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Lipidomic Analysis of *Clostridium cadaveris* and *Clostridium fallax*

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Abstract

The lipidomes of *Clostridium fallax* and *Clostridium cadaveris* were studied using thin-layer chromatography (TLC) and normal phase liquid chromatography/mass spectrometry (NPLC/MS). Both species contain diradylglycerol (DRG), monohexosyldiradylglycerol (MHDRG), monohexosyl monoacylglycerol (MHMAG), phosphatidylglycerol (PtdGro), and phosphatidylethanolamine (PtdEtn). DRG, MHDRG, PtdEtn, and PtdGro are present in both diacyl and alk-1-enyl acyl (plasmalogen) forms. Both species contain cardiolipin (Ptd₂Gro), which is present in tetraacyl, monoalkenyl-triacyl, and dialkenyl-diacyl forms. Both species contain small amounts of phosphatidylcholine (PtdCho). The presence of octadecadienoic (18:2) acyl chains in some PtdCho species indicates that they arise from the medium because no 18:2 is seen in the other lipids and clostridia generally lack the capacity to synthesize polyunsaturated fatty acids. The major lipidomic differences between these two species are that *C. fallax* contains a glycerolacetal of plasmenylethanolamine while *C. cadaveris* contains an ethanolamine-phosphate-modified diacylglycerol. The significance of these lipid compositions is discussed.

Keywords

Clostridium cadaveris; *Clostridium fallax*; Glycolipid; Lipidome; Phospholipid; Plasmalogens

Introduction

The genus *Clostridium* (*sensu stricto*) has been proposed to contain the species type *Clostridium butyricum* and related species (Lawson and Rainey, 2016), most of which were previously placed within rRNA clostridial cluster I (Collins et al., 1994). Even within this large group, there is considerable heterogeneity with respect to their physiology; some are proteolytic, others are saccharolytic, or neither. A relatively small number of these species

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are pathogenic to humans, including *Clostridium botulinum*, *C. perfringens*, *Clostridium novyi*, *Clostridium tetani*, and *Clostridium difficile*.

Clostridia are anaerobic, spore-forming, rod-shaped, and Gram-positive, although they may appear to be Gram-negative at times. Because life on earth is thought to have arisen during the period before oxygen appeared in the earth's atmosphere, it is likely that clostridia are related to some of the most ancient forms of life, which probably accounts for their great lipid diversity.

Although the basic building blocks of life, amino acids, deoxyribonucleotides, and ribonucleotides, are identical throughout the living kingdoms, there is considerable diversity with respect to bacterial lipids and cell wall structures. Studies of the complex lipids of members of the genus *Clostridium* have revealed several lipids that are found in most species, but others that are found in specific clusters of organisms (Goldfine and Johnston, 2005; Guan et al., 2011, 2012, 2013, 2014; Johnston et al., 2010; Oulevey et al., 1986). *Clostridium botulinum* has been divided into four subgroups (I to IV) based on physiological characteristics and 16S rRNA sequencing (Hatheway, 1990). Studies of the lipids of *C. botulinum* strains have revealed clear relationships with those clostridia that have been found to be related by DNA sequencing and studies on their physiology (Guan et al., 2012).

The lipidomes of many species of *Clostridium* have not been explored in detail. Among these are two closely related species *Clostridium cadaveris* and *Clostridium fallax*, which are not closely related to the clostridia previously studied. They represented a distinct gap in our knowledge of the lipids of clostridia. We here describe the lipid compositions of these two species. *C. cadaveris* was first isolated from decomposing human bodies. It is present in the human microbiota and disseminates from the gut during decomposition of tissues. Both species produce acetic and butyric acids. *Clostridium fallax* also produces lactic acid on fermentable sugars (Rainey et al., 2015). An unusual characteristic of both species is the presence of phosphatidylcholine (PtdCho), which is found in about 10–15% of Gram-negative organisms (Geiger et al., 2013), but up to now has only been found in one other Gram-positive species (Adams et al., 2017).

Materials and Methods

Bacterial Cultures

Clostridium cadaveris CC88A and *C. fallax* ATCC 19400 were obtained from the American Type Culture Collection. They were grown overnight in flasks at 37 °C in an anaerobic hood on reinforced clostridial medium without agar, which contained per liter: yeast extract, 10.5 g; peptone, 12.5 g; glucose, 5 g; soluble starch, 1 g; NaCl, 5 g; sodium acetate, 3 g; and cysteine HCl•H₂O, 0.6 g. The pH was adjusted to approximately 7.1 with NaOH. The glucose solution was autoclaved separately.

Lipid Extraction

Cells were harvested by centrifugation at 3000 ×g for 10 min and washed twice in 20 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer, pH 7.2. The wet cell pellets were extracted with chloroform/methanol/water using the method of Bligh and Dyer (1959) as

modified (Kates, 1986). The dried lipids were dissolved in chloroform, and stored under argon at -20°C .

Thin-Layer Chromatography

Two-dimensional thin-layer chromatography (2D-TLC) was performed on silica gel 60, 10×10 cm, thin-layer plates (Merck KGaA, Darmstadt, Germany). The solvents were chloroform/methanol/concentrated ammonia/water, 65:30:2.5:2.5 (v/v/v/v, solvent 1) in the first dimension and chloroform/methanol/acetic acid/water, 80:18:12:5 (v/v/v/v, solvent 2)) in the second dimension. Acid hydrolysis of lipids on TLC plates for detection of plasmalogens was performed by exposing the lipids separated in the first dimension to HCl fumes for 20 s, after which the residual HCl was removed first under a stream of argon and then under high vacuum for at least 30 min. Amine-containing lipids were detected by spraying with 0.3% ninhydrin in ethanol, followed by air-drying and heating at 120°C for 5 min. The same plates were then sprayed with 0.3% (w/v) molybdenum blue for detection of phospholipids. Finally, the plates were charred for 20 min at 120°C . Glycolipids were detected on separate 2D-TLC plates by spraying with α -naphthol 0.5% in methanol–water 1:1 (v/v) followed by sulfuric acid–water 95:5 (v/v) and heating at 120°C until dark purple color appears (Goldfine and Guan, 2017).

Liquid Chromatography/Mass Spectrometry (LC/MS)

Normal phase LC-ESI/MS of lipids was performed, as previously described (Guan et al., 2014) using an Agilent 1200 Quaternary LC system (Santa Clara, CA, USA), coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA, USA). The MS and MS/MS spectra acquired on the TripleTOF 5600 mass spectrometer are of high resolution (>25 K) and high accuracy (better than 5 ppm). For lipidomic analysis, the MS scan range is set at m/z 200–2000 and the MS/MS scan range is set at m/z 50–2000. The per scan accumulation times for MS and MS/MS are 1.0 and 0.5 s, respectively. A lipid standard mixture (SPLASH LIPIDOMIX by Avanti) is routinely used to monitor the LC/MS performance. A blank sample is run before each analysis to ensure no contamination or sample carry over. Each sample was run by LC/MS at least three times.

An Ascentis Si HPLC column ($5\text{ }\mu\text{m}$, $25\text{ cm} \times 2.1\text{ mm}$) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v/v). The elution program was as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B for 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C for 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was $300\text{ }\mu\text{L}/\text{min}$. MS settings were as follows: In the negative ion mode: IS = -4500 V , CUR = 20 psi, GS1 = 20 psi, DP = -55 V , and FP = -150 V ; In the positive ion mode: IS = 5500 V , CUR = 20 psi, GS1 = 20 psi, DP = 50 V , and FP = 150 V . Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the Analyst TF1.5 software (Sciex, Framingham, MA, USA).

Results

2D-TLC of the total lipids of *C. fallax* and *C. cadaveris* indicated the presence of mixtures of phospholipids and glycolipids as revealed by specific staining (Figs. 1 and 2). A number of lipids common to most species of *Clostridium* (*sensu stricto*) were tentatively identified as monohexosyldiradylglycerol (MHDRG), cardiolipin (Ptd₂Gro), phosphatidylethanolamine (PtdEtn), and phosphatidylglycerol (PtdGro). Several lipids were not readily identifiable by 2D-TLC. The presence of plasmalogens, which have been found in all clostridia studied up to now, was indicated by hydrolysis on exposure to HCl fumes on a 2D-TLC plate (data not shown). To confirm the expected lipids and structurally characterize the unknown lipids, NPLC/MS was carried out in the negative and positive modes for both organisms (Fig. 3a, b). Stronger signals for PtdCho and other minor lipids are seen on NPLC/MS in the positive mode (Fig. 3b). Molecular species of the major glycolipids lipids are given in Table 1 and those for the phospholipids are given in Table 2. In both organisms, diradylglycerol (DRG), MHDRG, cardiolipin (Ptd₂Gro), and phosphatidylethanolamine (PtdEtn) are major lipids. Monohexosyl monoacylglycerol (MHMAG) is also present in both organisms (Table 1, Fig. 4). PtdCho is a minor lipid in both species (Fig. 5). In both species, PC contain 18:2 chains. For example, PtdCho at *m/z* 758.57 in *C. cadaveris* and *C. fallax* is a mix of PtdCho (16:0/18:2) and PtdCho (16:1/18:1). The presence of 18:2 chains suggests that the acyl chains are derived from the medium, because no 18:2 is seen in the other lipids and clostridia in general do not produce polyunsaturated fatty acids, which requires oxygen in eukaryotic organisms (Goldfine, 1965; Goldfine and Johnston, 2005).

DRG, MHDRG, PtdEtn, PtdGro, and cardiolipin are present in both diacyl and plasmalogen forms (Tables 1 and 2). Cardiolipin in both species has both monoalkenyl and dialkenyl species. *Clostridium fallax* has plandman@aocs. orgless of the dialkenyl species. Phosphatidic acid (PtdOH) and phosphatidylserine (PtdSer), lipid precursors present in small amounts, were detected in *C. fallax* and *C. cadaveris*, respectively, but only in the diacyl forms (Table 2).

Two lipids previously found in clostridia, but not in other bacteria, have a small amount of a glycerol acetal of plasmenylethanolamine (PlsEtn) in *C. fallax* (Fig. 1a, Fig. 6, Table 2). An ethanolamine-phosphate-modified glycosyldiradylglycerol was seen in *C. cadaveris* (Table 1, Fig. 6).

Discussion

Glycerol acetals of PlsE and the plasmalogen form of phosphatidyl-N-monomethyl ethanolamine were first detected in *C. butyricum* and *C. beijerinckii* (Khuller and Goldfine, 1974; Matsumoto et al., 1971). They were shown to stabilize the lamellar phase of the membrane lipids of these species in response to increased unsaturation of the hydrocarbon chains or the presence of solvents (Goldfine et al., 1987; MacDonald and Goldfine, 1991). *Clostridium fallax*, although distantly related, has been classified among the butyric acid-producing clostridia (Cummins and Johnson, 1971). Thus, the presence of a glycerol acetal of PlsE is not entirely unexpected.

PtdOH and PtdSer were present in *C. fallax* and *C. cadaveris*, respectively. As has been noted previously for *C. tetani* and *C. novyi*, these precursor lipids have very little or no plasmalogen forms, consistent with the hypothesis that plasmalogens in clostridia are formed from the diacyl end-products of phospholipid biosynthesis (Goldfine, 2010, 2017; Guan et al., 2011; Johnston et al., 2010).

Among the phospholipids, PtdGro, PtdEtn, and cardiolipin are found in most clostridia (see references cited above). PtdCho, with a positively charged choline head group, was readily detected by NPLC/MS(+) in both species (Fig. 5). It was also readily seen on TLC in *C. fallax* lipids, but was difficult to detect by molybdate staining on TLC of the lipids of *C. cadaveris*. PtdCho, which is present in many Gram-negative species (Geiger et al., 2013), has not been found in clostridia up to now and has only been found in one other Gram-positive species, *Streptococcus mitis* (Adams et al., 2017). Ethanolamine-phosphate-modified glycosyldiradylglycerols were first identified in *C. tetanus*, in which the hexose is N-acetylglucosamine (GlcNAc) (Johnston et al., 2010). The same lipid was subsequently found in *C. sporogenes* and in related Group I strains of *C. botulinum* (Guan et al., 2012). Etn-P-modified monoglycosyl and diglycosyl-DRG have also been found in *Clostridium acetobutylicum* (Tian et al., 2013). Both Etn-PGlcNAc diradylglycerol and Etn-P-hexosylDRG have been found in *Clostridium psychrophilum*, an obligately anaerobic psychrophilic species from Antarctica (Guan et al., 2013).

In *C. acetobutylicum*, Etn-P-hexosylDRG was found to increase in amount when the total membrane lipids were enriched in a monounsaturated fatty acid (oleic acid) or when octanol was added to the growth medium (Johnston and Goldfine, 1992; Tian et al., 2013). *Clostridium acetobutylicum* also contains monoglycosyl and diglycosyl-DRG. Increases in diglycosyl-DRG were also observed upon increased unsaturation of the cellular lipids and upon addition of octanol to the growth medium. These changes are thought to represent responses that counteract the tendency to form nonlamellar phases when monohexosyl diacyl glycerols are highly enriched in unsaturated fatty acids or when cells are exposed to certain solvents, e.g. octanol. Many studies on the cellular lipids of *Acholeplasma laidlawii* have shown that the conversion of monoglycosyl-diacylglycerol (DAG) to diglycosyl-DAG is a response to increased unsaturation of the membrane lipids or the presence of solvents in the growth media and that this increase in the size of the polar head group stabilizes the lamellar phase of the membrane lipids (Lindblom and Rilfors, 1989; Morein et al., 1996; Wieslander et al., 1980, 1981, 1986).

PtdCho in bacteria can be synthesized in four different ways: (1) sequential methylation of PtdEtn, (2) the transfer of phosphocholine from CDP-choline to diacylglycerol, (3) condensation of choline with CDP-DAG to form PtdCho and CMP (De Rudder et al., 1999; Geiger et al., 2013; Sohlenkamp et al., 2000), and (4) a glycerylphosphorylcholine (GPC) acylation pathway (Moser et al., 2014). Because PtdCho in *C. cadaveris* and *C. fallax* contains 18:2 chains, the acyl chains presumably come from the growth medium that contains both animal and yeast materials. The pathway by which the precursors enter into the cells of *C. cadaveris* and *C. fallax* and their conversion to PtdCho is unknown and will require further investigation. It should be noted that we have not observed PtdCho in other clostridia grown in the same medium.

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Abbreviations

DAG	diacylglycerol
DRG	diradylglycerol
GAPIsEtn	glycerolacetal of plasmeneylethanolamine
GlcNAc	N-acetylglucosamine
GPC	glycerylphosphorylcholine
MHDRG	monohexosyldiradylglycerol
MHMAG	monohexosyl monoacylglycerol
PlsEtn	plasmeneylethanolamine
Ptd₂Gro	cardiolipin
PtdEtn	phosphatidylethanolamine
PtdGro	phosphatidylglycerol
PtdOH	phosphatidic acid
PtdSer	phosphatidylserine

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Fig. 1. 2D-TLC of the total lipids of *Clostridium fallax* as seen after charring. GL1, monohexosyldiradylglycerol (MHDRG); GL2, MGMAG; GL3, unknown; PL1, Ptd₂Gro; PL2, PtdGro; PL3, PtdEtn; PL4, unknown phospholipid; PL5, PtdCho; PL6, GAPIsEtn; PL7, PtdOH. The vertical arrow indicates the first dimension; the horizontal arrow indicates the second dimension. Standard lipids were spotted as shown; abbreviation, MGDG, monoglucosyldiacylglycerol

Fig. 2. Two-dimensional thin-layer chromatography (2D-TLC) of the total lipids of *Clostridium cadaveris* lipids stained with α -naphthol. GL 1, monohexosyldiradylglycerol (MHDRG); GL2, may be monohexosyl monoacylglycerol (MHMAG), GL 3, unknown, GL 4, EtnPMHDRG; PL1, cardiolipin (Ptd₂Gro); and PL2, phosphatidylethanolamine (PtdEtn). Phospholipids appear as yellow to brown spots, glycolipids appear as purple spots. The vertical arrow indicates the first dimension; the horizontal arrow indicates the second dimension. Standard lipids were spotted as shown

Fig. 3.

(a) Total ion current chromatogram of normal phase liquid chromatography/mass spectrometry (NPLC/MS)(-) of total lipids from *Clostridium fallax* and *Clostridium cadaveris*. (b) Total ion current chromatogram of NPLC/MS(+) of total lipids from *C. fallax* and *C. cadaveris* FA, fatty acids.

Fig. 4. Normal phase liquid chromatography/mass spectrometry (NPLC/MS) identification of monohexosyl monoacylglycerol (MHMAG) in *Clostridium fallax*. The structure is for illustrative purposes only. The stereochemistry of hexose and the position of the glycosidic bond have not been determined

Fig. 5. Positive ion ESI/mass spectrometry (MS) of phosphatidylcholine species emerging at 20–21 min from (a) *Clostridium cadaveris*; (b) *Clostridium fallax*. The p before the molecular species number indicates the plasmalogen form

(a) *C. cadaveris* (b) *C. fallax* **Fig. 6.**

Negative ion ESI/mass spectrometry (MS) of ethanolamine-P-monohexosyl diacylglycerol (EtnPMHDAG) emerging at 21.5–22.5 min from *Clostridium cadaveris* and the glycerol acetal of plasmenylethanolamine (GAPIsEtn) emerging at 19–20 min from *Clostridium fallax*. The identification of these species is supported by accurate mass and tandem MS measurements. The structure of EtnPMHDAG is for illustrative purposes only. The positions of the glycosidic bond and ethanolamine-P have not been determined

Molecular species of *Clostridium cadaveris* and *Clostridium fallax* diradylglycerols and glycolipids as detected by negative ion ESI/mass spectrometry (MS)

Table 1

Major molecular species	<i>C. cadaveris</i>		<i>C. fallax</i>	
	Diacyl [M + Cl] ⁻	Plasmalogen [M + Cl] ⁻	Diacyl [M + Cl] ⁻	Plasmalogen [M + Cl] ⁻
Diradylglycerol, 28:0	547.414	531.417	547.413	531.416
Diradylglycerol, 30:1	573.432	557.435	573.430	557.431
Diradylglycerol, 30:0	575.445	559.447	575.443	559.449
Diradylglycerol, 32:1	601.461	585.465	601.459	585.467
Diradylglycerol, 32:0			603.475	587.477
Diradylglycerol, 34:1			629.494	613.498
MHDRG, 28:0	709.466	693.470		
MHDRG, 30:1	735.482	719.489		
MHDRG, 30:0	737.497	721.502	737.497	721.502
MHDRG, 32:1	763.514	747.522	763.493	
MHDRG, 32:0			765.532	749.535
MHDRG, 34:1			791.549	775.552
MHDRG, 36:0				805.542
MHMAG, 14:0	499.269		499.268	
MHMAG, 16:1	525.284			
MHMAG, 16:0			527.300	
MHMAG, 18:1			553.314	
MHMAG, 18:0			555.329	
MHMAG, 19:cyt			567.330	
EmPMHDAG, 28:0	796.500 ^a			
EmPMHDAG, 30:1	822.516 ^a			
EmPMHDAG, 30:0	824.530 ^a			
EmPMHDAG, 32:1	850.547 ^a			
EmPMHDAG, 34:1	878.574 ^a			

MHDRG, monohexosyl diradylglycerol; MHMAG, monohexosyl monoacylglycerol; EmPMHDAG, ethanolamine-P-mono-hexosyl diacylglycerol.

$\cdot [H-M]_p$

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Molecular species of *Clostridium cadaveris* and *Clostridium fallax* phospholipids as detected by negative ion ESI/mass spectrometry (MS) (unless noted otherwise)

Table 2

Major molecular species	<i>C. cadaveris</i>		<i>C. fallax</i>	
	Diacyl [M-H] ⁻	Plasmalogen [M-H] ⁻	Diacyl [M-H] ⁻	Plasmalogen [M-H] ⁻
PtdOH, 32:0			647.467	
PtdOH, 34:1			673.483	
PtdGro, 28:0	665.440	649.444	665.440	649.444
PtdGro, 30:0	693.473	677.476	693.473	677.476
PtdGro, 32:1	719.488	703.491	719.488	703.495
PtdGro, 32:0	721.501	705.507	721.504	705.507
PtdGro, 34:1	747.520		747.521	731.524
PtdGro, 35:1	761.534		761.535	
PtdGro, 36:0	775.554		775.551	
PtdEtn, 28:0	634.445	618.449		618.449
PtdEtn, 30:1	660.459	644.466		644.465
PtdEtn, 30:0	662.474	646.482	662.477	646.481
PtdEtn, 32:1			688.494	672.496
PtdEtn, 32:0			690.507	674.512
PtdEtn, 34:1			716.523	700.530
PtdEtn, 34:2			714.544	
GAPlsEtn, 28:0				710.499
GAPlsEtn, 30:1				736.514
GAPlsEtn, 30:0				738.531
GAPlsEtn, 32:1				764.546
GAPlsEtn, 34:1				792.576
Ptd ₂ Gro, 60:2		1259.886 ^a		
Ptd ₂ Gro, 60:1		1261.896 ^a		
		1277.894		
Ptd ₂ Gro, 62:2	1315.944	1287.917 ^a	1315.946	1287.914

Major molecular species	<i>C. cadaveris</i>		<i>C. fallax</i>	
	Diacyl [M-H] ⁻	Plasmalogen [M-H] ⁻	Diacyl [M-H] ⁻	Plasmalogen [M-H] ⁻
Ptd ₂ Gro, 62:1	1317.962	1289.929 ^a	1317.964	1289.931 ^a
Ptd ₂ Gro, 64:4			1343.979	
Ptd ₂ Gro, 64:2			1345.993	
Ptd ₂ Gro, 66:4			1372.010	
PtdSer, 28:0	678.436			
PtdSer, 30:1	704.451			
PtdSer, 30:0	706.465			
PtdSer, 32:1	732.483			
PtdCho, 32:2 ^b	730.541		730.539	
PtdCho, 32:1 ^b	732.567		732.555	
PtdCho, 34:2 ^b	758.573		758.571	742.574
PtdCho, 34:1 ^b	760.584		760.587	744.587
C ₅₀ -P ^c	777.597		777.596	

PtdOH, phosphatidic acid; PtdGro, phosphatidylglycerol; GAPtsEtn, glycerolacetal of plasmenylethanolamine; PtdEtn, phosphatidylethanolamine; Ptd2Gro, cardiolipin; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine.

^aDialkenyl species.

^bPositive mode.

^cDecaprenyl phosphate.