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...
demonstrate that phosphatidyglycerol 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 S22 was provided by R.H. 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 MgSO4 · 7H2O, and 0.5 g K2HPO4 per liter, pH 7) at 30°C on a rotary shaker. Cells were harvested during logarithmetic 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 MgSO4 · 7H2O, and 0.5 g K2HPO4 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 logarithmetic phase of growth. Cells were resuspended in 5 mL of Buffer A (50 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM 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 mM Tris-HCl (pH 8.0), 0.2 mM cytidine 5'-diphosphate dipalmitoyl, 2.4 mM Triton X-100, 2.5 mM MnCl2, 2.5 mM MgCl2, and 100 µM myo-[2-3H]inositol (19,000 cpm/nanomole). Activity was determined by following the appearance of radioactivity into a chloroform-soluble form [7]. The conversion of myo-[2-3H]inositol to [3H]-phosphatidylinositol was confirmed by analysis of chloroform-soluble product by thin layer chromatography with solvent system A (see above).

**Chemicals.** Dipalmityl phosphatidylethanolamine, dipalmityl phosphatidyl-N-monomethylethanolamine, dipalmityl phosphatidyl-N,N-dimethylethanolamine, dipalmityl phosphatidylcholine, dipalmitoyl phosphatidylglycerol, dipalmitoyl phosphatidylserine, cardiolipin (bovine heart), and phosphatidylinositol (soybean) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Myo-[2-3H]inositol was purchased from 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