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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 *C. difficile* are closely related (>95% sequence identity).

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 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

Abbreviations: CL, cardiolipin; DHDRG, dihexosyldiacylglycerol; MHDRG, monohexosyldiacylglycerol; PG, phosphatidylglycerol; THDRG, trihexosyldiacylglycerol; TetraHDAG, tetrahexosyldiacylglycerol.

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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 105 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.

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 tetrahexosyldiacylglycerol, respectively.

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

Table 2
Major molecular 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	+	+	+
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* no mass spectral data are available.