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The Cell Envelope Structure of the Lipopolysaccharide-Lacking Gram-Negative Bacterium *Sphingomonas paucimobilis*

SATOKO KAWASAKI,[1] RYOZO MORIGUCHI,[2] KACHIKO SEKIYA,[3] TOYOTSUGU NAKAI,[4] ERIKO ONO,[4] KATSUMI KUME,[4] AND KAZUYOSHI KAWAHARA[1]*

Department of Bacteriology[1] and Department of Pathology,[2] The Kitasato Institute, and Department of Microbiology, School of Pharmaceutical Sciences, Kitasato University,[3] Minato-ku, Tokyo 108, and Research Center for Veterinary Science of the Kitasato Institute, Kitamoto, Saitama 364,[4] Japan

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From the cell envelope preparation of *Sphingomonas paucimobilis* two membrane fractions with different densities were separated by sucrose density gradient ultracentrifugation. The high-density fraction contained several major proteins, phospholipids, and glycosphingolipids, which are the only glycolipids of this lipopolysaccharide-lacking gram-negative bacterium. The low-density fraction showed many minor bands of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and NADH oxidase activity was localized in this fraction. Combined with morphological data of vesicles formed by these membrane fractions, the high-density and low-density fractions were proposed to be an outer membrane and a cytoplasmic membrane, respectively. The localization of the glycosphingolipid was investigated also by means of immunoelectron microscopic analysis using a glycosphingolipid-specific antibody. The glycosphingolipid was shown to localize at the cell envelope, and the antigenic sugar portion was exposed to the bacterial cell surface. From these results the glycosphingolipid was assumed to have a function similar to that of the lipopolysaccharide of other gram-negative bacteria.

Sphingomonas paucimobilis is an aerobic gram-negative rod formerly belonging to the genus *Pseudomonas*. The new genus *Sphingomonas* was recently proposed by Yabuuchi et al. (21) because many taxonomical data, including the base sequence of the 16S rRNA, revealed that *S. paucimobilis* is apart from the fluorescent group pseudomonads. The most remarkable difference of *S. paucimobilis* from other gram-negative bacteria is the presence of glycosphingolipid, which is usually a membrane component of eukaryotic cells (4), as the component of cellular lipids and the absence of lipopolysaccharide. Lipopolysaccharide is an amphiphilic molecule and a major component of the outer membrane of gram-negative bacteria, localizing at the outer leaflet of the membrane (17, 18). For this reason lipopolysaccharide was believed to be an essential component for all gram-negative bacteria. However, we reported in our previous paper (9) that *S. paucimobilis* harbored monosaccharide-type and tetrasaccharide-type glycosphingolipids and contained no lipopolysaccharide-like molecule. Those unexpected findings on the cellular lipids of *S. paucimobilis* prompted us to investigate the cell surface structure of this gram-negative bacterium and the physiological roles of the glycosphingolipids. In the present paper the separation of an outer and a cytoplasmic membrane and the biochemical and electron microscopic investigations for the localization of the glycosphingolipids are described.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. paucimobilis* IAM 12576 (originally NCTC 11030, type strain) was used in this study. *Escherichia coli* C600 (1) was used for the control experiment. Cells were grown in a medium and under conditions described previously (10).

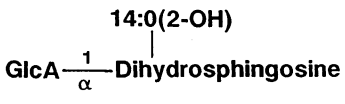
* Corresponding author. Mailing address: Department of Bacteriology, The Kitasato Institute, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan. Phone: 81-3-3444-6161, ext. 2128. Fax: 81-3-3444-6637.

Isolation and separation of cell membranes. Membrane fractions were prepared by the method of Yamada et al. (22) with some modifications. Cells were harvested at the late log phase by centrifugation at 7,000 × *g* for 10 min at 4°C. About 2.5 g (wet weight) of cell mass obtained from 1 liter of culture was washed with 0.2 M Tris acetate buffer (pH 7.8, at 4°C) and suspended in 20 ml of the same buffer. Subsequently, ice-cold reagents were slowly added into the cell suspension at 0°C in the following order: 7.5 ml of 2 M sucrose, 1.4 ml of 1% EDTA (pH 7.5), and 1.4 ml of 5-mg/ml lysozyme in 0.2 M Tris acetate buffer (pH 7.8). The mixture was then cooled in an ice-water bath for 30 min and warmed at 25°C for 15 min to form spheroplasts. The spheroplasts were disrupted with a French pressure cell (Aminco, Silver Spring, Md.), and the membrane fraction was separated by sucrose density gradient centrifugation according to the method of Yamada et al. (22). The amount of protein in the membrane fractions was determined by the method of Lowry et al. (13). Protein profiles of membrane fractions were analyzed by sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis with the buffer system of Laemmli (11).

Measurement of NADH oxidase activity. The activity of NADH oxidase was measured at 30°C as described in reference 14. The reaction mixture contained each membrane fraction, corresponding to 40 μg of protein, as the enzyme source.

Preparation and analysis of the glycosphingolipids. The monosaccharide-type and the tetrasaccharide-type glycosphingolipids of *S. paucimobilis* were designated GSL-1 and GSL-4A, respectively, in this study (Fig. 1). GSL-1 and GSL-4A were extracted with chloroform-methanol (1:3, vol/vol) after extraction with chloroform-methanol (2:1, vol/vol) and purified (9). For the analysis of total cellular lipids, thin-layer chromatography was carried out on silica gel 60 plates (Merck, Darmstadt, Germany) with the solvent system of chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol) as described in the previous paper (9).

GSL-1:



GSL-4A:

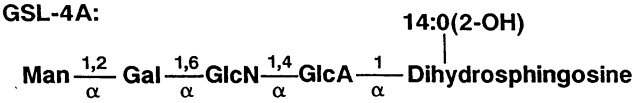


FIG. 1. Structures of the glycosphingolipids isolated from *S. paucimobilis*. Monosaccharide-type glycosphingolipid and tetrasaccharide-type glycosphingolipid (9) were designated GSL-1 and GSL-4A, respectively. Abbreviations: Man, D-mannose; Gal, D-galactose; GlcN, D-glucosamine; GlcA, D-glucuronic acid; 14:0(2-OH), (*S*)-2-hydroxymyristic acid.

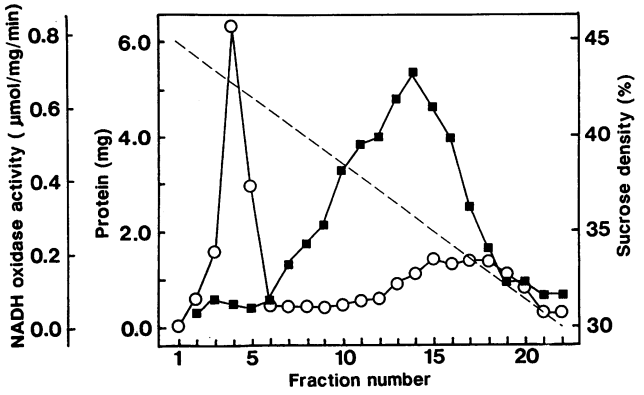


FIG. 2. Separation of membrane fractions by sucrose density gradient ultracentrifugation. Cells were disrupted with a French pressure cell (Aminco), and the membrane preparation was ultracentrifuged. ○, amount of total protein; ■, NADH oxidase activity, – – –, gradient of sucrose density.

Measurement of 2-OH-14:0. An aliquot of separated membrane fractions was lyophilized after dialysis in water and subjected to hydrolysis (4 M HCl, 100°C, 5 h). In the presence of heptadecanoic acid as an internal standard, 2-hydroxymyristic acid (2-OH-14:0) was measured by gas-liquid chromatography after methylesterification. The chemical-bonded capillary column (25-m CBP-1; Shimadzu, Kyoto, Japan) installed in a GC-14A (Shimadzu) instrument was used for the gas-liquid chromatographic analysis.

Measurement of phospholipids. Phospholipids in the membrane fraction were extracted with chloroform-methanol (2:1, vol/vol), and the phosphorus in the extracted lipids was measured by the method of Lowry et al. (12).

Immunization of rabbits with GSL-4A. Purified GSL-4A was mixed with bovine serum albumin (BSA) (Sigma, St. Louis, Mo.) in the water solution by sonication in a ratio of 1:2 (wt/wt) and lyophilized. Rabbits (New Zealand White, 3 kg; SLC, Hamamatsu, Japan) were immunized by subcutaneous injection of the mixture of GSL-4A and BSA (50 μg per rabbit) with complete Freund's adjuvant. After 2 weeks, the same dose of the antigen was injected as a booster. The rabbits were sacrificed 2 weeks after the final injection.

ELISA. Antibody titer was assayed by a solid-phase enzyme-linked immunosorbent assay (ELISA) system. ELISA plates were coated with 1 μg of purified GSL-1 or GSL-4A in ethanol per well by 2 h of incubation at 37°C, washed with phosphate-buffered saline (PBS), and used for the assay according to a previously described method (3). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) was used as the second antibody in a 1,000-fold dilution with PBS. The A_{492} was measured with a microplate reader (MRP-A4; Tosoh, Tokyo, Japan).

Preparation of glycosphingolipid-liganded affinity column and purification of anti-GSL-4A IgG from the antiserum. A glycosphingolipid-liganded affinity column was prepared by the method of Hirabayashi et al. (5) with some modifications. GSL-4A or GSL-1 (4 mg) was dissolved in an ethanol-methanol-water (2:1:1, vol/vol) solution containing 0.1 M KCl by warming and sonication. Two milliliters of Octyl-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) was equilibrated with the same solution and warmed at 70°C. The glycosphingolipid solution was added to the suspended resin and incubated for 1 h in a water bath at 70°C. After the incubation, the resin was washed with water and equilibrated with PBS. The resin was packed in a small column and used as a glycosphingolipid-liganded affinity column.

The crude antiserum (1 ml) was mixed with an equal amount of PBS and applied to the column of BSA-agarose (Sigma) to remove anti-BSA antibody. The pass-through fraction of the column was applied to the GSL-1-liganded column, and again the pass-through eluate was obtained and applied to the GSL-4A-liganded column. When the A_{280} of eluate (PBS) decreased to less than 0.05, the elution buffer was changed to PBS containing 3 M NaSCN. The eluate was desalted with a PD-10 Sephadex G-25M column (Pharmacia LKB) and applied to a protein A-agarose column (GIBCO BRL, Gaithersburg, Md.). The eluted IgG fraction was concentrated and stored in PBS containing 50% glycerol at −20°C before use. The final concentration was approximately 0.15 mg/ml, estimated by determining the A_{280}.

Fluorescence microscopy. Cells from an overnight culture were harvested and washed with PBS. Cell suspension in an appropriate concentration was dried on slide glass plate, fixed with acetone, and treated with purified anti-GSL-4A IgG for 30 min at room temperature. Next, it was treated with fluorescein-conjugated goat anti-rabbit IgG (The Binding Site Ltd.,

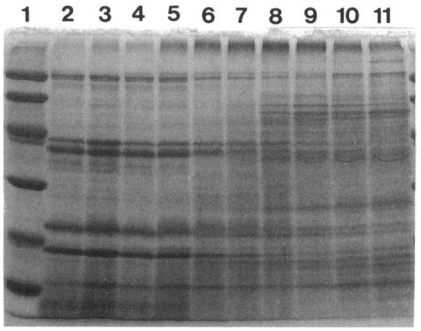


FIG. 3. Protein profiles of separated membrane fractions. Each membrane fraction (about 50 μg of protein) was treated with SDS at 100°C for 10 min and subjected to 12.5% polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. Lanes: 1, molecular weight markers; 2, fraction 2; 3, fraction 4; 4, fraction 6; 5, fraction 8; 6, fraction 10; 7, fraction 12; 8, fraction 14; 9, fraction 16; 10, fraction 18; 11, fraction 20.

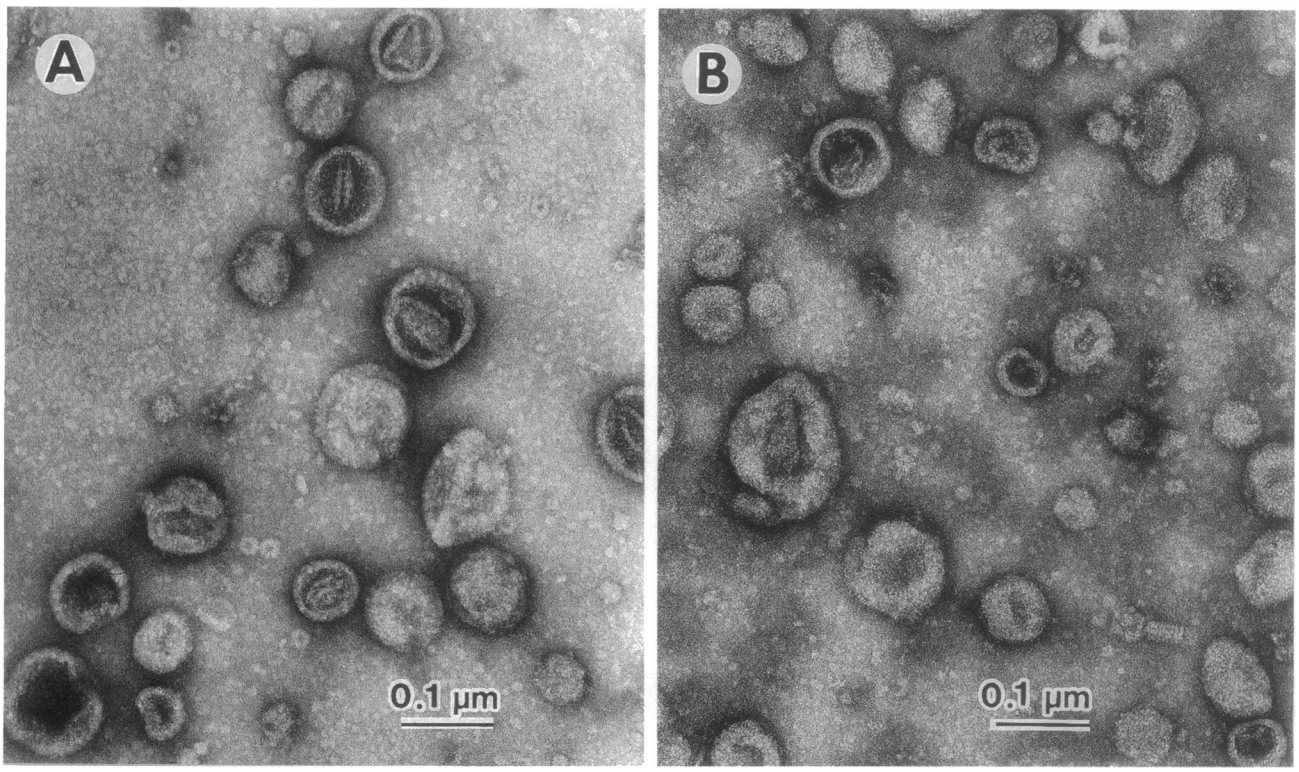


FIG. 4. Electron micrographs of vesicles formed by the separated membrane fractions. Separated membrane fractions were negatively stained with 4% uranyl acetate. (A) High-density fraction (F-4); (B) low-density fraction (F-15).

Birmingham, United Kingdom) in 100-fold dilution with PBS for 1 h at room temperature. After the reaction, the preparation was sealed under cover glass in the mounting medium, Immunon (Lipshaw, Pittsburgh, Pa.), and observed with a fluorescence microscope (BH-2; Olympus, Tokyo, Japan).

Immunogold-labeling electron microscopy. (i) Whole-mount method. The cell suspension was mounted on the carbon-Formvar-coated grid and dried. The grid was incubated in PBS containing 5% BSA for 30 min and then in PBS containing the purified anti-GSL-4A IgG for 30 min at room temperature. Subsequently the grid was incubated in PBS containing 3% goat anti-rabbit IgG conjugated to 10-nm gold particles (Enzymed Laboratories, Inc., San Francisco, Calif.) for 30 min at room temperature. The preparation was examined with electron microscopes, JEM-1200EX (JEOL, Tokyo, Japan) and H-500 (Hitachi, Tokyo, Japan).

(ii) Thin-section (postembedding) method. Cells were fixed first in 2.5% glutaraldehyde in PBS for 1 h and postfixed in 1% OsO_4 for 1 h, dehydrated in graded ethanols and propylene oxide, and embedded in epoxy resins. Ultrathin sections were preparated by an ultramicrotome (LKB, Stockholm, Sweden) and deposited on nickel grids. The same procedure used for the whole-mount method was applied, except for the additional pretreatment with 1% H_2O_2 for 3 min before gold-labeled anti-IgG staining. After the immunogold staining, the sections were stained with 4% uranyl acetate and 0.4% lead citrate in 0.1 M NaOH.

RESULTS

Separation of outer and cytoplasmic membranes. By sucrose density gradient ultracentrifugation, 22 fractions were separated from the membrane fraction of *S. paucimobilis* and designated F-1 to F-22 from the bottom to the top of the centrifuge tube. As shown in Fig. 2, the protein determination curve was bimodal (first peak, F-1 to F-5; second peak, F-13 to F-20), suggesting that two kinds of membrane with different densities were present. When the same procedure was applied to *E. coli* C600, the similar bimodal curve of protein determination was obtained (data not shown). The density of each fraction was calculated from the sucrose concentration, and the values for the fractions of *S. paucimobilis* and *E. coli* were compared. The high-density fraction of *S. paucimobilis* (F-1 to F-5) showed a value (1.19 mg/ml) smaller than that of the *E. coli* high-density fraction (1.23 mg/ml), while low-density fraction values were not significantly different (1.14 mg/ml for *S. paucimobilis* and 1.15 mg/ml for *E. coli*). As NADH oxidase was known to localize in the cytoplasmic membrane, the enzyme activity in each fraction was determined. As shown in Fig. 2, the activity was distributed from F-7 to F-18, but nearly no activity was detected in the high-density fraction (F-1 to F-5).

The protein profile of each membrane fraction was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). The high-density fraction (lanes 2 to 4) was found to contain major proteins with molecular weights of 19,000, 22,000, 37,000, and 40,000. In contrast, the low-density fraction (lanes 8 to 11) showed a large number of minor protein bands, but no major band was detected.

High- and low-density fractions were subjected to electron microscopic analysis, because a morphological difference was reported for vesicles formed by the outer membrane and the cytoplasmic membrane of *E. coli* (15) and *Pseudomonas aeruginosa* (14). As shown in Fig. 4, the vesicles formed by the high-density fraction were of a relatively constant size, whereas those of the low-density fraction showed more variation in size

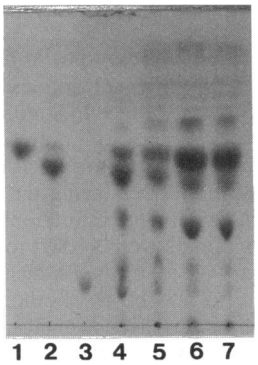


FIG. 5. Localization of the glycosphingolipids in separated membrane fractions. Cellular lipids were extracted from separated membrane fractions as described in Materials and Methods and analyzed by thin-layer chromatography. Lanes: 1, authentic phosphatidylethanolamine; 2, GSL-1; 3, GSL-4A; 4, fraction 4; 5, fraction 9; 6, fraction 14; 7, fraction 19.

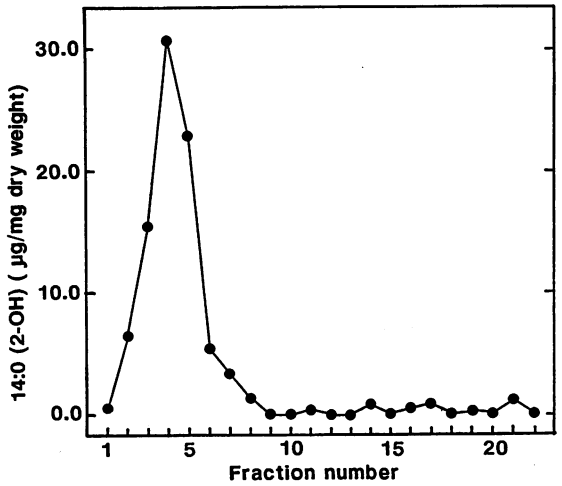


FIG. 6. Distribution of 2-OH-14:0 in separated membrane fractions. The amount of 2-OH-14:0 in membrane fractions was determined by gas-liquid chromatography after hydrolysis and methylesterification.

and morphology. However, these differences were not as clear as those reported for other bacteria.

Comparing those results for two membrane fractions of *S. paucimobilis* with the reported characteristics of the outer and the cytoplasmic membranes of *E. coli* (15, 19), the high-density fraction was proposed to be an outer membrane and the low-density fraction was proposed to be a cytoplasmic membrane of *S. paucimobilis*.

Distribution of the glycosphingolipids in separated membrane fractions. Distribution of GSL-1 and GSL-4A was investigated by thin-layer chromatographic analysis. As shown in Fig. 5, the F-4 fraction, representing the outer membrane fraction, contained large amounts of GSL-1 and GSL-4A (lane 4), but only small amounts of these lipids were detected in the intermediate fraction (lane 5) and the cytoplasmic membrane fraction (lanes 6 and 7). Instead, the amount of phospholipids was larger in the cytoplasmic membrane fraction than in the outer membrane fraction. The unknown spot migrating slightly faster than GSL-4A in lane 4 has not been identified. However, it has an R_f value similar to that of the trisaccharide-type glycosphingolipid extracted from other species of *Sphingomonas* (8a). Thus, it could be a degraded derivative of GSL-4A formed during preparation of membrane fractions.

The amount of 2-OH-14:0 in each membrane fraction was determined, because 2-OH-14:0 is the major component of the glycosphingolipids and not present in phospholipids. The distribution of 2-OH-14:0 was limited in F-2 to F-6, which corresponded to the outer membrane fraction, and this fatty acid was not detected in the cytoplasmic membrane fractions (Fig. 6). The content of 2-OH-14:0, phosphorus, and protein in both outer and cytoplasmic membrane fractions is shown in Table 1. 2-OH-14:0 was predominantly present in the outer membrane fraction, while reasonable amounts of phosphorus, representing the amount of phospholipids, and of protein were detected in both membrane fractions.

Those results clearly indicated that the glycosphingolipids of *S. paucimobilis* were localized in the outer membrane fraction.

Localization of the glycosphingolipids in the intact cell of *S. paucimobilis*. In order to investigate the localization of the glycosphingolipids in the intact cell, the antibody which recognized GSL-4A was prepared as described in Materials and Methods and used for immunoelectron microscopic analysis. The specificity of the antibody was examined by an ELISA system. As shown in Fig. 7, the antibody could recognize GSL-4A but binds neither to GSL-1 nor to BSA. Therefore, the antibody was assumed to recognize the terminal oligosaccharide of GSL-4A but not the glucuronic acid and lipid portions, which are common components of GSL-4A and GSL-1.

Binding of the antibody to the bacterial cells was first demonstrated by immunofluorescence study. Binding was observed on the entire surface of the cells of *S. paucimobilis* but was not seen on the cells of *E. coli* (data not shown).

Secondly, the immunogold-labeling method was applied to the cells of *S. paucimobilis* by the whole-mount method. The photograph in Fig. 8A clearly shows that the antibody bound constantly on the surface of the cell, suggesting that the oligosaccharide chain of GSL-4A was exposed to the outer surface of the cell. Also, by this method no binding of the antibody to *E. coli* cells was observed in the control experiment (Fig. 8B).

Binding of the antibody to ultrathin sections of the bacterial cells was investigated by the postembedding method. The gold particles were found in the cell envelope, where a bilayered outer membrane-like structure was observed; gold particles were rarely present in the cytosolic space (Fig. 9A). These results indicated that the glycosphingolipid was localized in the envelope of the intact cell. In the control experiment using *E. coli* cells, no gold staining was observed on the sectioned material (Fig. 9B).

From these results together with the results for separated membrane fractions, we came to the conclusion that *S. pauci-*

TABLE 1. Composition of membrane fractions of *S. paucimobilis*

Component[a]	Outer membrane[b] (µg/mg [dry wt])	Cytoplasmic membrane[c] (µg/mg [dry wt])
Protein	479.5	615.4
Phosphorus	6.3	16.9
2-OH-14:0	24.4	0.5

[a] Phosphorus was determined as representative of phospholipids, and 2-OH-14:0 was determined as representative of glycosphingolipids. Analytical methods are described in Materials and Methods.
[b] High-density fraction separated by sucrose density gradient centrifugation.
[c] Low-density fraction separated by sucrose density gradient centrifugation.

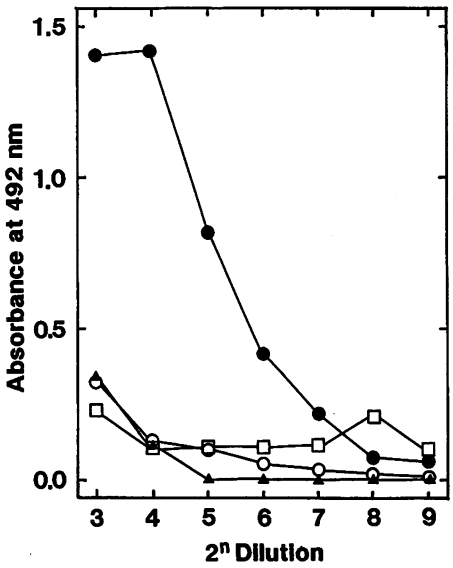


FIG. 7. Specificity of anti-GSL-4A antibody. The binding specificity of purified anti-GSL-4A IgG was examined by an ELISA system as described in Materials and Methods. ELISA plates were precoated with the following antigens with serial dilutions starting from 0.1 mg/ml: GSL-1 (○), GSL-4A (●), BSA (▲), and PBS (□).

mobilis has an outer membrane and a cytoplasmic membrane and that the glycosphingolipids were localized in the outer membrane with the sugar portion exposed to the outer surface of the bacterial cell.

DISCUSSION

In the present study we prove that *S. paucimobilis* harbors the outer and the cytoplasmic membranes and that the former is composed of glycosphingolipids in addition to phospholipids and membrane proteins. To our knowledge this is the first report of a bacterial outer membrane which contains glycosphingolipid and lacks lipopolysaccharide.

From both biochemical and immunoelectron microscopic analyses glycosphingolipids were shown to be present at least on the surface (outer leaflet) of the other membrane, although for the moment we do not know whether glycosphingolipids are placed also at the inner layer of the outer membrane. Our data strongly suggested that the glycosphingolipid plays a role similar to that of lipopolysaccharide, i.e., it maintains membrane fluidity, works as permeability barrier against bactericidal substances, or gives hydrophilicity and antigenicity to the surface of bacterial cell.

Although general characters of membranes of *S. paucimobilis* are similar to those of *E. coli*, the density of the outer membrane of *S. paucimobilis* was much lower than that of *E. coli*. This might be because of the structural differences of the lipid portion of lipopolysaccharide and the glycosphingolipid. Lipopolysaccharide contains about six molecules of fatty acids, and they are rather tightly gathered and stretched to the inside of the membrane (8). The glycosphingolipid has only two hydrocarbon chains (dihydrosphingosine and 2-OH-14:0), and probably they form a looser bundle of hydrocarbon chains with adjacent molecules compared with the fatty acids of lipopolysaccharide. The differences in membrane characteristics between the two bacteria were also found by the electron microscopic analysis. The electron densities of the outer and cytoplasmic membranes of *E. coli* are known to be different (19) (Fig. 9B), but the layers observed at the cell envelope of

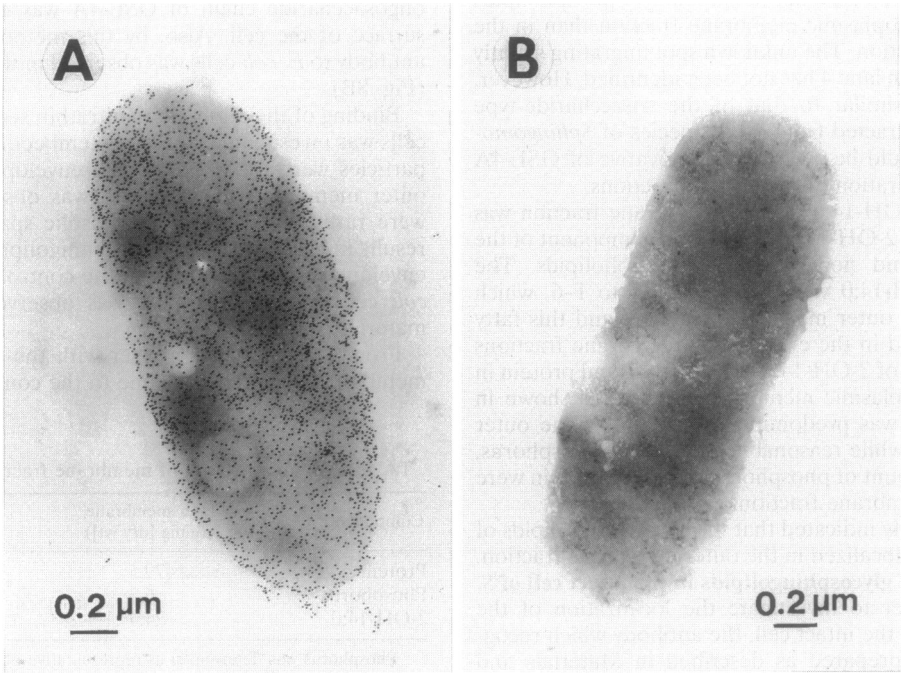


FIG. 8. Specific binding of the anti-GSL-4A antibody to the cell surface of *S. paucimobilis*, elucidated by immunogold staining. Binding of the anti-GSL-4A antibody to the cell surface was shown by the whole-amount method of immunoelectron microscopy. (A) *S. paucimobilis* IAM 12576; (B) *E. coli* C600.

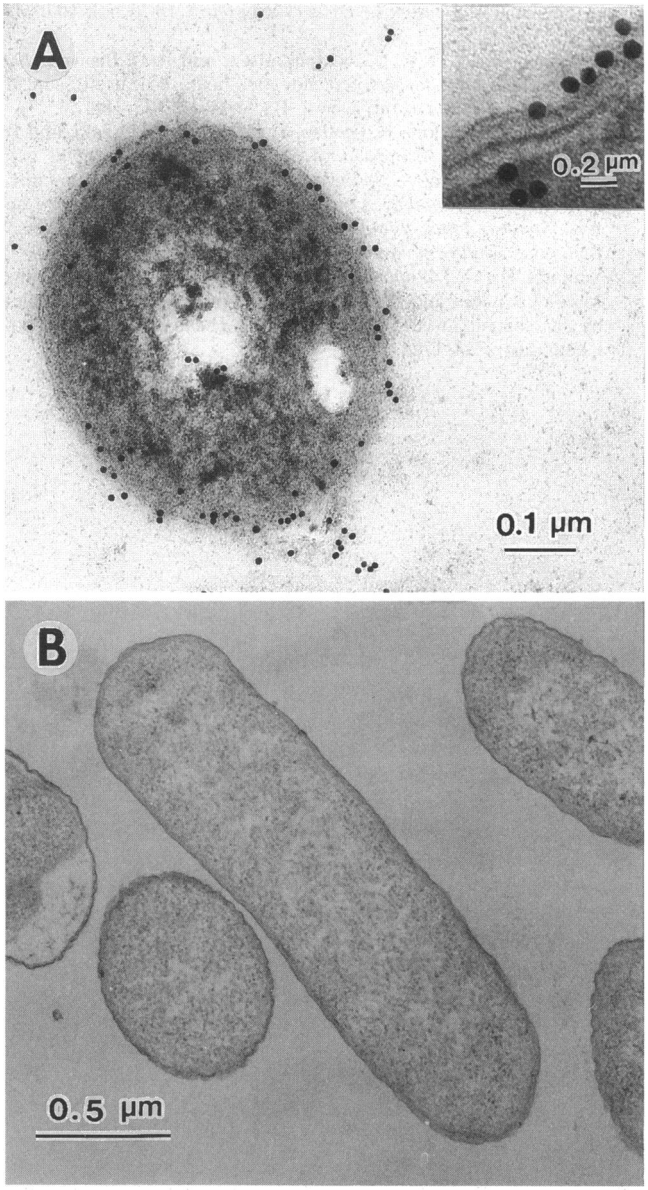


FIG. 9. Localization of the glycosphingolipid in the cell of *S. paucimobilis*. The localization of GSL-4A was investigated by the postembedding immunogold-labeling method of electron microscopy. The ultrathin section of bacterial cell was treated with anti-GSL-4A antibody and stained with gold-labeled secondary antibody. (A) *S. paucimobilis* IAM 12576; (B) *E. coli* C600.

S. paucimobilis did not exhibit much difference in electron density (Fig. 9A). This difference is also postulated to come from the difference of the glycosphingolipid and lipopolysaccharide, although further studies are necessary to clarify it.

S. paucimobilis was first isolated in clinical environment (6) and believed to be a unique and very rare bacterium. But recent studies revealed that many bacteria belonging to other genera have a close relationship with *S. paucimobilis*. The photosynthetic bacterium *Chlorobium limicola* was reported to contain aminoglycosphingolipid (7). *Rhizomonas suberifaciens*, reported by van Bruggen et al. as the pathogenic agent of corky root of lettuce (20), was shown to be taxonomically very close to *S. paucimobilis* and also contains glycosphingolipid (8a). From the rhizosphere and the ear of rice *S. paucimobilis* and related bacteria were isolated (2, 8a, 16). Those data may indicate that *S. paucimobilis* and related bacteria have a close relationship with plants and that therefore, the unusual outer membrane of *S. paucimobilis* has a physiological relevance for the bacterium to inhabit in the plant-associated environment.

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