

The regulator gene *phoB* mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N,N,N*-trimethylhomoserine in *Rhizobium (Sinorhizobium) meliloti*

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Summary

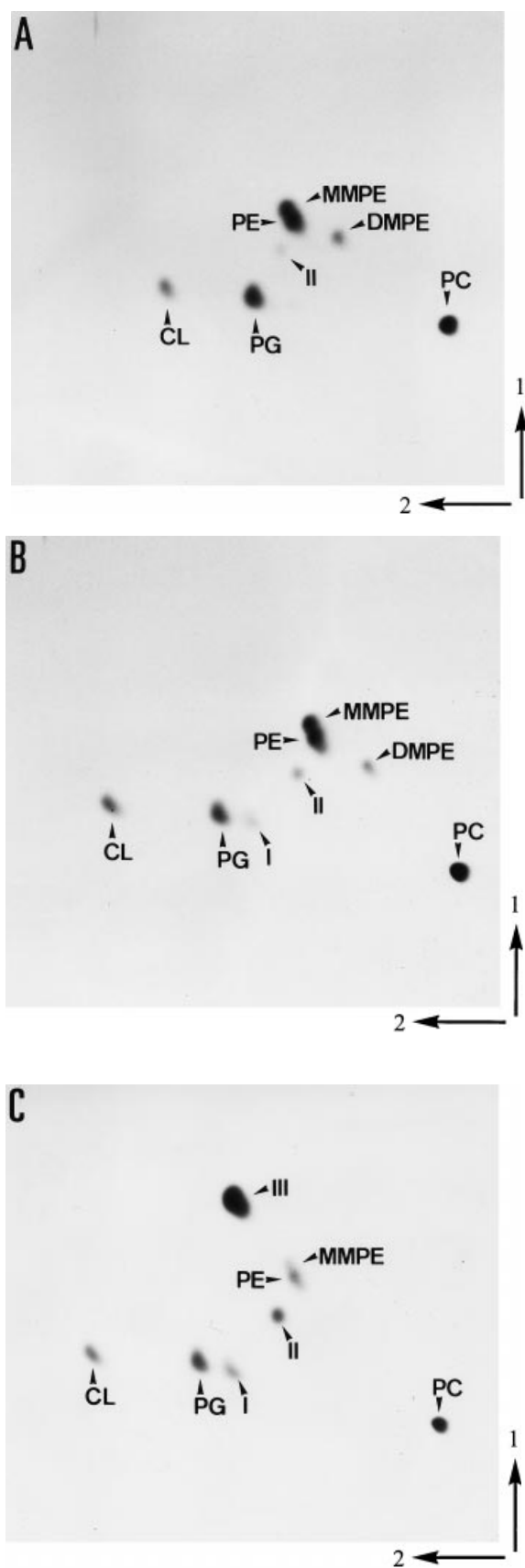
Bacteria react to phosphate starvation by activating genes involved in the transport and assimilation of phosphate as well as other phosphorous compounds. Some soil bacteria have evolved an additional mechanism for saving phosphorus. Under phosphate-limiting conditions, they replace their membrane phospholipids by lipids not containing phosphorus. Here, we show that the membrane lipid pattern of the free-living microsymbiotic bacterium *Rhizobium (Sinorhizobium) meliloti* is altered at low phosphate concentrations. When phosphate is growth limiting, an increase in sulpholipids, ornithine lipids and the *de novo* synthesis of diacylglyceryl trimethylhomoserine (DGTS) lipids is observed. *Rhizobium meliloti phoCDET* mutants, deficient in phosphate uptake, synthesize DGTS constitutively at low or high medium phosphate concentrations, suggesting that reduced transport of phosphorus sources to the cytoplasm causes induction of DGTS biosynthesis. *Rhizobium meliloti phoU* or *phoB* mutants are unable to form DGTS at low or high phosphate concentrations. However, the functional complementation of *phoU* or *phoB* mutants with the *phoB* gene demonstrates that, of the two genes, only intact *phoB* is required for the biosynthesis of the membrane lipid DGTS.

Introduction

Limitations of nitrogen and phosphate are physiological stress situations frequently encountered by free-living organisms. Higher plants have engaged in special symbiotic relationships leading to the formation of nitrogen-fixing nodules or phosphate-acquiring mycorrhiza to surmount such limiting conditions. Bacteria have developed their own mechanisms for reacting to phosphorus-limiting conditions. In *Escherichia coli*, for example, the two-component regulatory system PhoR–PhoB controls the Pho regulon, which consists of more than 30 but maybe up to 137 (van Bogelen *et al.*, 1996) genes that are induced or repressed by phosphate starvation. Some of these genes are involved in the transport and assimilation of inorganic phosphate and phosphorus-containing compounds. Similar systems have been described in *Bacillus subtilis* (Hulett *et al.*, 1994) and *Pseudomonas aeruginosa* (Anba *et al.*, 1990). The symbiotic soil bacterium *Rhizobium (Sinorhizobium) meliloti* forms nitrogen-fixing nodules with legumes of the genera *Medicago* and *Melilotus*. The *phoCDET* genes of *R. meliloti* encode a high-affinity phosphate uptake system, and mutants defective in these genes (formerly called *ndvF*) form 'empty' nodules, which contain few bacteria and fail to fix N₂ (Bardin *et al.*, 1996; Voegelé *et al.*, 1997). In *R. meliloti*, *phoB* is required for the expression of alkaline phosphatase (Al-Niemi *et al.*, 1997), the synthesis of exopolysaccharide II and the expression of the high-affinity phosphate uptake system PhoCDET, whereas it is required for the repression of the low-affinity phosphate uptake system OrfA-Pit (Bardin and Finan, 1998).

Some free-living soil bacteria have developed mechanisms in order to use less phosphorus for the biosynthesis of their cell constituents. Under phosphate-limiting conditions of growth, the cell wall composition of *Bacillus subtilis* changes drastically, and phosphorus-containing teichoic acid is quantitatively replaced by phosphorus-free teichuronic acid (Merad *et al.*, 1989). Also, under phosphate stress, membrane phospholipids are at least partially replaced by lipids containing no phosphorus, as demonstrated in *B. subtilis* (Minnikin *et al.*, 1972), *Pseudomonas diminuta* (Minnikin *et al.*, 1974), *Pseudomonas fluorescens* (Minnikin and Abdolrahimzadeh, 1974) and *Rhodobacter sphaeroides* (Benning *et al.*, 1995). The mechanism

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by which the expression of phosphorus-free membrane lipids is regulated was unknown before this study.

Here, we show that, under phosphate stress, *R. meliloti* produces the phosphorus-free lipids sulphoquinovosyl diacylglycerol, ornithine lipid and diacylglyceryl trimethylhomoserine. We demonstrate for the first time that reduced transport of phosphorus sources to the cytoplasm causes diacylglyceryl trimethylhomoserine synthesis and that this synthesis is controlled by the *phoB* regulatory gene.

Results

Rhizobium meliloti forms phosphorus-free lipids under phosphate limitation

The membrane of *Rhizobium (Sinorhizobium) meliloti* 1021 grown on complex TY medium is made up predominantly of the phospholipids phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylethanolamine (PE), monomethylphosphatidylethanolamine (MMPE), dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine (PC) (Fig. 1A). *R. meliloti* grown on minimal medium in the presence of high (1.3 mM) phosphate has a membrane lipid profile (Fig. 1B) similar to that after growth on complex medium. However, on quantification of the lipids synthesized (Table 1), an increase in the relative amounts of PC and of two, as yet unknown, lipids (lipid I and lipid II) is observed. In order to understand whether extra lipids are formed under phosphate-limiting conditions in *R. meliloti*, we compared the membrane lipid profiles produced after growth on high (1.3 mM) (Fig. 1B) and low (0.02 mM) phosphate-containing minimal medium (Fig. 1C). Quantification of the lipids (Table 1) demonstrates that, of the anionic phospholipids, the relative amount of cardiolipin is slightly increased and that of phosphatidylglycerol is slightly decreased by low phosphate, while that of the zwitterionic lipids (PE, MMPE, DMPE and PC) is drastically reduced. In contrast, the relative amounts of the two unknown lipids (lipid I and lipid II) are increased, and a very obvious synthesis of a third novel lipid (lipid III) is observed. Labelling experiments with [^{32}P]-phosphate at low phosphate concentrations show that, in contrast to the known phospholipids, radioactivity is not incorporated into any of the three newly formed, unknown lipids (data not shown), suggesting that lipids I, II and III do not contain phosphorus.

Fig. 1. Separation of [^{14}C]-acetate-labelled lipids from *Rhizobium meliloti* 1021 wild type after growth on complex TY medium (A) or after growth on minimal medium with high (B) or low phosphate concentrations (C) using two-dimensional thin-layer chromatography. The lipids phosphatidylcholine (PC), dimethylphosphatidylethanolamine (DMPE), monomethylphosphatidylethanolamine (MMPE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), lipid I (I), lipid II (II) and lipid III (III) are indicated.

Table 1. Membrane lipid composition of *Rhizobium meliloti* wild type after growth on different culture media.

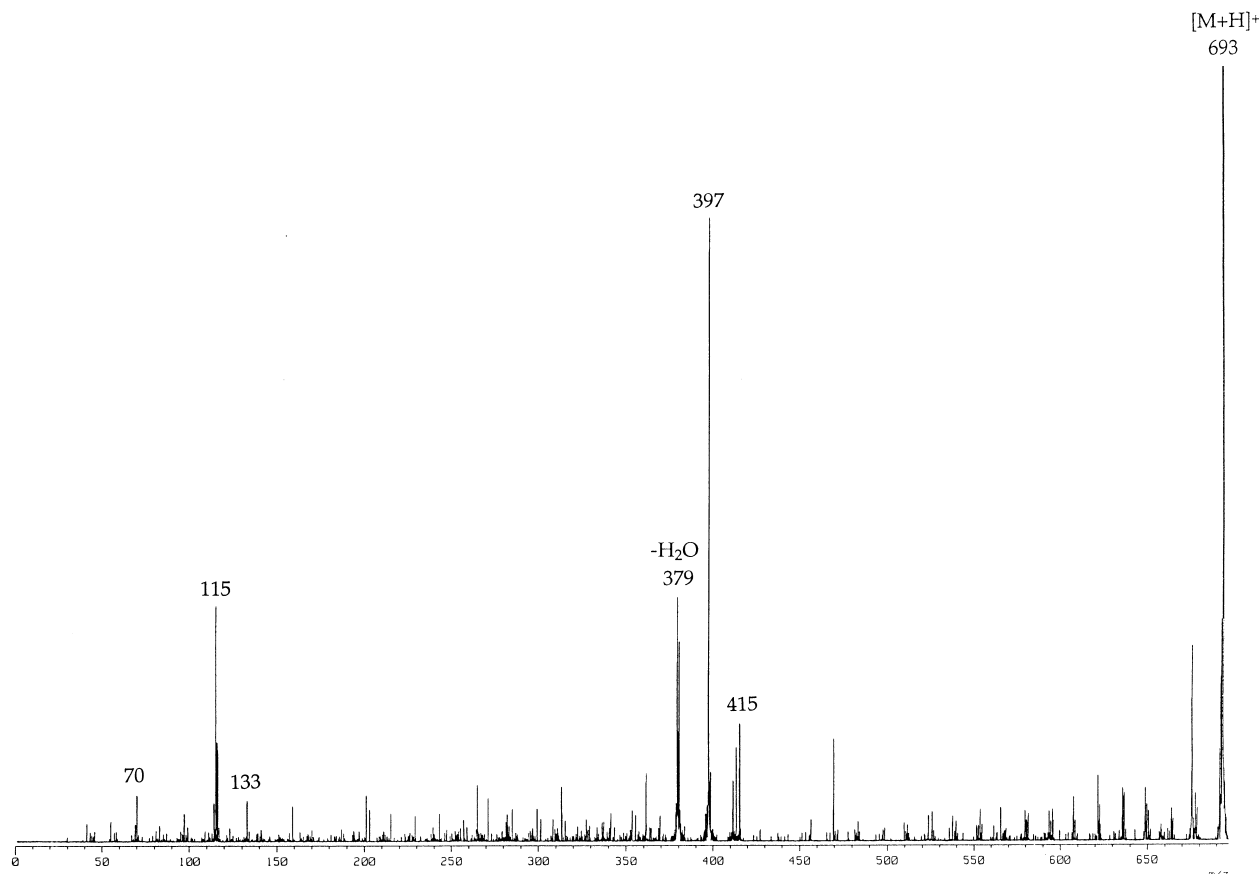
Lipid	Composition (% of total ^{14}C)		
	Complex TY	Minimal medium	
		(+ 1.3 mM Pi)	(+ 0.02 mM Pi)
PG	18	9.0	6.8
CL	4.3	4.7	6.1
PE + MMPE	35	19.5	8.0
DMPE	4.5	1.7	0.2
PC	37	60.1	10.3
Lipid I (SL)	0.3	2.8	5.4
Lipid II (OL)	0.9	2.2	5.9
Lipid III (DGTS)	0	0	57.3

Phosphorus-free lipids of R. meliloti are identified as sulphoquinovosyl diacylglycerol, ornithine lipid and diacylglycerol trimethylhomoserine

The three unknown lipids formed under phosphate limitation by *R. meliloti* 1021 wild type were separated and extracted from preparative two-dimensional thin-layer

chromatography (TLC) plates, and the fractions corresponding to lipids I, II and III were examined using mass spectrometry.

Lipid I was analysed using fast atom bombardment mass spectrometry (FAB-MS). While failing to form detectable ions in the positive ion mode, lipid I gave pseudomolecular ions in the negative ion mode at m/z 819, 833, 845, 847 (both weak), 859 (most intense) and 873 (weak), consistent with $[\text{M}-\text{H}]^-$ ions from sulphoquinovosyl diacylglycerols with the following fatty acyl combinations: C16:0 with C18:1; C16:0 with C19:1; two C18:1s; C18:1 with C18:0; C18:1 with C19:1; and C18:1 with C20:1 respectively. Consistent with these assignments, thioglycerol adduct ions (at m/z 927, 953, 955, 967 and 981) were observed for all of the species bearing a double bond in their acyl chains (Fukuda *et al.*, 1985), while the species (with $[\text{M}-\text{H}]^-$ at m/z 833) that has no double bonds (C16:0 with C19:1) did not form a thioglycerol adduct. Tandem mass spectrometric analysis of the major species (with $[\text{M}-\text{H}]^-$ at m/z 859) yielded fragment ions at m/z 80 (SO^-), 225 (SO-Hex^-), 281 (carboxylate anion of C18:1 fatty acid), 295 (weak) (C19:1 carboxylate anion), 563

**Fig. 2.** Positive-ion mode CID tandem mass spectrum of the major ornithine lipid ($[\text{M} + \text{H}]^+$ at m/z 693) from wild-type *R. meliloti* recorded in a matrix of thioglycerol. Spectrum is normalized to the most intense daughter ion.

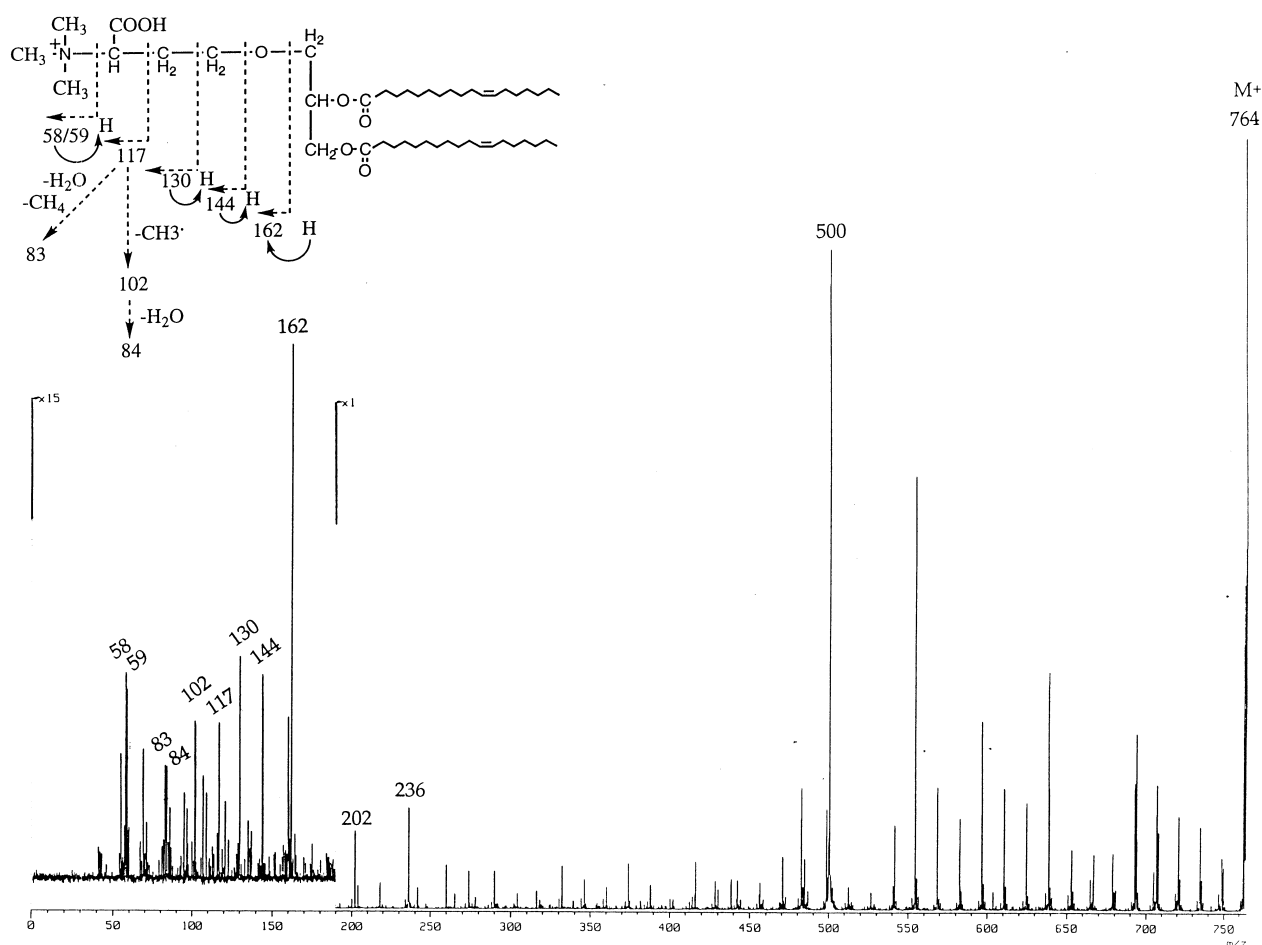


Fig. 3. Positive-ion mode CID tandem mass spectrum of a major diacylglycerol trimethylhomoserine (M^+ at m/z 764) from a wild-type *R. meliloti* recorded in a matrix of thioglycerol. Spectrum is normalized to the most intense daughter ion. The ions at m/z 59, 83 and 117 are assigned as radical cations, while all other ions correspond to closed-shell even electron species.

(arising by elimination of the C19:1 fatty acid) and 577 (elimination of the C18:1 fatty acid), consistent with the assignment of the major species in the lipid I preparation being a sulphoquinovosyl diacylglycerol with C18:1 and C19:1 fatty acyl chains.

Lipid II yielded an intense $[M + H]^+$ pseudomolecular ion at m/z 693, together with a less intense ion at m/z 679. Only the latter yielded a thioglycerol adduct ion at m/z 787, indicating that the major species bears no double bonds. Tandem mass spectrometric analysis of the ion at m/z 693 (Fig. 2) yielded an intense fragment ion at m/z 397, together with a much less intense ion at m/z 415, generated by elimination and direct cleavage, respectively, of a C19:1 fatty acid. Fragment ions in the low-mass region of the spectrum at m/z 133 ($[Orn + H]^+$), 115 (Orn B ion) and 70 (Orn immonium ion) are consistent with the presence of ornithine, the same three ions also being observed in the positive ion CID mass spectrum of *N*-acetyl ornithine (data not shown). The generation of ions by the facile loss of only the C19:1 fatty acid is consistent

with an *N*-acyl ornithine lipid in which a C18 hydroxy fatty acid is amide bound to ornithine, and to which a C19:1 fatty acid is ester linked to the hydroxyl group of the *N*-C18 fatty acid. Analogous ions are observed in the CID mass spectrum obtained from the less intense ion at m/z 679, consistent with it bearing an ester-linked C18:1 fatty acid on an *N*-C18 hydroxy ornithine. Lipid II is thus assigned as *N*-C18-OH ornithine to which a C19:1 fatty acid is ester linked via the OH group. A minor component corresponds to a species bearing a C18:1 fatty acyl ester.

Positive-ion FAB-MS of lipid III yielded intense molecular ions at m/z 764 and 778, each having a thioglycerol adduct ion (at m/z 872 and 886), consistent with both species bearing unsaturated fatty acids. Positive-ion CID mass spectrometric analysis (Fig. 3) of the ion with m/z 764 yielded an intense fragment ion at m/z 500, consistent with the loss of a C18:1 fatty acyl chain, with proton transfer. The fragment ion at m/z 236 is consistent with a similar loss of a second C18:1 fatty acid. The intensity of the molecular ions, together with the low-mass fragment ions at

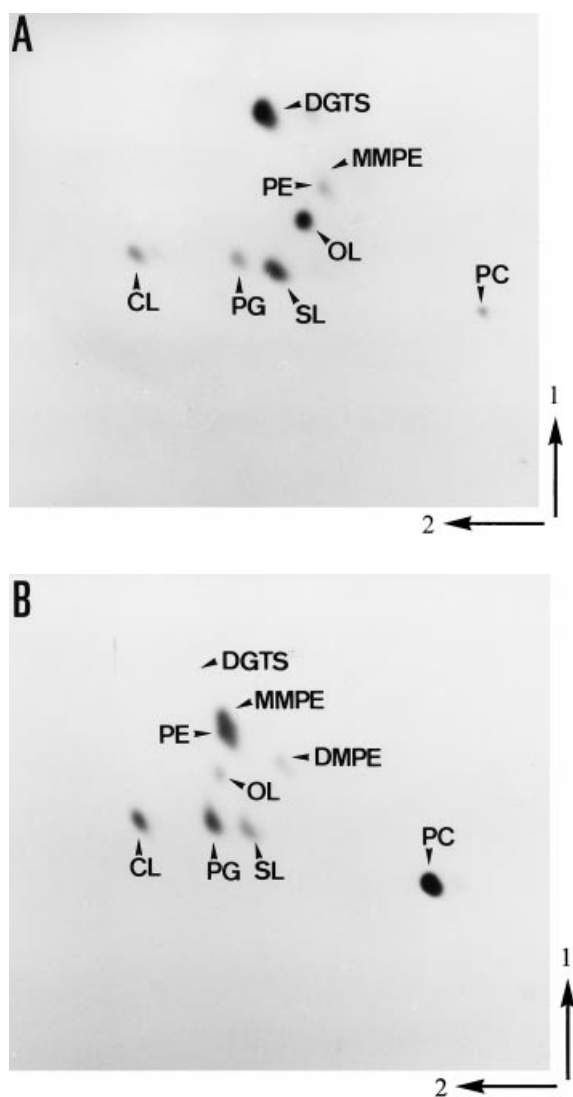


Fig. 4. Separation of [^{14}C]-acetate-labelled lipids from *R. meliloti* *pho* mutants using two-dimensional thin-layer chromatography. Lipid patterns of $\Delta\textit{phoCDET}$ mutant RmG439 after growth on culture medium with high phosphate concentration (A) and of *phoB*-deficient mutant RmH838 after growth on culture medium with low phosphate concentration (B) are shown. The lipids phosphatidylcholine (PC), dimethylphosphatidylethanolamine (DMPE), monomethylphosphatidylethanolamine (MMPE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), sulphoquinovosyl diacylglycerol (SL), ornithine lipid (OL) and diacylglycerol trimethylhomoserine (DGTS) are indicated.

m/z 58, 59, 83, 84, 102, 117, 130, 144, 162 and 202, are all consistent with a diacylglycerol trimethylhomoserine structure (see Fig. 3). These low-mass ions, together with the absence of an ion at m/z 74, are consistent with the assignment of a trimethylhomoserine head group rather than the isomeric 2-OH methyl *N*-trimethyl β -alanine head group. Comparable ions (at m/z 58, 84, 102, 117, 131 and 144) were observed in electron ionization spectra of diacylglycerol trimethylhomoserines (Eichenberger and

Boschetti, 1978), although the authors were unable to interpret them. The CID spectrum generated from the ion at m/z 778 was almost identical (data not shown) to that from the ion at m/z 764, but it contained an additional ion at m/z 514, arising from loss of a C18:1 fatty acid, in addition to the ion at m/z 500, in this case arising from loss of a C19:1 fatty acid. Thus, lipid III is assigned as being a mixture of two components, both diacylglycerol trimethylhomoserines, one bearing two C18:1 fatty acids, and the other bearing one C18:1 and one C19:1 fatty acid.

Rhizobium meliloti mutants deficient in phosphate transport synthesize phosphorus-free lipids even when high phosphate concentrations are present in the culture medium

In order to understand how phosphorus sources control the biosynthesis of phosphorus-free membrane lipids in *R. meliloti*, we compared the membrane lipid patterns of *R. meliloti* wild type with *R. meliloti phoCDET* mutants deficient in phosphate uptake to the cytoplasm (Bardin *et al.*, 1996). Two-dimensional TLC analysis of the lipids from the *R. meliloti phoCDET* deletion mutant RmG439 grown on high (1.3 mM) (Fig. 4A) and low (0.02 mM) phosphate-containing minimal medium (data not shown) reveals that phosphorus-free membrane lipids are formed under both low- and high-phosphate growth conditions. A quantitative comparison of the wild-type lipid pattern with that of mutant RmG439 grown on high-phosphate-containing minimal media (Table 2) demonstrates that phospholipids comprise about 95% of total membrane lipids in the wild type, whereas they account for only 16% in mutant RmG439. The relative amounts of sulpholipids are fivefold higher and ornithine lipids are eightfold higher in mutant RmG439 than in the wild type. No DGTS is formed in the wild type, whereas DGTS is the most prominent lipid formed in mutant RmG439, comprising more than half of the total lipids. Complementation of RmG439 with the *phoCDET*-carrying plasmid pTH38 restored the

Table 2. Membrane lipid composition of *Rhizobium meliloti* wild type, $\Delta\textit{phoCDET}$ mutant (RmG439) and complemented mutant (RmG439 \times pTH38) after growth on high (1.3 mM) phosphate-containing minimal medium.

Lipid	Composition (% of total ^{14}C)		
	Wild type	RmG439	RmG439 \times pTH38
PG	9.0	3.8	8.2
CL	4.7	4.8	4.5
PE + MMPE	19.5	5.0	15.3
DMPE	1.7	0	1.1
PC	60.1	2.6	60.5
SL (lipid I)	2.8	16.5	2.4
OL (lipid II)	2.2	17.3	5.6
DGTS (lipid III)	0	50.7	2.3

wild-type-like formation of membrane lipids (Table 2). Similar results were obtained with the *phoC* and *phoT* mutants RmG490 and RmG491 (data not shown). When RmG439 is grown in the presence of aminoethylphosphonate (1 mM) as phosphorus source, the relative amount of phospholipids formed exceeds 70% of the total membrane lipids (data not shown). Aminoethylphosphonate is transported by an alternative uptake system to PhoCDET and Pit in RmG439, and this can provide the signal controlling the phosphorus sensory system (Bardin and Finan, 1998). These results suggest that neither the culture medium concentration nor the periplasmic concentration of phosphate controls the formation of phosphorus-free membrane lipids. Rather, it must be the uptake of phosphorus sources to the cytoplasm or the cytoplasmic level of phosphate itself that is exerting control on DGTS expression.

Rhizobium meliloti mutants deficient in *phoU* or *phoB* are unable to synthesize diacylglycerol trimethylhomoserine under phosphate limitation

Regulation of some phosphate-controlled processes in *R. meliloti* occurs through the regulator gene *phoB* (Al-Niemi *et al.*, 1997; Bardin and Finan, 1998; Bardin *et al.*, 1998). In order to determine whether the *phoUB* operon is involved in the biosynthesis of phosphorus-free membrane lipids, the lipid patterns of the *phoU*-deficient mutant RmH399 and of the *phoB*-deficient mutant RmH838 were studied using two-dimensional TLC after growth on high (1.3 mM) or low (0.02 mM) phosphate-containing minimal medium. The *phoB*-deficient mutant RmH838 is unable to synthesize DGTS at high (data not shown) or low (Fig. 4B, Table 3) phosphate concentrations, suggesting that PhoB is essential for DGTS synthesis. Ornithine lipids are formed in the *phoB*-deficient mutant RmH838 in comparably low relative amounts after growth on both high (data not shown) or low phosphate concentrations (Table 3), suggesting that the increase in ornithine lipid synthesis when the wild type is shifted to low-phosphate medium is

mediated and controlled by PhoB. In contrast, the relative amounts of sulpholipids are still twofold higher at low than at high phosphate concentrations (data not shown). Quantitative analysis of the lipid pattern of RmH838 grown at low phosphate concentration (Table 3) demonstrates that this *phoB*-deficient mutant predominantly forms phospholipids and small amounts of phosphorus-free lipids, thereby showing a similar pattern to the wild type after growth at high phosphate concentration (Table 1).

Complementation of the *phoB* mutant *R. meliloti* H838 with the *phoB*-expressing plasmid pBW12 restored wild-type-like membrane lipid profiles. In high phosphate (1.3 mM) concentrations, phospholipids were mainly formed (data not shown), whereas after growth on low phosphate (0.02 mM), phosphorus-free membrane lipids were formed predominantly (Table 3), resembling the low-phosphate lipid profile of the wild type.

Quantification of the membrane lipids of the *phoU*-deficient mutant RmH399 grown at low phosphate (Table 3) demonstrates that it resembles that of the *phoB*-deficient mutant RmH838 (Table 3). However, minor amounts of DGTS are still expressed in the *phoU*-deficient mutant RmH399 (Table 3) grown at low phosphate. The introduction of a functional *phoB* gene into a *phoU*-deficient mutant (RmH399 × pBW12) reduced the production of phospholipids and increased the formation of phosphorus-free lipids at low phosphate concentrations (Table 3), yielding a lipid profile resembling that obtained in low phosphate from the wild type.

In order to define the functional sequence of gene products controlling the production of the phosphorus-free membrane lipids, we studied the lipid composition of double mutants defective in both *phoC* and *phoB*. Quantification of the lipids of a double mutant (RmH625) deficient in PhoB and PhoC shows that it contains more than 95% phospholipids, less than 5% phosphorus-free lipids and absolutely no DGTS after growth in high (data not shown) or low-phosphate-containing media (Table 3). The membrane lipid pattern of the *phoUB*-complemented *phoB*,

Table 3. Membrane lipid composition of *Rhizobium meliloti* wild type, a mutant deficient in the regulator PhoB (RmH838), a *phoB*-complemented mutant (RmH838 × pBW12), a mutant deficient in PhoU (RmH399), a *phoB*-complemented *phoU*-deficient mutant (RmH399 × pBW12), a double mutant deficient in PhoB and PhoC (RmH625) and a double mutant complemented with *phoUB* (RmH625 × pTH284) after growth on low (0.02 mM) phosphate-containing minimal medium.

Lipid	Composition (% of total ¹⁴ C)						
	Wild type	RmH838	RmH838 × pBW12	RmH399	RmH399 × pBW12	RmH625	RmH625 × pTH284
PG	6.8	12.2	5.5	9.2	6.1	9.8	10.8
CL	6.1	9.2	3.8	5.5	4.2	6.0	5.4
PE + MMPE	8.0	19.3	3.0	16.6	3.3	19.7	10.7
DMPE	0.2	1.6	0.1	1.5	0	1.5	1.0
PC	10.3	58.8	6.2	56.5	7.0	57.4	24.1
SL (lipid I)	5.4	3.8	9.6	4.6	7.6	3.9	7.1
OL (lipid II)	5.9	1.6	12.8	2.1	15.9	1.7	3.2
DGTS (lipid III)	57.3	0	59.1	3.2	55.9	0	38.8

phoC double mutant (RmH625 × pTH284) consists of about 50% phosphorus-free membrane lipids (Table 3), and this pattern therefore resembles that of a complemented *phoB* mutant (RmH838 × pTH284) (data not shown). These results suggest that *phoB* mutations are epistatic on *phoC* mutations, indicating that the signal controlling DGTS biosynthesis is first mediated by the *phoCDET* gene products and only further along in the signal transduction chain by the *phoB* gene product.

Discussion

Phospholipids are generally thought to be the major membrane-forming constituents in all living organisms. *R. meliloti* predominantly produces phospholipids under culture conditions in which sufficient phosphorus is supplied. However, as we have shown, when *R. meliloti* is growth limited by phosphorus, mainly phosphorus-free membrane lipids are formed. These phosphorus-free lipids of *R. meliloti* were isolated, analysed by mass spectrometry and determined to be sulphoquinovosyldiacylglycerols, ornithine lipids and diacylglyceryl-*N,N,N*-trimethylhomoserines. Representative structures of these phosphorus-free membrane lipids are shown in Fig. 5. Under phosphate limitation, the photosynthetic bacterium *Rhodobacter sphaeroides* replaces most of its phospholipids with phosphorus-free membrane lipids (sulphoquinovosyldiacylglycerol, an ornithine lipid, diacylglyceryl-*N,N,N*-trimethylhomoserine, glucosylgalactosyl diacylglycerol and a monohexosyl diacylglycerol), presumably in an attempt to minimize its requirement for phosphorus (Benning *et al.*, 1995).

Sulphoquinovosyldiacylglycerols have been described before in several members of the genus *Rhizobium*, among them *R. meliloti* (Cedergren and Hollingsworth, 1994). In *R. sphaeroides* sulpholipid formation is increased under phosphate-limiting growth conditions (Benning *et al.*, 1993). Here, we show that, in *R. meliloti*, sulpholipid production is also increased when the organism is grown under phosphate limitation. Surprisingly, this increase is not or not only controlled by PhoB, but presumably by an alternative system also mediating phosphorus limitation. One alternative system might be the two-component sensory transduction system ChvG (sensor) and ChvI (response regulator), which is important for virulence in *Agrobacterium tumefaciens* (Charles and Nester, 1993) and has also been described in *R. meliloti* (Østerås *et al.*, 1995). ChvI from *A. tumefaciens* is even able to complement *phoB* mutations in *E. coli* (Mantis and Winans, 1993).

Although ornithine-amide lipids are known from *Pseudomonas fluorescens* (Minnikin and Abdollahzadeh, 1974) and lysine-containing lipids have been reported from the close relative *Agrobacterium tumefaciens* (Tahara *et al.*,

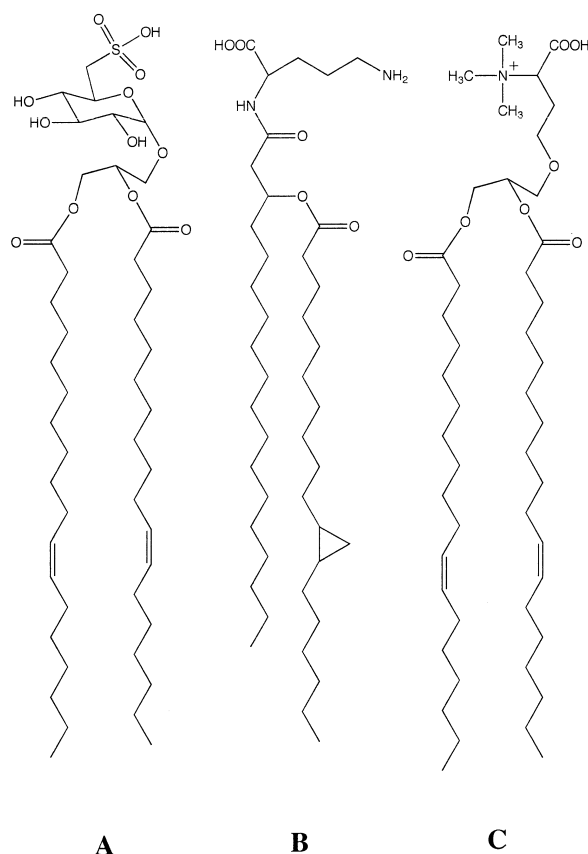


Fig. 5. Proposed structures of phosphorus-free lipids sulphoquinovosyl diacylglycerol (A), ornithine lipid (B) and diacylglyceryl trimethylhomoserine (C) in *R. meliloti*.

1976), our description of ornithine lipids in *R. meliloti* is the first in the *Rhizobiaceae* family.

Diacylglyceryl-*N,N,N*-trimethylhomoserines (DTGS) have been described in several algae, other lower eukaryotes (Thompson, 1996) and *R. sphaeroides* (Benning *et al.*, 1995). This is the first report of DGTS being present in the *Rhizobiaceae* and of becoming its major membrane lipid under phosphate limitation.

In order to learn about the mechanism by which the biosynthesis of phosphorus-free membrane lipids is controlled, we studied *R. meliloti ndvF* mutants deficient in the high-affinity phosphate uptake system *phoCDET* (Bardin *et al.*, 1996). Such mutants still contain a regulator PhoB, which mediates the lack of phosphorus under low phosphate concentrations. Surprisingly, the alternative low-affinity OrfA-Pit phosphate uptake system is repressed in a *phoCDET* mutant background, and this prevents efficient uptake of phosphate under low or high phosphate concentrations (Bardin and Finan, 1998). In comparison with the wild type, in which phospholipids comprise 95% of the total lipids, *phoCDET* mutants show drastically decreased relative amounts of phospholipid to as low as

16% (Table 2). Phosphatidylcholine undergoes the most dramatic decrease from 60% (wild type) to 2.6% (RmG439), and the possibility that phosphatidylcholine biosynthesis in *R. meliloti* is negatively regulated by PhoB is presently under investigation. In *phoCDET* mutants, the amounts of sulpholipids and ornithine lipids synthesized are raised, and DGTS is formed as the predominant membrane lipid.

In *E. coli*, phosphate starvation is mediated via the two-component system PhoR/PhoB, which controls genes of the Pho regulon (van Bogelen *et al.*, 1996). Although the exact role of PhoU is unclear, *E. coli* mutants deficient in *phoU* are constitutive for the expression of the Pho regulon (Wanner, 1996). In *R. meliloti*, no *phoR* gene has been detected, whereas *phoU* and *phoB* are found in one operon (Bardin and Finan, 1998; GenBank accession no. M96261, P. McLean, C. Liu, C. Sookdeo and F. Cannon, unpublished results). In order to understand whether PhoU and PhoB control the biosynthesis of phosphorus-free membrane lipids, we studied lipid profiles of *phoU*- or *phoB*-deficient mutants. At low phosphate concentrations, *phoU*-deficient mutants show slightly reduced formation of sulpholipids and ornithine lipids compared with the wild type. However, formation of DGTS is drastically reduced. The phenotype of a *phoB* insertion mutant is even more clear: sulpholipids are slightly reduced, ornithine lipids more strongly reduced, while DGTS is not formed at all. Also, there is no expression of the *phoCDET* operon in a *phoB*-deficient mutant and little *phoCDET* expression in a *phoU*-deficient mutant (Bardin and Finan, 1998), and we conclude that the *phoCDET* operon is controlled in a similar way to the formation of phosphorus-free membrane lipids. As *phoU* and *phoB* appear to form an operon, *phoUB*, an insertion in *phoU* probably has a polar effect on *phoB*. We therefore investigated the lipid profiles of *phoU*- or *phoB*-deficient mutants complemented with a functional *phoB* gene grown at low phosphate concentrations and found that *phoB* complements both *phoU*- or *phoB*-deficient mutants with regard to membrane lipid synthesis. These results suggest that PhoB is required as a positive regulatory element for DGTS biosynthesis, whereas PhoU is not.

Double mutants deficient in *phoB* and *phoC* predominantly form phospholipids and no DGTS and, therefore, show the same lipid profiles as *phoB* single mutants, suggesting that the sensing of reduced uptake of phosphorus sources to the cytoplasm is mediated via the PhoB regulation system.

The fact that a *phoB* insertion mutant is unable to direct DGTS synthesis under low phosphate suggests that activated, phosphorylated PhoB acts as a positive regulator of transcription of as yet unknown genes involved in DGTS biosynthesis. We therefore demonstrate here for the first time that the phosphate stress-induced change

in membrane lipid composition, and specifically DGTS biosynthesis, is controlled by the regulator PhoB. Mutants of *R. meliloti* (Al-Niemi *et al.*, 1997; Bardin and Finan, 1998) or *Bradyrhizobium japonicum* (Minder *et al.*, 1998) deficient in *phoB* can form nodules on their respective host plants. Therefore, it is unlikely that DGTS, which needs PhoB for its production, is required for nodule formation by rhizobia on their legume hosts. However, the role of the phosphorus-free membrane lipids in *Rhizobium* is expected to be important when the bacterium exists as a free-living organism in soils, environments that usually contain growth-limiting concentrations of inorganic phosphate (0.1–10 μM) (Bielecki, 1973). We suggest that the ability to form such phosphorus-free membrane lipids is a significant advantage in the soil when phosphate is growth limiting and that such an ability might lead to increased competitiveness.

Experimental procedures

Bacterial strains, media, and growth conditions

Rhizobium (Sinorhizobium) meliloti 1021 wild type (Meade *et al.*, 1982), mutants and complemented mutants (Table 4) were grown in either complex TY medium that contained 4.5 mM CaCl_2 (Beringer, 1974) or minimal medium (Sherwood, 1970) with succinate (8.3 mM) replacing mannitol as carbon source at 29°C on a gyratory shaker.

For quantitative determination of lipids after growth at high or low phosphate, strains were first grown on TY plates containing 1 mM aminoethylphosphonate in order to prevent selection for suppressor mutants in the case of *R. meliloti pho* mutants. Cells were then washed with phosphate-free minimal medium and resuspended at densities of 9×10^7 cells ml^{-1} in minimal medium containing either high (1.3 mM) phosphate or low (0.02 mM) phosphate. Cell suspensions were grown for 24 h and, during this growth period on minimal media, cultures containing high phosphate grew much faster than cultures containing low phosphate, indicating that the low phosphate concentration in the medium is growth limiting and that internal (poly)phosphate reserves (Shiba *et al.*, 1997) of the bacteria must have been exhausted. Suspensions of such cultures that had adapted to their respective available phosphate concentrations were transferred to fresh media to obtain initial densities of 9×10^7 cells ml^{-1} and grown for an additional 24 h in the presence of [^{14}C]-acetate. At the end of the growth phase, cell suspensions were streaked on low osmolarity glutamate–yeast extract–mannitol–salts (GYM) agar for single colonies in order to ensure that *phoCDET*-deficient mutants still showed a mucoid colony phenotype and not the dry-colony phenotype of the wild type or of *sfx1* or *sfx2* suppressor mutants (Oresnik *et al.*, 1994).

Cloning of the rhizobial phoB gene into a broad-host-range vector

Using polymerase chain reaction (PCR) and specific oligonucleotides (GGAATACATATGTTGCCGAAGATTGCCGT-

Table 4. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference
<i>Rhizobium meliloti</i> 1021	SU47 <i>str-21</i>	Meade <i>et al.</i> (1982)
Rm 1021 derivatives		
RmG439	12 kb <i>Hind</i> III fragment containing <i>ndvF</i> locus, replaced with neomycin resistance <i>Hind</i> III fragment of Tn5	Charles <i>et al.</i> (1991)
RmG490	<i>phoC490::ΩSp^r</i>	Charles <i>et al.</i> (1991)
RmG491	<i>phoT491::ΩSp^r</i>	Charles <i>et al.</i> (1991)
RmH399	<i>ΩphoU10::TnV</i>	This work
RmH625	<i>phoC490::ΩSp^r ΩphoB3::TnV</i>	Bardin and Finan (1998)
RmH838	<i>ΩphoB3::TnV</i>	Bardin and Finan (1998)
Plasmids		
pTH22	pLAFR1 clone carrying Rm1021 <i>ndvF</i>	Charles <i>et al.</i> (1991)
pTH38	7.3 kb <i>Bam</i> HI subclone of pTH22 in pRK7813, carries <i>ndvF</i>	Charles <i>et al.</i> (1991)
pTH284	pLAFR1 clone carrying Rm1021 <i>phoUB</i>	Finan laboratory strain collection
pET9a	ColE1, T7 promoter-controlled expression vector, kanamycin-resistant	Studier <i>et al.</i> (1990)
pMP3510	IncP, expression vector containing the promoter of <i>nodA</i> , tetracycline-resistant	Spaink <i>et al.</i> (1995)
pBW10	<i>phoB</i> as <i>Nde</i> I– <i>Bam</i> HI insert in pET9a	This study
pBW12	<i>phoB</i> as <i>Xba</i> I– <i>Bam</i> HI fragment from pBW10 in pMP3510	This study

AGTC and AAAGGATCCTCAGCTCTCCAGCGAATAGCC-CG), the *phoB* gene was amplified from genomic DNA of *R. meliloti* 1021 with *Pfu* polymerase. Suitable restriction sites (underlined) for cloning the *phoB* gene were introduced by PCR with the oligonucleotides. After restriction with *Nde*I and *Bam*HI, the PCR-amplified DNA fragment was cloned into a pET9a vector (Studier *et al.*, 1990) to obtain the expression plasmid pBW10, in which the *phoB* gene can be over-expressed under control of the T7 promoter (data not shown). The correct in frame cloning and the correct published sequence (GenBank accession no. M96261) was demonstrated by DNA sequencing (data not shown). The *phoB*-containing *Xba*I–*Bam*HI fragment of pBW10 was recloned into the broad-host-range vector pMP3510 (Spaink *et al.*, 1995), in which *phoB* can be expressed under the control of the *nodA* promoter.

In vivo labelling of *R. meliloti* with [¹⁴C]-acetate and quantitative analysis of lipid extracts

The lipid compositions of *R. meliloti* 1021 wild type, mutant strains and complemented mutants were determined after labelling with [1-¹⁴C]-acetate. Cultures (1 ml) in TY or minimal medium were inoculated from phosphate-adapted precultures. After the addition of 0.4 μCi [1-¹⁴C]-acetate (60 mCi mmol⁻¹) to each culture, the cultures were incubated for 24 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 Bligh and Dyer (1959). The chloroform phase was used for lipid analysis on TLC plates and, after two-dimensional separation, the individual lipids were quantified as described previously (de Rudder *et al.*, 1997).

Isolation of phosphorus-free lipids using preparative thin-layer chromatography

Cells of *R. meliloti* wild type were grown in 1 l of minimal medium (Sherwood, 1970) containing low phosphate

(0.02 mM). After harvesting, lipids were extracted from the cells according to Bligh and Dyer (1959). Concentrated lipid preparations were separated on high-performance (HP)TLC silica gel 60 plates in two dimensions as described previously (de Rudder *et al.*, 1997). Iodine-stained material with an R_f value between 0.25 and 0.30 in the first dimension and with an R_f value between 0.45 and 0.51 in the second dimension was scraped off, and the lipid (lipid I) was repeatedly extracted from the silica gel using a mixture of chloroform–methanol–water (1:2:0.8, v/v). After the addition of 1 volume of chloroform and 1 volume of water, the unknown lipid material partitioned into the lower chloroform phase and was analysed using mass spectrometry. Lipid II, with an R_f value between 0.37 and 0.40 in the first dimension and with an R_f value between 0.49 and 0.51 in the second dimension, as well as lipid III, with an R_f value between 0.54 and 0.60 in the first dimension and with an R_f value between 0.60 and 0.65 in the second dimension, were extracted as described above for lipid I.

Mass spectrometry

Fast atom bombardment mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at ±10 kV accelerating voltage. The fab gun was operated at 4 kV accelerating voltage with an emission current of 10 mAmps and using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP 9000 data system running JEOL COMPLEMENT software.

Collision-induced dissociation mass spectra of lipids II and III were recorded using the same machine, with helium as the collision gas in the third field free region collision cell, at a pressure sufficient to reduce the parent ion to one-third of its original intensity. Unimolecular decomposition spectra of lipid I were obtained similarly, but without the use of collision gas. Samples (1 μl aliquots) were loaded into a matrix of monothioglycerol.

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