Primary Structure of a New Phosphocholine-containing Glycoglycerolipid of *Mycoplasma fermentans*

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The chemical structure of a novel phosphocholine-containing glycoglycerolipid, the major polar lipid in the cell membrane of *Mycoplasma fermentans* PG18, was investigated by chemical analyses, gas-liquid chromatography-mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, as well as one- and two-dimensional homonuclear NMR spectroscopy and identiﬁed as 6'-O-(3'-phosphocholine-2'-amino-1'-phospho-1,3'-propanediol)-a-D-glucopyranosyl-(1'→3)-1,2-diacylglycerol (MGFL-II). Palmitate (16:0) and stearate (18:0), in a 3.6:1 molar ratio, constitute the major fatty acids present. MALDI-TOF mass spectrometry revealed two major pseudomolecular ions at m/z 1049.5 [M+H]+ and 1077.3 [M+H]+ representing a dipalmitoyl as the major component and a palmitoyl-stearoyl structure as a minor component. This is the first report of 2-amino-1,3-propanediol-1,3-bisphosphate present in a natural product. This glycoglycerolipid is the second phosphocholine-containing glycoglycerolipid found in *M. fermentans*.

The human pathogen *Mycoplasma fermentans* PG18 was isolated from the urogenital tract several decades ago (1). Because of reports indicating its possible role as a cofactor accelerating the progression of human immunodeficiency virus disease, its signiﬁcance as a pathogen in other immunocompromised patients (2), and its role in the pathogenesis of rheumatoid arthritis, interest in *M. fermentans* has recently increased (3). Although little is known of the molecular mechanisms underlying *M. fermentans* pathogenicity (4), it has been shown that human immunodeﬁciency virus-associated cytopathic effects could be increased by the presence of *M. fermentans* (2) and that *M. fermentans* is capable of fusing with T-cells and peripheral lymphocytes (5).

It is reasonable to assume that *Mycoplasma* membrane components are involved in the attachment and fusion of the microbe with eukaryotic host cells. Salman et al. (6) isolated an unusual phospholipid from the cell membranes of *M. fermentans* and showed that this material (compound X) was capable of enhancing the fusion of small, unilamellar vesicles with MOLT-3 lymphocytes in a dose-dependent manner.

Matsuda et al. (4) isolated two glycoglycerolipids (GGPL-I and GGPL-III) from *M. fermentans*. GGPL-I structure was shown to be 6'-O-phosphocholine-a-D-glucopyranosyl-1,2-diacyl-sn-glycerol (7) as elucidated by mass and NMR spectroscopy. It was later shown (4) that the structure of a more polar glycolipid (GGPL-III) isolated from the same strain of *M. fermentans* was very similar to that of GGPL-I. The chemical structure of GGPL-III, however, has so far remained obscure. The only distinguishing structural feature known is that it differs from GGPL-I in having an additional amino residue (4). Both GGPLs were shown to be species-specific major lipid antigens of *M. fermentans* (4).

Here we describe the structural analysis of a new type of polar lipid isolated from *M. fermentans*, and we present the complete structural analysis of MGFL-II. Furthermore, we show that both glycolipids of *M. fermentans*, GGPL-I and GGPL-III, share the basic structure of 6'-O-phospho-a-D-glucopyranosyl-(1'→3)-1,2-diacylglycerol (7) but differ in their polar head groups.

**MATERIALS AND METHODS**

**Growth of the Organism—** Cultures of *M. fermentans* strain PG18 and strain Incognitus (provided by S.-C. Lo, Armed Forces Institute of Pathology, Washington, D.C.) were grown in a modiﬁed Channock medium (8) inoculated with a 48-h culture at an inoculum level of 2% and incubated statically at 37 °C. After 68 h the cells were harvested, washed twice, and freeze-dried as described previously (8) with yields ranging from 130 to 160 mg dry weight per liter of medium.

**Lipid Extraction and Purification—** Freeze-dried cells were suspended in 25 mM Tris/HCl, pH 7.5, containing 0.25 M NaCl to a ﬁnal concentration of 25 mg of cells per ml. Lipids were extracted from cell suspensions by the method of Bligh and Dyer (9) and concentrated to near dryness on a rotary evaporator. Quantitative separation of MGFL-II was achieved by silica gel column chromatography. Total lipid was redissolved in 2 ml of chloroform and loaded onto a silica gel column (1.5 × 3 cm; Kieselgel 60, 230–400 mesh, Merck), equilibrated with

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1 The abbreviations used are: GGPL, glycoglycerophospholipid; MGFL, *M. fermentans* glycoclipid; GLC-MS, gas-liquid chromatography-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MALDI-LIN-TOF, matrix-assisted laser desorption/ionization time-of-flight recorded in the linear mode; EI-MS, electron impact mass spectrometry; CI-MS, chemical ionization mass spectrometry; COSY, correlated spectroscopy; ROESY, rotating frame Overhauser enhancement spectroscopy; HMBC, heteronuclear multiple-quantum coherence; Gro, glycerol; Glc, glucose; AP, 2-amino-1,3-propanediol; Cho, choline; HF, hydrofluoric acid.

2 Unfortunately there exists a confusing terminology in the literature concerning these biologically significant polar glycolipids. We propose replacing the equivalent terms GGPL-I (also called lipid v (4)) with MGFL-I and replacing the equivalent terms GGPL-III = lipid vi (4) with compound X (6) with MGFL-II. The Roman numerals indicate the sequence of their discovery and structural description.
choloroform and sequentially eluted with four bed volumes of choloroform (fraction 1), choloroform/methanol, 1:4 (v/v, fraction 2), choloroform/methanol/water, 1:4.0:7 (fraction 3), choloroform/methanol/water, 1:4.1 (fraction 4), choloroform/methanol/water, 1:4.1:5 (fraction 5), choloroform/methanol/water, 1:8.3:0 (fraction 6), methanol (fraction 7), methanol/water, 1:1 (fraction 8), and methanol. Fractions were vacuum evaporated to dryness, redissolved in 0.5 ml of elution solution, and analyzed by thin layer chromatography. Since fractions 4–7 contained pure MfGL-II they were combined and dialyzed in 10 mM EDTA against water for 4 days at 4 °C.

**Mild Methanolysis—**MfGL-II (370 μg) was dissolved in 2 ml HCl/MeOH, incubated at 85 °C for 16 h, and peracetylated with acetanhydride/pyridine (1:2, v/v) for 60 min at 85 °C. Carbohydrates and other components of MfGL-II were analyzed as their peracetylated derivatives by GLC and GLC-MS.

**Strong Methanolysis and Peracetylation—**Following mild methanolysis, samples were dissolved in 2 ml HCl/MeOH, incubated at 85 °C for 16 h, and peracetylated with acetaldehyde/pyridine (1:2, v/v) for 60 min at 85 °C. Carbohydrates and other components of MfGL-II were analyzed as their peracetylated derivatives by GLC and GLC-MS.

**Dephosphorylation—**MfGL-II (290 μg) and derived phosphomethyl ester were dephosphorylated by treatment with 48% aqueous HF at 4 °C for 36 h. Following solvent removal in vacuo over KOH, the product was peracetylated and analyzed as described earlier.

**Synthesis of 2-Amino-1,3-propanediol—**Serine (Sigma) was methyolated with diazomethane (CH2N2) treatment at room temperature for 15 min. The resulting methyl ester was reduced with NaBH4 in methanol/water (1:1, v/v) to the corresponding 2-amino-1,3-propanediol which was then peracetylated and analyzed by GLC-MS.

**Methylation Analysis—**MfGL-II (1.3 mg, 1.2 μmol) was per-O-acetylated with acetaldehyde/pyridine (1:2, v/v) at room temperature for 16 h in the dark followed by O-deacylation with 0.1 M NaOH, precipitated with acetone (3 ml) and washed twice with 2 ml of water, 7:3 (fraction 8), and methanol/water, 6:4 (fraction 9). Fractions 1–7 contained pure MfGL-II they were combined and dialyzed in 10 mM EDTA against water for 4 days at 4 °C for 36 h. Following solvent removal in vacuo over KOH, the product was peracetylated and analyzed as described earlier.

**Synthesis of 2-Amino-1,3-propanediol—**Serine (Sigma) was methylated with diazomethane (CH2N2) treatment at room temperature for 15 min. The resulting methyl ester was reduced with NaBH4 in methanol/water (1:1, v/v) and the phosphates were transformed to methyl esters with ethereal diazomethane (CH2N2) at room temperature for 15 min. The solvents were removed under a stream of nitrogen, and the product was washed three times with ether/n-hexane (10:90, v/v) to remove liberated fatty acids. The sample was dried in vacuo and methylated (10). The methylated sample was extracted with water/chloroform (7 ml, 6:1, v/v); the aqueous layer was washed twice with 1 ml of chloroform, and the combined organic phases were washed again with 100 ml of water and taken to dryness under a stream of nitrogen. After methylation the product was dephosphorylated as described earlier, and an aliquot was directly peracetylated (see above) and analyzed by GLC-MS.

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the synthetic 2-amino-1,3-propanediol described above, thus indicating that 2-amino-1,3-propanediol is involved in the structure of MfGL-II as a phosphoester.

Methylation analysis of native MfGL-II (N-acetylation, deacylation, methylation (10), dephosphorylation (48% HF), hydrolysis (trifluoroacetic acid), and reduction (NaBD₄)) was done in two steps. First, after methylation and dephosphorylation an aliquot of the sample was peracetylated and subjected to GLC-MS analysis. The EI-MS spectra revealed a fragmentation pattern corresponding to 1,2-di-O-methyl-3-O-(O-acetyl-tri-O-methyl-glucopyranosyl)glycerol (peaks at m/z 247 assigned to the glucopyranoside moiety and at m/z 103 assigned to the glycerol moiety with the typical McLafferty rearrangement at m/z 163 (spectra not shown)) indicating that glucose was substituted with one phosphate. In addition, 1,3-di-O-acetyl-2-(N-methylacetamido)propanediol was detected suggesting that the 2-amino-1,3-propanediol moiety was symmetrically substituted with two phosphates (spectra not shown). To identify the position of the phosphate residue on the glucopyranosyl moiety, the sample was hydrolyzed and reduced yielding 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-glucitol (spectra not shown), indicating the presence of a 6-phosphate on the glucopyranosyl moiety.

These findings suggested a linear structure of diacyl-glyceryl-glucopyranoside with 6-O-(2-O-amino-1,3-diphospho-1,3-propanediol)-α-D-glucopyranosyl-(1→3)-glycerol as the hydrophilic backbone (Fig. 2).

Because the phosphocholine moiety in native MfGL-II was not accessible by methylation analysis and GLC-MS spectrometry other analytical procedures were used.
MALDI-TOF Mass Spectrometry—The molecular size of underivatized MfGL-II was investigated by MALDI-LIN-TOF mass spectrometry in the positive ion mode. Native MfGL-II showed two major pseudomolecular ions (m/z 1049.5 [M+H]+ and m/z 1077.3 [M+H]+) (Fig. 3) with various pseudomolecular ions having Na+ attached to M+ and M+, respectively. The difference between the molecular weights (Δm/z = 28) suggested the presence of two molecular species expressing variability in the fatty acid components, representing a dipalmitoyl derivative of MfGL-II as the major component and a palmitoyl-stearoyl derivative as a minor component, respectively. The molecular weight of M+ ([M+H]+ = 1049.5) is consistent with the formula C₄₉H₉₉O₁₇N₂P₂ and also with the structure shown in Fig. 8.

The presence of different fatty acid residues in the glycerol moiety was further supported by detecting methyl palmitate and methyl stearate in the GLC-MS analysis (see above). These findings were in good agreement with previous reports on fatty acid composition of MfGL-II (8).

NMR Spectroscopy—The structure of the MfGL-II was further elucidated by different NMR experiments on native as well as on O-deacylated MfGL-II. The results are summarized in Table I for 1H signals and in Table II for 13C signals. The assignment of various signals produced by one- and two-dimensional homo- and heteronuclear NMR spectroscopy (Fig. 4, Fig. 5) were in good agreement with those of the diacyl-(glycosyl-6-phospho)glycerol moiety recently identified in M. fermentans by Matsuda et al. (7).

In the O-deacylated MfGL-II, the resonances of H-1αGro, H-1βGro, and H-2Gro were shifted upfield (Table I), whereas the positions of resonances of the other glycerol proton signals (H-3αGro and H-3bGro) were unchanged. These findings indicated that the glycosyl moiety is linked to O-3Gro as is found in an aminophosphoglycolipid of Clostridium innocuum (13).

The α-anomeric configuration of the hexose was inferred by the coupling constant J₁,₂ of 3.4 Hz. The other coupling constants of the hexose (J₁,₃, J₂,₄, and J₃,₄) were larger than 8 Hz, indicating an α-glucopyranoside configuration. The chemical shifts of C-3Glc, C-3Glc, C-4Glc, C-5Glc, and C-6Glc as well as the coupling constant J₆α,₆β were similar to those of α-glucose 6-phosphate (14), providing evidence that a phosphate group was bonded to the O-6 hydroxyl of glucose. A ROESY experiment with O-deacylated MfGL-II showed nuclear Overhauser effects between H-1Glc and both H-3αGro and H-3βGro indicating a close proximity of these protons consistent with an α-1′→3′ linkage (Fig. 6).

The assignment of the 1H NMR and 13C NMR signals was done by 1H,1H COSY and 1H,13C COSY experiments in which
the assignment of C-1<sup>AP</sup> and C-3<sup>AP</sup> may be interchanged because it was not possible to distinguish clearly between these carbon signals. The C-2<sup>AP</sup> signal in the native MfGL-II was a doublet of doublet with two similar heteronuclear coupling constants of $J_{C-2,P}$ 7.1 Hz and $J_{C-2,P}$' 7.7 Hz, respectively, indicating that the 2-amino-1,3-propanediol is symmetrically substituted by two phosphate residues. The chemical shifts, except those for C-3<sup>AP</sup> and H-3<sub>a,b</sub><sup>AP</sup>, approximated the values reported for a phosphoglycolipid isolated from the taxonomically closely related *C. innocuum*, which contained a 1-phosphate-2-amino-1,3-propanediol at O-6 of a-<i>galactopyranosyl</i> moiety (13). The differences for C-3<sup>AP</sup> and H-3<sub>a,b</sub><sup>AP</sup> indicated that in the MfGL-II the 2-amino-1,3-propanediol moiety is substituted at O-3, whereas in the phosphoglycolipid of *C. innocuum* it was not.

The H-1<sup>Cho</sup> signal at 4.21 ppm coupled with the H-2<sup>Cho</sup> signal appeared as a superposition with other signals. The -N<sub>1</sub>(CH<sub>3</sub>)<sub>3</sub> group was assigned to an intense signal (integral 9H) at 3.12 ppm. The chemical shifts were very close to the values reported for GGPL-I containing a choline phosphate group at O-6 of α-<i>glucosyl</i> moiety (7).

The 1H and 13C NMR signals of the ester-linked fatty acids,
summarized in Tables I and II, were in the expected range (13). Also present in the 1H NMR spectrum were signals of olefinic protons (5.25–5.26 ppm) corresponding to unsaturated fatty acids. This finding agrees well with data from GLC and GLC-MS indicating trace amounts of unsaturated fatty acids (18:1).

Different experiments were performed to determine the position of the phosphate groups in MfGL-II. Various one-dimensional and two-dimensional 31P NMR experiments of native MfGL-II measured in 100:100:30 (CDCl3:MeOD:D2O, v/v) gave no satisfactory resolution for the 31P signals. Therefore, the phosphate linkages were determined with O-deacylated MfGL-II. The broad band-decoupled 31P NMR spectrum of the O-deacylated MfGL-II showed two singlets at 1.09 and 0.08 ppm, respectively (spectrum not shown). However, in the proton-coupled gated 31P NMR spectrum, the two phosphate groups gave identical multiplets due to the coupling with two structurally related methylene protons (O-CH2\textsubscript{Glc} and O-CH2\textsubscript{AP} versus O-CH2\textsubscript{AP} and O-CH2\textsubscript{Cho}). The signal intensities upon integration matched precisely the theoretically expected binomial coefficient of 1:4:6:4:1:3 (found, 1.0:4.3:6.4:4:4:1.3). This splitting

FIG. 5. Two-dimensional H-detected 1H,13C HMQC spectrum of the native MfGL-II. A, the whole spectrum with the assignment of the signals of the glycerol moiety (A) and fatty acids (FA); B, extension of the area shown in A by rectangle with the assignment of the signals of the glucosyl (B), 2-amino-1,3-propanediol (C), and choline (D) residues. The corresponding parts of the 1H and 13C NMR spectra are displayed along the horizontal and vertical axes, respectively. For numbering of atoms see Tables I and II.
is typical for phosphate groups symmetrically substituted with two methylene groups. From these data it was concluded that in MfGL-II four methylene groups are attached to two phosphate residues: one from choline, two from the 2-amino-1,3-propanediol, and one from the Glc residue. The $^3$P, $^1$H HMQC spectrum (Fig. 7) revealed unambiguously the linkage of the phosphate groups. Both $^3$P signals (P-1 and P-2) showed highly complex cross-peaks. The $^3$P signal at 1.09 ppm (P-1) correlated with a multiplet of H-1a,b (3.96 ppm) and with the H-6a,Glc and H-6b,Glc signals (4.08 and 4.12 ppm, respectively). The other $^3$P signal (P-2) at 0.08 ppm showed cross-peaks with a multiplet of H-3a,b (3.96 ppm) and with the H-1a,b,cho signal (4.33 ppm).

Taken together these findings confirmed the structure of the MfGL-II as 6'-O-(3'-phosphocholine-2'-amino-1'-phospho-1',3'-propanediol)-α-D-glucopyranosyl-(1'→3)-1,2-diacyl-glycerol. The results of the chemical analyses, GLC-MS analyses with synthetic reference compounds, MALDI-TOF mass spectrometry and various NMR experiments, described above were identical with those obtained by analyzing the MfGL-II of *M. fermentans* incognitus strain. The best structure suggested by these results is shown in Fig. 8.

**DISCUSSION**

The role of AIDS-associated *M. fermentans* in the pathogenesis of the disease has not been yet defined. An interesting hypothesis is based on the ability of *M. fermentans* to fuse with lymphocytes (5). It has been suggested that *Mycoplasma* com-
ponent released to the lymphocyte upon *Mycoplasma*-lymphocyte fusion adversely affected lymphocyte function (6). The fusogenicity of *M. fermentans* cells is correlated with a membrane-associated MfGL-II (6); although several studies have investigated the chemical nature of this lipid, there is presently no consensus on the chemical structure. We have addressed this issue with the aim to establish unequivocally the primary structure of the MfGL-II previously designated as compound X (6, 8) or GGPL-III (7).

MfGL-II was isolated from dried cells of *M. fermentans* PG18 using the extraction protocol described previously (8). Using various analytical procedures (GLC, MALDI-TOF mass spectrometry, and NMR spectrometry), we showed that while both compounds were isolated by identical procedures, the structure proposed (8) has to be revised. We have unequivocally identified the structure of the major polar membrane lipid of *M. fermentans* PG18 as 6'-O-[3'-phosphocholine-2'-amino-1',3'-propanediol]-α-D-glucopyranosyl-(1'→3)-1,2-dipalmitoyl-sn-glycerol (MfGL-II).

MfGL-II shows high structural homology to GGPI-I (MfGL-I), a phosphocholine-containing glyceroglycolipid from *M. fermentans* (7). The main difference between GGPI-I and MfGL-II is the presence of a 2-amino-1,3-propanediol moiety and an additional phosphate residue. Matsuda et al. (4) described another phosphocholine-containing glycoalkalolipid which they termed GGPL-III and postulated to possess a structure very similar to GGPI-I but harboring an additional amino group (4). Our data and those presented by Matsuda et al. (4) show that GGPL-III and the MfGL-II could be structurally identical compounds.

It is of great interest that in both MfGL-I and MfGL-II the phosphocholine moiety is the terminal, exposed structural motif. As the two glycosphingolipids constitute the major lipid fraction of the *M. fermentans* PG18 membrane, it appears likely that phosphocholine is a key structure in cellular adhesion of *M. fermentans* to host cells.

Phosphocholine has recently been reported to be a constituent of the glycoalkalolipid of the anideid *Phereutica hilaenderi* (15), of lipoteichoic acid (16), and of the cell wall associated teichio acid of *Streptococcus pneumoniae* (17). In *Haemophilus influen-
zae phosphocholine was also identified on the surface and was found to be attached to the lipopolysaccharide (18, 19). All bacteria having phosphocholine as part of their surface structures colonize the human nasopharynx. This observation supports the assumption that phosphocholine plays a key role in potentiating microorganism-host interaction.

There are reports that the human C-reactive protein, an acute phase protein, has the ability to bind to phosphocholine-containing pneumococcal C-polysaccharide (20). This complex activates the complement system. Thus, bacteria-associated choline appears to play an important role not only in cellular adhesion but also in subsequent inflammatory reactions.

The 2-aminol-1,3-diphosphate-1,3-propanedio group has so far not been identified in nature. Fischer et al. (13) described a 2-amino-1,3-propanediol-3-phosphate-carrying diradylglycerolipid as a major membrane lipid of C. innocuum. Interestingly, Clostridia are taxonomically closely related to Mycoplasma (21), and it is tempting to speculate that in both organisms the 2-amino-1,3-propanediol is formed by transamination of dihydroxyacetone, a known intermediate of glycolysis (13). This indicates that in M. fermentans glucose has at least two possible fates as follows: catabolic, for energy metabolism, and anabolic, for the biosynthesis of structural molecules.

Recently 2-amino-1,3-propanediol has been detected as an O-antigen component of the lipopolysaccharide of Vibrio cholerae H11. Here, however, this moiety is linked by an amide bond to the carboxyl group of D-galacturonosyl residues (22).

While studies are needed to elucidate the molecular basis for mycoplasmal adhesion to host cells, the finding of phosphocholine as a terminal structure represents an important step toward understanding the molecular mechanism of the pathogenicity of M. fermentans.

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