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The polar lipids of *Clostridium psychrophilum*, an anaerobic psychrophile

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Abstract

We have examined the polar lipids of *Clostridium psychrophilum*, a recently characterized psychrophilic *Clostridium* isolated from an Antarctic microbial mat. Lipids were extracted from cells grown near the optimal growth temperature (+5 °C) and at −5 °C, and analyzed by two-dimensional thin layer chromatography and liquid chromatography coupled with mass spectrometry. The major phospholipids of this species are: cardiolipin, phosphatidylethanolamine, and phosphatidylglycerol. Phosphatidylserine and lyso-phosphatidylethanolamine were found as minor components. The most abundant glycolipids are a monoglycosyldiradylglycerol (MGDRG) and a diglycosyldiradylglycerol (DGDRG). The latter was only seen in cells grown at −5 °C. An ethanolamine-phosphate derivative of N-acetylglucosaminyldiradylglycerol was seen in cells grown at −5 °C and an ethanolamine-phosphate derivative of MGDRG was found in cells grown at +5 °C. All lipids were present in both the all acyl and plasmalogen (alk-1 -enyl acyl) forms with the exception of PS and MGDRG, which were predominantly in the diacyl form. The significance of lipid changes at the two growth temperatures is discussed.

Keywords

Phospholipid; Glycolipid; Phosphoglycolipid; Plasmalogen; Mass spectrometry

1. Introduction

Studies on microbial lipids and those of higher organisms have shown that cells must maintain their membrane lipids in a liquid-crystalline state at the growth temperatures of a given organism [1–3]. There have been a number of investigations of the polar lipids of psychrophilic aerobic and facultative anaerobic organisms [4–6], but similar studies have not been conducted on the lipids of psychrophilic obligate anaerobes, which have unique features. Most earlier studies focused on the effects of growth temperature on the degree of unsaturation of bacterial membrane lipids, with little information on the lipid class composition [4]. The recent isolation and characterization of four psychrophilic clostridia from an Antarctic microbial mat [7] have presented an opportunity to explore the nature of

their polar lipids. Based on 16SrRNA sequencing these organisms were placed in cluster I of clostridia and were related to *Clostridium estertheticum* and *Clostridium laramiense*. The temperature optimum of growth of the four new species ranged from 4 to 16 °C. We have chosen to study *Clostridium psychrophilum* which had the lowest optimum growth temperature, 4 °C. Examination of the cellular fatty acids of *C. psychrophilum* has shown that the most abundant species were a relatively short chain saturated fatty acid, 14:0, and positional isomers of the unsaturated fatty acid, 16:1. The presence of dimethylacetals of the fatty acids after saponification and methylation indicated the presence of plasmalogens in all four species [7].

The lipids of clostridia are unusual among bacteria in that they contain plasmalogens, which have an 1-*O*-alk-1-enyl chain instead of an acyl chain attached to the *sn*-1 position of the glycerol backbone [8]. These ether lipids evolved first in anaerobes and later in animals, in which they are made by an oxygen-dependent mechanism. Their presence in anaerobes and a number of other studies show that the mechanism for their formation is unique to this group of microorganisms [9–12]. During the evolution of respiring organisms the anaerobic mechanism for plasmalogen biosynthesis appears to have been lost and replaced by an aerobic mechanism in animal cells [13]. Furthermore, various groups of clostridia have distinct polar lipids as shown by recent work with the lipids of physiologically distinct groups of *Clostridium botulinum* [14]. In this paper we show that the lipids of *C. psychrophilum* are rich in plasmalogens and that they contain phospholipids, glycosyldiradylglycerols and ethanolamine phosphate derivatives of N-acetylglucosaminyl-diradylglycerol and a monoglycosyldiradylglycerol.

2. Materials and methods

2.1. Bacterial strain and growth conditions

C. psychrophilum DSM 14207 was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). These bacteria were grown for one month at +5 °C or –5 °C on medium DSM 339. For incubation at –5 °C anaerobically prepared glycerol was supplemented at a concentration of 5% (v/v). The cells were harvested by centrifugation at 9900 ×g at 10 °C for 10 min and washed three times with cold PBS buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride), pH 7.4. The cell pellet was freeze-dried overnight at a pressure of 0.1 mbar and temperature of –80 °C and stored at –20 °C sealed in a container under nitrogen atmosphere.

2.2. Lipid extraction and thin-layer chromatography

The lipids were extracted from lyophilized cells using chloroform and methanol [15] as modified by Kates [16]. Two dimensional thin layer chromatography (2D-TLC) was performed on silica gel 60, 10×10 cm, thin-layer plates. The solvents were chloroform/methanol/concentrated ammonia/water, 65:30:2.5:2.5 (by vol.) in the first dimension and chloroform/methanol/acetic acid/water, 80:18:12:5 (by vol.) in the second dimension. Acid hydrolysis of lipids with HCl fumes on TLC plates for detection and quantification of plasmalogens has been described [17]. Phospholipids or amine-containing lipids were detected using 0.3% (w/v) molybdenum blue or 0.3% ninhydrin in ethanol, followed by heating at 120 °C for 10 min, respectively. Glycolipids were detected by -naphthol staining [18].

2.3. Liquid chromatography/mass spectrometry(LC/MS)

Normal phase LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system (Santa Clara, CA), coupled to a QSTAR XL quadrupole time-of-flight tandem mass

spectrometer (Applied Biosystems, Foster City, CA) as previously described [11]. Data acquisition and analysis were performed using the Analyst QS software (Applied Biosystems, Foster City, CA).

3. Results

3.1. Preliminary identification of *C. psychrophilum* lipids by thin layer chromatography

Initial analysis of the lipids from *C. psychrophilum* grown at -5°C was done by 2D-TLC followed by spraying with reagents specific for amino groups (ninhydrin), phosphate (molybdenum blue) and sugars (1-naphthol). The presence of several glycolipids including a monoglycosyl diradylglycerol (spot 3), a diglycosyl diradylglycerol (spot 5) and a very polar phosphoglycolipid which stained positive for an amino group (spot 8), and has been identified as Etn-P-GlcNAcDRG by LC/MS (see below) (Fig. 1). Spot 4 corresponded to both PG and cardiolipin, spot 6 corresponded to PE and spot 7 occupied a position close to that of PS in this 2D-TLC system, the latter two were ninhydrin-positive [11]. The presence of plasmalogens was detected for most of the polar lipids, by a 2D-TLC method in which the plates were treated with HCl fumes between the first and second dimensions. Plasmalogens, which are exquisitely sensitive to acid, are hydrolyzed on the plate and the resulting lyso-lipids are readily detected by staining (data not shown).

3.2. LC/ESI-MS of total *C. psychrophilum* lipids

Total *C. psychrophilum* lipids were subjected to LC-ESI/MS using a silica column for lipid separation. The chromatograms for cells grown at -5°C and $+5^{\circ}\text{C}$ are shown in Fig. 2. Spectra of the compounds emerging between 2 and 5 min are shown in Fig. 3. As seen in Fig. 2, cells grown at both temperatures have diradylglycerol, cells grown at $+5^{\circ}\text{C}$ (Fig. 3B) have relatively more MGDG than cells grown at -5°C (Fig. 3A), but cells grown at the lower temperature have DGDG, which are not evident in cells grown at the higher temperature. The major molecular species of these lipids are presented in Table 1.

Mass spectrometry confirmed the presence of cardiolipin, PG, PE and PS (Table 2) in *C. psychrophilum*. Lyso-PE was also observed emerging at 21.7 min (Table 2). We noted the presence of PS 31:1, containing 17:1 chains in cells grown at $+5^{\circ}\text{C}$ with very little of the corresponding peak in cells grown at -5°C . PG 31:1 and PE 31:1 were also more abundant at $+5^{\circ}\text{C}$. As expected, CL in these cells was also found to contain 17:1 chains.

A minor lipid in *C. psychrophilum*, Etn-P-GlcNAcDRG, was recently identified in *Clostridium tetani*, *Clostridium sporogenes* and in strains of *C. botulinum* closely related to *C. sporogenes* [14,17]. Upon 2D-TLC we observed a phosphorus containing glycolipid at a location corresponding to that of Etn-P-GlcNAcDRG (Fig. 1). The presence of this lipid in *C. psychrophilum* grown at -5°C was confirmed by LC-ESI/MS (Fig. 4A). It is seen to be almost completely in the plasmalogen form. The peak at 21 min seen in cells grown at -5°C is much diminished in the lipids from cells grown at $+5^{\circ}\text{C}$ (Fig. 2). Instead, the material emerging between 20.5 and 22 min produced the spectra shown in Fig. 4B. MS/MS of this material confirmed the presence of an Etn-P as evidenced by the presence of a product ion at m/z 140. However, the molecular masses of the major components were approximately 41 mass units lower, indicating the absence of the N-acetyl group on a hexose. These compounds corresponded to EtnP-MGDRGs of the species shown in Fig. 4B. Thus, EtnPGlcNAcDRG in cells grown at -5°C is replaced by EtnPMGDRG in cells grown at $+5^{\circ}\text{C}$, a significant chemical difference. We have also observed an EtnPMGDRG in *Clostridium acetobutylicum* (unpublished). This recently described modification of glycolipids appears to be a common theme in clostridia [14,17]. It should be noted that the medium used for growth at -5°C contained 5% glycerol, which was not added to the

medium for growth at +5 °C. *C. psychrophilum* did not grow at +5 °C with added 5% glycerol. Thus there are two variables in the growth at the two temperatures.

4. Discussion

C. psychrophilum is one of the four newly identified truly psychrophilic clostridia isolated from a mat retrieved from a shallow, moated area around Lake Fryxell, a fresh-water lake in Antarctica. The other clostridia isolated including *Clostridium frigidis*, *Clostridium lacusfryxellense* and *Clostridium bowmanii*, had higher optimal growth temperatures. The mat also contained bacteria from a wide range of psychrotolerant aerobic organisms [19]. The maximum temperatures at this site normally do not exceed 6 °C. These new species were found to be closely affiliated with cluster I clostridia and to be most closely related to *C. estertheticum*, which is reported to have a growth temperature optimum of 6–8 °C [7].

C. psychrophilum has a growth temperature optimum of 4 °C. It was found to contain almost exclusively short chain saturated (14:0) and monounsaturated acyl and alk-1-enyl chains, which would confer a low melting transition ($L \rightarrow L_c$) to the membrane lipids [1,2]. Since the physical behavior of membrane lipids depends greatly on both their polar head groups and their hydrocarbon chains, it is of considerable interest to learn more about the structures of the polar lipids of this organism. No analyses of polar lipids of the newly characterized psychrophilic clostridia have been reported and there have been no studies on the polar lipids of *C. estertheticum*. In addition to the major phospholipids, PG, PE and cardiolipin, which are present in both the diacyl and plasmalogen forms (Table 2), we observed that PS, a minor lipid in *C. psychrophilum*, is almost entirely in the diacyl form consistent with the hypothesis that plasmalogens are formed from the final products of phospholipid biosynthesis, PE, PG and cardiolipin [11,17]. The aerobic pathway found in animal cells differs in that the ether bond is formed at an early step of plasmalogen biosynthesis resulting in intermediates containing first a saturated ether and ultimately an ω -unsaturated ether [20]. Notably, MGDG, a precursor of DGDG is also predominantly in the diacyl form (Fig. 2), but DGDG is mostly plasmalogen (Fig. 3), indicating that the alk-1-enyl acyl form is derived from the diacyl form in the glycolipids.

The presence of mono- and diglycosyldiacylglycerols in *C. psychrophilum*, suggests that it has the capacity to adjust to the relatively low phosphorus levels found in Lake Fryxell [21]. Bacteria can shift the relative levels of phospholipids and glycolipids depending on the abundance of *phosphorus* in the environment [22,23]. DGDG a bilayer-prefering lipid can substitute for PG and MGDG, which in isolation has a preference for non-bilayer phases such as the reversed cubic (I_H) and reversed hexagonal (H_H) phases, can substitute for PE which has similar phase preferences [2,24]. Most clostridia have glycolipids of the glycosyldiacylglycerol type. In some these are only a few percent of the total lipid. In a few species, including *C. acetobutylicum* [25], and *Clostridium innocuum* [26] there are larger amounts. As noted above Etn-P-GlcNAcDRG, was recently identified in *C. tetani*, *C. sporogenes* and in strains of *C. botulinum* closely related to *C. sporogenes* [14,17].

The major challenge for organisms like *C. psychrophilum* is to maintain a fluid, flexible membrane at temperature below 0 °C. One way this can be accomplished is to maintain a high degree of unsaturation of the membrane lipids, as seen in *C. psychrophilum* [7]. Another possible adjustment could reside in alterations of the polar lipid classes. The largest changes in cells grown at or below the optimal temperature were seen in the glycolipids and phosphoglycolipids. As shown in Figs. 2 and 3, MGDG is more abundant at +5 °C near the optimal growth temperature and DGDG, which is present at –5 °C, is essentially absent at this temperature. The phase behavior of a monoglucosyldiacylglycerol (MGDG) and a diglucosyldiacylglycerol (DGDG) have been studied intensively in *Acholeplasma*

laidlawii [27–29]. In a classic study on phase equilibria of total membrane lipids from this organism, it was shown that as the degree of unsaturation of the membrane lipids was varied from approximately 20% to over 90% *cis*-9–18:1 (oleic acid), the ratio of DGDG/MGDG increased from 0.59 to 5.6. Near the higher ratio of DGDG to MGDG, completion of the formation of the gel phase was reduced to below –10 °C [27]. This lowering of the temperature of gel phase boundary is a function of both increased unsaturation and the ratios of the glycolipids. From these studies it is clear that with the high content of unsaturated and short chain fatty acids seen in *C. psychrophilum* lipids (63% unsaturated plus cyclopropane, 30% short chain) and a mixture of DGDG and MGDG, the formation of the gel phase at –5 °C is likely to be suppressed in *C. psychrophilum*.

Another significant difference between the lipids from cells grown at the two temperatures is the presence of EtnPGlcNAcDRG in cells grown at the lower growth temperature and its near absence at the optimal growth temperature. Notably, at the optimal growth temperature, we observe the presence of an EtnP derivative of MGDG, presumably containing the same sugar present in MGDG. The phase behavior of these recently described lipids is unknown. The interplay between these modifications will only be understood once they have been purified and their individual and ensemble physical properties have been analyzed.

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Abbreviations

DGDG	diglucosyldiacylglycerol
DGDRG	diglycosyldiradylglycerol (diradyl indicates the presence of diacyl and alk-1 -enyl, acyl glycerolipids)
EtnP-GlcNAcDRG	ethanolaminephosphate-N-acetylglucosaminyldiradylglycerol
EtnPMGDRG	ethanolaminephosphate monoglycosyldiradylglycerol
LC-ESI/MS	liquid chromatography-electrospray ionization mass spectrometry
MGDG	monoglucosyldiacylglycerol
MGDRG	monoglycosyldiradylglycerol
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PS	phosphatidylserine
TLC	thin layer chromatography

Fig. 1. Analysis by 2D-TLC of the total lipids from *C. psychrophilum*. Based on their positions relative to standards and specific staining the major lipids were tentatively identified as: (1) unknown, (2) artifact, (3) monoglycosyldiradylglycerol, (4) cardiolipin plus PG, (5) diglycosyldiradylglycerol, (6) PE, (7) PS and/or lyso-PE, and (8) ethanolaminephosphate N-acetylglucosamine diradylglycerol. The symbols indicate: * -naphthol-positive, † molybdenum blue-positive, and # ninhydrin-positive. Arrows indicate the first and second dimensions of the chromatogram.

Fig. 2. Total ion current from LC/MS of lipids from *C. psychrophilum* grown at (A) -5°C and (B) $+5^{\circ}\text{C}$. Abbreviations: DRG, diradylglycerol; MGDRG, monoglycosyldiradylglycerol; DGDRG, diglycosyldiradylglycerol; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; EtnPMGDRG, ethanolaminephosphate monoglycosyldiradylglycerol and EtnPGlcNAcDRG, ethanolaminephosphate N-acetylglucosamine diradylglycerol. The intensity of the peaks for different lipids is not correlated with the abundance of lipids as they have different ionization efficiencies.

Fig. 3. Negative ion ESI/MS of the lipids emerging at 2.0 to 5.0 min from *C. psychrophilum* grown at (A) −5 °C and (B) +5 °C. Abbreviations: DRG, diradylglycerol; MGDRG, monoglycosyldiradylglycerol; and DGDRG, diglycosyldiradylglycerol.

Fig. 4.

Negative ion ESI/MS of the lipids emerging at 20.5 to 22.0 min from *C. psychrophilum* grown at (A) -5°C and (B) $+5^{\circ}\text{C}$. Abbreviations: EtnPGlcNAcDRG, ethanolaminephosphate N-acetylglucosamine diradylglycerol and EtnPMGDRG, ethanolaminephosphate monoglycosyldiradylglycerol. Pla indicates the plasmalogen species and the number before the colon indicates the total number of carbons in the two chains and the number after the colon indicates the number of double bonds.

Table 1

Molecular species of *C. psychrophilum* diradylglycerol and glycolipids lipids seen by ESI/MS. The number before the colon indicates the total number of carbons in the two chains and the number after the colon indicates the number of double bonds.

Major molecular species	Diacyl forms [M+Cl] ⁻	Plasmalogen forms [M+Cl] ⁻
Diradylglycerol, 30:2	571.47 ^a	
Diradylglycerol, 30:1	573.44	557.45
Diradylglycerol, 30:0	575.44	559.45
Diradylglycerol, 31:0	587.47 ^a	
Diradylglycerol, 32:2	599.46	583.46
Diradylglycerol, 32:1	601.50 ^c	585.47
MGDRG, 30:1	735.50 ^a	719.60 ^b
MGDRG, 32:0	749.52 ^a	
MGDRG, 31:2	761.52	
DGDRG, 32:0		911.70 ^c
DGDRG, 34:1	953.73 ^{b,c}	937.72 ^c
DGDRG, 34:0		939.72 ^c
DGDRG, 36:0	983.76 ^c	
EtnPGlcNAcdiradylglycerol, 30:0		849.53 ^{c,d}
EtnPGlcNAcdiradylglycerol, 32:1		875.56 ^{c,d}
EtnPGlcNAcdiradylglycerol, 34:1		903.58 ^{c,d}
EtnPGlcNAcdiradylglycerol, 34:2		901.57 ^{c,d}
EtnPMGDRG, 31:0		822.56 ^{a,d}
EtnPMGDRG, 32:0		836.58 ^{a,d}
EtnPMGDRG, 33:1		848.58 ^{a,d}
EtnPMGDRG, 34:1		862.58 ^{a,d}

^a In cells grown at +5 °C.

^b Minor species.

^c In cells grown at -5 °C. Absent or minor in cells grown at +5 °C.

^d [M-H]⁻.

Table 2

Molecular species of *C. psychrophilum* phospholipids seen by ESI/MS. The number before the colon indicates the total number of carbons in the two chains and the number after the colon indicates the number of double bonds.

Major molecular species	Diacyl or tetracyl forms	Plasmalogen forms
	[M-H] ⁻	[M-H] ⁻
PE, 30:2	658.48 ^a	
PE, 30:1	660.47	644.48
PE, 31:1	674.50 ^b	
PE, 32:2	686.49	670.50
PE, 32:0	690.52	
PE, 34:2		698.00 ^a
PG, 30:1	691.48	675.49 ^c
PG, 30:0	693.47	
PG, 31:1	705.48 ^b	
PG, 32:1	719.47 ^c	
PG, 32:0	721.51	
PG, 34:2	745.52	731.51
Cardiolipin, 60:1	1293.91	1277.90
Cardiolipin, 60:0	1295.89	1279.90
Cardiolipin, 62:2	1319.90	1303.91 ^d
Cardiolipin, 62:1	1321.91	1305.87
Cardiolipin, 64:3	1345.93	1331.95
Cardiolipin, 64:2	1347.96	1333.95 ^d
PS, 30:1	704.45	
PS, 30:0	706.48	
PS, 31:1	718.47 ^b	
PS, 32:1	732.45	
PS, 32:0	730.47	
Lyso-PE 14:0	424.25	
Lyso-PE 16:1	450.27	

^a More abundant in cells grown at +5 °C than in cells grown at -5 °C.

^b Much more abundant in cells grown at +5 °C than in cells grown at -5 °C. The presence of a 17:1 chain was confirmed by MS/MS (data not shown).

^c More abundant in cells grown at -5 °C than in cells grown at +5 °C.

^d Species containing 17:1 chains were also found in cells grown at +5 °C.