Published in final edited form as:

Biochim Biophys Acta. 2013 July ; 1831(7): 1239–1249. doi:10.1016/j.bbalip.2013.03.012.

Structural Identities of Four Glycosylated Lipids in the Oral Bacterium *Streptococcus mutans* UA159

Larry Sallans^a, José-Luis Giner^b, David J. Kiemle^b, Jenny E. Custer^c, and Edna S. Kaneshiro^{c,*}

Larry Sallans: Larry.Sallans@uc.edu; José-Luis Giner: jlginer@syr.edu; David J. Kiemle: dkiemle@esf.edu; Jenny E. Custer: custerje@mail.uc.edu

^aDepartment of Chemistry, University of Cincinnati, Cincinnati, Ohio, 45221, USA

^bDepartment of Chemistry, State University of New York, ESF, Syracuse, New York, 13210, USA

^cDepartment of Biological Sciences, University of Cincinnati, Cincinnati, Ohio, 45221, USA

Abstract

The cariogenic bacterium *Streptococcus mutans* is an important dental pathogen that forms biofilms on tooth surfaces, which provide a protective niche for the bacterium where it secretes organic acids leading to the demineralization of tooth enamel. Lipids, especially glycolipids are likely to be key components of these biofilm matrices. The UA159 strain of *S. mutans* was among the earliest microorganisms to have its genome sequenced. While the lipids of other *S. mutans* strains have been identified and characterized, lipid analyses of UA159 have been limited to a few studies on its fatty acids. Here we report the structures of the four major glycolipids from stationary-phase *S. mutans* UA159 cells grown in standing cultures. These were shown to be monoglucosyldiacylglycerol (MGDAG), diglucosyldiacylglycerol (DGDAG), diglucosylmonoacylglycerol (DGMAG) and, glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG). The structures were determined by high performance thin-layer chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy. The glycolipids were identified by accurate, high resolution, and tandem mass spectrometry. The identities of the sugar units in the glycolipids were determined by a novel and highly efficient NMR method. All sugars were shown to have α -glycosidic linkages and DGMAG was shown to be acylated in the *sn*-1 position by NMR. This is the first observation of unsubstituted DGMAG in any organism and the first mass spectrometry data for GPDGDAG.

Keywords

Bacterium; glycolipids; lipidomics; dental pathogen

1. Introduction

The oral bacterium *Streptococcus mutans* synthesizes and releases a number of phospholipids and glycolipids [1,2] believed to play a role in the formation of dental plaque biofilms. *S. mutans* also produces organic acids within these biofilms covering teeth

© 2013 Elsevier B.V. All rights reserved.

*Corresponding author. Tel: +1-513-556-9712; Fax: +1 513-556-5280 Edna.Kaneshiro@uc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

surfaces and causes demineralization of the tooth enamel that contributes to the formation of caries [3,4]. Pieringer and his colleagues [1,2] earlier identified the major lipids in the *S. mutans* BHT and FA-1 strains grown in a chemically defined medium, and reported that as much as 30% of the lipids synthesized by *S. mutans* were released from the cell and that the relative percent composition of the cellular and extracellular lipid classes were similar. They identified diacylglycerol (DAG), phosphatidylglycerol, (PG), diphosphatidylglycerol (cardiolipin, CL), aminoacyl-PG and three glycolipids. The glycolipids identified were monoglucosyldiacylglycerol (MGDAG), diglucosyldiacylglycerol (DGDAG) and glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG). That the bacteria synthesized these lipids was demonstrated by metabolic radiolabeling experiments in which [¹⁴C]glycerol was incorporated into each of these lipid components [1]. Since those reports, the genome of *S. mutans* strain UA159 was sequenced [3] and this strain is now being studied in detail with respect to gene expression, biochemistry, and physiology [4-11]. Since it is important to document the biochemical nature of this strain, in this report we focus on detailed characterizations of four glycolipids of the UA159 strain providing definitive structural identifications by high performance thin-layer chromatography (HPTLC), high resolution mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Other detailed studies on the effects of culture age and pH on lipid class and fatty acid compositions are part of a separate report (Custer, J.E., Goddard, B.D., Kaneshiro, E.S., in preparation).

2. Materials and methods

2.1 Bacterial cultures and lipid extraction

Streptococcus mutans strain UA159 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown at 37 °C in 5% CO₂ in Fernbach flasks containing 500 mL of 3.7 % brain heart infusion (BHI) medium (Becton, Dickinson and Company; Sparks, MD). Culture growth was monitored spectrophotometrically at Abs_{600 nm}. After 8 h, cultures were in log phase and after 20 h they were in stationary phase. Most structural analyses in the present study were performed on stationary-phase cells (20-h cultures) when most lipid classes were detectable and sufficient amount of material could be isolated. Organisms were harvested by vigorous mixing to suspend cells attached to the bottom of flasks in biofilms. The suspended cells were pelleted by centrifugation at 5,000 × *g* for 15 min at 4 °C. After centrifugation, the supernatant was discarded and the cell pellet was washed twice with double-distilled water (ddH₂O). Total lipids were extracted by adding chloroform and methanol to the packed cell pellet according to the protocol of Bligh and Dyer [12]. To test whether the BHI culture medium contain the lipids found in *S. mutans*, 18.5 g of BHI powder (equivalent to that in a 500-ml culture) was extracted for lipids with CHCl₃:MeOH (2:1, v/v) and analyzed as a control.

All solvents for lipid extraction, fractionation and isolation, except those used for final MS and NMR analysis, contained the antioxidant butylated hydroxytoluene (BHT). After extraction for 2 h at room temperature, the suspension was centrifuged at 5,000 × *g* for 15 min then the supernatant containing total extracted lipids was removed and purified by biphasic partitioning [13]. The volume of the total lipid fraction was reduced (Brinkmann Rotavapor, BÜCHI, Flawil, Switzerland) and transferred to a 4-dram, screw-capped, Teflon-lined shell vial and dried under N₂ (N-Evap, Organomation, Shrewsbury, MA).

2.2 Isolation of lipid classes

Total lipids were fractionated by adsorption column chromatography (Unisil, Clarkson Chemical, South Williamsport, PA) by first eluting with CHCl₃ followed by elution of the polar lipid fraction with CH₃OH. The components in the polar lipid fraction were isolated

by 1- or 2- dimensional thin-layer chromatography (TLC) using high-performance HPTLC Silica gel 60, (EMD Chemicals, Darmstadt, Germany) or Al-backed Silica gel 60 (Alltech Associates Inc., Deerfield, IL) plates. The solvent system for development in the first dimension was $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{ddH}_2\text{O}$ (65:25:4, v/v/v); CHCl_3 :acetone: CH_3OH : acetic acid (HAc): ddH_2O (50:20:10:10:5, v/v/v/v/v) was used for the second dimension. Initial identification of lipid spots was performed by 5% phosphomolybdate in ethanol and I_2 vapor as a general lipid stains, orcinol for sugars, ninhydrin for amino and imino groups, the Dragendorff reagent for choline and a reagent described by Dittmer & Lester [14] for phosphorus. To isolate individual components, plates were wet with ddH_2O to locate spots in 2-D HPTLC or bands in 1-D HPTLC and the lipid components were isolated by scraping, eluting with $\text{CHCl}_3:\text{CH}_3\text{OH}$ (1:2, v/v) and dried under N_2 .

2.3 Mass spectrometry

Analyses of the isolated glycolipids were performed with a Thermo Scientific LTQ-FT™ using the static nanospray source and New Objective PicoTip™ emitters (2 mm tip). Nanospray samples were prepared by first dissolving the dried lipids in CHCl_3 followed by the addition of MeOH for negative ion, or MeOH containing 0.5 mM NaI for positive ion giving a final ratio of CHCl_3 :MeOH of between 1:1 or 1:2 volume to volume. The glycolipids gave strong Na^+ adducts under positive ion conditions. Negative ion mode was used for analyzing the phosphoglycolipid.

The source voltage was held at 1.70 kV with a capillary temperature of 175 °C. High mass accuracy measurements of both the nanosprayed ions and product ions arising from collision-induced dissociation was performed in the Fourier transform ion cyclotron resonance (FT-ICR) portion of the LTQ-FT™ with the resolution generally set at 100,000 (at m/z 400) although some situations required using 500,000 resolving power. Errors in mass accuracy were below 1 ppm with 235 ± 129 ppb as the average of absolute errors. Collision-induced dissociation (MS^n) was performed in the linear ion trap portion of the system. FT-ICR MS^n mass errors were slightly higher due to the lower product ion intensities (484 ± 255 ppb).

2.4 NMR spectroscopy

2.4.1 General—NMR spectra were measured using a 600 MHz Bruker Avance instrument at 30 °C. The chemical shifts in solvents containing CDCl_3 were calibrated to residual CHCl_3 (7.260 ppm); in solvents containing d_6 -DMSO, they were calibrated to residual d_5 -DMSO (2.500 ppm); in 2M $\text{D}_2\text{SO}_4/\text{D}_2\text{O}$, DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid, 0.015 ppm) was used for calibration.

NMR procedures employed included COrelation SpectroscopY (COSY), Nuclear Overhauser Effect SpectroscopY (NOESY), Rotating-Frame Nuclear Overhauser Effect SpectroscopY (ROESY) and TOtal Correlation Spectroscopy (TOCSY).

2.4.2 Sugar Analysis—A sample was digested with 2M $\text{D}_2\text{SO}_4/\text{D}_2\text{O}$ at 100 °C for 1 h. The resulting hydrolysate was analyzed directly by ^1H -NMR. The spectrum, in particular the anomeric region, was compared to reference spectra of different sugars and was found to match that of glucose [15]. Under these conditions, glucose exhibits signals at 5.26 ppm (d, 3.6 Hz, α -anomer) and 4.68 ppm (d, 7.9 Hz, β -anomer) in a 38:62 ratio.

3. Results and discussion

3.1 Identification of *S. mutans* UA159 lipids: high performance thin-layer chromatography

Approximately 10-13 lipid components were resolved and detected by 2-D HPTLC of *S. mutans* UA159 cells grown to stationary-phase of culture growth (Fig. 1A). There were four lipid components that stained positive for sugars (Fig. 1B), one of which was also positive for phosphorus (Fig. 1C). Thus, these analyses established that this bacterium contained four glycolipids.

2-D HPTLC analysis of the BHI extract showed no detectable spots on the plates indicating that the lipid components analyzed in *S. mutans* cell extracts did not originate from the culture medium. The *S. mutans* UA159 lipids identified in the present study are consistent with those reported by Cabacungan and Pieringer [1] who found similar lipid compositions in *S. mutans* BHT and FA-1 strains grown in a chemically defined medium.

3.2 Identification of *S. mutans* UA159 glycolipids: mass spectrometry

3.2.1 General—Spots identified as glycolipids were extracted from TLC plates and were directly nanosprayed into the instrument. Under the assumption that members within a glycolipid class differing only by the length of the acyl groups would ionize with similar efficiency this technique offers a facile comparison of the relative intensities of the various component members within a group. The analysis is however complicated by overlap between isotopic contributions that are isobaric at lower mass resolution. This necessitated that the data be acquired under the higher resolving power of the FT-ICR portion of the instrument (Fig. 2). For example diglucosyl monoacylglycerol (DGMAG) where the nominal masses for the octadecenyl and octadecanyl acyl groups are 703 and 705 respectively and denoted 18:1 and 18:0, the m+2 contribution for 18:1 resulting from two ^{13}C or an ^{18}O (and to a lesser extent ^{13}C with ^2H) overlaps with the nominal mass 18:0 component such that 57,000 (57K) resolving power at m/z 705 begins to suggest that at least two components are present (Fig. 2B). Resolving power of 113K clearly separates 18:1 from 18:0 components verifying the presence of 18:0 (Fig. 2C) and increasing the resolving power to 284K separated the various isotopic contributions for the m+2 component of 18:1 (Fig. 2D). Under collision-induced dissociation (CID) conditions, DGMAG loses a sugar group which will be discussed in detail below. The resulting MS/MS product ions show contributions from both 18:1 and 18:0 (Fig. 2E) in which m/z 541 is the nominal mass contribution for 18:1; m/z 542 is the 18:1 contribution still retaining one ^{13}C atom; and the m/z 543 product ion has various isotopic contributions from both 18:1 and 18:0 components. These contributions are easily resolved and assigned with a resolving power of 294K (Fig. 2F). For this study, spectra were routinely acquired for full scans at a resolving power of 100K (m/z 400) and at 500K (m/z 400) under SIM conditions when better resolution was required.

For each glycolipid class a representation of the relative abundance of the various components contributing to the class is provided (Fig. 3). While not rigorously quantitative, this comparison still permits a useful survey of the major acyl contributors within each lipid class. To support this claim one must assume that the ionization and instrument transfer characteristics are not greatly influenced by the small differences in acyl chain length, generally spanning less than 200 Da for the group. Support that ionization is not significantly influenced by acyl length can be seen by comparing the positive and negative ion spectra of GPDGDAG, Figures 3D and 3E respectively. Although involving different solutions analyzed days apart, the sodiated adducts (most likely involving the sugar and carbonyl moieties) show a pronounced similarity to the anion pattern which is most likely centralizing the charge at the phosphate group. Both techniques represent ions differing

greatly in type and charge local. Regarding ion transmission especially within a narrow mass range of less than 200 Da, the LTQ-FT™ utilizes multipoles which characteristically demonstrate a broad transfer range. In addition, such narrow ranges also do not exhibit “time-of-flight” effects between the linear ion trap and the FT-ICR; the LTQ-FT™ can trap ions an order of magnitude broader in range. It is informative to note that the largest acyl contributions for each glycolipid class investigated by MS were 16:0, 18:1, and 20:1. Quantitation of the fatty acid compositions of the major lipid classes in *S. mutants* UA159 by GLC confirmed that 16:0, 18:1 and 20:1 as major acyl groups (Custer, J.E., Goddard, B.D., Kaneshiro, E.S., in preparation). The observed acyl distribution extended as low as decyl (10:0 and 10:1) and as high as tetracosyl (24:0 and 24:1) with higher unsaturation observed with the longer, more abundant acyl groups (e.g. 18:3 and 20:3). Table 1 compliments Figure 3 providing accurate mass data confirming the elemental composition for each component. Tandem mass spectrometry data was used to determine the acyl distribution for each observed mass (see below). The use of “trace” for the acyl groups observed is typically used for acyl components that are under 2% of the intensity of the largest acyl component. Since the major acyl groups observed were 16:0 and 18:1, the parent ion containing these two groups was chosen as the representative example showing relative product ion distributions for each glycolipid class (Table 2).

3.2.2 Monoglucosyldiacylglycerol and diglucosyldiacylglycerol—Sodium adducts of monoglucosyldiacylglycerol (MGDAG) and diglucosyldiacylglycerol (DGDAG) form similar collision-induced dissociation (CID) profiles. An example of each is given in Table 2 with 16:0 and 18:1 as the two acyl groups. The fragmentation is dominated by acid loss as illustrated in (Fig. 4A); acid loss from the *sn*-2 position can also be observed in a similar manner. The other major fragmentation pathway involves hydrogen transfer and loss of the sugar moiety resulting in a characteristic Y-ion for glycoconjugates as designated by Domon and Costello ([17]).

The MGDAG components are summarized (Table 1), which can be used with data in Figure 3A to show relative abundance of the various components in this lipid class. There are other components present including a significant number of oxidation products. Although effort was made to prevent oxidation of the sample, it is presently unknown whether the oxidation products originated from the organism or due to sample handling. Collision-induced dissociation was performed on each species listed in Table 1 to ensure that these components are MGDAG and to determine their acyl distributions. The most predominant product ions correspond to acid losses (the larger acid loss is listed first in the pair). Other observable but less abundant acyl pairs are listed in the table in the order of relative abundance. Acid loss has been shown to favor the *sn*-1 position for sodiated mono- and digalactosyldiacylglycerols [18]. In the present study, C₆H₁₀O₅ loss from the sugar portion of the molecule was also observed.

3.2.3 Diglucosylmonoacylglycerol—While characterizations of diglucosylmonoacylglycerols (DGMAG) are rare in the literature, examples have been reported. Two possible basic structures are possible with either the two sugars linked together or separately attached to the glycerol portion (Fig. 5). It has been reported that the cyanobacterium, *Synechocystis*, contains a DGMAG similar to that shown in Figure 5A except that the sugar linkage is 1,6 with a palmitoyl substitution at the terminal sugar's C-6 position; it also has the acyl group substituted at the *sn*-2 position [19]. Colebroside A is an example with structure similar to that shown in Figure 5B [20]. The present report describes the first observation and tandem mass spectrometry for unsubstituted DGMAG, which is a minor glycolipid found in *S. mutants* UA159. NMR (see below) clearly indicates that the sugars are linked together with a 1,2'- α-linkage and that acylation of glycerol is at the *sn*-1 position (Fig. 5A). Even though the diacyl glycolipids DGDAG and MGDAG show loss of

the sugar moiety, acid loss is the major product ion observed. In contrast DGMAG showed almost exclusive sugar loss in both the MS² and MS³ experiments (Table 2). The fact that acid loss is observed two orders of magnitude lower in product ion abundance may be indicative of a stronger sodium ion preference for the carbonyl group thus hindering proton transfer and subsequent acid loss. Although present <1% relative abundance in MS² spectra (Fig. 6), there are two product ions that also support the linked sugar structure shown in Figure 5A as opposed to the separated sugar structure of Figure 5B. These product ions are B₂ as a sodium adduct with a H transfer to the glycerol (m/z 347) and C₂ as a sodium adduct with a H-transfer from the glycerol (m/z 365), using the ion nomenclature designated by Domon and Costello [17].

3.2.4 Glycerophosphoryldiglucosyldiacylglycerol—In

glycerophosphoryldiglucosyldiacylglycerides (GPDGDAG) the diacylglyceride is not bound through a phosphate group to the sugar. While there are many studies of phosphatidylglucosides especially regarding lipid rafts and other lipid domains [21, 22] GPDGDAG has received considerably less attention. GPDGDAG has been identified in *Acholeplasma laidlawii* [23--26] by NMR but without mass spectrometry data. Also, GPDGDAG had been tentatively identified in another strain of *S. mutans* but MS was not performed [1].

CID of the sodium adduct is dominated by hydrogen transfer to the sugar followed by loss of C₃H₆O₅PNa to produce a product ion similar in structure to the parent ions of DGDAG (Fig. 4B). This is further supported by MS³; comparison of the m/z 941 product ion from m/z 1117 for GPDGDAG (Table 2) with the MS² of the m/z 941 parent for DGDAD. Not only are the observed losses identical but the relative intensities are also strongly similar. MS³ generates strong acid losses and is used in preference to MS² in determination of the acyl components.

In addition to the sodium ion adducts similar to the glycolipids, the phosphate group contributes to intense anions that are also useful in the identification and characterization of GPDGDAG. The major product ion observed for the GPDGDAG anions was C₃H₆O₂ loss most likely directly involving the charge site (Fig. 4C). The anions also show strong acid and ketene losses characteristic of phosphatidyl lipids but remote from the charge site. The acid and ketene losses can also be used to determine the acyl components. The major product ions obtained through low energy CID of these anion components in the ion trap, presents very little evidence regarding the sugar structure with the exception of m/z 315 (Fig. 7). This ion has an elemental composition suggesting a B₁ ion that also contains a phosphate group with an unsubstituted glyceryl; the positive ion fragmentation supports the glycerol molecule attached to the phosphate group which is in agreement with the NMR data (see below).

3.3 Identification of *S. mutans* UA159 glycolipids: nuclear magnetic resonance spectroscopy

3.3.1 Monoglucosyldiacylglycerol (MGDAG)—This fraction was characterized by ¹H-NMR and COSY using CDCl₃ as the solvent (Table 3). The linkage of the sugar was shown to be alpha by the small coupling constant of the anomeric signal (3.6 Hz). Positions 4-6 of the sugar were difficult to assign because of overlapping signals. Assignment of the sugar as glucose was determined by hydrolysis with 2 M D₂SO₄/D₂O at 100 °C for 1 h, followed by direct ¹H-NMR measurement.

3.3.2 Diglucosyldiacylglycerol (DGDAG)—This fraction was characterized by ¹H-NMR, COSY, TOCSY and NOESY using CDCl₃ / *d*₄-CH₃OH 3:1 as the solvent (Fig. 8).

The linkages of the sugars were shown to both be alpha by the small coupling constants of the anomeric signals (*ca.* 3.6 Hz). The 1,2'-linkage of the sugars was determined by NOESY which showed a cross peak between 4.78 and 3.41 ppm. This was confirmed by 1D ROESY. Linkage of the other sugar and the glyceryl backbone was evident from a cross peak between 4.81 and 3.47 ppm by NOESY. Assignment of the sugar as glucose was determined by hydrolysis with 2 M D₂SO₄/D₂O at 100 °C for 1 h, followed by direct ¹H-NMR measurement (Fig. 9).

3.3.3 Diglycosylmonoacylglycerol (DGMAG)—This fraction was characterized by ¹H-NMR and COSY using *d*₆-DMSO containing 2% D₂O as the solvent. The signals of the glyceryl group were detected by COSY. The glyceryl group was determined to be acylated at position-1 (*sn*-1) by the higher chemical shifts of the corresponding signals.

The linkages of the sugars were shown to be alpha by the small coupling constants of the anomeric signals (*ca.* 3.5 Hz). The ¹H-NMR signals of the C2 positions of the sugars were evident from their correlation to the signals of the anomeric H-atoms by COSY. The 1,2'-linkage of the sugars was determined by 1D ROESY. Irradiation of the anomeric signal at 4.83 led to enhancement of a signal at 3.35 ppm (the C2 H-atom of the other sugar) as well as the signal of the coupled C2 H-atom. When applied to the anomeric signal at 4.92 ppm, 1D ROESY did not enhance the signal of C2 H-atom at 3.18 ppm. Because signal of the C2 H-atom at 3.35 ppm was overlapping with other signals, the outcome of the 1D ROESY experiments was confirmed using CDCl₃ /*d*₄-CH₃OH 3:1 as the solvent. In this solvent, the anomeric signals at 4.84 ppm (d, 3.2 Hz) and 4.81 ppm (d, 3.8 Hz) were shown by COSY to be coupled to signals of C2 H-atoms at 3.46 and 3.30 ppm, respectively, while NOE was confirmed by 1D ROESY between the 4.81 and 3.46 ppm signals.

The assignment of the sugar groups as glucose was determined by hydrolysis with 2M D₂SO₄/D₂O at 100 °C for 1 h, followed by direct ¹H-NMR measurement.

3.3.4 Glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG)—This fraction was characterized by ¹H-NMR and COSY using *d*₆-DMSO containing 2% D₂O as the solvent. The spectra correlated well with those of compound 1 described by Hauksson et al. [23] (Fig. 10).

3.4 *S. mutans* glycolipids

The abundance of glycolipids and their accumulation with culture age in *S. mutans* is correlated with the acidification as well as depletion of nutrients in the culture medium suggests that these compounds participate in adhesion and inclusion in teeth biofilms [27]. Although glycolipids and/or other lipids might confer greater resistant to acidic conditions, there is currently insufficient evidence showing that these compounds specifically serve that function in *S. mutans*.

Other strains of *S. mutans* were shown to synthesize these glycolipids [1] and the present study on UA159 provides detailed MS and NMR confirming the nature of their structures. Glucosyltransferases have been identified on the *S. mutans* surface and in the extracellular milieu released by the bacteria [4-6, 25]. Hence it is possible, that the formation of these glycolipids first involves de novo synthesis of glycerolipids (i.e., PG) followed by remodeling by the additions of glucose to PG at the cell surface and/or in the extracellular environment. This would be consistent with previous observations of lipid synthesis in or by this organism and the presence of these glycolipids in the culture medium [1, 2].

Since sucrose is a particularly cariogenic disaccharide and can either be taken up by *S. mutans* cells or hydrolyzed extracellularly [29-31], it might serve as a source of glucose

molecules that are transferred to PG thus giving rise to these glycolipids. Future studies might include testing whether incubation of PG with “conditioned” media in which *S. mutans* was grown and presumably containing the necessary enzymes, leads to the production of these same glycolipids.

4. Conclusions

Three glycolipids and one phosphoglycolipid were detected in the oral pathogen *Streptococcus mutans* strain UA159 grown in standing cultures to late stationary phase. Definitive structural identities were obtained by specific staining of 2-D high-performance thin-layer chromatography plates, high-resolution mass spectrometric analyses and nuclear magnetic resonance spectroscopy. The glycolipids included monoglucosyldiacylglycerol, diglucosyldiacylglycerol, diglucosylmonoacylglycerol, and glycerophosphoryldiglucosyldiacylglycerol (Fig. 11). The two glucose moieties in the later three compounds were connected via an α -1,2 glycosidic linkage. Galactosyl lipids were not detected as reported in *S. mutans* strain FA-1 [32]. We hypothesize that some phosphatidylglycerol molecules synthesized de novo are modified by extracellular or externally located cellular glucosyltransferases to form these glycolipids found associated with the cell as well as in the culture medium. More research is required to determine the role of these compounds in the physiology of *S. mutans*, whether they are involved in enabling the bacterium to live and proliferate under changing environmental conditions and whether these glycolipids play a role in oral disease.

Acknowledgments

We thank Bryan Goddard for helpful comments on the manuscript. This study was supported in part by NIH grants RO1 AI064084 and NIH RR19900.

References

1. Cabacungan E, Pieringer RA. Excretion of extracellular lipids by *Streptococcus mutans* BHT and FA-1. *Infect Immun*. 1980; 27:556–562. [PubMed: 7380539]
2. Cabacungan E, Gustow E, Pieringer RA. In vivo studies on stereospecificity of the monoglyceride kinase, lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase and CDP-diglyceride synthase of *Streptococcus mutans* BHT using the stereoisomers of the ether lipid, dodecylglycerol. *Biochim Biophys Acta*. 1988; 962:241–247. [PubMed: 2844276]
3. Ajdi D, McShan WM, McLaughlin RE, Savi G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Lin HS, Qian Y, Li S, Zhu H, Najjar F, Lai H, White J, Roe BA, Ferretti JJ. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci USA*. 2002; 99:14434–14439. [PubMed: 12397186]
4. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev*. 1986; 50:353–380. [PubMed: 3540569]
5. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Mol Biol Rev*. 1980; 44:331–384.
6. van Hoogmoed CG, van der Kuijl-Booij M, van der Mei HC, Busscher HJ. Inhibition of *Streptococcus mutans* NS adhesion to glass with and without salivary conditioning film by biosurfactant-releasing *Streptococcus mitis* strains. *Appl Environ Microbiol*. 2000; 66:659–663. [PubMed: 10653732]
7. Quivey RG Jr, Faustoferri R, Monahan K, Marquis R. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiol Lett*. 2000; 189:89–92. [PubMed: 10913871]
8. Fozo EM, Quivey RG. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Appl Environ Microbiol*. 2003; 70:929–936. [PubMed: 14766573]

9. Fozo EM, Quivey RG. The *fabM* gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. *J Bacteriol.* 2004; 186:4152–4158. [PubMed: 15205416]
10. Fozo EM, Kajfasz JK, Quivey RG Jr. Low pH-induced membrane fatty acid alterations in oral bacteria. *FEMS Microbiol Lett.* 2004; 238:291–295. [PubMed: 15358413]
11. Fozo EM, Scott-Anne K, Koo H, Quivey RG Jr. Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*. *Infect Immun.* 2007; 75:1537–1539. [PubMed: 17220314]
12. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959; 37:911–917. [PubMed: 13671378]
13. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226:497–509. [PubMed: 13428781]
14. Dittmer JC, Lester RL. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res.* 1964; 15:126–127. [PubMed: 14173318]
15. Kiemle DJ, Stipanovic AJ, Mayo KE. Proton NMR methods in compositional characterization of polysaccharides. *ACS Symposium Series.* 200; 864:122–139.
16. James PF, Perugini MA, O'Hair RAJ. Sources of artifacts in the electrospray ionization mass spectra of saturated diacylglycerophosphocholines: from condensed phase hydrolysis reactions through to gas phase intercluster reactions. *J Am Soc Mass Spectrom.* 2006; 17:384–394. [PubMed: 16443367]
17. Domon B, Costello CE. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J.* 1988; 5:397–409.
18. Guella G, Frassanito R, Mancini I. A new solution for an old problem: the regiochemical distribution of the acyl chains in galactolipids can be established by electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom.* 2003; 17:1982–1994. [PubMed: 12913862]
19. Kim YH, Choi K-S, Hong J, Yoo JS, Kim MS. Identification of acylated glycolipids from a cyanobacterium, *Synechocystis* sp. by tandem mass spectrometry. *Lipids.* 1999; 34:847–853. [PubMed: 10529096]
20. Yang H, Jiang B, Hou A-J, Lin Z-W, Sun H-D. Colebroside A, a new diglucoside of fatty acid ester of glycerin from *Clerodendrum cobrookianum*. *J Asian Nat Prod Res.* 2000; 2:177–185. [PubMed: 11256691]
21. Nagatsuka Y, Hirabayashi Y. Phosphatidylglucoside: A new marker for lipid rafts. *Biochim Biophys Acta.* 2008; 1780:405–409. [PubMed: 17933468]
22. Murate M, Hayakawa T, Ishii K, Inadome H, Greimel P, Watanabe M, Nagatsuka Y, Ito K, Ito Y, Takahashi H, Hirabayashi Y, Kobayashi T. Phosphatidylglucoside forms specific lipid domains on the outer leaflet of the plasma membrane. *Biochemistry.* 2010; 49:4732–4739. [PubMed: 20433166]
23. Hauksson JB, Lindblom G, Rilfors L. Structures of glucolipids from the membrane of *Acholeplasma laidlawii* Strain A-EF22.1. Glycerophosphoryldiglucoacyldiacylglycerol and Monoacylbisglycerophosphoryldiglucoacyldiacylglycerol. *Biochim Biophys Acta.* 1994; 1214:124–130. [PubMed: 7918591]
24. Danino D, Kaplun A, Lindblom G, Rilfors L, Orädd G, Hauksson JB, Talmon Y. Cryo-TEM and NMR studies of a micelle-forming phosphoglucolipid from membranes of *Acholeplasma laidlawii* A and B. *Chem Phys Lipids.* 1997; 85:75–89. [PubMed: 9032946]
25. Andersson AS, Rilfors L, Lewis RN, McElhaney RN, Lindblom G, Lindblom G. Occurrence of monoacyl-diglucoacyl-diacyl-glycerol and monoacyl-bis-glycerophosphoryl-diglucoacyl-diacyl-glycerol in membranes of *Acholeplasma laidlawii* Strain B-PG9. *Biochim Biophys Acta.* 1998; 1389:43–49. [PubMed: 9443602]
26. Orädd G, Andersson AS, Rilfors L, Lindblom G, Standberg E, Andrén PE. α -Methylene ordering of acyl chains differs in glucolipids and phosphatidylglycerol from *Acholeplasma laidlawii* membranes: ^2H -NMR quadrupole splittings from individual lipids in mixed bilayers. *Biochim Biophys Acta.* 2000; 468:329–344. [PubMed: 11018677]
27. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* 2011; 45:69–86. [PubMed: 21346355]

28. Burt BA. Relative consumption of sucrose and other sugars: has it been a factor in reduced caries experience? *Caries Res.* 1993; 27(Suppl):56–63. [PubMed: 8500127]
29. Woodward M, Walker AR. Sugar consumption and dental caries: evidence from 90 countries. *Br Dent J.* 1994; 176:297–302. [PubMed: 8186040]
30. Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. *J Dent Educ.* 2001; 65:1028–1037. [PubMed: 11699974]
31. Zeng L, Burne RA. Comprehensive mutational analysis of sucrose-metabolizing pathways in *Streptococcus mutans* reveals novel roles for sucrose phosphotransferase system permease. *J Bacteriol.* 2013; 195:833–843. [PubMed: 23222725]
32. Chiu T-H. Biosynthesis of galactosyl lipids and polysaccharide in *Streptococcus mutans*. *Biochim Biophys Acta.* 1988; 963:359–366. [PubMed: 3196739]

Abbreviations

BHI	brain-heart infusion
BHT	butylated hydroxytoluene
CID	collision-induced dissociation
CL	cardiolipin, diphosphatidylglycerol
COSY	correlation spectroscopy
DAG	diacylglycerol
ddH₂O	double-distilled water
DGDAG	diglucosyldiacylglycerol
DGMAG	diglucosylmonoacylglycerol
DMSO	dimethylsulfoxide
FT-ICR	Fourier transform ion cyclotron resonance
GPDGDAG	glycerophosphoryldiglucosyldiacylglycerol
HPTLC	high performance thin-layer chromatography
MS	mass spectrometry
MGDAG	monoglucosyldiacylglycerol
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
PG	phosphatidylglycerol
ROESY	rotating frame nuclear Overhauser effect spectroscopy
TLC	thin-layer chromatography
TOCSY	Total correlation spectroscopy

Highlights

- Larry Sallans BBA
- *Streptococcus mutans* UA159 synthesizes four glycolipids.
- Definitive structural identities were determined by HPTLC, MS and NMR
- *S. mutans* glycolipids were comprised of glucosyl, not galactosyl moieties.
- This is the first observation of unsubstituted DGMAG in any organism.
- These glycolipids might enhance biofilm formation involved in dental pathogenesis

Fig. 1. Staining of *Streptococcus mutans* UA159 polar lipids isolated from stationary-phase cultures and separated by 2-D HPTLC. (A) General lipids, iodine. (B) Sugars, orcinol. (C) Phosphorus [12]. Four spots stained for sugars and one for both sugar and phosphorus.

Fig. 2.

Overlap of diglycosylmonoacylglycerols with octadecenyl and octadecanyl acyl groups (18:1-DGMAG and 18:0-DGMAG) demonstrates the need for high resolution mass spectrometry. (A) Expanded region of the FT-ICR spectrum for the DGMAG sample. (B) Expansion of the m/z 705 region at 57K resolving power; the 18:1 and 18:0 components are barely distinguishable. (C) At 113K resolving power, the resolution is sufficient to clearly separate the 18:1 and 18:0 components thus confirming the presence of the 18:0 component. (D) At 284K resolving power, the multiple isotopic components contributing to the 18:1 $m+2$ ion are resolved. (E) Tandem mass spectrum of m/z 705 obtained in the linear ion trap expanding the mass region corresponding to $C_6H_{10}O_5$ (sugar) loss; refer to text for details. (F) Transfer of m/z 705 product ions into the FT-ICR. Expansion of the m/z 543 product ion region obtained at 294K resolving power, distinguishing sugar loss from 18:0-DGMAG and 18:1-DGMAG still retaining various stable isotopes in the production.

Fig. 3.

FT-ICR spectra for the glycolipids presented in this study. All are positive ion spectra of sodium adducts with the exception of the deprotonated, negative ion spectrum E. (A) Sodiated monoglucosyldiacylglycerol (MGDAG). (B) Sodiated diglucosyldiacylglycerol (DGDAG). (C) Sodiated diglucosylmonoacylglycerol (DGMAG). (D) Sodiated glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG). (E) Deprotonated glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG).

Fig. 4.

Proposed mechanisms consistent with accurate mass product ion data and similar to other reported mechanisms. A. Mechanism common for carboxylic acid loss from glycolipids similar to Scheme 2 in Guella et al. [18]. B. Mechanism accounting for the $C_3H_6O_5PNa$ losses observed in the sodiated GPDGDAG product ion spectra. C. Mechanism accounting for the prevalent $C_3H_6O_2$ losses observed in the deprotonated GPDGDAG product ion spectra similar to Scheme 4 in P.F. James, et. al. [17].

Fig. 5. Possible structures for diglucosylmonoacylglycerol (DGMAG). (A) Glucose rings linked together; the DGMAG observed in this study has this structure with the 1-2' linkage and the *sn*-1 acylation as shown. (B) Glucose rings separated; this structure has also been observed for other bacteria (see text) and was not observed in *S. mutans* UA159 DGMAG.

Fig. 6. Product ion spectrum (LTQ) of the DGMAG parent containing an octyldecenyl acyl (18:1) group, m/z 703. While the product ions are dominated by sugar loss, two small ions labeled B_2 and C_2 support the linked sugar structure shown in Figure 5A (refer to text).

Fig. 7.

Proposed structure for the m/z 315 product ion arising from glucosyldiacylglycerol loss from deprotonated GPDGDAG. The presence of this ion supports the glycerophosphoryl group being attached to a different glucose than the diacylglycerol.

Fig. 8.
COSY ¹H-NMR spectrum of diglycosyl diacylglycerol. Solvent: CDCl₃ / *d*₄-CH₃OH 3:1.

Fig. 9. Sugar analysis by ¹H-NMR. A. Anomeric region of the hydrolysate of diglucosyl diacylglycerol;. B. Anomeric region of glucose. Solvent: 2M D₂SO₄/D₂O.

Fig. 10. COSY ¹H-NMR spectrum of glycerophosphoryl diglucosyl diacylglycerol. Solvent: *d*₆-DMSO + 2% D₂O.

Monoglucosyldiacylglycerol
(MGDAG)

Diglucosyldiacylglycerol
(DGDAG)

Diglucosylmonoacylglycerol
(DGMAG)

Glycerophosphoryldiglucosyldiacylglycerol
(GPDGDAG)

Fig. 11.
Structures of *Streptococcus mutans* UA159 glycolipids.

Table 1

Mass spectra of *Streptococcus mutans* UA159 glycolipids. Mass spectra are shown in Figure 3. See figure 11 for structures.

Observed Mass	Elemental Composition	Theoretical Mass	Error (ppb)	Major Acyl Groups	Other Acyl Groups Observed
Monoglucosyldiacylglycerol (MGDAG)					
669.45504	C ₃₅ H ₆₆ O ₁₀ Na ⁺	669.45482	329	10:0/16:0;	14:0/12:0
695.47086	C ₃₇ H ₆₈ O ₁₀ Na ⁺	695.47047	562	16:1/12:0; 16:0/12:1	
697.48625	C ₃₇ H ₇₀ O ₁₀ Na ⁺	697.48612	187	16:0/12:0	
723.50199	C ₃₉ H ₇₂ O ₁₀ Na ⁺	723.50177	305	16:0/14:1; 16:1/14:0	18:1/12:0
725.51762	C ₃₉ H ₇₄ O ₁₀ Na ⁺	725.51742	276	16:0/14:0	18:0/12:0
749.51739	C ₄₁ H ₇₆ O ₁₀ Na ⁺	749.51742	-40	16:1/16:1; 16:0/16:2	18:0/14:2; 18:2/14:0; 14:1/18:1
751.53319	C ₄₁ H ₇₈ O ₁₀ Na ⁺	751.53307	160	16:0/16:1	18:1/14:0; 18:0/14:1; tr ^d 20:1/12:0
753.54891	C ₄₁ H ₈₀ O ₁₀ Na ⁺	753.54872	252	16:0/16:0	18:0/14:0; tr 20:0/12:0
777.54888	C ₄₃ H ₇₈ O ₁₀ Na ⁺	777.54872	206	18:1/16:1	18:2/16:0; tr 14:1/20:1
779.56462	C ₄₃ H ₈₀ O ₁₀ Na ⁺	779.56437	321	16:0/18:1	18:0/16:1; 20:1/14:0
781.58019	C ₄₃ H ₈₂ O ₁₀ Na ⁺	781.58002	218	16:0/18:0	
805.58032	C ₄₅ H ₈₂ O ₁₀ Na ⁺	805.58002	372	18:1/18:1	20:1/16:1; 16:0/20:2; 18:0/18:2
807.59576	C ₄₅ H ₈₄ O ₁₀ Na ⁺	807.59567	111	20:1/16:0	18:0/18:1
833.61151	C ₄₇ H ₈₆ O ₁₀ Na ⁺	833.61132	228	18:1/20:1	16:0/22:2; ; tr 20:0/18:2; tr 18:0/20:2
835.62726	C ₄₇ H ₈₈ O ₁₀ Na ⁺	835.62697	347	18:0/20:1	16:0/22:1; 18:1/20:0; tr 14:0/24:1
Diglucosyldiacylglycerol (DGDAG)					
831.50781	C ₄₁ H ₇₆ O ₁₅ Na ⁺	831.50764	201	10:0/16:0	14:0/12:0
857.52345	C ₄₃ H ₇₈ O ₁₅ Na ⁺	857.52329	183	16:1/12:0	12:1/16:0
859.53904	C ₄₃ H ₈₀ O ₁₅ Na ⁺	859.53894	113	16:0/12:0	tr 14:0/14:0; tr 18:0/10:0
883.53888	C ₄₅ H ₈₀ O ₁₅ Na ⁺	883.53894	-71	16:0/14:2	16:1/14:1; tr 16:2/14:0
885.55461	C ₄₅ H ₈₂ O ₁₅ Na ⁺	885.55459	19	16:0/14:1	16:1/14:0; 18:1/12:0
887.57047	C ₄₅ H ₈₄ O ₁₅ Na ⁺	887.57024	256	16:0/14:0	18:0/12:0
911.57015	C ₄₇ H ₈₄ O ₁₅ Na ⁺	911.57024	-102	16:1/16:1	16:0/16:2; 18:2/14:0; 18:0/14:2; 18:1/14:1
913.58607	C ₄₇ H ₈₆ O ₁₅ Na ⁺	913.58589	194	16:0/16:1	18:1/14:0; 18:0/14:1; tr 20:1/12:0

Observed Mass	Elemental Composition	Theoretical Mass	Error (ppb)	Major Acyl Groups	Other Acyl Groups Observed
915.60175	C ₄₇ H ₈₈ O ₁₅ Na ⁺	915.60154	226	16:0/16:0	18:0/14:0; 20:0/12:0
937.58558	C ₄₉ H ₈₆ O ₁₅ Na ⁺	937.58589	-334	18:2/16:1	18:1/16:2; 18:3/16:0; tr 20:1/14:2
939.60169	C ₄₉ H ₈₈ O ₁₅ Na ⁺	939.60154	156	18:1/16:1	18:2/16:0; tr 20:1/14:1
941.61742	C ₄₉ H ₉₀ O ₁₅ Na ⁺	941.61719	241	16:0/18:1	tr 18:0/16:1; tr 20:1/14:0
943.63307	C ₄₉ H ₉₂ O ₁₅ Na ⁺	943.63284	240	18:0/16:0	tr 20:0/14:0
965.61727	C ₅₁ H ₉₀ O ₁₅ Na ⁺	965.61719	79	18:2/18:1	tr 20:2/16:1; tr 20:1/16:2; tr 20:3/16:0
967.63299	C ₅₁ H ₉₂ O ₁₅ Na ⁺	967.63284	152	18:1/18:1	20:1/16:1; 20:2/16:0
969.64873	C ₅₁ H ₉₄ O ₁₅ Na ⁺	969.64849	244	20:1/16:0	18:0/18:1
971.66462	C ₅₁ H ₉₆ O ₁₅ Na ⁺	971.66414	490	20:0/16:0	18:0/18:0
995.66403	C ₅₃ H ₉₆ O ₁₅ Na ⁺	995.66414	-114	20:1/18:1	
997.67985	C ₅₃ H ₉₈ O ₁₅ Na ⁺	997.67979	57	18:0/20:1	12:0/18:1; tr 22:1/16:0
1023.69565	C ₅₅ H ₁₀₀ O ₁₅ Na ⁺	1023.69544	202	20:1/20:1	22:1/18:1
1025.71028	C ₅₅ H ₁₀₂ O ₁₅ Na ⁺	1025.71109	-793	20:0/20:1	
Diglycosylmonoacylglycerol (DGMAG)					
591.26227	C ₂₅ H ₄₄ O ₁₄ Na ⁺	591.26233	-97	10:1	
593.27811	C ₂₅ H ₄₆ O ₁₄ Na ⁺	593.27798	224	10:0	
617.27783	C ₂₇ H ₄₆ O ₁₄ Na ⁺	617.27798	-239	12:2	
619.29368	C ₂₇ H ₄₈ O ₁₄ Na ⁺	619.29363	85	12:1	
621.30938	C ₂₇ H ₅₀ O ₁₄ Na ⁺	621.30928	165	12:0	
645.30925	C ₂₉ H ₅₀ O ₁₄ Na ⁺	645.30928	-42	14:2	
647.32504	C ₂₉ H ₅₂ O ₁₄ Na ⁺	647.32493	174	14:1	
649.34067	C ₂₉ H ₅₄ O ₁₄ Na ⁺	649.34058	142	14:0	
673	C ₃₁ H ₅₄ O ₁₄ Na ⁺	673.34058	too low	16:2	
675.35639	C ₃₁ H ₅₆ O ₁₄ Na ⁺	675.35623	240	16:1	
677.37200	C ₃₁ H ₅₈ O ₁₄ Na ⁺	677.37188	181	16:0	
699.35618	C ₃₃ H ₅₆ O ₁₄ Na ⁺	699.35623	-68	18:3	
701.37198	C ₃₃ H ₅₈ O ₁₄ Na ⁺	701.37188	146	18:2	

Observed Mass	Elemental Composition	Theoretical Mass	Error (ppb)	Major Acyl Groups	Other Acyl Groups Observed
703.38770	C ₃₃ H ₆₀ O ₁₄ Na ⁺	703.38753	245	18:1	
705.40329	C ₃₃ H ₆₂ O ₁₄ Na ⁺	705.40318	159	18:0	
729.40326	C ₃₃ H ₆₂ O ₁₄ Na ⁺	729.40318	113	20:2	
731.41896	C ₃₃ H ₆₄ O ₁₄ Na ⁺	731.41883	181	20:1	
733.43464	C ₃₃ H ₆₆ O ₁₄ Na ⁺	733.43448	221	20:0	
Glycerophosphoryldiacylglycerol (GPDGDAG) - Positive Ions					
1007.49305	C ₄₄ H ₈₂ O ₂₀ PNa ₂ ⁺	1007.49270	350	10:0/16:0	12:0/14:0
1033.50874	C ₄₆ H ₈₄ O ₂₀ PNa ₂ ⁺	1033.50835	380	16:1/12:0	12:1/16:0
1035.52432	C ₄₆ H ₈₆ O ₂₀ PNa ₂ ⁺	1035.52400	312	16:0/12:0	14:0/14:0
1059.52420	C ₄₈ H ₈₆ O ₂₀ PNa ₂ ⁺	1059.52400	191	16:1/14:1	16:0/14:2; 12:1/18:1; 14:0/16:2; 12:0/18:2
1061.54007	C ₄₈ H ₈₈ O ₂₀ PNa ₂ ⁺	1061.53965	398	16:0/14:1; 16:1/14:0	18:1/12:0; 12:1/18:0
1063.55561	C ₄₈ H ₉₀ O ₂₀ PNa ₂ ⁺	1063.55530	294	16:0/14:0	18:0/12:0
1087.55576	C ₅₀ H ₉₀ O ₂₀ PNa ₂ ⁺	1087.55530	425	16:1/16:1	18:1/14:1; 16:0/16:2; 18:2/14:0; tr 20:1/12:1
1089.57131	C ₅₀ H ₉₂ O ₂₀ PNa ₂ ⁺	1089.57095	333	16:0/16:1	18:1/14:0; tr 18:0/14:1
1091.58693	C ₅₀ H ₉₄ O ₂₀ PNa ₂ ⁺	1091.58660	304	16:0/16:0	18:1/4:0
1115.58701	C ₅₂ H ₉₄ O ₂₀ PNa ₂ ⁺	1115.58660	370	18:1/16:1	18:2/16:0; tr 20:1/14:1
1117.60254	C ₅₂ H ₉₆ O ₂₀ PNa ₂ ⁺	1117.60225	261	18:1/16:0	18:0/16:1; 20:1/14:0
1119.61832	C ₅₂ H ₉₈ O ₂₀ PNa ₂ ⁺	1119.61790	377	18:0/16:0	20:0/14:0; tr 12:0/22:0
1143.61841	C ₅₄ H ₉₈ O ₂₀ PNa ₂ ⁺	1143.61790	448	18:1/18:1	20:1/16:1; 20:2/16:0
1145.63396	C ₅₄ H ₁₀₀ O ₂₀ PNa ₂ ⁺	1145.63355	360	20:1/16:0	18:0/18:1
1147.64967	C ₅₄ H ₁₀₂ O ₂₀ PNa ₂ ⁺	1147.64920	411	20:0/16:0	18:0/18:0; 22:0/14:0; tr 24:0/12:0
1169.63404	C ₅₆ H ₁₀₀ O ₂₀ PNa ₂ ⁺	1169.63355	421	20:2/18:1	22:3/16:0; 18:2/20:1
1171.64970	C ₅₆ H ₁₀₂ O ₂₀ PNa ₂ ⁺	1171.64920	429	20:1/18:1	tr 22:2/16:0
1173.66537	C ₅₆ H ₁₀₄ O ₂₀ PNa ₂ ⁺	1173.66485	445	20:0/18:1; 18:0/20:1	22:0/16:1; 22:1/16:0
Glycerophosphoryldiacylglycerol (GPDGDAG) - Negative Ions					
961.51400	C ₄₄ H ₈₂ O ₂₀ P ⁻	961.51426	-265	10:0/16:0	
987.52973	C ₄₆ H ₈₄ O ₂₀ P ⁻	987.52991	-177	16:1/12:0; 16:0/12:1	
989.54529	C ₄₆ H ₈₆ O ₂₀ P ⁻	989.54556	-268	16:0/12:0	tr 14:0/14:0

Observed Mass	Elemental Composition	Theoretical Mass	Error (ppb)	Major Acyl Groups	Other Acyl Groups Observed
1013.54540	C ₄₈ H ₈₆ O ₂₀ P ⁻	1013.54556	-153	16:1/14:1	16:0/14:2
1015.56099	C ₄₈ H ₈₈ O ₂₀ P ⁻	1015.56121	-212	16:1.14:0; 16:0/14:1	18:1/12:0
1017.57669	C ₄₈ H ₉₀ O ₂₀ P ⁻	1017.57686	-163	16:0/14:0	18:0/12:0
1041.57658	C ₅₀ H ₉₆ O ₂₀ P ⁻	1041.57686	-264	16:1/16:1	18:1/14:1
1043.59243	C ₅₀ H ₉₂ O ₂₀ P ⁻	1043.59251	-72	16:0/16:1	18:1/14:0; tr 18:0/14:1
1045.60802	C ₅₀ H ₉₄ O ₂₀ P ⁻	1045.60816	-130	16:0/16:0	18:0/14:0
1069.60795	C ₅₂ H ₉₄ O ₂₀ P ⁻	1069.60816	-192	18:1/16:1	18:2/16:0; tr 20:1/14:1
1071.62350	C ₅₂ H ₉₆ O ₂₀ P ⁻	1071.62381	-285	18:1/16:0	18:0/16:1; tr 20:1/14:0
1073.63930	C ₅₂ H ₉₈ O ₂₀ P ⁻	1073.63946	-145	18:0/16:0	20:0/14:0
1097.63935	C ₅₄ H ₉₈ O ₂₀ P ⁻	1097.63946	-96	18:1/18:1	20:1/16:1; 20:2/16:0
1099.65500	C ₅₄ H ₁₀₀ O ₂₀ P ⁻	1099.65511	-96	20:1/16:0	18:0/18:1
1101.67103	C ₅₄ H ₁₀₂ O ₂₀ P ⁻	1101.67076	249	20:0/16:0	18:0/18:0
1123.65492	C ₅₆ H ₁₀₀ O ₂₀ P ⁻	1123.65511	-165	too low ^a	
1125.67051	C ₅₆ H ₁₀₂ O ₂₀ P ⁻	1125.67076	-218	20:1/18:1	tr 22:2/16:0
1127.68625	C ₅₆ H ₁₀₄ O ₂₀ P ⁻	1127.68641	-138	18:0/20:1; 20:0/18:1	tr 22:1/16:0; tr 16:1/22:0

^aThe ion was too low to be observed in the FT-ICR and determine an accurate mass and the mass error (error relative to the theoretical mass).

Table 2

Product ions of glycolipids from *Streptococcus mutans* UA159. Structures are shown in Figure 11.

Product Ion	Relative Abundance	Accurate Mass	Elemental Composition	Error (ppb)	Description of loss
MGDAG MS²: C₄₃H₈₀O₁₀Na⁺ (779)					
523	100	523.32427	C ₂₇ H ₄₈ O ₈ Na ⁺	248	16:0 acid
497	75.1	497.30862	C ₂₅ H ₄₆ O ₈ Na ⁺	261	18:1 acid
617	9.2	617.51172	C ₃₇ H ₇₀ O ₃ Na ⁺	280	sugar (C ₆ H ₁₀ O ₅)
495	8.9	495.29293	C ₂₅ H ₄₄ O ₈ Na ⁺	182	18:0 acid
525	4.0	525.33998	C ₂₇ H ₅₀ O ₈ Na ⁺	361	16:1 acid
469	1.8	469.27721	C ₂₃ H ₄₂ O ₈ Na ⁺	43	20:1 acid
551	1.1	551.35508	C ₂₉ H ₅₂ O ₈ Na ⁺	-654	14:0 acid (buried)
DGDAG MS²: C₄₉H₉₀O₁₅Na⁺ (941)					
685	100	685.37729	C ₃₃ H ₅₈ O ₁₃ Na ⁺	476	16:0 acid
659	88.7	659.36160	C ₃₁ H ₅₆ O ₁₃ Na ⁺	434	18:1 acid
779	22.6	779.56477	C ₄₃ G ₈₀ O ₁₀ Na ⁺	512	sugar (C ₆ H ₁₀ O ₅)
657	8.6	657.34586	C ₃₁ H ₅₄ O ₁₃ Na ⁺	299	18:0 acid
523	6.2	523.32439	C ₂₇ H ₄₈ O ₈ Na ⁺	477	16:0 acid & sugar
497	5.5	497.30878	C ₂₅ H ₄₆ O ₈ Na ⁺	583	18:1 acid & sugar
687	4.1	687.39295	C ₃₃ H ₆₀ O ₁₃ Na ⁺	489	16:1 acid
631	1.9	631.33033	C ₂₉ H ₅₂ O ₁₃ Na ⁺	501	20:1 acid
713	1.0	713.40886	C ₃₅ H ₆₂ O ₁₃ Na ⁺	836	14:0 acid
495	0.6	495.29242	C ₂₅ H ₄₄ O ₈ Na ⁺	-848	18:0 acid & sugar
525	0.3	too low ^d	too low	too low	16:1 acid & sugar
617	0.3	too low	too low	too low	both sugars
551	0.2	too low	too low	too low	14:0 acid & sugar
469	0.1	too low	too low	too low	20:1 acid & sugar
DGMAG MS²: C₃₁H₅₈O₁₄Na⁺ (677 - contains 16:0)					
515	100	515.31902	C ₂₅ H ₄₈ O ₉ Na ⁺	-68	sugar (C ₆ H ₁₀ O ₅)

Product Ion	Relative Abundance	Accurate Mass	Elemental Composition	Error (ppb)	Description of loss
353	1.8	353.26610	C ₁₉ H ₃₈ O ₄ Na ⁺	-372	both sugars
659	0.8	too low	too low	too low	water
421	0.6	too low	too low	too low	16:0 acid
347	0.6	too low	too low	too low	B ₂ - H sugar ion ^b
365	0.3	too low	too low	too low	C ₂ + H sugar ion ^b
259	0.2	too low	too low	too low	sugar & 16:acid
DGMAG MS ³ : C ₂₅ H ₄₈ O ₉ Na ⁺ (515 from 677 - contains 16:0)					
353	100	353.26617	C ₁₉ H ₃₈ O ₄ Na ⁺	-173	sugar (C ₆ H ₁₀ O ₅)
352	4.6	too low	too low	too low	sugar w/o H-transfer
497	2.4	too low	too low	too low	water
185	1.7	too low	too low	too low	monoacylglycerol
259	1.7	too low	too low	too low	16:0 acid
DGMAG MS ² : C ₃₃ H ₆₀ O ₁₄ Na ⁺ (703 - contains 18:1)					
541	100.0	541.33435	C ₂₇ H ₅₀ O ₉ Na ⁺	-656	sugar (C ₆ H ₁₀ O ₅)
379	2.0	379.28181	C ₂₁ H ₄₀ O ₄ Na ⁺	-188	both sugars
685	0.8	too low	too low	too low	water
365	0.6	too low	too low	too low	C ² + H sugar ion ^b
347	0.5	too low	too low	too low	B ₂ - H sugar ion ^b
421	0.3	too low	too low	too low	18:1 acid
259	0.1	too low	too low	too low	sugar & 18:1 acid
DGMAG MS ³ : C ₂₇ H ₅₀ O ₉ Na ⁺ (541 from 703 - contains 18:1)					
379	100.0	379.28179	C ₂₁ H ₄₀ O ₄ Na ⁺	-241	sugar (C ₆ H ₁₀ O ₅)
523	1.5	too low	too low	too low	water
185	0.9	too low	too low	too low	monoacylglycerol
378	0.6	too low	too low	too low	sugar w/o H-transfer
259	0.5	too low	too low	too low	18:1 acid
GPDGDAG MS ² : C ₅₂ H ₉₆ O ₂₀ PNa ₂ ⁺ (1117)					
941	100	941.61733	C ₄₉ H ₉₉ O ₁₅ Na ⁺	144	C ₃ H ₆ O ₃ PNa
923	4.7	923.60710	C ₄₉ H ₈₈ O ₁₄ Na ⁺	509	C ₃ H ₈ O ₆ PNa

Product Ion	Relative Abundance	Accurate Mass	Elemental Composition	Error (ppb)	Description of loss
835	4.4	835.34687	C ₃₄ H ₆₂ O ₁₈ PNa ₂ ⁺	598	18:1 acid
861	4.3	861.36250	C ₃₆ H ₆₄ O ₁₈ PNa ₂ ⁺	557	16:0 acid
659	4.2	659.36158	C ₃₁ H ₅₆ O ₁₃ Na ⁺	404	C ₃ H ₆ O ₅ PNa & 18:1 acid
685	3.9	685.37725	C ₃₃ H ₅₈ O ₁₃ Na ⁺	418	C ₃ H ₆ O ₅ PNa & 16:0 acid
1025	0.9	1025.55553	C ₄₉ H ₈₈ O ₁₇ PNa ₂ ⁺	608	C ₃ H ₈ O ₃
1043	0.8	1043.56605	C ₄₉ H ₉₀ O ₁₈ PNa ₂ ⁺	555	C ₃ H ₆ O ₂
833	0.4	833.33123	C ₃₄ H ₆₀ O ₁₈ PNa ₂ ⁺	612	18:0 acid
657	0.4	657.34599	C ₃₁ H ₅₄ O ₁₃ Na ⁺	497	C ₃ H ₆ O ₅ PNa & 18:0 acid
863	0.1	too low	too low	too low	16:1 acid
807	0.1	too low	too low	too low	20:1 acid
889	0.1	too low	too low	too low	14:0 acid
687	0.1	too low	too low	too low	C ₃ H ₆ O ₅ PNa & 16:1 acid
631	0.1	too low	too low	too low	C ₃ H ₆ O ₅ PNa & 20:1 acid
731	0.0	too low	too low	too low	C ₃ H ₆ O ₅ PNa & 14:0 acid
GPDGDAG MS³: C₄₉H₉₀O₁₅Na⁺ (941 from 1117)					
659	100	659.36108	C ₃₁ H ₅₆ O ₁₃ Na ⁺	-354	18:1 acid
685	97.3	685.37672	C ₃₃ H ₅₈ O ₁₃ Na ⁺	-356	16:0 acid
779	25.5	779.56410	C ₄₃ H ₈₀ O ₁₀ Na ⁺	-347	sugar (C ₆ H ₁₀ O ₅)
657	8.1	657.34544	C ₃₁ H ₅₄ O ₁₃ Na ⁺	-340	18:0 acid
497	6.4	497.30843	C ₂₅ H ₄₆ O ₈ Na ⁺	-121	18:1 acid & sugar
523	6.1	523.32405	C ₂₇ H ₄₈ (O) ₈ Na ⁺	-172	16:0 acid & sugar
687	3.6	687.39225	C ₃₃ H ₆₀ O ₁₃ Na ⁺	-529	16:1 acid
631	2.5	631.32999	C ₂₉ H ₅₂ O ₁₃ Na ⁺	-37	20:1 acid
713	1.2	713.40787	C ₃₅ H ₆₂ O ₁₃ Na ⁺	-552	14:0 acid
495	0.6	too low	too low	too low	18:0 acid & sugar
617	trace - buried	617.51114	C ₃₇ H ₇₀ O ₄ Na ⁺	-659	both sugars
525	trace	too low	too low	too low	16:1 acid & sugar
469	trace	too low	too low	too low	20:1 acid & sugar

Product Ion	Relative Abundance	Accurate Mass	Elemental Composition	Error (ppb)	Description of loss
551	trace	too low	too low	too low	14:0 acid & sugar
GPDGDAG MS²: C₅₂H₉₆O₃₀P⁺ (1071)					
997	100	997.58818	C ₄₉ H ₉₀ O ₁₈ P ⁺	1154	C ₃ H ₆ O ₂
789	64.3	789.36863	C ₃₄ H ₆₂ O ₁₈ P ⁺	890	18:1 acid
815	63.7	815.38418	C ₃₆ H ₆₄ O ₁₈ P ⁺	739	16:0 acid
833	32.9	833.39480	C ₃₆ H ₆₆ O ₁₉ P ⁺	789	16:0 ketene
807	32.3	807.37925	C ₃₄ H ₆₄ O ₁₉ P ⁺	938	18:1 ketene
315	21.3	315.04884	C ₉ H ₁₆ O ₁₀ P ⁺	575	glucosyldiacylglycerol
979	6.4	979.57732	C ₄₉ H ₈₈ O ₁₇ P ⁺	874	C ₃ H ₈ O ₃
787	5.4	787.35271	C ₃₄ H ₆₀ O ₁₈ P ⁺	549	18:0 acid
459	4.4	459.09132	C ₁₅ H ₂₄ O ₁₄ P ⁺	876	C ₃ H ₆ O ₂ & both acids
715	4.4	715.33182	C ₃₁ H ₅₆ O ₁₆ P ⁺	939	C ₃ H ₆ O ₂ & 18:1 acid
741	4.2	741.34738	C ₃₃ H ₅₈ O ₁₆ P ⁺	785	C ₃ H ₆ O ₂ & 16:0 acid
477	3.6	477.10124	C ₁₅ H ₂₈ O ₁₅ P ⁺	-508	C ₃ H ₆ O ₂ & acid & ketene
759	2.8	759.35756	C ₃₃ H ₆₀ O ₁₇ P ⁺	260	C ₃ H ₆ O ₂ & 16:0 ketene
733	2.5	733.34181	C ₃₁ H ₅₈ O ₁₇ P ⁺	133	C ₃ H ₆ O ₂ & 18:1 ketene
817	2.2	too low	too low	too low	16:1 acid
835	2.0	too low	too low	too low	16:1 ketene
533	1.8	533.12745	C ₁₈ H ₃₀ O ₁₆ P ⁺	-464	both acids
805	1.6	805.36302	C ₃₄ H ₆₂ O ₁₉ P ⁺	221	18:0 ketene
761	1.4	too low	too low	too low	20:1 acid
551	0.8	too low	too low	too low	acid & ketene
843	0.7	too low	too low	too low	14:0 acid
861	0.6	too low	too low	too low	14:0 ketene
495	0.4	too low	too low	too low	C ₃ H ₆ O ₂ & both ketenes
779	0.4	too low	too low	too low	20:1 ketene
713	0.3	too low	too low	too low	C ₃ H ₆ O ₂ & 18:0 acid
743	trace	too low	too low	too low	C ₃ H ₆ O ₂ & 16:1 acid
687	trace	too low	too low	too low	C ₃ H ₆ O ₂ & 20:1 acid

Product Ion	Relative Abundance	Accurate Mass	Elemental Composition	Error (ppb)	Description of loss
769	trace	too low	too low	too low	C ₃ H ₆ O ₂ & 14:0 acid
731	trace	too low	too low	too low	C ₃ H ₆ O ₂ & 18:0 ketene
705	trace	too low	too low	too low	C ₃ H ₆ O ₂ & 20:1 ketene

^a too low: the product ion from collision induced dissociation of the parent ion given in bold for each section, was too low to be observed in the FT-ICR and thus determine an accurate mass which would have also generated an elemental composition and mass error value.

^b Not losses but sodiated glycoconjugates according to the nomenclature designated by Domon and Costello [14].

Table 3

¹H-NMR analysis of *S. mutans* UA159 glycolipids. See figure 11 for structures and designation of positions.

Position	¹ H-NMR chemical shifts	Hauksson et al. [13]
Monoglucosyldiacylglycerol (MGDAG)		
Glucose, 1	4.86 ppm (d, 3.6 Hz)	
Glucose, 2	3.52 ppm (dd, 3.6, 9.6 Hz)	
Glucose, 3	3.76 ppm (t, 9.4 Hz)	
Glycerol, 1	4.11 ppm (dd, 5.8, 11.6 Hz)	
Glycerol, 1'	4.37 ppm (dd, 3.9, 11.6 Hz)	
Glycerol, 2	5.23 ppm (m)	
Glycerol, 3	3.84 ppm*	
Glycerol, 3'	3.61 ppm*	
Diglucosyldiacylglycerol (DGDAG)		
Glucose A, 1	4.81 ppm (d, 3.5 Hz)	
Glucose A, 2	3.41 ppm*	
Glucose B, 1	4.78 ppm (d, 3.7 Hz)	
Glucose B, 2	3.27 ppm (dd, 3.6, 9.5 Hz)	
Glycerol, 1	4.26 ppm (dd, 3.2, 12.0 Hz)	
Glycerol, 1'	4.02 ppm (dd, 6.5, 12.0 Hz)	
Glycerol, 2	5.05 (m)	
Glycerol, 3	3.67 ppm*	
Glycerol, 3'	3.47 ppm (dd, 5.2, 10.9 Hz)	
Diglucosylmonoacylglycerol (DGMAG)		
Glucose A, 1	4.92 ppm (d, 3.4 Hz)	
Glucose A, 2	3.35 ppm*	
Glucose B, 1	4.83 ppm (d, 3.6 Hz)	
Glucose B, 2	3.18 ppm (dd, 3.6, 9.5 Hz)	
Glycerol, 1	3.96 ppm*	
Glycerol, 1'	4.03 ppm*	
Glycerol, 2	3.85 ppm*	
Glycerol, 3	3.34 ppm*	
Glycerol, 3'	3.57 ppm*	
Glycerophosphoryldiglucosyldiacylglycerol (GPDGDAG)		
Glucose A, 1	4.92 ppm (d, 3.7 Hz)	4.93 ppm
Glucose A, 2	3.36 ppm*	3.38 ppm
Glucose B, 1	4.82 ppm (d, 4.2 Hz)	4.84 ppm
Glucose B, 2	3.20 ppm*	3.23 ppm
Glycerol A, 1	4.35 ppm*	4.39 ppm
Glycerol A, 1'	4.16 ppm*	4.15 ppm
Glycerol A, 2	5.11 ppm*	5.14 ppm
Glycerol A, 3	3.57 ppm*	3.59 ppm
Glycerol A, 3'	3.70 ppm*	3.71 ppm

Position	¹ H-NMR chemical shifts	Hauksson et al. [13]
Glycerol B, 1, 1'	3.28 ppm*	3.31 ppm
Glycerol B, 2	3.49 ppm*	3.52 ppm
Glycerol B, 3, 3'	3.65 ppm*	3.67 ppm

**
Chemical shifts determined by COSY.