A phosphoethanolamine-modified glycosyl diradylglycerol in the polar lipids of Clostridium tetani

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Abstract The polar lipids of the anaerobic bacterium Clostridium tetani, the causative agent of tetanus, have been examined by two-dimensional thin layer chromatography, ESI mass spectrometry, and NMR spectroscopy. Plasmalogen and di- and tetra-acylated species of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and N-acetylglucosaminyl diradylglycerol were the major lipids present in most strains examined except for strain ATCC 10779, the parent of strain E88, the first C. tetani strain to have its genome sequenced. This strain contained the same di- and tetra-acylated species but did not contain plasmalogens. All strains contained a novel derivative of N-acetylglucosaminyl diradylglycerol in which a phosphoethanolamine unit is attached to the 6′-position of the sugar, as judged by selective 31P-decoupled, 1H-detected NMR difference spectroscopy. The N-acetylglucosamine (GlcNAc) residue is presumably linked to the 3-position of the diradylglycerol moiety, and it has the β-anomeric configuration. Very little plasmalogen component was detected by mass spectrometry in the precursors phosphatidic acid and phosphatidylether, consistent with the idea that plasmalogens are formed from diacylated phospholipids at a late stage of phospholipid assembly in anaerobic clostridia. —Johnston, N. C., S. Aygun-Sunar, Z. Guan, A. A. Ribeiro, F. Daldal, C. R. H. Raetz, and H. Goldfine. A phosphoethanolamine-modified glycosyl diradylglycerol in the polar lipids of Clostridium tetani. J. Lipid Res. 2010. 51: 1953–1961.

Supplementary key words glycolipid • mass spectrometry • nuclear magnetic resonance spectroscopy • phospholipid • plasmalogen

Clostridium tetani is the cause of tetanus, which is still widely prevalent among humans. It often occurs in the form of neonatal tetanus of which there are approximately 400,000 cases per year according to the World Health Organization. The disease is caused by a neurotoxin that blocks the release of neurotransmitters from presynaptic membranes of inhibitory neurons, leading to spastic paralysis. The toxin is encoded on a large plasmid in toxigenic strains of C. tetani. The neonatal disease is endemic in 90 developing countries, resulting in approximately 200,000 deaths in 1997 (www.who.int/immunization/topics/tetanus/en/index.html). Vaccination with toxoid is widespread and is a valuable preventative. The disease cannot be eradicated because C. tetani spores are widespread in the environment.

The genus Clostridium contains more than 110 recognized species, but published information is available on the polar lipids of only nine of them (1). There is no published information on the polar lipids of the pathogen C. tetani. rRNA sequencing suggests that this genus should be reclassified into a number of new genera (2), but this notion has not been officially accepted. The diversity of lipids found in the members of this genus further supports the concept that its classification requires revision (1). The complete DNA sequence of the genome of C. tetani E88 was published in 2003. Several phospholipid biosynthetic genes were annotated, and these predicted that the bacterial pathways leading from phosphatidic acid to phosphatidylethanolamine (PtdEtn), phosphatidylglycerol (PtdGro), and cardiolipin should be present (3).

Abbreviations: COSY, correlation spectroscopy NMR; GlcNAc, N-acetylglucosamine; HMBC, heteronuclear multiple-bond correlation NMR; HMQCNMR, heteronuclear multiple-quantum-correlation NMR; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdSer, phosphatidylserine.

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We initially examined the polar lipids of *C. tetani* ATCC 10779, the parent strain of E88 (G. Gottschalk and H. Brüggemann, unpublished observations), and another strain of *C. tetani* from the collection of the Department of Microbiology, University of Pennsylvania School of Medicine. We found by two-dimensional thin layer chromatography that they both contained PtdEtn, PtdGro, cardiolipin, and several other polar lipids. However, the two strains differed in that the University of Pennsylvania strain contained plasmalogens, whereas the ATCC 10779 strain did not. We therefore examined four additional strains from the American Type Culture Collection and found that all contained plasmalogens. The lipids were further characterized by ESI mass spectrometry and NMR spectroscopy. All strains of *C. tetani* contained a novel phosphoethanolamine-derivatized glycosyldiacylglycerol, which we identified as phospholipids in anaerobic bacteria.

Although many glycosyl diradylglycerols have been identified previously in bacterial and plant systems, we are not aware of any GlcNAc-diradylglycerols modified with phosphoethanolamine. Although each of the major polar lipids contained a large proportion of plasmalogen, very little plasmalogen was detected using 0.3% (w/v) molybdenum blue or 0.3% aldehydes. A similar formula was used for the other lipids. The % plasmalogen = 100 – % diacyl lipid.

**Lipid analysis**

For quantification of the polar lipids of *C. tetani* strains ATCC 454 and ATCC 10779, the cells were labeled during growth in 10 ml reinforced clostridial medium with 10 µCi [1-13C] acetate (60 mCi/mmol, Perkin Elmer Life Sciences, Waltham, MA) at 37°C for 24 h. Cells were harvested by centrifugation and washed, and the lipids extracted as described above. As needed, cellular lipids were also extracted according to Benning and Somerville (6) to be added as carrier for TLC. 2D-TLC was performed using 14C-labeled material (7,500 cpm) with added carrier, as described above. Radiolabeled lipids were visualized and quantified with a Phospholmager (Typhoon 9410, Amersham Biosciences, Arlington Heights, IL), equipped with ImageQuant software. Percent diacyl PtdEtn was calculated using the formula: % diacylPtdEtn = % (of total cpm) in diacylPtdEtn after acid hydrolysis / % diacylPtdEtn + % LysophEtn + % aldehydes.

**Materials and Methods**

**Bacterial cultures**

*C. tetani* strains ATCC 454, 9441, 10709, 10779, and 19406 were obtained from the American Type Culture Collection (Manassas, VA). For lipid isolation all strains of *C. tetani* were grown on reinforced clostridial medium without agar, which contained the following per liter: yeast extract, 10.5 g; peptone, 12.5 g; glucose, 5 g; soluble starch, 1 g; NaCl, 5 g; sodium acetate, 3 g; and cysteine HCl·H2O, 0.6 g. The pH was adjusted to ~7.1 with NaOH. The glucose solution was autoclaved separately.

**Lipid extraction**

Cells were harvested by centrifugation at 2000 g for 10 min and washed twice in 20 mM MOPS buffer, pH 7.2. The wet cell pellets were extracted with chloroform/methanol/water by the method of Bligh and Dyer (4), as modified (5). The lipid extracts were dried under a stream of nitrogen while being warmed in a heating block. They were weighed, dissolved in chloroform, and stored at −20°C.

**TLC**

Two-dimensional thin-layer chromatography (2D-TLC) was performed on silica gel 60 and 10 × 10 cm thin-layer plates. The solvents were chloroform/methanol/concentrated ammonia/water, 65:30:2:5:2.5 (v/v/v/v) in the first dimension and chloroform/methanol/acetic acid/water, 80:18:12:5 (v/v/v/v) in the second dimension. For acid hydrolysis of lipids on TLC plates after separation in the first dimension, the area of the plates containing the lipids was suspended over boiling HCl for 40 s, after which the plates were dried under a stream of nitrogen and reactivated under vacuum. The plates were then chromatographed in the second dimension. Phospholipids or amine-containing lipids were detected using 0.3% (w/v) molybdenum blue or 0.3% ninhydrin in ethanol, respectively, followed by heating at 120°C for 10 min. Glycolipids were detected with α-naphthol.

**High-resolution ESI/MS**

High-resolution ESI mass spectra were acquired on a QSTAR XL quadrupole-time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray source. For ESI/MS analysis, the lipid extracts from 500-ml cultures were each redissolved in 200 µl of chloroform/methanol (2:1, v/v). Typically, 10 µl of this solution was diluted into 200 µl of chloroform/methanol (1:1, v/v) containing piperdine (15%, v/v), and then immediately infused into the ESI source at 5–10 µl/min. The negative and positive electrospray voltages were set at −4200 V and +5500 V, respectively. Other MS settings were as follows: CUR = 20 psi (pressure), GS1 = 20 psi, DP = −55 V, and FP = −265 V. For MS/MS, collision-induced dissociation was performed with collision energy ranging from 40 V to 70 V (laboratory frame of energy) with nitrogen as the collision gas. Data acquisition and analysis were performed using the Analyst QS software.

**LC/MS**

LC/MS of lipids was performed using a Shimadzu LC system, which comprised a solvent degasser, two LC-10A pumps, and a SCL-10A system controller, coupled to a QSTAR XL quadrupole-time-of-flight tandem mass spectrometer (as above). LC was operated at a flow rate of 200 µl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min, and then linearly increased to 100% mobile phase B over 14 min, and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 µm, 2.1 × 50 mm) was obtained from Agilent (Palo Alto, CA). The postcolumn splitter diverted ~10% of the LC flow to the ESI source of the mass spectrometer.

**Purification of the unknown lipid**

*C. tetani* ATCC 10779 was grown in anaerobic jars in twelve 500-ml cultures and harvested as described above. The total yield of cells was 12.6 g wet weight. Total lipids were extracted with chloroform/methanol/water as described above, yielding 61.0 mg of dry lipid. The lipids, dissolved in chloroform/methanol (7:3, v/v), were fractionated on DEAE-cellulose. The DEAE-cellulose column was packed in chloroform/methanol/2.4 M aqueous ammonium acetate (2:3:1, v/v/v) to give a bed volume of 1954 Journal of Lipid Research Volume 51, 2010
15 ml. The column was then washed with chloroform/methanol, 7:3 (by volume). After application of the lipids, the column was washed with the same solvent mixture: 50 ml (fraction 1). The column was then eluted with chloroform/methanol 1:1, 50 ml, and the same solvent, 60 ml (designated fractions 2 and 3). The column was then eluted with chloroform/methanol 2:1, containing 0.25% ammonium acetate, 100 ml (fraction 4). All fractions were evaporated in a rotary evaporator and transferred to weighed tubes. A portion of each fraction (50 µg) was applied to a TLC plate and chromatographed in chloroform/methanol/acetic acid/water, 200:40:30:12.5 (v/v/v/v) (solvent 1), sprayed with 10% sulfuric acid in ethanol, and charred on a hot plate. Almost all of the unknown lipid was found in fraction 2.

A silicic acid column, 1.0 x 18 cm, was packed in Solvent I, and the lipid from fraction 2 was applied in the same solvent. The column was eluted with 60 ml of the same solvent, and 1-mL fractions were collected. Lipids from every other fraction were chromatographed on a TLC plate, as described for the DEAE-cellulose fractions, and the unknown lipid was eluted in fractions 15–25, as judged by charring. Fractions 15–25 were pooled, dried, and taken up in CDCl3/CD3OD/D2O for NMR spectroscopy.

NMR spectroscopy

Approximately 5 mg of the purified unknown lipid was dissolved in 0.6 ml of CDCl3/CD3OD/D2O (2:3:1, v/v/v) in a 5 mm NMR tube. Spectra were obtained at 25°C using a Varian Inova 500 spectrometer equipped with a Dell 390 computer and a 5-mm Varian probe. Proton and carbon chemical shifts are reported relative to internal tetramethylsilane (TMS) at 0.00 ppm. The 1H signal of CD3OD was used as a field frequency lock with the residual signal of CD3OD serving as the secondary reference at 49.5 ppm for carbon spectra. 31P NMR chemical shifts were referenced to 85% H3PO4 at 0.00 ppm. Use of the CDCl3/CD3OD/D2O solvent system introduced four solvent 1H resonances. The signals from CH3OD (3.3 ppm) and CHCl3 (7.6 ppm) did not overlap with the lipid resonances. The HOD (4.5 ppm) and CD3OH (4.8 ppm) signals were removed with a presaturation sequence. 1H NMR spectra at 500 MHz were obtained with spectral width (SW) of 4.5 kHz, a 67° pulse flip angle (6 msec), a 5.0 s acquisition time (AT), and a 1.2 s relaxation delay (RD); they were digitized into 45k points yielding a digital resolution of 0.2 Hz/pt. 1H-decoupled 13C NMR spectra were recorded at 125.7 MHz with a spectral window of 34212.8 Hz digitized into 80,000 data points (digital resolution: 0.78 Hz/point or ~0.006 ppm/point), a 60° pulse flip angle (8 µs), and a 2.0 s repeat time. 1H-decoupled 31P NMR spectra were recorded at 202.3 MHz with a spectral window of 12143.3 Hz digitized into 25,280 data points (digital resolution: 1 Hz/point or ~0.005 ppm/point), a 60° pulse flip angle (8 µs), and a 1.6 s repeat time. Two-dimensional NMR experiments [correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC), heteronuclear multiple-bond correlation (HMBC)] and 31P-detected difference spectra derived from selective 31P-decoupled NMR experiments were performed as previously described (7, 8).

RESULTS

TLC and acid sensitivity of C. tetani polar lipids

Two-dimensional TLC of the total lipids of C. tetani ATCC 10779 revealed five major polar lipids, four of which were tentatively identified as PtdEtn, PtdGro, cardiolipin, and a GlcNAc-diacylglycerol (see below), based on their relative mobility and staining with phosphomolybate, ninhydrin, or α-naphthol. The fifth compound, labeled 6, stained with both phosphomolybate and α-naphthol and was, therefore, identified as a phosphorus-containing glycolipid of unknown structure (Fig. 1A).

Because plasmalogens are commonly found in anaerobic bacteria, including members of the genus Clostridium, we carried out exposure to HCl fumes between the first and second dimensions of the 2D-TLC (9). Plasmalogens are acid-labile, and treatment with HCl fumes hydrolyzes the alk-1-enyl bond, releasing a long-chain aldehyde and a lysolipid. Treatment of the lipids from C. tetani ATCC 10779 with acid fumes resulted in no change in the mobility of the polar lipids (data not shown); however, treatment of the similar polar lipids from a strain of C. tetani from the collection of the Department of Microbiology, University of Pennsylvania School of Medicine revealed extensive hydrolysis of all the major lipids, indicating that all contained both all acyl and alk-1-enyl acyl species.

We obtained four additional ATCC strains of C. tetani: ATCC 19406, ATCC 10709, ATCC 454, and ATCC 9441. The 2D-TLC analysis of the lipids extracted from these cells with and without acid hydrolysis indicated that all of these strains contained plasmalogens. Thus, strain ATCC 10779 appeared to be an exception. A representative 2D-TLC with acid hydrolysis of the lipids between dimensions from strain ATCC 454 is shown in Fig. 1B, demonstrating the extensive formation of lyso-phospholipids compared with Fig. 1A.

Quantification of C. tetani polar lipids

The polar lipid composition of C. tetani strains ATCC 10779 (Fig. 1A) and ATCC 454 (Fig. 1B) could be quantified isotopically, given that the cells had been grown for 24 h with [1-14C] acetate (Table 1). Aside from the presence of plasmalogens in strain ATCC 454 and their absence in strain ATCC 10779, there are other differences in the lipid class compositions. Strain ATCC 454 contains almost twice as much PtdEtn as ATCC 10779, but it has very little GlcNAc-diacylglycerol, compared with 21.5% of total polar lipid in the latter strain. However, the other plasmalogen-rich strains ATCC 9441, ATCC 10709, and ATCC 19406 contain larger amounts of GlcNAc-diacylglycerol than ATCC 454, as visualized after 2D-TLC (data not shown). Strains ATCC 10779 and ATCC 454 have comparable amounts of the unknown phosphorus-containing glycolipid.

ESI/MS of total C. tetani polar lipids

To confirm the identities of the major lipids and obtain additional information about the structure of the unknown compound, we subjected the total polar lipids of all C. tetani strains to ESI/MS. The identities of all major lipids of strains ATCC 10779 and ATCC 454 assigned by TLC were confirmed by mass spectroscopy (Table 2 and supplementary Figs. I–III). In the other strains (data not shown), similar results were obtained for the all-acyl lipids and the corresponding plasmalogens, which were observed at 16 amu lower than those for the all-acyl lipids (Table 1). For cardiolipin from ATCC 454, in addition to the species that contain a single alkyl chain, there is evidence for cardiolipin containing two alkylen chains. TLC after HCl treatment shows a lipid
more polar than lyso-cardiolipin, which may represent dilyso-cardiolipin. Mass spectrometry shows prominent peaks at 1207.8, 1233.9, 1235.8, 1261.9, 1287.9, and 1289.9, which correspond to the dialkenyl species of cardiolipin, 56:0, 58:1, 58:0, 60:1, 62:2, and 62:1, respectively (Table 2).

Phosphatidic acid and phosphatidylserine (PtdSer) were detected in strain ATCC 454 (and other strains), but in contrast to the major lipids, no plasmalogen species were detected (supplementary Figs. V and VI), consistent with previous studies indicating that plasmalogens are formed from diacylated phospholipids at a late stage of phospholipid assembly in clostridia (10–13).

An ethanolamine-P-containing glycosyl diradylglycerol in C. tetani

A series of singly charged peaks seen during ESI/MS in the negative ion mode at m/z 837.526, 863.54, 865.55, 891.58, and 893.59 were observed in the total lipids of strain ATCC 10779 (Table 2 and supplementary Fig. 1). The corresponding plasmalogens were also seen in strain ATCC 454 (Table 2 and supplementary Fig. 1) and in other ATCC strains we examined (data not shown). The observed m/z suggested the presence of a phosphoethanolamine derivative of GlcNAc-diacylglycerol. The MS/MS analysis of the peak at m/z 837.5 gave product ions at m/z 627.32, 325.06, and 140.01, consistent with phosphoethanolamine linked to an acetylated amino sugar (Fig. 2, inset).

Evaluation of the covalent structure of the unknown lipid by 31P and 1H NMR spectroscopy

31P NMR spectroscopy of the 5-mg sample of purified unknown lipid from C. tetani ATCC 10779, which contained no plasmalogen (Table 2), revealed large (76%) and small (24%) phosphorus resonances near 1.511 and 5.1 ± 0.47, 5.7 ± 2.7.

| Strain      | PtdEtn | PtdGro | Cardiolipin | GlcNAcDAG | EmPGlcNAcDAG | LysPtdEtn | Unknown A |
|-------------|--------|--------|-------------|-----------|--------------|-----------|------------|
| ATCC 10779  | 25.5 ± 10.5 | 11.2 ± 1.1 | 12.3 ± 8.6 | 21.4 ± 2.3 | 10.3 ± 4.1 | 2.7 ± 1.8 | 12.7 ± 4.6 |
| ATCC 454    | 45.5 ± 4.3 | 12.7 ± 4.6 | 23.0 ± 8.4 | 1.29 ± 0.47 | 5.2 ± 1.9 | 5.1 ± 0.47 | 5.7 ± 2.7 |

Abbreviations: GlcNAc, N-acetylgalactosamine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol.

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1.242 ppm, respectively (Fig. 3A). These $^{31}\text{P}$ NMR shifts are consistent with the presence of two distinct phosphodiester linkages. The larger signal arises from the major diacyl component, while the smaller signal arises from contaminating lysophosphatidylethanolamine (lysoPE), which elutes together with the unknown compound. Reverse phase chromatography allows separation of the unknown lipid from lysoPE (not shown).

The 500-MHz $^1\text{H}$ NMR spectrum of the unknown lipid, dissolved in CDCl$_3$/CD$_3$OD/D$_2$O (2:3:1, v/v) (Fig. 3C, Fig. 4, and Table 3), shows relatively well-resolved resonances in the sugar (3.2–5.8 ppm) and acyl chain regions (0.8–2.7 ppm). An expansion of the sugar region (Fig. 3C) reveals a prominent envelope signal integrating to five protons near 4.1 ppm, three overlapped multiplets each integrating to two protons near 3.6, 3.5, and 3.2 ppm, respectively, and five resolved single proton resonances.

The positions of the individual protons of the glycerol moiety were derived from 2D-COSY NMR (Fig. 4 and Table 3). The multiplet at 5.18 ppm arises from the glycerol H-2 proton (see Fig. 2 inset for the numbering scheme). A pair of COSY cross peaks from H-2 (Fig. 4) locate the H-1a signal at 4.33 ppm (dd, $J_{12,1a}=8.4$, $J_{ab,1a}=12.0$ Hz) and the H-1b multiplet as one of five overlapped signals near 4.1 ppm. A second pair of COSY cross peaks from H-2 connect to H-3a at 3.93 ppm (dd, $J_{12,3a}=5.1$, $J_{ab,3a}=10.7$ Hz) and H-3b as one of two protons signals near 3.63 ppm.

The 2D-COSY analysis also revealed a strong cross peak between the resolved β-CH$_2$ (3.19 ppm) of the phosphethanolamine moiety (8) and the α-CH$_2$ for the same residue, which contributes to two of the five overlapped proton signals near 4.1 ppm (Fig. 3C, Fig. 4, and Table 3).

The positions of the individual protons of the putative N-acetylglucosamine residue were also derived from 2D-COSY (Fig. 4 and Table 3). The doublet at 4.41 ppm ($J_{12,8.4}$) arises from the glucosamine anomeric H-1′ proton (Fig. 2, inset). The large $J_{12}$ coupling (8.4 Hz) indicates that H-1 of the GlcNAc unit is in the β configuration and the axial orientation, so that the glycerol group is linked equatorially (Fig. 2, inset). The COSY cross peak from H-1′ (Fig. 4) locates the H-2′ signal at 3.62 ppm (which overlaps with the glycerol H3b). A second COSY cross peak from H-2′ connects to overlapped H-3′ (3.49 ppm) and H-4′ (3.50 ppm) multiplets. H-4′, in turn, is coupled to H-5′ at 3.40 ppm (a broad multiplet). Further tracing of the COSY connectivities locate H-6′a and H-6′b as the remaining two overlapped proton signals near 4.1 ppm.

The 2D-COSY (Fig. 4) also revealed strong cross peaks between the resolved α-CH$_2$ (≈2.32 ppm) and the β-CH$_2$ (≈1.59 ppm) resonances, as well as the terminal ω-CH$_3$ (0.86 ppm) and the bulk CH$_2$ units (1.25 ppm) of the acyl chains (see Fig. 2 inset for labeling). The CH multiplet at 5.32 ppm connects to overlapped H-3′ (3.49 ppm) and H-4′ (3.50 ppm) multiplets. H-4′, in turn, is coupled to H-5′ at 3.40 ppm (a broad multiplet). The 2.0 ppm CH$_2$ multiplet also overlaps with an N-acetyl CH$_3$ singlet resonance at 1.99 ppm.

The location of the linkage between the phosphethanolamine moiety and the proposed GlcNAc unit of the unknown lipid was evaluated by subtraction of two $^1\text{H}$ NMR spectra, obtained with on- and off-resonance selective decoupling of the 1.511 ppm phosphate signal. This strategy revealed simultaneous changes at the H-6 CH$_3$ signal of the putative GlcNAc residue and the α-CH$_2$ signal of the phosphethanolamine moiety (Fig. 3B), establishing the presence of a single phosphate group linking the glucosamine C-6′ carbon and the α-CH$_2$ carbon of the phosphethanolamine unit, as shown in the inset of Fig. 2.
Evaluation of the carbon structure of unknown lipid by 13C and HMQC spectroscopy

To confirm the assignments derived from the 1H NMR analysis, 13C data for the unknown lipid (5 mg) were obtained both directly through 1D 13C NMR spectroscopy, and indirectly through 1H-detected 2D-HMQC and 2D-HMBC NMR experiments (Table 3). The partial 2D-HMQC 1H-13C correlation map (Fig. 5A) reveals the five direct 1H-13C single-bond correlations in the glycerol unit. The glycerol H-2 proton signal correlates to the glycerol CH carbon resonance at 70.87 ppm (C-2). The H-3a and H-3b proton multiplets correlate to a carbon signal at 68.31 ppm (C-3), while the H-1a and H-1b multiplets connect to a carbon resonance at 63.67 ppm (C-1). The α-CH2 and the β-CH2 1H signals of the phosphoethanolamine moiety correlate to CH2 carbon resonances at 62.57 and 41.17 ppm, respectively. The upfield shift of the β-CH2 carbon reflects the nitrogen substitution of the methylene carbon by the NH2 group.

The anomeric H-1′ proton signal of the presumed GlcNAc residue correlates to the anomeric carbon resonance at 102.6 ppm (C-1′). This carbon position further verifies

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**Fig. 2.** ESI-MS/MS of [M-H]⁻ at m/z 837.53, a major molecular species of the ethanolamine-phosphate containing glycosyldiacylglycerol from *C. tetani* ATCC 10779. The proposed structure of a typical phosphoethanolamine GlcNAc-diacylglycerol (Fig. 1A, spot 6) is shown in the inset, along with the numbering scheme used for the NMR analysis.

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**Fig. 3.** 31P NMR spectrum, selective inverse 31P-decoupled 1H-detected difference spectroscopy, and partial 1H NMR spectrum of the purified novel lipid. A: Two 31P NMR signals in 75:25 ratio are detected. The large 31P NMR signal arises from the purified novel lipid, while the small signal arises from lyso-phosphatidylethanolamine, which elutes with the purified lipid. The chemical shifts (1.511 and 1.242 ppm) are consistent with the presence of one monophosphodiester in each compound. B: The selective inverse decoupled difference 1H NMR spectrum obtained with on and off resonance 31P decoupling of the 1.511 ppm phosphorus signal showed that the 6-CH2 group of the putative N-acetylglucosamine is linked via a phosphodiester to the α-methylene unit of the phosphoethanolamine group. C: The partial one-dimensional 500 MHz 1H NMR spectrum of the donor lipid shows six GlcNAc and various glycerol and phosphoethanolamine proton signals (see Table 3). The resonances at 4.6 ppm and 3.9 ppm are due to residual water and methanol solvent signals. The small resolved multiplets at 3.99 and 3.86 ppm arise from impurities, presumably from co-eluting lysophosphatidylethanolamine, the presence of which is well detected in the 31P nmr spectrum of Panel A. Note that an impurity peak also overlaps H-5 near 3.66 ppm. The proposed structure of the novel lipid is shown in the inset to Fig. 2 along with the numbering scheme.
the sugar as glucosamine because the C1 of β-D-N-acetyl glucosamine is reported at 102.5 ppm, whereas the C1 of β-D-N-acetyl galactosamine is reported at 96.3 ppm (14). The H-6aβ' proton multiplet correlates to a carbon signal at 65.07 ppm (C-6''), while the H-3', H-4', and H-5' multiplets connect to carbon resonances at 74.45 (C-3''), 70.49 (C-4''), and 75.96 (C-5'') ppm, respectively, which correspond to oxygen-substituted carbons of sugars. The nitrogen-substituted carbons of amino sugars resonate near 50–55 ppm (14). The H-2' multiplet shows a prominent cross peak near 56.58 ppm. This C-2' chemical shift is diagnostic evidence confirming C-2' as the site of the amino group substitution. The results of the HMQC correlations, along with data for the acyl chains (not discussed), are summarized in Table 3.

**TABLE 3.** 1H and 13C NMR assignments of the novel lipid from *C. tetani*

| Residue/Position | 1H, [shape, J(Hz)] | 13C                  |
|-----------------|--------------------|----------------------|
| Glycerol        |                    |                      |
| 3a              | 3.925 [dd, 10.7,5.1] | 68.311               |
| 3b              | 3.625 [m]          | 70.867               |
| 2               | 5.175 [m]          | 63.677               |
| 1a              | 4.328 [dd, 12.0,2.5] | 4.1 [m]             |
| 1b              | 4.1 [m]            |                      |
| Glucosamine     |                    |                      |
| 1'              | 4.413 [dd, 8.4]    | 102.362              |
| 2'              | 3.624 [m]          | 56.583               |
| 3'              | ~3.49             | 74.456               |
| 4'              | ~3.50             | 70.489               |
| 5'              | 3.40 [m]          | 75.963               |
| 6'              | 4.105 [m]         | 65.069               |
| N-Acetyl        | 1.989 [s]          | 23.163               |
| Phospho-ethanolamine |                |                      |
| α-CH₂           | 4.083 [m]         | 62.571               |
| β-CH₂           | 3.190 [m]         | 41.173               |
| Acyl chains     |                    |                      |
| α-CH₂           | ~2.32 [m]         | 34.910               |
| β-CH₂           | 1.59 [m]          | 25.695               |
| (CH₂)₉          | 1.25 [m]          | 30.436               |
| (β-2)-CH₂       | ~1.25 [m]         | 32.690               |
| (ω-1)-CH₂       | ~1.25 [m]         | 23.410               |
| α-CH₃           | 0.863 [t]         | 14.558               |
| HC = CH         | ~5.33 [m]         | 130.769              |
| C = C-CH        | ~2.05 [m]         | 27.930               |

1H and 13C chemical shifts at 25°C in CDCl₃/CD₃OD-D₂O (2:3:1, v/v) are recorded relative to an internal tetramethylsilane (TMS) standard. The numbering scheme is shown in Fig. 2.

**DISCUSSION**

The lipids of *C. tetani* are characteristic of the genus *Clostridium* in having PtdEtn, PtdGro, and cardiolipin in both the acyl forms and plasmalogens as major components. Absent are the glycerol acetals of plasmalogens which are characteristic of the solvent-producing species, such as *C. acetobutylicum*, *C. beijerinckii*, and the closely related *C. butyricum* (1). Plasmalogens are present in all strains of *C. tetani* examined with the exception of ATCC 10779. This strain is sometimes referred to as strain Massachusetts, which has been used in vaccine production, and is the parent strain of E88, a nonsporulating variant that has had its genome sequenced (3).

The major fatty acids (15) and alk-1-enyl chains (16) of *C. tetani* have been identified as 14:0, 16:0, and 16:1. The 18:0 and 18:1 were found to be minor species of the

![Fig. 4. Two-dimensional 1H-1H COSY analysis of the purified novel lipid at 500 MHz. The COSY experiment establishes the connectivities among the key glycerol, phosphoethanolamine, GlcNAc sugar, and acyl 1H resonances (also see Table 3). COSY, correlation spectroscopy NMR; GlcNAc, N-acetylgalactosamine.](image)
acyl, but not the alk-1-enyl, chains in strains E4222 and 34946A. Analysis of the molecular species of the polar lipids by MS (Table 2 and supplementary Figs. I–III) is consistent with these earlier analyses, which were done by gas chromatography.

Glycosyl diradyl glycerols containing glucose and galactose in either monoglycosyl or diglycosyl forms were previously identified in C. acetobutylicum (17) and C. innocuum (18, 19). An unusual feature of the polar lipids of C. tetani is the presence of N-acetyl glucosaminyl diradylglycerol. An α-D-GlcNAc diacylglycerol has been found in Streptococcus hemolyticus strain D-58 (20), and a β-D-Glc(1-4)-β-D-GlcN diacylglycerol with an N-acyl substitutant has been described in Bacillus acidocaldarius (21). There have been no previous reports of a lipid containing an ethanolamine-phosphate addition to a N-acetyl glucosaminyl diradylglycerol. Fischer et al. identified an S2-amino-1,3-propanediol-3-phospho-6-α-D-galactopyranosyl (1, 2)-α-D-glycopyranosyl-(1-3)-diradylglycerol and a derivative with an acetyl residue on the 2-amino-1,3-propanediol in C. innocuum (18).

Evidence for the ethanolamine-phosphate-GlcNAc diradylglycerol came initially from negative ion ESI/MS analysis of the total lipids of C. tetani ATCC 10779, which revealed prominent peaks at m/z 837.52, 863.54, and 891.57 (Table 2 and supplementary Fig. I). The lipid giving rise to m/z 837.52 produced fragmentsoverlapping those of phosphatidic acid and phosphatidylserine, which serve as precursors of the major diacylglycerol lipids, were not enriched in the corresponding plasmalogen species (supplementary Figs. V and VI). This finding is consistent with previous studies based on isotopic labeling, which indicated that the glycerol-phosphate backbones of the diacyl lipids may serve as precursors for the plasmalogens (10–13). All of the C. tetani strains we examined contain plasmalogens, with the exception of ATCC 10779, which was used for toxoid production and was presumably passaged many times in the past. It will be interesting to study the ability of these strains to cause tetanus. Eventually, when the genes for plasmalogen biosynthesis have been discovered, a study of the pathogenicity of knockout strains will be instructive.

The biosynthesis and function of our novel phosphoethanolamine-GlcNAc diradylglycerol will require further investigation. In principle, the phosphoethanolamine moiety might be derived from CDP-ethanolamine, or alternatively, by transfer of the phosphoethanolamine unit from phosphatidylethanolamine, as occurs during lipid A modification in Gram-negative bacteria (22). Identification of the relevant enzyme and the corresponding structural gene, in conjunction with studies of the phenotypes of specific deletion mutants, should facilitate the elucidation of the function of this novel lipid.

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