipid X and is required for lipid A biosynthesis. It was noticed previously that at least two LpxH subfamilies exist in many β- and γ-proteobacteria, LpxH1 and LpxH2 (23). *Pseudomonas aeruginosa* and *Ralstonia solanacearum* have good homologs for both. Whereas LpxH1 from *P. aeruginosa* can complement a *lpxH*-deficient mutant of *E. coli*, LpxH2 cannot do so (23). LpxH2 from *P. aeruginosa* is a close homolog of PlcP (SMc00171) from *S. meliloti* (Fig. S4). We, therefore, suggest that LpxH2 homologs are, in fact, phospholipases C that degrade the bacteria's own phospholipids under phosphorus-limiting conditions. Good homologs of sinorhizobial PlcP are also encountered in most α-proteobacteria but are notably absent in *Bartonella, Brucella*, and the Rickettsiales, which are human or animal pathogens that are usually not confronted with low phosphorus conditions.

PlcP is regulated by PhoB (13, 14), and on phosphorus limitation, the *plcP* (*smc00171*) transcript is increased about 20-fold (13). The genes encoding for PlcP/SMc00171/LpxH2 homologs from *Agrobacterium tumefaciens* (Atu1649), *Bradyrhizobium japonicum* (BLL5904), *Caulobacter crescentus* (CC_3344), *Mesorhizobium loti* (MLL0806), and *Pseudomonas putida* (PP4510) (14), as well as *P. aeruginosa* (PA3219), all contain predicted PhoB binding sites in their upstream regions (Fig. S4 and Table S3), suggesting that they also might be induced under phosphorus-limiting conditions. In *Rhodopseudomonas palustris*, *plcP* (RPE_1465) seems to be the second gene in a PhoB-regulated operon in which phospholipid *N*-methyltransferase (RPE_1464) is encoded by the first gene and a putative glycosyltransferase (RPE_1466) is encoded by the third gene. This sequence in genes might reflect the biochemical events occurring in *R. palustris* on phosphorus limitation, which is initiated by an increased de novo biosynthesis of PC, continues with its degradation to DAG, and ends with the conversion of the latter into a phosphorus-free glycolipid. Remarkably, also *pmtA* (*smc00414*) (24) of *S. meliloti* is induced about fourfold (13) under phosphorus-limiting conditions. Similarly, genes encoding for enzymes of the initial steps of SL and DGTS biosynthesis (Fig. 4), SqdB (SMc03961) and BtaA (SMc01848), respectively, are preceded by a Pho box that mediates PhoB-controlled expression under phosphorus limitation (13).

Phosphorus limitation is encountered by many organisms in different environments. For example, many soils and lakes have limited phosphorus (1), and plants as well as microbes have to cope with phosphorus-limitation stress. Although phosphorus scarcity in the Sargasso Sea provokes phytoplankton of the ocean to use nonphosphorus lipids in response, a similar response has not been observed with marine heterotrophic *Pelagibacter* bacteria of that location (25). However, in other natural phosphorus-poor environments, such as the Cuatro Ciénegas Basin in Mexico, *Bacillus coahuilensis* is a major endemic species of the location and replaces most of its phospholipids by phosphorus-free sulfolipids (26). PlcP (SMc00171) from *S. meliloti* degrades zwitterionic phospholipids of its own membrane to DAG and phosphoalcohol (Fig. 4). Liberation of Pi from phosphoalcohols, such as phosphocholine (27), is a well-known process. The sinorhizobial phospholipid membrane, therefore, constitutes a phosphorus reservoir from which Pi can be mobilized under phosphorus-limiting conditions, and the liberated Pi can be used for the synthesis of essential phosphorus-containing biomolecules such as nucleic acids. We suggest that the mechanism of using endogenous membrane phospholipids as a phosphorus pool is widespread among Gram-negative environmental bacteria. Polyphosphate is another major reserve for phosphorus and energy in all living organisms (28). The relative importance of these two phosphorus pools in *S. meliloti* is not clear and needs further investigation.

The fact that DAG kinase-deficient mutants of *E. coli* show reduced growth when DAG accumulates to >10% of the total lipid (29) has led to the idea that larger amounts of DAG might inhibit bacterial growth by causing bilayer instability. In eukaryotes, DAG functions as an important phospholipase C-generated second messenger that modulates several proteins, among them members of the protein kinase C family (30). However, little is known about potential functions of DAG in bacteria, although several DAG-generating cycles are known. During adaptation to hypoosmotic conditions, *S. meliloti* synthesizes periplasmic cyclic β-1,2-glucans, which can be substituted with *sn*-1-phosphoglycerol moieties derived from PG (31). The phosphoglycerol transferase CgmB

(SMc04438) of *S. meliloti* (32) is thought to catalyze a reaction similar to the *sn*-1-phosphoglycerol transferase MdoB from *E. coli*, and produce DAG as a byproduct. Given the multiple sources of DAG in bacteria, including the SMc00171-encoded phospholipase C PlcP reported herein, it will be of great interest to determine whether or not DAG or DAG-derived molecules function as stress signals in bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used and their relevant characteristics are shown in Table S4. The construction of sinorhizobial mutants deficient in putative phospholipases or phosphatases is described in Table S5. *S. meliloti* strains were grown either in complex tryptone/yeast extract (TY) medium that contained 4.5 mM $CaCl_2$ (33) or in minimal medium (34) with succinate (8.3 mM) replacing mannitol as the carbon source at 30°C on a gyratory shaker. For determination of growth rates, strains were first grown on TY plates. Then, cells were resuspended at cell densities of 9×10^7 cells/mL in minimal medium and grown for 20 h during such a first growth cycle. During a second subcultivation in minimal medium, again inoculating with 9×10^7 cells/mL, growth rates of *S. meliloti* wild type and SMc00171-deficient mutant PHO00171 were determined. No differences in growth rates or final optical densities were observed between *S. meliloti* wild type and mutant PHO00171 when cultivated in minimal medium under high (1.3 mM) or low (0.02 mM) concentrations of Pi.

E. coli strains were cultured on Luria-Bertani (LB) medium (35) at 37°C or 30°C when the SMc00171 protein was expressed. Antibiotics were added to media in the following concentrations when required: in the case of *S. meliloti*, spectinomycin = 300 μg/mL, gentamicin = 70 μg/mL, nalidixic acid = 40 μg/mL, tetracycline = 10 μg/mL, neomycin = 50 μg/mL, and chloramphenicol = 60 μg/mL, and in the case of *E. coli*, spectinomycin = 200 μg/mL, carbenicillin = 100 μg/mL, tetracycline = 20 μg/mL, gentamicin = 70 μg/mL, kanamycin = 50 μg/mL, and chloramphenicol = 20 μg/mL. Plasmids were mobilized into *S. meliloti* strains by diparental mating.

In Vivo Labeling of Bacterial Strains with [^{14}C]acetate. The lipid composition of *S. meliloti* strains was determined as described previously (12). In pulse–chase experiments, 1-mL cultures of *S. meliloti* with an initial cell density of 2×10^8 cells/mL were labeled during a 24-h period of pulse with 10 μCi [1-^{14}C]acetate (60 mCi/mmol) in minimal medium containing high (1.3 mM) concentrations of Pi. After this pulse period, cultures were split in equal volumes, and the respective 0.5-mL cell suspensions were washed two times with 1-mL portions of minimal medium containing either high (1.3 mM) or low (0.02 mM) concentrations of Pi before being resuspended in 10 mL of the respective fresh medium. During the chase period, 1-mL aliquots were taken from the cultures at different time points, and the radioactivity of individual membrane lipids was quantified after separation by two-dimensional thin-layer chromatography.

Preparation of Cell-Free Extracts for Analysis of the SMc00171 Phospholipase. Cultures (0.5 L) of exponentially growing *E. coli* BL21(DE3) pLysS, also harboring pET9a or pC00171, were induced with 0.1 mM isopropyl-β-D-thiogalactoside at a density of 4×10^8 cells/mL and incubated for another 3 h at 30°C. After harvesting cells by low speed centrifugation at 4°C, each cell pellet was resuspended in 5 mL of 50 mM Tris/HCl buffer with a pH of 8.0. Cell suspensions were passed three times through a cold French pressure cell at 20,000 lb/in^2. Unbroken cells and cell debris were removed by centrifugation at $4,000 \times g$ for 10 min at 4°C to obtain cell-free extracts as supernatants. Protein concentrations were determined by the method of Dulley and Grieve (36).

Phospholipase Assays. To determine the activity of phospholipase SMc00171 in various extracts, assays were generally performed at 30°C. The standard reaction mixture contained 50 mM diethanolamine/HCl, (pH 9.8), 40 mM NaCl, 3 mM $MnCl_2$, 0.02% Triton X-100, PC (10 nCi 1,2-di[^{14}C]palmitoyl-L-3-PC with a specific radioactivity of 112 mCi/mmol [Amersham], 1,2-dipalmitoyl-D62-*sn*-glycero-3-phosphocholine [Avanti Polar Lipids], or [^{32}P]-labeled PC; see *SI Materials and Methods*), and 100 μg of protein in a final volume of 100 μL. After a certain time, the reaction was stopped by the addition of 250 μL of methanol and 125 μL of chloroform, and the lipid fraction was extracted as described previously (37). For analysis of the DAG formed, the lipid-containing chloroform phase was separated with n-hexane:diethyl ether:glacial acetic acid (30:70:1, vol/vol) by thin-layer chromatography (TLC). Analysis of the aqueous phase by TLC after extraction according to Bligh and Dyer (37) was performed using water:ethanol:ammonium hydroxide (91:95:6, vol/vol) as a mobile system. For phospholipase C from *Clostridium perfringens* (Sigma), the described assay (38) was modified using 10 mM $CaCl_2$. Standard *sn*-1,2-diolein, *sn*-1,3-diolein and phosphocholine (phosphorylcholine) were obtained from Sigma.

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