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The Phospholipid Composition of *Bradyrhizobium* spp.

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Abstract. The phospholipid composition of two strains of *Bradyrhizobium* is reported. In contrast to previous studies [Bunn CR, Elkan GH (1970) Can J Microbiol 17:291–295; and Gerson T, Patel JJ (1975) Appl Microbiol 30:193–198], we determined that phosphatidylglycerol is a major phospholipid within this bacterial genus. Furthermore, neither phosphatidylserine nor phosphatidylinositol was detected within lipid extracts derived from these bacteria. In addition to phosphatidylglycerol, other major phospholipids of *Bradyrhizobium* were shown to include phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. Possible explanations for the discrepancies between the present study and those of previous investigations are discussed.

Bacteria of the family *Rhizobiaceae* are characterized by their ability to infect higher plants. Infection caused by species of two genera of this family, *Rhizobium* and *Bradyrhizobium*, leads to the development of beneficial nitrogen-fixing nodules on the roots of susceptible leguminous plant hosts. Although the infection process induced by *Rhizobium* and *Bradyrhizobium* appears to be fundamentally similar, several significant distinctions have been noted between species of these two bacterial genera. These distinctions include rate of growth, extracellular polysaccharide structure, nucleic acid homology, as well as pathways of carbohydrate utilization and metabolism [6, 14]. In addition, our laboratory has recently determined that the cell-associated oligosaccharides of *Bradyrhizobium* spp. are substantially different in structure from those synthesized by species of *Rhizobium* [20].

The cell surface composition of rhizobial and bradyrhizobial species has been the subject of intensive research because it is generally believed to provide essential signaling functions during the plant infection process [see reference 12 for review]. Such functions may include the determination of plant–host specificity as well as possible involvement in the attachment of bacterium to plant. Indeed, studies of various bacterial mutants have provided evidence for essential functions of cell surface polysaccharides and oligosaccharides during legume nodulation [5, 9, 15, 16].

A relatively little-studied cell surface component of the *Rhizobiaceae* is the membrane phospholipid composition of these bacteria. For example, only two previous studies have been concerned with the determination of the phospholipid composition of species of *Bradyrhizobium* [1, 10]. The results of these studies have indicated that the phospholipid composition of species of *Bradyrhizobium* shares one significant characteristic with that of species of *Rhizobium*: both bacterial genera have been found to contain substantial amounts of phosphatidylcholine [see references 11 and 23 for review]. However, aside from the presence of phosphatidylcholine, several unusual features of the phospholipid composition of species of *Bradyrhizobium* have been reported. These features include the presence of significant levels of phosphatidylserine and phosphatidylinositol as well as the apparent absence of phosphatidylglycerol [1, 10]. On the basis of these previous studies, the phospholipid composition of bacteria within the genus *Bradyrhizobium* would appear to be quite unusual for the following reasons: (a) phosphatidylserine, an intermediate in the biosynthesis of phosphatidylethanolamine, is not normally present as a major phospholipid within bacterial membranes [11]; (b) phosphatidylinositol is rarely found within Gram-negative bacteria [11]; and (c) phosphatidylglycerol is an abundant phospholipid within most bacterial membranes [11].

For the above reasons, our laboratory initiated a reexamination of the phospholipid composition of species of *Bradyrhizobium*. In the present study we

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demonstrate that phosphatidylglycerol is a major phospholipid within *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1. In addition, we report the absence of detectable levels of phosphatidylserine and phosphatidylinositol within these two bradyrhizobial strains. Possible explanations for the apparent discrepancies between our results and previous studies are discussed.

Materials and Methods

Bacterial and yeast strains. *Bradyrhizobium japonicum* USDA 110 was provided by R.F. Griffin of the Nitrogen Fixation and Soybean Genetics Laboratory (Agricultural Research Service, Beltsville, Maryland). *Bradyrhizobium* sp. strain 32H1 (ATCC 33848) was obtained from the American Type Culture Collection (Rockville, Maryland). *Rhizobium meliloti* 1021 was provided by F.M. Ausubel (Harvard Medical School, Boston, Massachusetts). *Saccharomyces cerevisiae* strain 522 was provided by R.B. Beelman (The Pennsylvania State University, University Park, Pennsylvania).

Extraction of lipids. Six-liter cultures of each bacterial strain were grown in YM medium (0.4 g yeast extract, 10 g mannitol, 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.5 g K_2HPO_4 per liter, pH 7) at 30°C on a rotary shaker. Cells were harvested during logarithmic growth at a density of approximately 50 μg total cell protein per mL. Pellets were washed once with YM salts (0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.5 g K_2HPO_4 per liter, pH 7), and lipids were extracted by a modified Bligh and Dyer procedure [19]. Lipid extracts were stored under nitrogen at −20°C.

Phospholipid identification. Phospholipids were separated by both one-dimensional and two-dimensional thin-layer chromatography with silica gel 60 aluminum-backed plates (E. Merck, Darmstadt, FRG). Several different solvent systems were employed: A, chloroform/methanol/water (65 : 25 : 4, vol/vol); B, chloroform/acetone/methanol/acetic acid/water (6 : 8 : 2 : 2 : 1, vol/vol); C, *n*-butanol/acetic acid/water (6 : 2 : 2, vol/vol); and D, chloroform/methanol/7 *M* ammonia (60 : 35 : 5, vol/vol). Lipids were detected with the following spray reagents: molybdenum blue, periodate-Schiff, Dragendorff reagent (Sigma Chemical Co., St. Louis, Missouri), ninhydrin, and cupric acetate in sulfuric acid as previously described [18]. Phospholipid identification was based on the relative mobilities of the various components in each solvent system compared with phospholipid standards as well as by the reactivity with specific detection reagents.

Quantitative analysis of phospholipids. Phospholipids were separated by two-dimensional thin layer chromatography. The first dimension was performed with solvent system A. After first-dimension separation, plates were allowed to dry at room temperature for approximately 30 min. Plates were then turned 90° and developed in the second dimension with solvent system B. After development of the second dimension, plates were allowed to dry overnight at room temperature.

Phospholipids were revealed by spraying with the general detection reagent cupric acetate in sulfuric acid as described previously [18]. After spraying, the plates were heated to approximately 170°C for 3 min. Phospholipids appeared dark brown on a white background. Quantitative analysis of phospholipids was subsequently performed with the LeMont Scientific OASYS Image Analysis System (LeMont Scientific, State College, Pennsylvania). An image of each thin layer plate was captured with a video camera, and the area of each lipid spot was defined with a digitizing pen and tablet. Relative percentage of phospholipid composition was determined from the numerical products of area and average intensity for each spot.

Assay for phosphatidylinositol synthase activity within cell extracts. Bacterial cell extracts were prepared as follows: Bacterial cultures (1-liter volume) were harvested during the logarithmic phase of growth. Cells were resuspended in 5 mL of Buffer A [50 m*M* Tris-HCl (pH 8.0), 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 10 m*M* 2-mercaptoethanol], cooled in a salt-ice bath, and subjected to sonic disruption with a high intensity (250-watts) ultrasonic processor (Sonics and Materials, Danbury, Connecticut). The samples were treated for 6 min with 1-s bursts, interrupted by 1-s periods of cooling. After sonication, unbroken cells were removed by centrifugation at 3000 *g* for 10 min. The supernatant was stored at −20°C.

Yeast cell extracts were prepared with a Mini-BeadBeater (Biospec Products, Bartlesville, Oklahoma) by methods previously described by Fischl and co-workers [8]. The disruption buffer was buffer A, to which 0.3 *M* sucrose was added. After removal of unbroken cells by centrifugation at 3000 *g* for 10 min, cell extracts were stored at −20°C.

Total cell extracts were examined for phosphatidylinositol synthase activity by the method described by Fischl and co-workers [8]. Briefly, 50 μg of cell extract protein was incubated at 30°C for 30 min in a final assay volume of 100 μL. The assay mixture contained 50 m*M* Tris-HCl (pH 8.0), 0.2 m*M* cytidine 5′-diphosphate dipalmitoyl, 2.4 m*M* Triton X-100, 2.5 m*M* $MnCl_2$, 2.5 m*M* $MgCl_2$, and 100 μM *myo*-[2-^{3}H]inositol (19,000 cpm/nanomole). Activity was determined by following the appearance of radioactivity into a chloroform-soluble form [7]. The conversion of *myo*-[2-^{3}H]inositol to [^{3}H]-phosphatidylinositol was confirmed by analysis of chloroform-soluble product by thin layer chromatography with solvent system A (see above).

Chemicals. Dipalmitoyl phosphatidylethanolamine, dipalmitoyl phosphatidyl-*N*-monomethylethanolamine, dipalmitoyl phosphatidyl-*N*,*N*-dimethylethanolamine, dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidylglycerol, dipalmitoyl phosphatidylserine, cardiolipin (bovine heart), and phosphatidylinositol (soybean) were purchased from Sigma Chemical Co (St. Louis, Missouri). *Myo*-[2-^{3}H]inositol was purchased fron NEN Research Products (Boston, Massachusetts).

Results and Discussion

Two-dimensional thin layer chromatographic analysis revealed the presence of four major phospholipids within extracts of both *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 (Fig. 1A and 1B). For comparison, we examined the phospholipid composition of *Rhizobium meliloti* 1021 and found it also to contain the same four major phospholipids (Fig. 1C). Based on relative mobilities

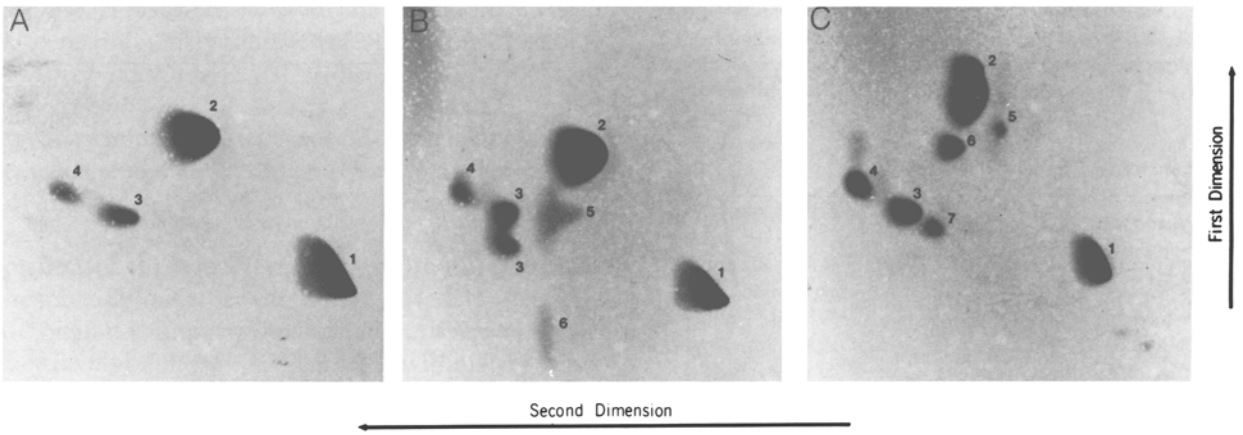

Fig. 1. Two-dimensional thin layer chromatographic analysis of the phospholipids of *Bradyrhizobium japonicum* USDA 110 (A), *Bradyrhizobium* sp. strain 32H1 (B), and *Rhizobium meliloti* 1021 (C). First-dimension separation was performed with solvent system A, and second dimension separation was performed with solvent system B (as described in Materials and Methods). Spots were revealed with the cupric acetate/sulfuric acid general detection reagent. Spot identification is described in Table 1.

in various solvent systems as well as specific staining behaviors, these four phospholipids were identified as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Fig. 1 and Table 1).

The results of the quantitative analysis of the relative amounts of each phospholipid are shown in Table 2. Consistent with previous studies of the phospholipid composition of members of the *Rhizobiaceae* family, our results demonstrate that phosphatidylcholine is an abundant phospholipid within both bradyrhizobial strains as well as *Rhizobium meliloti* 1021. However, in contrast to the results of previous researchers [1, 10], the present study demonstrates that phosphatidylglycerol is a major phospholipid within species of *Bradyrhizobium*. As shown in Table 2, this lipid was found to represent 11% and 18% of the total phospholipid of *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1, respectively. Indeed, the relative amount of phosphatidylglycerol within the two strains of *Bradyrhizobium* was found to be very similar to that present within *Rhizobium meliloti* 1021 (Table 2).

In addition to the four major phospholipids identified within the bradyrhizobial lipid extracts, two unidentified components were detected within the extract derived from *Bradyrhizobium* sp. strain 32H1 (Fig. 1B and Table 1). However, these components did not react with the molybdenum blue indicator reagent and are, therefore, not phospholipids (Table 1). It may be noted that the unusual appearance of phosphatidylglycerol on the two-dimensional thin layer chromatogram of the extract derived from *Bradyrhizobium* sp. strain 32H1 probably results from the presence of one of these unidentifed components (component 5, Fig. 1B).

A fifth phospholipid was detected within lipid extracts derived from *R. meliloti* 1021 (Fig. 1C). This lipid was determined to be phosphatidyl-*N*,*N*-dimethylethanolamine. Although phosphatidyl-*N*,*N*-dimethylethanolamine is likely to be an intermediate in the synthesis of phosphatidylcholine within species of *Bradyrhizobium*, this phospholipid was not detected within lipid extracts derived from *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1. It should be noted that phosphatidyl-*N*-monomethylethanolamine may also be present within the lipid extracts of all three strains examined; however, this lipid was not completely resolved from phosphatidylethanolamine by two-dimensional thin layer chromatography.

It is of interest that neither phosphatidylserine nor phosphatidylinositol was detected within the lipid extracts derived from the two strains of *Bradyrhizobium* examined in the present study. Although it is possible that these lipids are present at trace levels, these results are in contrast to those of previous studies that have indicated that phosphatidylserine [1, 10] and phosphatidylinositol [10] are major phospholipids within species of *Bradyrhizobium*.

Further evidence consistent with a lack of detectable phosphatidylinositol in these preparations was obtained when cell-free extracts were examined

Table 1. Identification of phospholipids present within species of *Bradyrhizobium* and *Rhizobium*

Bacterial strain	Spot[a]	Identity	Indicator reagent[b] N	M	P[c]	D
Bradyrhizobium japonicum USDA 110	1	Phosphatidylcholine	−	+	−	+
	2	Phosphatidylethanolamine[d]	+	+	−	−
	3	Phosphatidylglycerol	−	+	+	−
	4	Cardiolipin	−	+	−	−
Bradyrhizobium sp. strain 32H1	1	Phosphatidylcholine	−	+	−	+
	2	Phosphatidylethanolamine[d]	+	+	−	−
	3	Phosphatidylglycerol	−	+	+	−
	4	Cardiolipin	−	+	−	−
	5	?	+	−	−	−
	6	?	+	−	−	−
Rhizobium meliloti 1021	1	Phosphatidylcholine	−	+	−	+
	2	Phosphatidylethanolamine[d]	+	+	−	−
	3	Phosphatidylglycerol	−	+	+	−
	4	Cardiolipin	−	+	−	−
	5	Phosphatidyl-N,N-dimethylethanolamine	−	+	−	−
	6	?	+	−	−	−
	7	?	−	−	−	−

[a] Refer to Fig. 1 for the assignment of numbers for each spot resolved by two-dimensional thin layer chromatography.
[b] Indicator reagents are as follows: N, ninhydrin; M, molybdenum blue; P, periodate-Schiff; D, Dragendorff.
[c] A positive ("+") reaction after treatment with the periodate-Schiff reagent refers to the immediate development of a purple color.
[d] Phosphatidylethanolamine and phosphatidyl-*N*-monomethylethanolamine were not resolved by two-dimensional thin layer chromatography.

Table 2. Quantitative analysis of the phospholipid composition of species of *Bradyrhizobium* and *Rhizobium*

Bacterial strain	Phospholipid	Relative composition[a] (percent)
Bradyrhizobium japonicum USDA 110	Phosphatidylcholine	42
	Phosphatidylethanolamine[b]	40
	Phosphatidylglycerol	11
	Cardiolipin	8
Bradyrhizobium sp. strain 32H1	Phosphatidylcholine	29
	Phosphatidylethanolamine[b]	47
	Phosphatidylglycerol	18
	Cardiolipin	7
Rhizobium meliloti 1021	Phosphatidylcholine	23
	Phosphatidylethanolamine[b]	48
	Phosphatidylglycerol	15
	Cardiolipin	12
	Phosphatidyl-*N*,*N*-dimethylethanolamine	2

[a] The phospholipid relative percentage composition was determined with the LeMont Scientific OASYS Image Analysis System as described in Materials and Methods.
[b] Phosphatidylethanolamine and Phosphatidyl-*N*-monomethylethanolamine were not resolved by two-dimensional thin layer chromatography.

Table 3. Phosphatidylinositol synthase activity[a] within cell-free extracts of *Bradyrhizobium*, *Rhizobium*, and *Saccharomyces*

Organism	Phosphatidylinositol synthase activity
Bradyrhizobium japonicum USDA 110	ND[b]
Bradyrhizobium sp. strain 32H1	ND
Rhizobium meliloti 1021	ND
Saccharomyces cerevisiae 522	300

[a] Activity is defined as the number of picomoles of phosphatidylinositol formed per minute per milligram of total protein. Phosphatidylinositol formation was confirmed by thin layer chromatographic analysis as described in the text.
[b] ND = none detected.

for phosphatidylinositol synthase activity. As shown in Table 3, no phosphatidylinositol synthase activity was detected within cell extracts derived from *B. japonicum* USDA 110, *Bradyrhizobium* sp. strain 32H1, or *R. meliloti* 1021.

One possible explanation for the discrepancies between the results of the present study and those previously reported [1, 10] is that different bradyrhizobial strains have been examined among the three studies. Additionally, it should be noted that previous results that have indicated an absence of phosphatidylglycerol within lipid extracts of *Bradyrhizobium* spp. [1, 10] may derive from the possibility that this phospholipid was not completely resolved from other components by thin layer chromatographic analysis.

Our inability to detect phosphatidylinositol or phosphatidylinositol synthase activity within two bradyrhizobial species and *R. meliloti* 1021 is of in-

terest, particularly in view of the fact that this lipid has been previously reported to occur within species of *Agrobacterium* [2, 3], *Rhizobium* [4, 10], and *Bradyrhizobium* [10]. However, it should be noted that there appears to be a general discrepancy among previous studies regarding the presence of phosphatidylinositol within species of the *Rhizobiaceae*. For example, in several previous studies of the lipid composition of these bacterial genera, the presence of phosphatidylinositol was not reported [1, 17, 22]. Although it is impossible to rule out the presence of trace levels of phosphatidylinositol within the strains examined in the present study, previous reports of significant levels of this phospholipid within species of the *Rhizobiaceae* would appear to be questionable.

Finally, the present study confirms that phosphatidylcholine is a major phospholipid within species of *Bradyrhizobium*. Indeed, as shown above, this phospholipid may comprise over 40% of the total phospholipid of *B. japonicum* USDA 110. Because the presence of phosphatidylcholine within bacterial membranes is unusual and appears to be characteristic of the *Rhizobiaceae* family, it has previously been proposed by Goldfine [11] that this phospholipid may provide an important function during plant infection. Goldfine's suggestion is of particular interest because of the recent discovery by Rolin and coworkers [D.B. Rolin, P.E. Pfeffer, S.F. Osman, B.S. Swergold, and F. Kappler, Abstr. 12th North American Symbiotic Nitrogen Fixation Conference, 1989, P-51, p. 113] of a phosphocholine-substituted glucan synthesized by *Bradyrhizobium japonicum* USDA 110. By analogy to the pathways of synthesis of the phosphoglycerol-substituted glucans of *Escherichia coli* [13] and *R. meliloti* [19], it is tempting to propose that phosphatidylcholine may be the source of the phosphocholine substituent present on the bradyrhizobial glucans. Furthermore, in view of the apparent requirement for rhizobial glucans during plant infection [5, 9, 21], it is possible that the phosphocholine-substituted glucans of *Bradyrhizobium* may provide an essential role during nodulation. However, in order to further evaluate the role(s) of phosphatidylcholine (and/or phosphocholine-substituted glucans) in the plant infection process, it will be necessary to isolate bacterial mutants selectively blocked for the biosynthesis of this phospholipid.

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