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Dialkylether polar lipids in *Leucobacter exalbidus*

Harumi Ue[1,2)]*, Akira Yokota[2)] and Akinori Ohta[3)]

[1)]Medicinal Chemistry Research Laboratories, KYOWA HAKKO KIRIN Co., Ltd.
3-6-6, Asahi-machi, Machida-shi, Tokyo, 194-8533, Japan
[2)]Institute of Molecular and Cellular Biosciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[3)]Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

For the chemotaxonomic classification of microorganisms, polar lipid patterns are often used to distinguish different types of viable microorganisms. In this study, the cellular polar lipid composition of *Leucobacter exalbidus* was analyzed by Liquid Chromatography ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS). By LC-ESI-MS analysis, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) as well as several unknown compounds were found. Furthermore, the results from LC-ESI-MS showed that two dialkylether polar lipids were present in *Leucobacter exalbidus*. Dialkylether polar lipids are primarily found in hyperthermophilic bacteria, archaea, and sulfate-reducing bacteria. Based on the data obtained from LC-ESI-MS, it was determined that two ether phospholipids were dialkyl (33:0) phosphatidylglycerol, and dialkyl (32:0) phosphatidylglycerol. In this study, dialkyl ether polar lipids were found in the phylum Actinobacteria for the first time.

Key words: Ether polar lipids, *Leucobacter*, Polar lipids, LC-ESI-MS, Actinobacteria

INTRODUCTION

For the chemotaxonomic classification of microorganisms, polar lipid patterns obtained by separation with thin layer chromatography have been widely applied. The resulting data about polar lipid patterns allow taxonomic differentiation. In recent years, direct structural analysis of intact polar lipids has become possible with Liquid Chromatography ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS), which can be applied to characterize bacterial isolates. LC-ESI-MS is a powerful tool for the determination of accurate molecular masses. Using this technique, information about phospholipid types and their corresponding side chains is obtained. In addition, the binding between side chains and the head groups of polar lipids can be directly estimated.

The characteristics unique to archaeal lipids compared with bacterial lipids have been reported by Kates (1993) and Koga *et al*., (1993). Differences in characteristics between bacterial and archaeal polar lipids are shown below. First, the glycerophosphate backbone of bacterial polar lipids are composed of *sn*-glycerol-3-phosphate (G-3-P), which is an enantiomer of *sn*-glycerol-1-phosphate (G-1-P) present in archaeal polar lipids. In bacterial polar lipids, hydrocarbon chains are bonded to the glycerol moiety exclusively by ester linkages between fatty acids and the glycerol moiety, in contrast to archaeal polar lipids, most of which have ether linkages. The hydrocarbon chains of polar lipids of bacteria are mostly straight or iso or anteiso methyl-branched chain fatty acids, whereas they are highly methyl-branched isoprenoids and isoprenoids in archaea. Significant numbers of archaea species contain bipolar lipids with a tetraether core that spans through the membrane (De Rosa *et al*., 1983). Such bipolar tetraether lipids are also found, albeit rarely, in bacteria (Clarke *et al*., 1980).

The presence of ether linkages in the membrane lipids (linking the fatty acids to the glycerol backbone) of bacteria have only been observed a few times, whereas all described archaea possess polar lipids with ether-linked isoprenoidal side chains (Kates, 1997; Koga *et al*., 1998). The characteristic ether polar lipids of anaerobic eubacterium are plasmalogen, which in their natural form are mainly found as 1-o-alkyl-2-acyl glycerophosphatides, and were detected in strict anaerobe, *Clostridium spp*.

*Corresponding author
E-mail: harumi.ue@kyowa-kirin.co.jp
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and the rumen bacteria (Kamio *et al*., 1969; Goldfine & Hagen, 1972; Fischer *et al*., 1994).

Although diether polar lipids are rare amongst bacteria, they have been detected in thermophiles (Langworthy *et al*., 1983; Huber *et al*., 1992, 1996), in sulfate-reducing bacteria capable of anaerobic oxidation of methane (Hinrichs *et al*., 2000; Pancost *et al*., 2001), and in the myxobacterium *Stigmatella aurantiaca* (Caillon *et al*., 1983). Mixed ether/ester polar lipids were found in deep-branching thermophilic bacteria such as Aquifex (Huber *et al*., 1992) and *Thermotoga* (De Rosa *et al*., 1988; Carballeira *et al*., 1997), in two mesophilic sulfate-reducing bacteria (Rütters *et al*., 2001), in *S. aurantiaca* (Caillon *et al*., 1983) and in *Propionibacterium propionicum* (Pasciak *et al*., 2003). The ether polar lipids are always not limited to, species representing early branches of bacteria. We recently have described the strain K-540B as a new species '*Leucobacter exalbidus*' (Ue, 2011), therfore we tried to identify the structure of the polar lipids in the strain K-540B in detail the determining of accurate molucular masses by LC-ESI-MS. We discovered that the polar lipid composition of strain K-540B belongs to the phylum *Actinobacteria* using LC-ESI-LS.

METHODS AND MATERIALS

Materials (Organism)

The actinobacterial strain used in this study was strain K-540B (=NBRC 106062^{T}). It was separated from mixed culture isolated from compost, and is a Gram-positive, non-motile, short rod bacterium, which grows at 4℃ and not at 37℃ on Luria-Bertani (LB) agar (Difco Laboratories, Detroit, Mich). The strain grows in a pH range of 6-10 and the optimum pH is 7.0-7.5. The strain K-540B represents a novel species of the genus *Leucobacter* in the family *Microbacteriaceae* within the suborder *Micrococcineae* (Ue, 2011).

Extraction of polar lipids

Cells were cultured in LB medium at 28℃ and harvested at the end of the exponential growth phase by centrifugation, washed with distilled water, freeze dried, and stored at −80℃. Total polar lipid was extracted as per the method described by Minnikin *et al*. (1984). Two milliliter of methanol-water mixture (0.3% aqueous sodium chloride) (100:10 v/v) and 2 m*l* of petroleum ether were added to the dry organism (50 mg), and were mixed on tube rotator for 15 min. After phase separation, the upper layer was removed, and 1 m*l* of petroleum ether was transferred to the lower layer and mixed. The upper layer was removed by centrifugation. The lower layer was boiled in a water bath for 5 min, cooled at 37℃ for 5 min, and mixed for 60 min by adding 2.3 m*l* chloroform-methanol-water mixture (90:100:30 v/v/v). After centrifugation the supernatant was transferred to 8.5 m*l* tube (step 1). A 0.75 m*l*-volume of chloroform methanol-water mixture (50:100:40 v/v/v) was added to the precipitate, mixed for 30 min, and the supernatant was combined with above (step 2). Step 2 was repeated (step 3). Chloroform (1.3 m*l*) and water (1.3 m*l*) were added to the combined supernatants from steps 1-3, mixed for 30 min, and the upper layer was removed by centrifugation. The lower layer was evaporated with N_2.

LC-ESI-MS analysis of polar lipids

The LC-ESI-MS analysis of polar lipids was performed on the Qstar XL mass spectrometer (Applied Biosystems, CA, USA). HPLC separation was achieved on the 5-μm, 4 mm×125 mm Lichrospher 100 Diol column (Agilent Technologies, CA, USA). A flow rate of 1.0 m*l* min^{-1} was employed with the following solvent gradient: 0 min 0% B, increasing over 15 min to 65% B using a concave curvature, holding isocratically for 5 min, followed by 40 min of reconditioning. Eluent A was a mixture of n-hexane/i-propanol/formic acid/ammonia (25% solution on water) (79:20:1.2:0.04 by volume). Eluent B was a mixture of i-propanol/water/formic acid/ammonia (25% solution on water) (88:10:1.2:0.04 by volume). The mass spectrometer was set to the positive-ion mode with a spray voltage of 4.0 kV and the negative-ion mode with a spray voltage of −3.5 kV and a capillary temperature of 350℃.

RESULTS

The HPLC conditions were optimized using polar lipid standards to separate different polar lipid types, indicating that the head group was the main factor in controlling retention times. Differences in fatty acid side chains led to minor shifts in retention times, so that compounds with longer side chains eluted earlier than those with shorter substituents. In *Leucobacter exalbidus*, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) was the major type of phospholipid (Table 1). In the only negative

mode of LC-ESI-MS, a base ion $[M-H]^-$ for PG (33:0) was detected in m/z 735.5209, and base ions $[M-H]^-$ for several unknown polar lipids were detected in m/z 909.5732, 923.5831, 937.6059, 977.5761, 991.5930, 1005.6097 in addition to PG and DPG. Substitutions of an ester bond to an ether bond shifted earlier retention times for PG. Thus, retention times allowed distinguishing different phospholipid types, and dialkyl side chains from diacyl side chains. In the positive mode, sodium adduct ions $[M+Na]^+$ for PG (33:0) and PG (32:0) with dialkyl ether were observed in m/z 731.5608 and 717.5484, respectively (Table 1; Fig. 1). A hydrogen adduction $[M+H]^+$ for PG (32:0) with dialkyl ether was observed in m/z 695.5577. These values were in good agreement with those of the calculated values (Table 1). Ether and ester-lipids of equal nominal mass cannot be differentiated by their retention times only, but also by their different accurate masses, which are easily distinguishable by modern MS instruments (717.5410 for C38 H79 O8 Na1 P1 (dialkyl ether lipid with 2 additional carbon units) and 717.4683 for C36 H71 O10 Na1 P1 (diacyl ester lipid).

DISCUSSION

The polar lipid patterns of *Leucobacter exalbidus* are PG, DPG, and several unknown compounds, which display the characteristics of the genus *Leucobacter*. Furthermore, the results from LC-ESI-MS showed that two dialkylether phosphatidylglycerols are present in *Leucobacter exalbidus*. Previously, only *P. propionicum* (Pasciak *et al*., 2003) was reported to have a monoalkyl ether polar glycolipid, but till date dialkyl ether phospholipids have not been found in the phylum *Actinobacteria*. In this study, dialkyl ether polar lipids were found in the phylum Actinobacteria for the first time. An example of an ether polar lipid that was not limited to species representing early branches in the bacterial domain is shown.

The presence of ether bonds in place of ester bonds has been considered as an adaptation for existence at high temperatures, however an ether bond has only a small effect on polar lipid packing

Table 1 Measured and calculated m/z values for the molecular ions of the polar lipids from *Leucobacter exalbidus* in the positive mode

Retention time on LC (min)	Ion	Observed m/z	Calculated m/z	Δ m[a] mDa	Error[b] ppm	Identification[c]	Identification[d] linkages
9.15	$[M+Na]^+$	731.5608	731.5567	4.10	5.60	PG (33:0)	**EE**
9.31	$[M+H]^+$	695.5577	695.5591	−1.40	−2.01	PG (32:0)	**EE**
	$[M+Na]^+$	717.5484	717.5410	7.40	10.31		
10.99	$[M+H]^+$	723.5200	723.5176	2.40	3.32	PG (32:0)	**AA**
	$[M+Na]^+$	745.4984	745.4996	−1.20	−1.61		
11.09	$[M+H]^+$	709.5049	709.5020	2.90	4.09	PG (31:0)	**AA**
	$[M+Na]^+$	731.4835	731.4839	−0.40	−0.55		
11.29	$[M+H]^+$	695.4849	695.4863	−1.40	−2.01	PG (30:0)	**AA**
	$[M+Na]^+$	717.4697	717.4683	1.40	1.95		
11.43	$[M+H]^+$	681.4709	681.4707	0.20	0.29	PG (29:0)	**AA**
	$[M+Na]^+$	703.4526	703.4526	0.00	0.00		
12.17	$[M+H]^+$	1353.9773	1353.9801	−2.80	−2.07	DPG (64:0)	**AAAA**
	$[M+Na]^+$	1375.9569	1375.9620	−5.10	−3.71		
12.23	$[M+H]^+$	1339.9615	1339.9644	−2.90	−2.16	DPG (63:0)	**AAAA**
	$[M+Na]^+$	1361.9402	1361.9463	−6.10	−4.48		
12.34	$[M+H]^+$	1325.9408	1325.9488	−8.00	−6.03	DPG (62:0)	**AAAA**
	$[M+Na]^+$	1347.9273	1347.9307	−3.40	−2.52		
12.46	$[M+H]^+$	1311.9251	1311.9331	−8.00	−6.10	DPG (61:0)	**AAAA**
	$[M+Na]^+$	1333.9096	1333.9150	−5.40	−4.05		
12.63	$[M+H]^+$	1297.9185	1297.9175	1.00	0.77	DPG (60:0)	**AAAA**
	$[M+Na]^+$	1319.8942	1319.8994	−5.20	−3.94		
12.70	$[M+H]^+$	1283.8932	1283.9018	−8.60	−6.70	DPG (59:0)	**AAAA**
	$[M+Na]^+$	1305.8713	1305.8837	−12.40	−9.50		

[a] observed mass − calculated mass, [b] [(observed mass − calculated mass)/calculated mass] × 100, [c] PG: phosphoglycerol; DPG: diphosphatidylglycerol, [d] A: acyl linkage; E: ether linkage

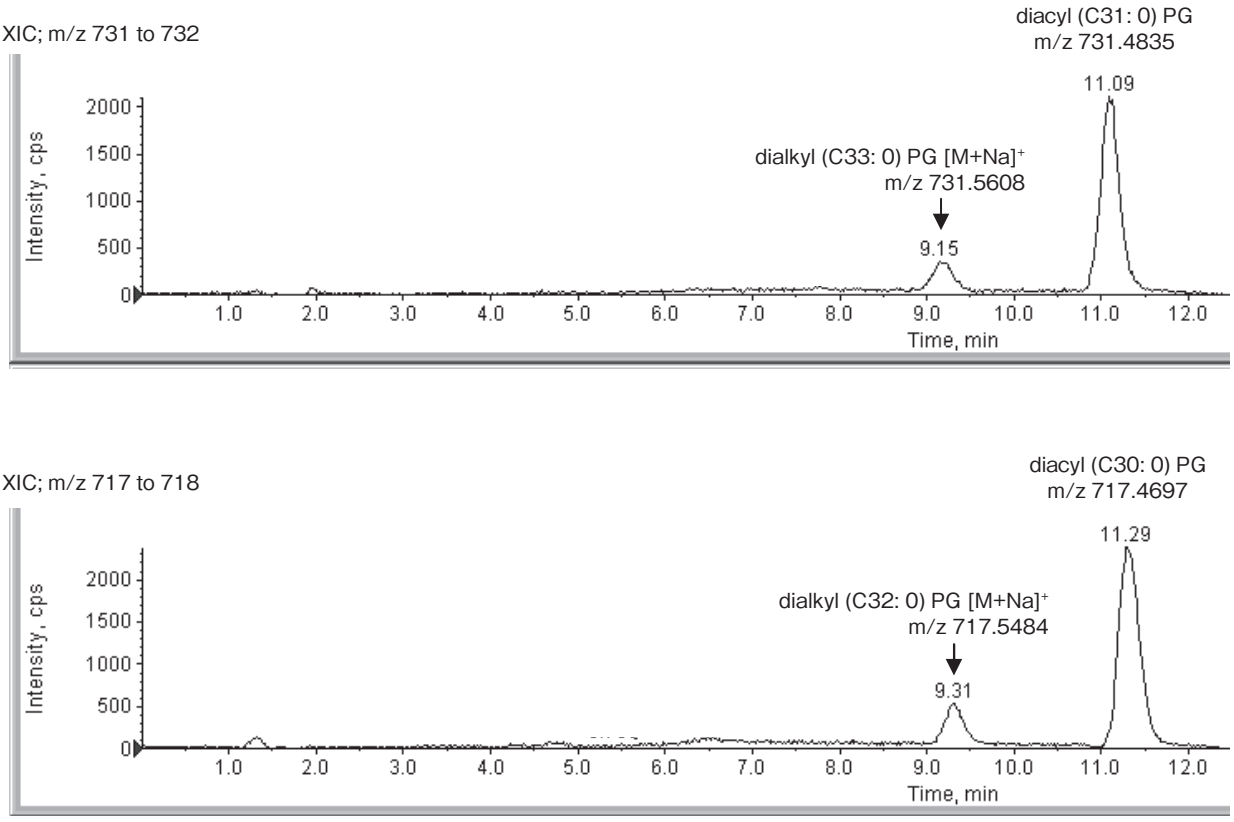

Fig. 1 The extracted ion chromatograms of polar lipids fraction of *Leucobacter exalbidus*

(Paltauf, 1983). The main difference between ester and ether bonds is that ether bonds have greater resistance to chemical or enzymatic attack, which would be significant in extremely acidic or alkaline conditions in which several extreme thermophiles live. The universal presence of ether-linked phytanyl lipids in both methanogens and extreme halophiles, as well as in extreme thermophiles, is an indication that these lipids are a phylogenetic trait that provide membrane stability in a range of extreme environments, not just hot ones (Russell & Fukunaga, 1990). *Leucobacter exalbidus* does not grow in extreme environments but contains dialkyl ether polar lipids. The detection of alkyl ether polar lipids in mesophilic sulfate-reducing bacteria, the myxobacterium, *S. aurantiaca*, *P. propionicum* (Pasciak *et al*., 2003), and *Leucobacter exalbidus* shows that these ether lipids may be much more common membrane lipids in bacteria than previously thought. Some of the soil bacteria secrete lipase and separate the fatty acid of the membrane polar lipid causing lyses. Furthermore, when the bacteria are eaten by predators such as nematodes they are exposed to enzymes. The ether bonds may be regarded as an adaptation for this type of enzymatic decompositions. The alkyl ether polar lipids might be found from more microorganisms by the development of the analysis technique. Moreover, the meaning of the existence of ether bonds might become clear as a result.

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Leucobacter exalbidus のジアルキルエーテル型リン脂質

植　晴美[1,2]，横田　明[2]，太田明徳[3]

[1]協和発酵キリン株式会社創薬化学研究所，[2]東京大学分子細胞生物学研究所，[3]東京大学生物生産工学研究センター

微生物の化学分類において，リン脂質のパターンは，様々な微生物の異なるタイプを識別するのによく利用される．本研究では *Leucobacter exalbidus* のリン脂質の組成を液体クロマトグラフィー・エレクトロスプレーイオン化マススペクトロメトリー（LC-ESI-MS）で分析した．その結果，*Leucobacter exalbidus* のリン脂質成分は，未知物質以外に，主成分として phosphatidylglycerol 及び diphosphatidylglycerol を含んだ．さらに詳細な解析により，*Leucobacter exalbidus* にはジアルキルエーテル型リン脂質が存在することが示された．ジアルキルエーテル型リン脂質は，主に高度好熱菌，古細菌及び硫酸還元菌で見出されている．LC-ESI-MS のデータ解析により，2 つのエーテル型リン脂質 dialkyl（33:0）phosphatidylglycerol 及び dialkyl（32:0）phosphatidylglycerol が同定された．本研究において初めて *Actinobacteria* 門からジアルキルエーテル型リン脂質が見出されたことになる．

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