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Lipid analysis of *Methanopyrus kandleri*

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Abstract

Non-polar and polar lipids were isolated from *Methanopyrus kandleri*. Non-polar lipids accounted for 50% w/w of total lipids, with a high proportion of 2,3-di-*O*-geranylgeranyl-sn-glycerol, 2,3-di-*O*-phytanyl-sn-glycerol and geranylgeraniol. The core lipids prepared by mild acid methanolysis consisted exclusively of 2,3-di-*O*-phytanyl-sn-glycerol. Two-dimensional TLC showed mostly glycolipids, and minor amounts of aminophospholipids, phosphoglycolipids and phospholipids. The purification yielded three diglycosyl-lipids (50% of total polar lipids), one triglycosyl-lipid (5%) and six glycosyl-lipids with five glycosyl-groups (36%), which consisted of glucose, galactose and mannose. The lipid analysis supports the unique position of *Methanopyrus kandleri* within the 16S rRNA-based phylogenetic tree.

Keywords: *Methanopyrus kandleri*; 2,3-di-*O*-geranylgeranyl-*sn*-glycerol; Lipid composition; Glycolipid

1. Introduction

Methanopyrus kandleri is a submarine hot vent organism with an upper temperature border of growth at 110°C [1,2]. Phylogenetic studies based on 16S rRNA sequences indicate that this organism represents the deepest phylogenetic branch-off in the *Euryarchaeota* kingdom within the archaeal domain [3,4]. Based on its deep, short phylogenetic lineage and its exceedingly high growth temperature, *Methanopyrus* is a rather primitive organism [4,5]. So far no complete lipid study from *Methanopyrus kandleri* has been reported [2]. Recently we described the novel fully unsaturated ether lipid 2,3-di-*O*-geranylgeranyl-*sn*-glycerol as a major compound in the membrane of *Methanopyrus kandleri* [6]. In the biosynthesis of polar lipids the fully unsaturated pre-diethers are linked stepwise with polar groups and finally reduced to the saturated membrane lipids. Therefore we expected to find polar ether lipids with 2,3-di-*O*-geranylgeranyl-*sn*-glycerol as core lipid. Here we describe the analysis of non-polar and complex membrane lipids of *Methanopyrus kandleri*.

2. Materials and methods

Methanopyrus kandleri isolates AV19 (Guayamas Basin, Mexico), KOL6 (Kolbensey, Iceland), GC34 (Guayamas basin, Mexico) were grown in 300 l batches as described [2]. Cells were harvested using a continuous flow centrifuge and stored under liquid nitrogen.

For the extraction of all lipids 400 g of cells (wet weight) were divided into two parts. Two hundred

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grams of the cells were extracted as described previously to get the unpolar fraction [6]. Polar lipids were extracted by the TCA method of Nishihara and Koga [7].

The following solvents (composition in volume ratio) were used for HPTLC on Silica gel 60 plates: solvent A: chloroform-methanol-7 M aqueous ammonia (60:35:8), solvent B: chloroform-methanol-acetic acid-water (85:30:15:5), solvent C: *n*-hexane-ethyl acetate (4:1) and solvent D: isopropanol-water-ammonia (8:2:1). Polar lipids were separated by two-dimensional TLC with solvent A in the vertical direction and solvent B in the horizontal direction. Core lipids were chromatographed with solvent C. Lipid spots on TLC plates were detected by using the following spray reagents: ninhydrin for amino groups, acid molybdate for phospholipids, 0.5% α-naphthol reagent for glycolipids and anisaldehyde for all lipids. Hydrolyzed sugars were chromatographed with solvent D using anisaldehyde for visualization.

For the purification of the non-polar lipids the dried cell extract was dissolved into 10 ml of chloroform. For preparative scale purification, the chloroform solution was mixed with 10 g of silica gel (silica gel 60, 0.04–0.063 mm, Merck, Germany) and evaporated to dryness. This material was added to a column (30 × 5 cm). Gradient elution with *n*-hexane/ethyl acetate gave 669 mg of non-polar lipids. The non-polar lipids were purified by silica gel chromatography using chloroform as eluent (30 × 5 cm, silica gel 0.015–0.04 mm, Merck, Germany).

For the purification of the polar lipids the dried TCA-extract was dissolved into 10 ml chloroform/methanol (1:1, v/v) mixed with 10 g of silica gel (silica gel 60, 0.04–0.063 mm, Merck, Germany) and evaporated to dryness. This material was added to a 30 × 5 cm glass column. Chromatography was performed in gradient steps using chloroform methanol mixtures. For further purification of the polar lipid–compound flash chromatography was used, eluting with chloroform and methanol in various ratios.

Acid hydrolysis of the lipids was performed using 2 M HCl at 100°C for 2 h. After drying, the hydrolysis products were extracted with $CHCl_3$ and H_2O. The $CHCl_3$ and H_2O and soluble fractions were examined chromatographically.

HPLC analysis of hydrolyzed sugars was performed on a Aminex ion exclusion (HPX-87H, 300mm × 7.8 mm, BioRad, CA, USA) with a flow rate of 0.6 ml/min using 0.005 M H_2SO_4 as solvent. For detection a RI-detector (ERC, Germany) was used.

Mass spectra were taken with Finnigan MAT 95 (FAB; matrix: methanol/TEA).

3. Results and discussion

The cellular content of total lipids from *Methanopyrus kandleri* (type strain AV19) was in the range of 1.8% of dry cells. Non-polar lipids accounted for 50% of total lipids. TLC of the non-polar lipids showed the presence of 2,3-di-*O*-geranylgeranyl-*sn*-glycerol, 2,3-di-*O*-phytanyl-glycerol and geranyl-geraniol as main components. Purification of the non-polar lipid fraction by chromatography on silica gel gave 44% 2,3-di-*O*-geranylgeranyl-*sn*-glycerol, 16% 2,3-di-*O*-phytanyl-glycerol, 14% geranylgeraniol and 26% minor unidentified compounds. Similar percentages of non-polar lipids, in particular the significant quantity of 2,3-di-*O*-geranylgeranyl-*sn*-glycerol, was found in the *Methanopyrus* isolates KOL6 and GC34 from different sample sites. The non-polar lipid fraction from the related *Methanobacterium thermoautotrophicum* (21% of total lipids) consisted of mostly acyclic isoprenoid hydrocarbons. The major components were squalene and a continuous range of hydrosqualene derivatives [8]. The other neutral lipids, with less than 5% relative percentage concentration consisted of isoprenoid alkenes, alkanes, and small quantities of free phytanyl diethers and dibiphytanyl-diglycerol tetraethers. In some other methanogens a nonpolar lipid content up to 30% was reported in which isoprenoids and hydroisoprenoid hydrocarbons can comprise up to 95% of the non-polar lipid fraction [8–10].

Core lipids of *Methanopyrus kandleri* prepared by mild acid hydrolysis consisted exclusively of 2,3-di-*O*-phytanyl-*sn*-glycerol (archaeol). According to these investigations no 2,3-di-*O*-geranylgeranyl-*sn*-glycerol was found as a core lipid. Archaeol is present in all species of methanogens examined so far. Tetraether core lipids were found in the

Methanobacteriaceae, thermophilic *Methanococcaceae*, and *Methanomicrobiaceae* but not in *Methanosarcina* and mesophilic *Methanococcus* species. Hydroxyarchaeol core lipids were present in *Methanosarcina*, *Methanotrix*, and mesophilic *Methanococcus* species [11].

The polar lipids of *Methanopyrus kandleri* were separated by two-dimensional TLC (Fig. 1). They were classified on the basis of specific staining responses into four groups, glycolipids (5 spots, designated as GL 1–5), aminophospholipids (3 spots, designated as APGL), phosphoglycolipids (2 spots, designated as PGL) and phospholipids (1 spot, designated as PL). The predominant polar lipids (more than 92%) were glycolipids.

The purification of the glycolipids yielded 10 fractions (Table 1). The major component of the polar lipids (lipid I) was subjected to negative FAB-MS and identified as C_{20}-C_{20} species with two glycosidic groups: $[M\text{-}H]^-$, m/z 975. The lipid showed ^{1}H-NMR signals (in CD_3OD) expected for saturated isopranoid ether linked chains and glucosyl groups: 0.8 ppm (isopranoid CH_3), 1.0–1.1 ppm ($(CH_3)_2C-$), 1.2–1.3 ppm (CH_2), 3.4–3.9 ppm (glycosidic CH and OH). Acid hydrolysis of lipid I yielded galactose and mannose as the water soluble products identified by TLC in solvent D and HPLC. Diphytanylglycerol was confirmed as the hydrophobic product, identified by TLC in solvent C. In contrast, the glycolipids of most methanogens contain mono- or diglucose residues. Only a few species were known to contain sugars other than glucose in their glycolipids.

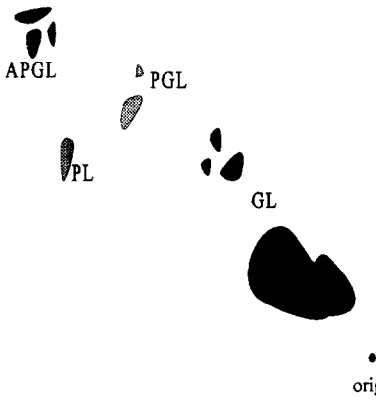

Fig. 1. Two-dimensional thin-layer chromatogram of polar lipids of *Methanopyrus kandleri*. Solvents A (chloroform-methanol-7 M aqueous ammonia, 60:35:8) and B (chloroform-methanol-acetic acid-water, 85:30:15:5) were used in the vertical and horizontal directions, respectively. PL, phospholipid; PGL, phosphoglycolipid; APGL, aminophosphoglycolipid; GL, glycolipid.

Table 1
Composition and chromatographic mobilities of purified glycolipids from *Methanopyrus kandleri*

Component	Total polar lipids (wt.%)	R_F-value	Solvent system
Lipid I	45	0.7	$CHCl_3$/MeOH (3:1)
Lipid II	5	0.8	$CHCl_3$/MeOH (2:1)
Lipid III	1.5	0.35	$CHCl_3$/MeOH (1:1)
Lipid IV	13.5	0.3	$CHCl_3$/MeOH (1:1)
Lipid V	4	0.2	$CHCl_3$/MeOH (1:1)
Lipid VI	5	0.1	$CHCl_3$/MeOH (1:1)
Lipid VII	4	0.8	$CHCl_3$/MeOH (1:2)
Lipid VIII	3	0.7	$CHCl_3$/MeOH (1:2)
Lipid IX	4	0.7	$CHCl_3$/MeOH (1:3)
Lipid X	7	0.5	$CHCl_3$/MeOH (1:3)

Negative FAB-MS, TLC and HPLC analysis of the glycolipids showed the presence of two other diglycosyl-lipids (lipid III and lipid IX) and one triglycosyl-lipid (lipid II) with galactose, glucose and mannose as glycosyl groups.

Negative FAB-MS of lipids IV-XIII and lipid X showed a molecular ion peak, $[M\text{-}H]^-$, with m/z 1461.5 and the fragmentation of 1299.7, 1137.5, 975.6, 813.6. This corresponds to C_{20}-C_{20} molecular species with five glycosyl groups. After hydrolysis, the water soluble products were identified by TLC and HPLC as mannose, galactose and glucose in different combinations. Further investigations are in progress to elucidate the structures of these glycolipids.

Methanogens display a high diversity in their mostly phosphate-containing polar head groups and core lipids. This variety could be used for a chemotaxonomy of methanogens. Sets of core lipids, polar head groups and glycolipid sugars were correlated to a family-level taxonomy of methanogens based on 16S rRNA [11,12]. *Methanopyrus kandleri* is unique among methanogens because of the presence of only one core-lipid and the huge quantity of simple glycolipids. This lipid composition supported the outlying position of *Methanopyrus kandleri* within the 16S rRNA-based phylogenetic tree [4,5]. In addition,

the high level of non-polar glycerol ether lipids in the membrane distinguishes *Methanopyrus kandleri* from all investigated *archaea*.

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