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The cellular lipids of *Romboutsia*

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Abstract

We have examined the lipids of three isolates, *Romboutsia lituseburensis, Romboutsia ilealis*, and *Romboutsia* sp. strain FRIFI, of the newly described genus *Romboutsia* by two-dimensional thin-layer chromatography (2D-TLC) and by liquid chromatography/mass spectrometry (LC/MS). We have found three phospholipids, phosphatidylglycerol (PG), cardiolipin and phosphatidic acid in all three species. A fourth phospholipid, lysyl-PG, was found in *R. lituseburensis* and strain FRIFI. Polyprenyl-phosphates were identified in the lipid extracts of all three species. Three glycolipids, mono-, di- and tri-hexosyldiacylglycerol, were common to all three species. An additional glycolipid, tetrahexosyl-diacylglycerol was identified in strain FRIFI. Acylated trihexosyldiacylglycerol and acyl-tetrahexosydiacylglycerol were also found in *R. ilealis* and strain FRIFI. Remarkably, no alk-1-enyl ether lipids (plasmalogens) were present in *Romboutsia* as distinct from bacteria of the related genus *Clostridium* in which these ether lipids are common. We have compared the lipidome of *Romboutsia* with that recently described for *Clostridium difficile*, which has plasmalogens, no lysyl-PG, and no tetrahexosyl-diacylglycerol. According to 16S rRNA gene sequencing, *Romboutsia* spp. and *Cosmarium difficile* are closely related (>95% sequence identity).

Keywords

Romboutsia; Glycolipid; Mass spectrometry; Phospholipid; Thin-layer chromatography

1. Introduction

A Gram-positive, rod-shaped, non-motile, spore-forming obligately anaerobic bacterium isolated from the rat gastro-intestinal tract was recently characterized and classified in a new genus, *Romboutsia*. On the basis of 16S rRNA gene sequencing and its low G + C content, 28.1 mol%, the new strain, named *Romboutsia ilealis*, was considered to be closely related to several species of the genus *Clostridium*, including *Clostridium lituseburense* (97.2%), *Clostridium glycolicum* (96.2%), *Clostridium irregulare* (95.5%) and two other species of clostridia. It was therefore decided to transfer *C. lituseburense* to this new genus and to

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reclassify *C. glycolicum* and *C. irregulare* into new genera [1]. Furthermore, strain FRIFI was recently isolated from human ileostoma effluent and was shown to represent a new species within the genus *Romboutsia* [2]. As part of this study, the polar lipids were examined by two-dimensional thin-layer chromatography (2D-TLC) with appropriate staining. Two of the major phospholipids were tentatively identified as cardiolipin (diphosphatidylglycerol) and phosphatidylglycerol (PG). Several other phospholipids and a number of glycolipids were detected, but not identified. It was noted that unlike species of *Clostridium* cluster I previously studied [3–9], there was no phosphatidylethanolamine. Since we have recently described the polar lipids of *C. difficile* which is related to the newly reclassified *R. lituseburensis* [10], we have carried out a 2D-TLC and mass spectrometric lipidomic analysis of three species in this new genus. We have identified PG, cardiolipin (CL) and phosphatidic acid (PA) in all three species. In addition we have identified lysyl-PG in two of the three species, *Romboutsia* sp. strain FRIFI and *R. lituseburensis*, but not in *R. ilealis*. Monohexosyldiacylglycerol (MHDAG), dihexosyldiacylglcyerol (DHDAG) and trihexosyldiacylglycerol (TriHDAG) were observed in all three species. Strain FRIFI contains a tetrahexosyldiacylglycerol (TetraHDAG) in addition to the other three glycosyldiacylglycerols. Acylated trihexosyldiacylglycerol and acyl-tetrahexosyldiacylglycerol were also identified in *R. ilealis* and strain FRIFI. Mass spectrometry revealed the presence of decaprenyl and/or nonaprenyl (C_{50} and C_{45})-P in all three species.

2. Methods

2.1. Bacterial strains and growth conditions

R. lituseburensis DSM 797^T, *R. ilealis* CRIB^T (DSM 25109) and *Romboutsia* sp. strain FRIFI (DSM 28814) were grown in liquid DSM medium 104b at 37 °C. This medium consisted of (per litre distilled water): 5 g trypticase peptone, 5 g peptone from meat (pepsin-digested), 10 g yeast extract, 5 glucose, 1 mg resazurin, 40 ml salt solution [11] and 0.5 g L-cysteine hydrochloride (pH 7). Cells were harvested in mid-exponential to end-exponential phase and lyophilized.

2.2. Lipid extraction and thin-layer chromatography (TLC)

Total lipids were extracted from the lyophilized cells after addition of 0.5 to 1.0 ml of water using chloroform-methanol [12] with minor modifications [13]. Two-dimensional TLC was carried out on silica gel 60, 10 × 10 cm, thin-layer plates using the following solvent systems: System A, chloroform/methanol/concentrated ammonia/water, 65:30:2.5:2.5 (by vol.) in the first dimension and System B, chloroform/methanol/acetic acid/water, 80:18:12:5 (by vol.) in the second dimension. Amine-containing lipids were detected with 0.3% ninhydrin in ethanol, followed by heating at 120 °C for 10 min. On the same plates, phospholipids were detected with 0.3% (w/v) molybdenum blue. After recording the results at each step, the lipids were charred by heating at 120 °C for 15 min. On separate plates, glycolipids were detected by α-naphthol staining [14]. All reagents were obtained from Sigma-Aldrich, St. Louis, MO.

Preparative TLC of strain FRIFI total lipids was performed on a 10 × 10 cm silica gel 60 TLC plate. The lipids were chromatographed in solvent system A and all but the left and right edges was scraped in 1 cm bands which were eluted as described above for extraction of cellular lipids. The remaining left edge was stained for glycolipids with α-naphthol and the remaining right edge was stained for phospholipids with molybdenum blue reagent and charred.

2.3. Liquid chromatography/tandem mass spectrometry (LC/ESI–MS/MS)

Methods for LC/ESI–MS/MS have been described [3,15]. Briefly, normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μm, 25 cm × 2.1 mm (Sigma-Aldrich, St. Louis, MO) as described. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Applied Biosystems, Foster City, CA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: IS = −4500 V; CUR = 20 psi; GSI = 20 psi; DP = −55 V; and FP = −150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Applied Biosystems, Foster City, CA).

3. Results

3.1. Identification of diacylglycerol and phospholipids

Diacylglycerol was identified by LC/MS in all three species of *Romboutsia* (Tables 1 and 2). As described previously, 2DTLC revealed the presence of several phospholipids and glycolipids in *R. ilealis* and *R. lituseburensis*, but some differences between species were noted [1]. We have now analyzed the polar lipids of three species, *R. lituseburensis, R. ilealis* and strain FRIFI, by 2D-TLC using solvent systems that differ from those used in the previous study. PG and CL were identified in all three species (Fig. 1) and confirmed by LC/MS (Table 2). A major ninhydrin and molybdate-positive spot was seen in *R. lituseburensis* and strain FRIFI, but not in *R. ilealis*. Its position relative to other lipids corresponds to that of lysyl-PG (Fig. 1) [7]. The presence of lysyl-PG was confirmed by LC/ESIMS and by MS/MS (Fig. 2). As found by Gerritsen et al. [1], no phosphatidylethanolamine is present in any of the three species. Fig. 1 also shows a spot (PL5) corresponding to a lipid that was previously identified as PA [7]. The presence of PA was confirmed by mass spectrometry of strain FRIFI and *R. ilealis* lipids (Table 2). Mass spectrometry revealed the presence of decaprenyl-P (C_{50}-P) or nonaprenyl-P (C_{45}-P) in all three species. The latter was predominant in *R. lituseburensis*. A representative spectrum of the lipids from strain FRIFI is shown in Fig. 3.

3.2. Identification of glycolipids

Three glycolipids, mono-, di- and tri-hexosyldiacylglycerol, were common to all three species of *Romboutsia* (Fig. 1, GL1, GL2 and GL4). All three species had another glycolipid designated as GL3 (Fig. 1), with only a faint hint of this lipid in *R. lituseburensis* visible after α-naphthol staining. Preparative TLC of strain FRIFI lipids followed by LC/ESI–MS revealed the presence of an acyl-TriHDAG in the band corresponding to GL3 (Fig. 4). After preparative TLC a tetrahexosyldiacylglycerol (TetraHDAG) was identified by accurate mass

measurements and MS/MS (Fig. 5) in the fraction corresponding to GL6 in Fig. 1C. Lastly, a glycolipid isolated by preparative TLC in the band corresponding to GL5 (Fig. 1C) was identified as an acylated (primarily C16:0) derivative of TetraHDAG (Fig. 6) by accurate mass measurement. Some unacylated TetraHDAG also appears to be present in *R. ilealis*. The major molecular species of *Romboutsia* glycolipids are given in Table 3.

4. Discussion

The lipids of currently known members of the new genus *Romboutsia* differ from those of most clostridia in the absence of plasmalogens and of phosphatidylethanolamine. Like clostridia, *Romboutsia* spp. have low DNA G + C content (27–28.1 mol%) [1]. *R. lituseburensis*, previously *C. lituseburense*, was placed in *Clostridium* Cluster XI and seen to be closely related to *C. difficile* by 16S rRNA gene sequencing [10]. Lawson and Rainey have recently proposed to restrict the genus *Clostridium* to *Clostridium butyricum* and related species in cluster I [17]. Our recent work showed that *C. difficile* has several phospholipids and glycolipids most of which were present as both diacyl and plasmalogen species [3]. These include PG, CL, MHDAG, DHDAG and TriHDAG. Previous 2D-TLC studies on the lipids of *R. lituseburensis* and *R. ilealis* showed the presence of a number of phospholipids, two of which were tentatively identified as PG and CL, and a number of unidentified glycolipids. In order to shed more light on the lipidome of *Romboutsia*, we undertook a 2D-TLC and ESI/MS examination of the lipids of three species. Our work confirms the presence of three phospholipids, PG, CL and PA in *R. ilealis*. An additional phospholipid, lysyl-PG, was identified in *R. lituseburensis* and strain FRIFI. Decaprenyl and/or nonaprenyl (C50 and C45)-P are detected in all three species. In comparison, undecaprenyl (C55)-P is found in *Escherichia coli* and Francisella novicida [18].

Three glycolipids, MHDAG, DHDAG and TriHDAG, were identified in all three species by LC/ESI–MS, and a fourth, TetraHDAG, was identified in strain FRIFI. A fifth glycolipid (Fig. 1, GL3) was observed in all three species by 2D-TLC and was identified in strain FRIFI, by preparative TLC followed by LC/ESI–MS as an acylated TriHDAG. A sixth glycolipid observed in both strain FRIFI and *R. ilealis*, was similarly identified as an acylated TetraHDAG. We have not studied the linkages of the acyl chains and the sugars in these glycolipids since that would require isolation of each lipid from large amounts of cells of each species followed by NMR and chemically-based linkage studies. Mass spectrometry alone, cannot determine these linkages.

Lysyl-PG has previously been identified in a variety of Gram-positive bacteria including aerobic and anaerobic species. Among clostridia containing this lipid are *Clostridium novyi* [7] and two strains of *Clostridium botulinum* Group III [6]. One of these strains, D1873, like *C. novyi*, also contains alanyl-PG. *Clostridium perfringens* also has both lysyl-PG and alanyl-PG [19,20]. The formation of amino-acyl PG is catalyzed by amino-acyl-PG synthetase (MprF), an enzyme that catalyzes the transfer of an amino acid from aminoacyl-tRNA to PG [21,22]. In *C. perfringens* two phylogenetically distinct MprF paralogs have been identified; MprF1 responsible for the formation of alanyl-PG and MprF2 responsible for the formation of lysyl-PG [23]. Genes encoding homologs of MprF2 in *C. perfringens* were identified in the genomes of *R. lituseburensis* and strain FRIFI, but not in *R. ilealis*,

supporting the observation that lysyl-PG can be produced by the first two strains, but not the latter. The gene *mprF* (multi peptide resistance factor) confers resistance to cationic peptides. In tissue cultures and in mouse models of infection, *Listeria monocytogenes* mutants defective in *mprF* were found to be attenuated in virulence [24]. In a recent study *mprF* was found also to confer resistance to osmolytes such as urea, sodium chloride and sodium sulfate and to bile salts [25]. Thus, the presence of amino-acyl PG can provide protection from a variety of environmental insults encountered by both pathogenic and non-pathogenic species [26]. *R. lituseburensis* was originally isolated from mud [27], *R. ilealis* was isolated from ileal digesta of rats [1] and strain FRIFI was isolated from human ileostoma effluent [2]. Thus the multiple functions of amino-acyl PG could serve to protect these species in both the soil and within the mammalian intestine. PA is an intermediate in the biosynthesis of phospholipids in bacteria. It is usually either undetectable or present in trace amounts, as appears to be the case in the three species of *Romboutsia* we have investigated.

A large variety of glycolipids has been found in Gram-positive bacteria [16,28]. Recent studies of clostridia have demonstrated that these are major components of the lipidome in *C. difficile* [3], *Clostridiumtetani* [8], *Clostridium sporogenes*, some strains of *C. botulinum* Group I that are related to *C. sporogenes* [6], *Clostridium psychrophilum* [4], and *Clostridium acetobutylicum* [5,29,30]. We note that three of the glycolipids common to *Romboutsia*, MHDAG, DHDAG and TriHDAG, were also found in *C. difficile*. The unusual glycolipids TetraHDAG, and acylated forms of TriHDAG and TetraHDAG found in *Romboutsia*, were not seen in *C. difficile*. Acylated tri- and tetra-hexoses have been found as lipid anchors of the lipoteichoic acid of *Lactobacillus casei* [31] and *Lactobacillus gasseri* [32]. Acyl-TriHDAG and TetraHDAG were previously isolated as free lipids from *L. casei* [33]. Their potential role in the assembly of lipoteichoic acids has been considered [31]. The lipid composition of strain FRIFI has a striking resemblance to that of *L. casei* with the exception of free acyl-tetraHDAG, which was not found in *L. casei*.

It should be noted that our picture of the complex lipidome of *Romboutsia* is only a snapshot. We have only analyzed the lipidome of cells grown in a single medium and harvested at one time. The relative amounts of the lipids will undoubtedly change when cells are grown under different conditions and harvested at different stages of growth. Our lipidomic analysis of *Romboutsia* suggests that the major difference between *C. difficile* and *Romboutsia* is not in the compositions of phospholipids and glycolipids, but rather in the presence (*C. difficile*) or absence (*Romboutsia*) of plasmalogens. So far, all species of *Clostridium* that have been studied contain plasmalogens. These and previous studies on the lipidomes of *Clostridium*, including those species that may be considered to belong to new genera [10], indicate that they can shed light on bacterial taxonomy as an adjunct to the powerful technique of 16S rRNA gene sequencing.

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Abbreviations

CL	cardiolipin
DHDRG	dihexosyldiacylglycerol
MHDRG	monohexosyldiacylglycerol
PG	phosphatidylglycerol
THDRG	trihexosyldiacylglycerol
TetraHDAG	tetrahexosyldiacylglycerol

References

1. Gerritsen J, Fuentes S, Grievink W, van Niftrik L, Tindall BJ, Timmerman HM, Rijkers GT, Smidt H. Characterization of *Romboutsia ilealis* gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus *Clostridium* into the genera *Romboutsia* gen. nov., *Intestinibacter* gen. nov., *Terrisporobacter* gen. nov and *Asaccharospora* gen. nov. Int. J. Syst. Evol. Microbiol. 2014; 64:1600–1616. [PubMed: 24480908]
2. Gerritsen, J. The Genus *Romboutsia* — Genomic and Functional Characterization of Novel Bacteria Dedicated to Life in the Intestinal Tract. Wageningen University; 2015.
3. Guan Z, Katzianer D, Zhu J, Goldfine H. *Clostridium difficile* contains plasmalogen species of phospholipids and glycolipids. Biochim. Biophys. Acta. 2014; 1841:1353–1359. [PubMed: 24983203]
4. Guan Z, Tian B, Perfumo A, Goldfine H. The polar lipids of *Clostridium psychrophilum*, an anaerobic psychrophile. Biochim. Biophys. Acta Mol. Cell Biol. Lipids. 2013; 1831:1108–1112.
5. Tian B, Guan Z, Goldfine H. An ethanolamine-phosphate modified glycolipid in *Clostridium acetobutylicum* that responds to membrane stress. Biochim. Biophys. Acta Mol. Cell Biol. Lipids. 2013; 1831:1185–1190.
6. Guan Z, Johnston NC, Raetz CRH, Johnson EA, Goldfine H. Lipid diversity among botulinum neurotoxin-producing clostridia. Microbiology. 2012; 158:2577–2584. [PubMed: 22837302]
7. Guan Z, Johnston NC, Aygun-Sunar S, Daldal F, Raetz CR, Goldfine H. Structural characterization of the polar lipids of *Clostridium novyi* NT. Further evidence for a novel anaerobic biosynthetic pathway to plasmalogens. Biochim. Biophys. Acta. 1811; 2011:186–193.
8. Johnston NC, Aygun-Sunar S, Guan Z, Ribeiro AA, Daldal F, Raetz CR, Goldfine H. A phosphoethanolamine-modified glycosyl diradylglycerol in the polar lipids of *Clostridium tetani*. J. Lipid Res. 2010; 51:1953–1961. [PubMed: 20173213]
9. Goldfine, H.; Johnston, NC. Membrane lipids of Clostridia. In: Dürre, P., editor. Hand- book on Clostridia. Boca Raton, FL: Taylor & Francis; 2005. p. 297-310.
10. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE. The phylogeny of the genus *Clostridium*; proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 1994; 44:812–826. [PubMed: 7981107]
11. Holdeman, LV.; Cato, EP.; Moore, WEC. Anaerobe Laboratory Manual. Blacksburg, VA: Virginia Polytechnic Institute and State University; 1977.
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. Kates M. Techniques of Lipidology. Isolation, Analysis and Identification of Lipids, Techniques of Lipidology. Isolation Analysis and Identification of Lipids. 1990; 106
14. Siakotos AN, Rouser G. Analytical separation of nonlipid water soluble substances and gangliosides from other lipids by dextran gel column chromatography. J. Am. Chem. Soc. 1965; 42:913–919.

15. Goldfine, H.; Guan, Z. Lipidomic analysis of bacteria by thin layer chromatography and liquid chromatography/mass spectrometry. In: McGenity, TJ.; Timmis, KN.; Nogales-Fernández, B., editors. Hydrocarbon and Lipid Microbiology Protocols. Berlin Heidelberg: Springer Verlag; 2015.
16. Shaw N. Bacterial glycolipids. Bacteriol. Rev. 1970; 34:365–377. [PubMed: 4924864]
17. Lawson PA, Rainey FA. Proposal to restrict the genus *Clostridium* (Prazmowski) to *Clostridium butyricum* and related species. Int. J. Syst. Evol. Microbiol. 2016
18. Guan Z, Eichler J. Liquid chromatography/tandem mass spectrometry of dolichols and polyprenols, lipid sugar carriers across evolution. Biochim. Biophys. Acta. 2011; 1811:800–806. [PubMed: 21570481]
19. Macfarlane MG. Characterization of lipoamino-acids as O-amino-acid esters of phosphatidylglycerol. Nature (London). 1962; 196:136–138.
20. Johnston NC, Baker JK, Goldfine H. Phospholipids of *Clostridium perfringens*: a re- examination. FEMS Microbiol. Lett. 2004; 233:65–68. [PubMed: 15043870]
21. Lennarz WJ, Bonsen PPM, Vandeene LL. Substrate specificity of O-L-Lysylphosphatidylglycerol synthetase. Enzymatic studies on structure of O-L-Lysylphosphatidylglycerol. Biochemistry. 1967; 6 (2307-&).
22. Lennarz WJ, Nesbitt JA, Reiss J. Biosynthesis of Lysyl phosphatidylglycerol. Fed. Proc. 1966; 25 (521-&).
23. Roy H, Ibba M. RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:4667–4672. [PubMed: 18305156]
24. Thedieck K, Hain T, Mohamed W, Tindall BJ, Nimtz M, Chakraborty T, Wehland J, Jansch L. The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. Mol. Microbiol. 2006; 62:1325–1339. [PubMed: 17042784]
25. Roy H, Dare K, Ibba M. Adaptation of the bacterial membrane to changing environments using aminoacylated phospholipids. Mol. Microbiol. 2009; 71:547–550. [PubMed: 19054327]
26. Goldfine H. Charge counter charge. Bacterial response to antimicrobial cationic peptides and more. Virulence. 2014; 5:451–453. [PubMed: 24705072]
27. Prevot AR. Étude des bactéries anaerobes d'AFrique occidentale française (Senégal, Guinée, Côte d'Ivoire). Ann. Inst. Pasteur (Paris). 2015; 74:157–170.
28. Kates, M. Glyco-, phospho-, and sulfoglycoglycerolipids of bacteria. In: Kates, M., editor. Handbook of Lipid Research, Glycolipids, Phosphoglycolipids, and Sulfoglycolipids. Vol. 6. New York: Plenum Press; 1990. p. 1-122.
29. Oulevey J, Bahl H, Thiele OW. Novel alk-1-enyl ether lipids isolated from *Clostridium acetobutylicum*. Arch. Microbiol. 1986; 144:166–168.
30. Johnston NC, Goldfine H. Replacement of the aliphatic chains of *Clostridium acetobutylicum* by exogenous fatty acids: regulation of phospholipid and glycolipid composition. J. Bacteriol. 1992; 174:1848–1853. [PubMed: 1548233]
31. Nakano M, Fischer W. Trihexosyldiacylglycerol and acyltrihexosyldiacylglycerol as lipid anchors of the lipoteichoic acid of *Lactobacillus casei* DSM20021. Hoppe Seylers Z. Physiol. Chem. 1978; 359:1–11. [PubMed: 414986]
32. Shiraishi T, Yokota S, Morita N, Fukiya S, Tomita S, Tanaka N, Okada S, Yokota A. Characterization of a *Lactobacillus gasseri* JCM1131(T) lipoteichoic acidwith a novel glycolipid anchor structure. Appl. Environ. Microbiol. 2013; 79:3315–3318. [PubMed: 23503311]
33. Nakano M, Fischer W. The glycolipids of *Lactobacillus casei* DSM 20021. Hoppe Seylers Z. Physiol. Chem. 1977; 358:1439–1453. [PubMed: 411727]

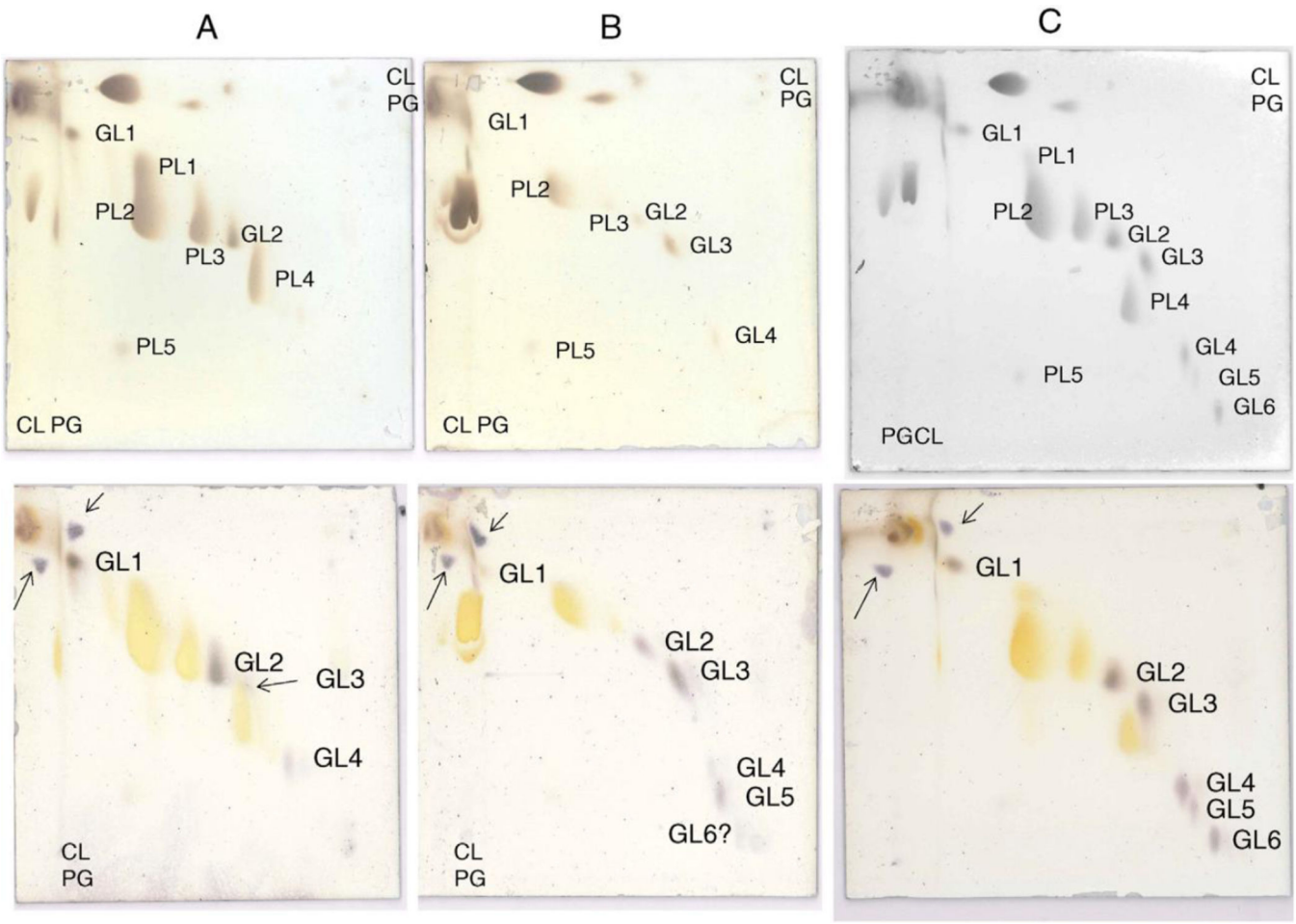

Fig. 1.

Analysis by 2D-TLC of lipids from: A) *R. lituseburensis*, B) *R. ilealis*, and C) strain FRIFI. The upper plates were stained with ninhydrin and after recording, with molybdenum blue followed by charring and the lower plates were stained with α-naphthol. The major lipids were identified by their staining properties and confirmed by LC/MS as follows: PL1, PG; PL2, CL; PL3, PG; PL4, lysyl-PG, and PL5, PA. The glycolipids were initially shown by staining with α-naphthol and their identities revealed by LC/MS as follows: GL1, MHDAG, GL2, DHDAG; GL3, acyl-TriHDAG, GL4, TriHDAG; GL5, acyl-TetraHDAG; and GL6, TetraHDAG. Lysyl-PG was ninhydrin-positive. Standards of PG and CL were applied to the left margin and in the upper right hand corner, upper plates and a standard of MHDAG was applied to the left margin and upper right hand corner of the lower plates stained with α-naphthol. The resulting spots are shown with arrows.

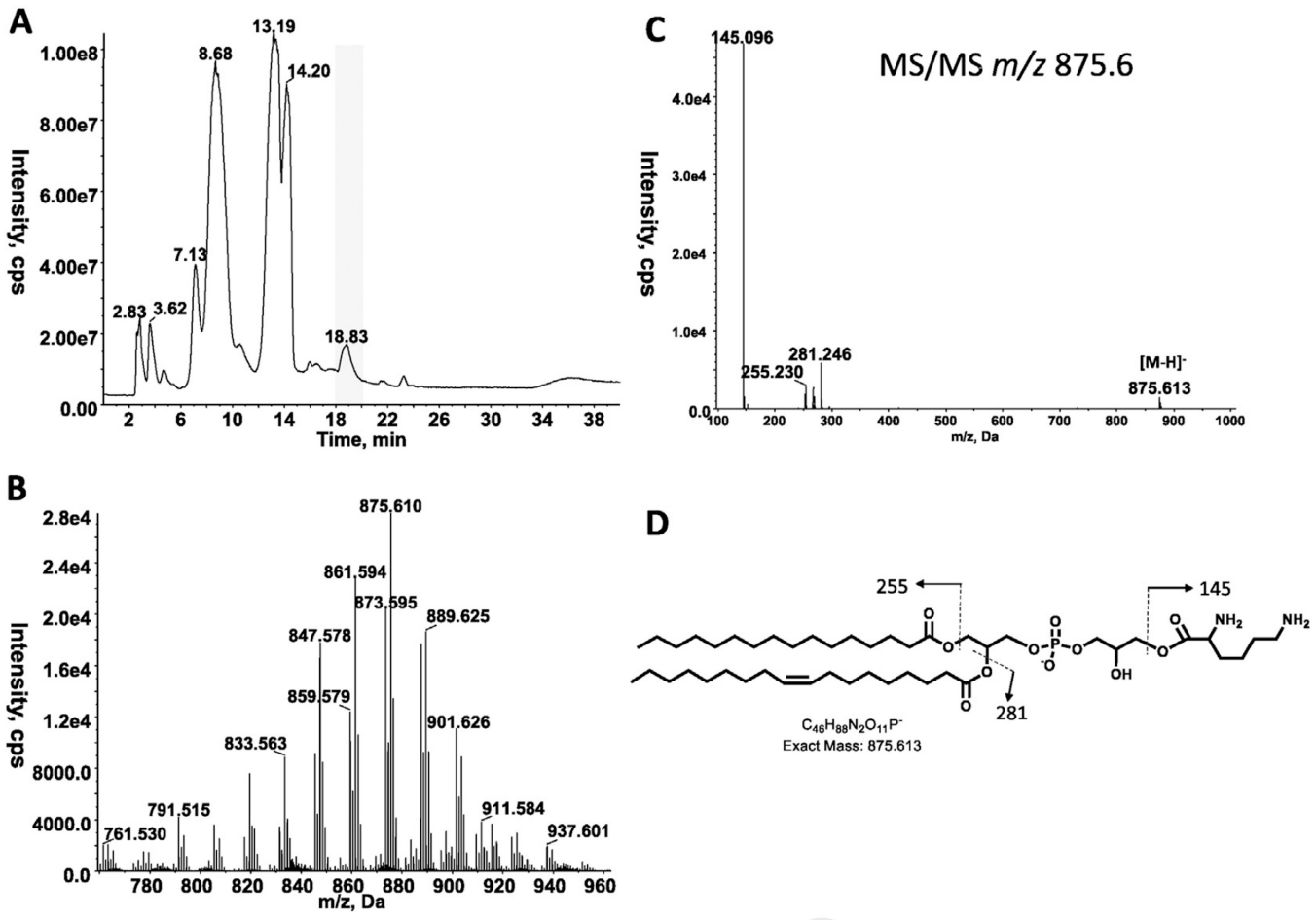

Fig. 2.

LC/MS/MS identification of lysyl-PG. A. Total ion chromatogram of lipids from *R. lituseburensis*. B. Negative ion ESI/MS spectrum showing the $[M-H]^-$ ions of the lipid emerging at 18 to 20 min. C. MS/MS spectrum of $[M-H]^-$ at *m/z* 875.6. D. The product ion at *m/z* 145 corresponds to the lysine residue.

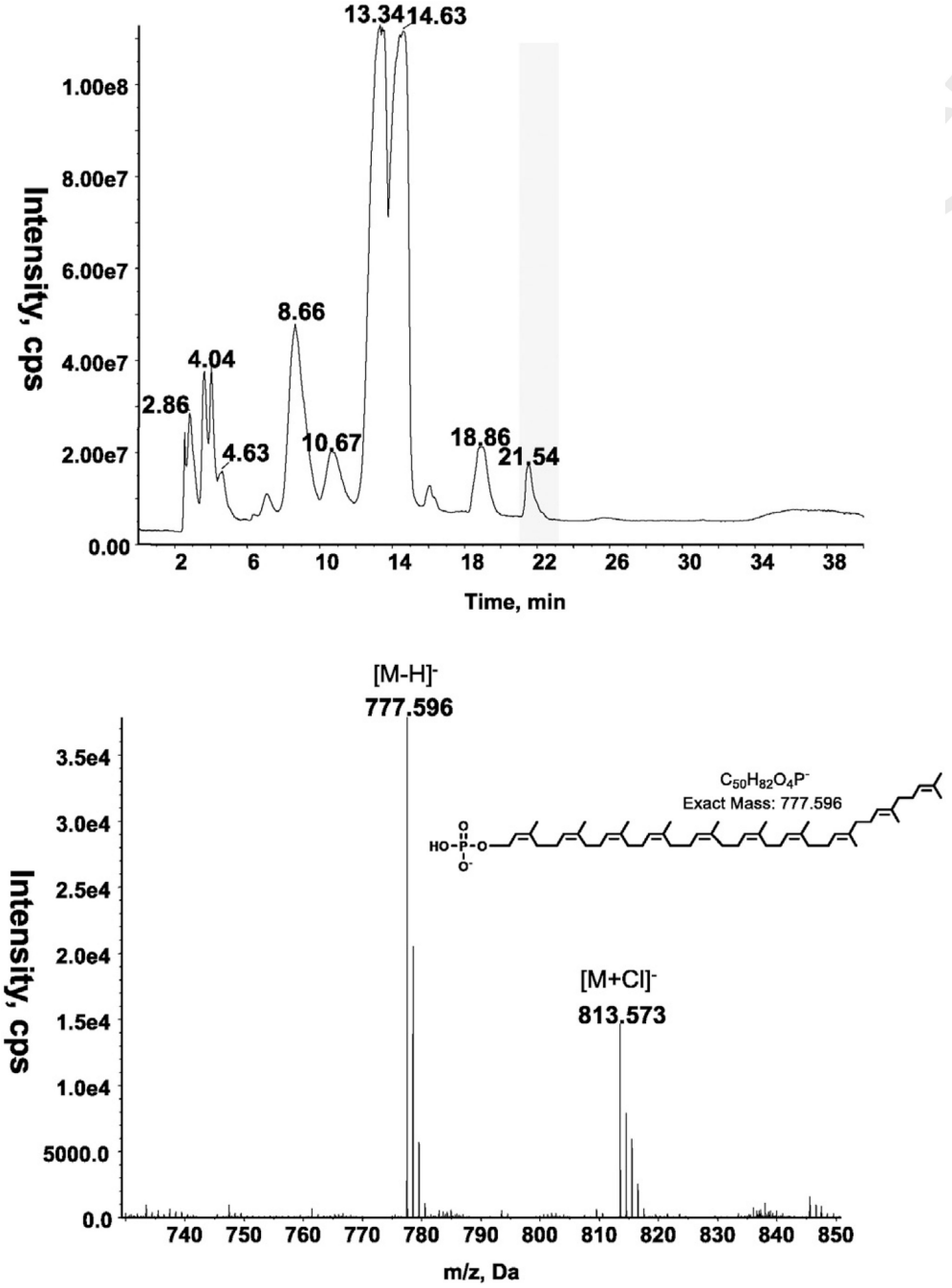

Fig. 3.

LC/MS identification of decaprenyl (C50)-P from strain FRIFI. A. Total ion chromatogram of lipids from strain FRIFI (top) and negative ion ESI/MS spectrum showing the $[M-H]^-$ and $[M+Cl]^-$ ions of the lipid emerging at 21 to 22.5min (bottom).

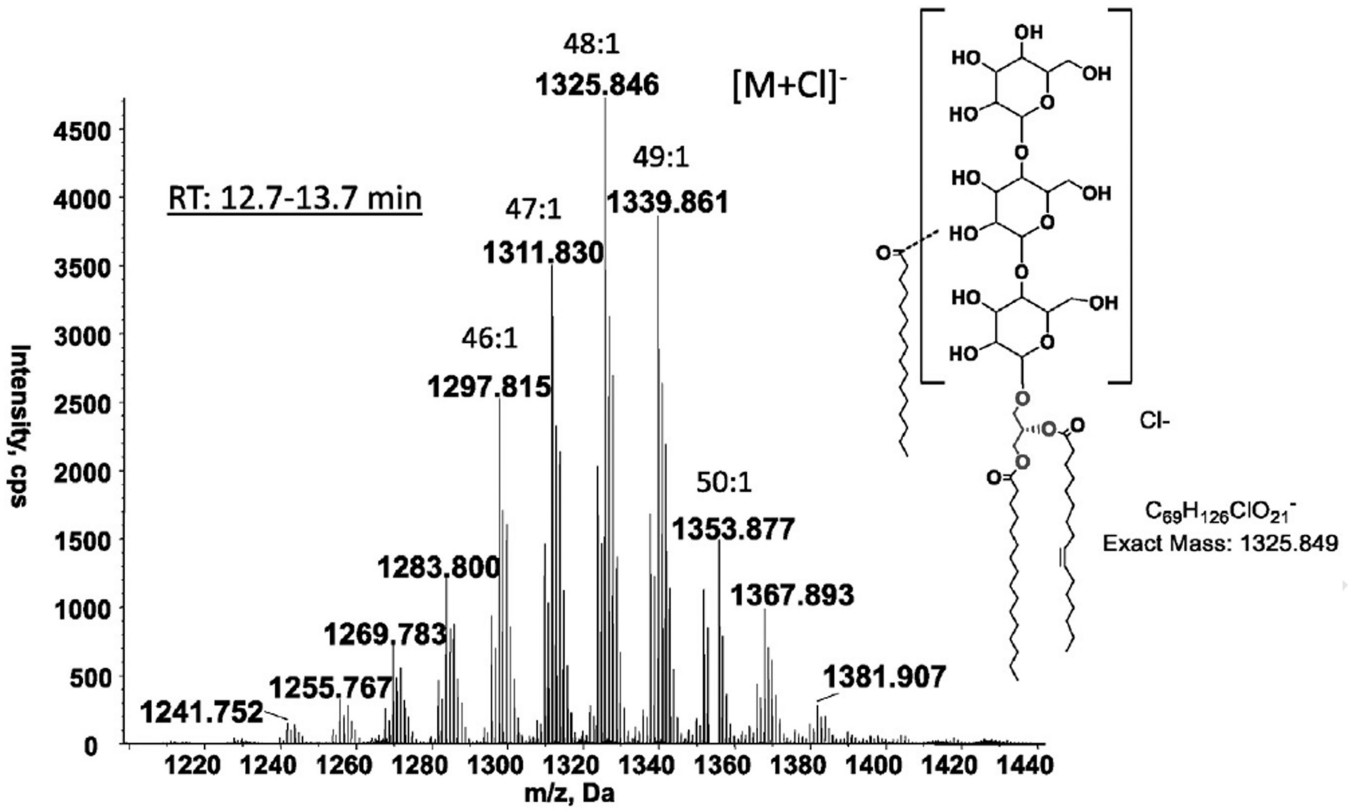

Fig. 4.

LC/MS identification of acyl-triHDAG from strain FRIFI. Negative ion ESI/MS showing the $[M + Cl]^-$ ions of the lipid emerging at 12.7 to 13.7 min. The structural drawings of carbohydrates are for the illustration of chemical formula, and are based on previous reports that bacterial glycolipids, at least majority of them, contain hexopyrnoses (glucose, galactose and mannose) [16].

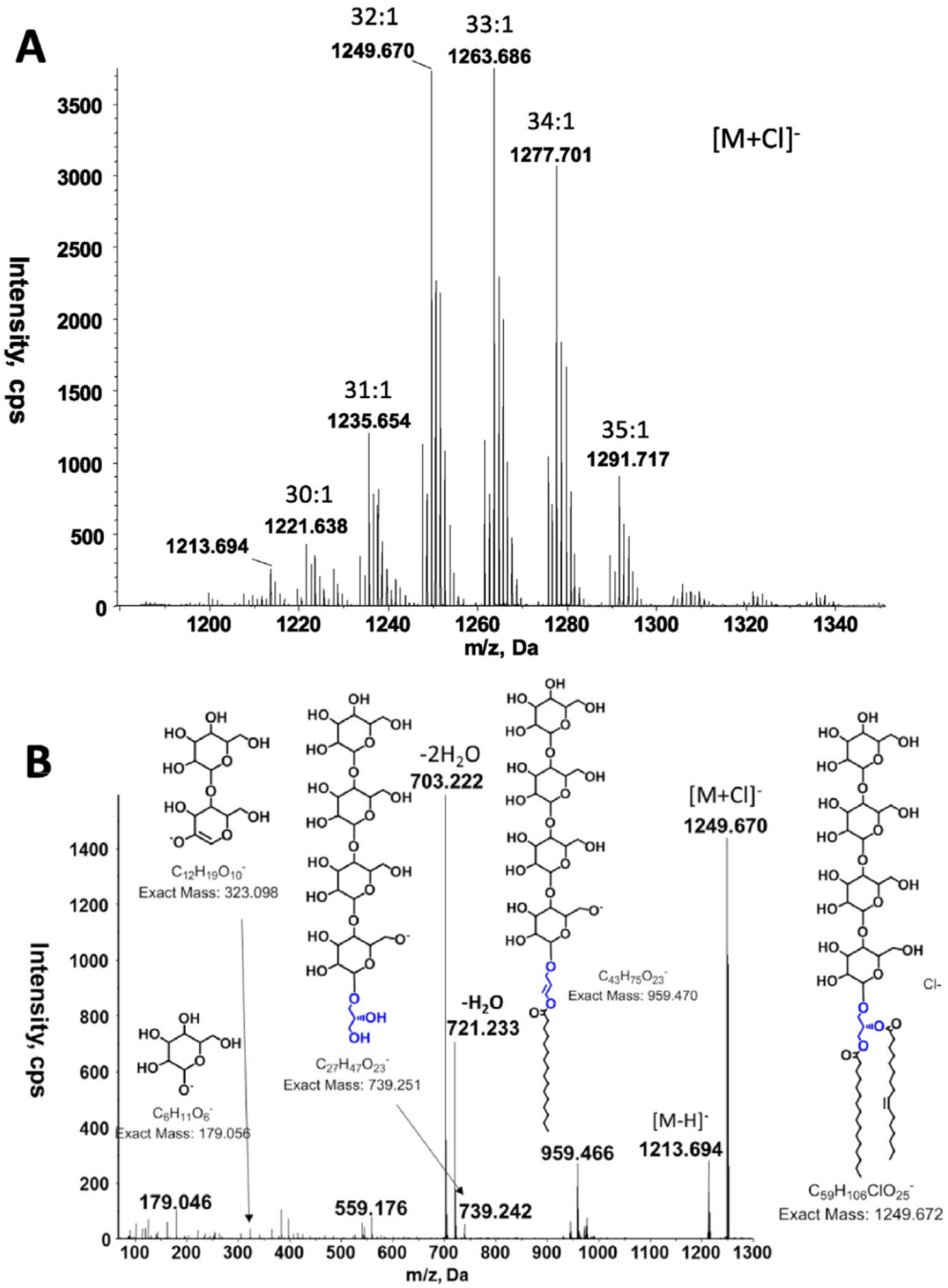

Fig. 5.

LC/MS/MS identification of tetraHDAG from strain FRIFI. A. Negative ion ESI/MS showing the $[M + Cl]^-$ ions of the lipid emerging at 25.5 to 26.5 min. B. MS/MS spectrum of the $[M + Cl]^-$ at *m/z* 1249.670.

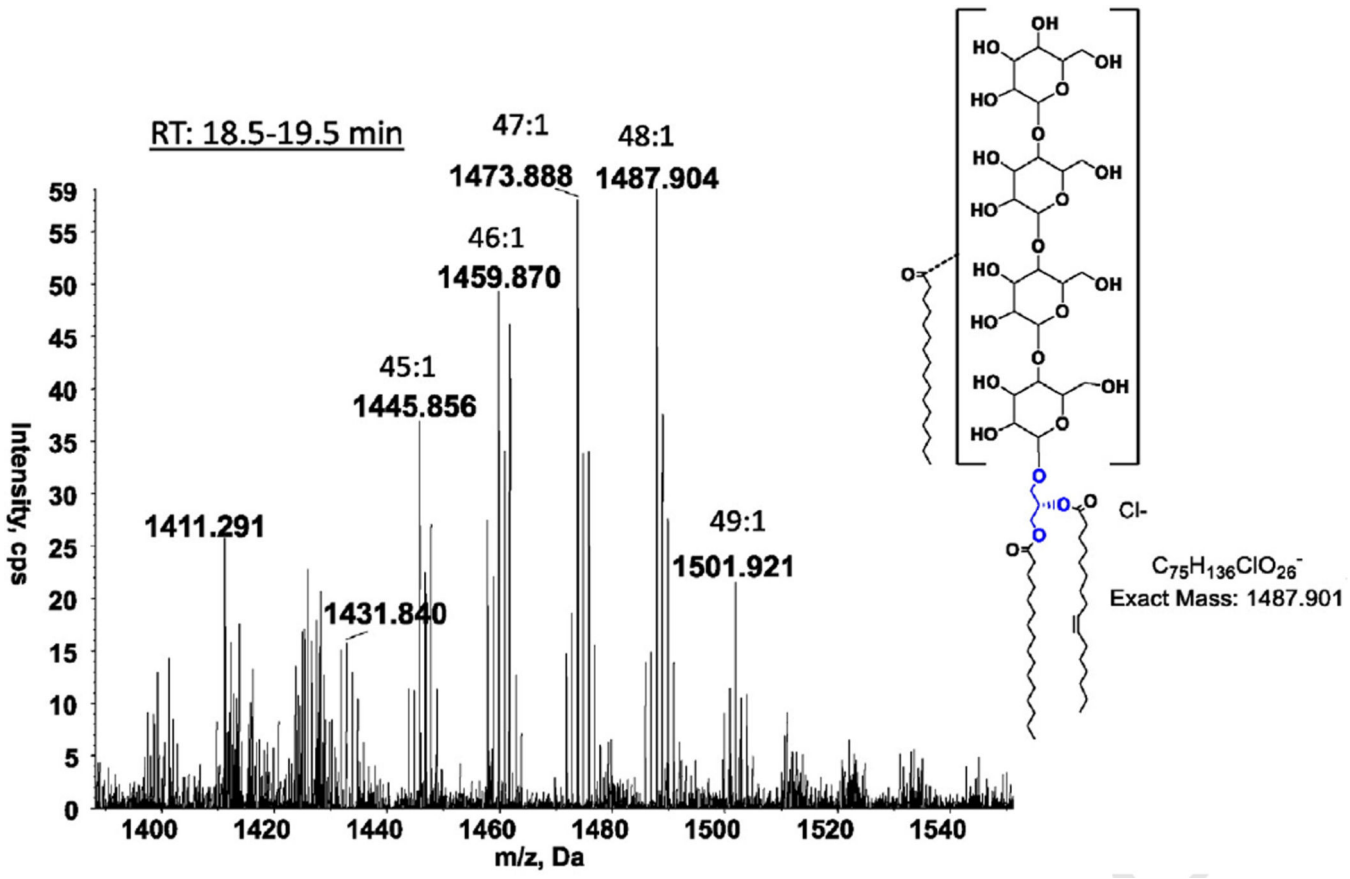

Fig. 6.

LC/MS identification of acyl-tetraHDAG from strain FRIFI. Negative ion ESI/MS showing the $[M+ Cl]^-$ ions of the lipid emerging at 18.5 to 19.5 min.

Table 1

Romboutsia lipids identified by 2D-TLC and LC/MS/MS.

Lipid	*R. lituseburensis*	*R. ilealis*	strain FRIFI
Diacylglycerol	+	+	+
Phosphatidylglycerol	+	+	+
Cardiolipin	+	+	+
Lysyl-PG	+	–	+
MHDAG[a]	+	+	+
DHDAG[a]	+	+	+
TriHDAG[a]	+	+	+
TetraHDAG[a]	–	tr	+
Acyl-TriHDAG	+	+	+
Acyl-TetraHDAG	–	+	+
Polyprenyl-P	+	+	+
Phosphatidic acid	+	+	+

[a]MHDAG, DHDAG, TriHDAG and TetraHDAG are mono-, di-, tri-, and tetra-hexosyldiacylglycerol, respectively.

Table 2

Majormolecular species of *Romboutsia* diacylglycerol and phospholipids seen by negative ion ESI/MS.

Major molecular species[a]	[M-H]$^{-}$[b]	*R. lituseburensis*	*R. ilealis*	strain FRIFI
DAG, 31:1	587.443	+	+	+
DAG, 32:1	601.459	+	+	+
DAG, 33:1	615.474	+	+	+
DAG, 34:1	629.490	+	+	+
DAG, 35:1	643.505		+	+
PA, 31:0	633.449	*c*		+
PA 32:1	645.450	*c*	+	+
PA 33:1	659.465	*c*	+	
PA 33:0	661.481	*c*		+
PA 34:1	673.481	*c*	+	+
PA 34:0	675.496	*c*		+
PA 35:1	687.497	*c*	+	+
PG 32:1	719.488[a]	+	+	+
PG 33:1	733.503	+	+	+
PG 34:1	747.518	+	+	+
PG 35:1	761.533		+	+
CL 60:1	1293.886	+		
CL 61:1	1307.902	+		
CL 63:2	1333.916	+		+
CL 64:2	1347.933	+	+	+
CL 65:2	1361.949	+	+	+
CL 66:2	1375.964	+	+	+
CL 67:2	1389.980		+	
CL 68:2	1403.995		+	+
CL 69:1	1418.011		+	
CL 69:3	1415.996			+
Lysyl-PG 31:1	833.563			+
Lysyl-PG 32:1	847.578	+		+
Lysyl-PG 33:1	861.540	+		+
Lysyl-PG 34:1	875.610	+		+
Lysyl-PG 35:2	887.611	+		
Lysyl-PG 35:1	889.625	+		+

[a] DAG, diacylglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin.

[b] These *m/z* values are for strain FRIFI. The values for other species may vary slightly.

[c] Although PA has been detected in *R. lituseburensis* nomass spectral data are available.

Table 3

Major molecular species of *Romboutsia* glycolipids seen by negative ion ESI/MS.

Major molecular species[a]	[M + Cl]$^{-}$[b]	*R. lituseburensis*	*R. ilealis*	strain FRIFI
MHDAG 31:1	749.498	+	+	+
MHDAG 32:1	763.513	+	+	+
MHDAG 33:1	777.529	+	+	+
MHDAG 34:1	791.544	+	+	+
DHDAG 31:1	911.550	+	+	+
DHDAG 32:1	925.566	+	+	+
DHDAG 33:1	939.561	+	+	+
DHDAG 34:1	953.596		+	+
TriHDAG 31:1	1073.602	+	+	+
TriHDAG 32:1	1087.618	+	+	+
TriHDAG 33:1	1101.634	+	+	+
TriHDAG 34:1	1115.650		+	+
TriHDAG 35:1	1129.665		+	+
TetraHDAG 31:1	1235.654		tr	+
TetraHDAG 32:1	1249.670		tr	+
TetraHDAG 33:1	1263.686		tr	+
TetraHDAG 34:1	1277.701		tr	+
TetraHDAG 35:1	1291.717		tr	+
Acyl-TriHDAG 46:1	1297.815	*c*	*c*	+
Acyl-TriHDAG 47:1	1311.830	*c*	*c*	+
Acyl-TriHDAG 48:1	1325.846	*c*	*c*	+
Acyl-TriHDAG 49:1	1339.861	*c*	*c*	+
Acyl-TriHDAG 50:1	1353.877	*c*	*c*	+
Acyl-TetraHDAG 45:1	1445.856		*c*	+
Acyl-TetraHDAG 46:1	1459.870		*c*	+
Acyl-TetraHDAG 47:1	1473.888		*c*	+
Acyl-TetraHDAG 48:1	1487.904		*c*	+
Acyl-TetraHDAG 49:1	1501.921		*c*	+

[a] MHDAG, DHDAG, TriHDAG and TetraHDAG are mono-, di-, tri-, and tetra-hexosyldiacylglycerol, respectively.

[b] These *m/z* values are for strain FRIFI. The values for other species may vary slightly.

[c] Although these lipids have been detected in *R. lituseburensis* and *R. ilealis*, nomass spectral data are available.