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# Novel sulfated phosphoglycolipids from *Natronomonas moolapensis*

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ABSTRACT

Polar lipid pattern determination is often used for the taxonomic classification of halophilic *Archaea* in addition to a genomic characterization. During the analysis of polar lipid extracts from the recently described haloarchaeon *Natrononomonas moolapensis*, an unknown glycolipid was detected. Fragmentation patterns observed from preliminary mass spectrometric analysis initially suggested the presence of a sulfo-hexosyl-phosphatidylglycerol. However, by NMR spectroscopy and enzymatic assays the existence of two isomeric molecules with different hexoses (1-(6-sulfo-D-glcp/galf-β1,2-glycero)-phospho-2,3-diphytanylglycerol) could be shown. The structural origin from phosphatidylglycerol distinguishes these glycolipids within *Archaea*, because all other characterized haloarchaeal glycolipids consist of diphytanylglycerol directly linked to an oligoglycosyl moiety. Now the door is open to investigate the physical and functional consequences of these architectural differences of the head groups.



## 1. Introduction

*Archaea* of the family *Halobacteriaceae* populate habitats with extremely high salinity. An overall increase in the fraction of polar lipids and the presence of some unique molecular species in the cell membrane, e.g., phosphatidylglycerol sulfate (PGS), allow them to survive at sodium chloride concentrations almost up to saturation (Russell, 1989; Tenchov et al., 2006). Besides phospholipids, the glycolipids are an important membrane constituent of several halophilic *Archaea* in this context (Oren, 2006). The explicit physiological role of the glycolipids is not completely clear. In liposomes reconstituted from polar lipids of *Halobacterium salinarum*, the presence of the sulfated triglycosylarchaeol S-TGA-1 lowers the ion permeablility of the membrane (Höjeberg et al., 1982). At the same time, S-TGA-1 is a hitherto exclusive case of an archaeal glycolipid shown to specifically affect membrane protein stability and function. In the cell membrane of *H. salinarum*, S-TGA-1 is almost exclusively associated to the light-activated proton pump bacteriorhodopsin (BR) (Weik et al., 1998). This protein is arranged in crystalline lattices of homotrimers resulting in purple membrane patches. The glycolipid–protein interaction appears to be involved in trimer stabilization in vivo (Essen et al., 1998) and is also required for optimal reconstitution of BR in proteo-liposomes in vitro (Höjeberg et al., 1982). Moreover, the charged head group allows participation in long-range proton conduction on the membrane surface away from BR (Teissie et al., 1990). Yet, not all glycolipid-containing halophilic *Archaea* produce BR or related proteins implying alternative glycolipid functions.

All glycolipids observed so far in *Halobacteriaceae* share a common core structure based on archaeol, a diether glycerol, glycosylated with a di-, tri- or tetrahexose chain (Oren, 2006; Kates, 1993). The diglycosyl residue α-D-mannosyl(1-2)-α-D-glucose resembles the parental pattern for the majority of the known glycolipid modifications. Frequently, sulfation of the sugar moieties is observed. Since species of the same haloarchaeal genus usually contain the same glycolipids they can be used as biochemical marker for taxonomic classification (Kamekura and Kates, 1999). Of the genus *Natronomonas* so far only three species

*Abbreviations:* BR, bacteriorhodopsin; DEPT, distortionless enhancement by polarization transfer; DMF, dimethylformamide; HMBC, heteronuclear multiple bond coherence; HSQC, heteronuclear single quantum coherence; MWCO, molecular weight cut-off; NOE(SY), nuclear overhauser effect (spectroscopy); PA, phosphatidic acid; PG, phosphatidylglycerol; PGS, phosphatidylglycerol sulfate; PHA, polyhydroxyalkanoic acids; S-DGA-3, sulfated diglycosylarchaeol-3; S-TGA-1, sulfated triglycosylarchaeol-1; TOCSY, total correlation spectroscopy.

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i.e., the alkaliphilic *Natronomonas pharaonis* and the non-alkaliphilic *Natronomonas moolapensis* and *gomsonensis* were characterized. In *N. pharaonis*—analogous to other alkaliphilic species of *Halobacteriaceae*—glycolipids are absent (Oren, 2006; Tindall et al., 1984). In contrast, in *N. moolapensis* and *gomsonensis* the presence of unidentified glycolipids was detected (Burns et al., 2010; Kim et al., 2013). Interestingly, these glycolipids contain a phosphate moiety (Burns et al., 2010; Kim et al., 2013). Phosphoglycolipids occur rarely in *Halobacteriaceae* and were only previously found in *Halococcus* (Moldoveanu et al., 1990), *Halorientalis* (Amoozegar et al., 2014), and *Natronomonas* strains (Burns et al., 2010; Kim et al., 2013). Herein, the chemical structures of two novel phosphoglycolipids from *N. moolapensis* were resolved by mass spectrometry, enzymatic analysis and NMR spectroscopy.

## 2. Materials and methods

### 2.1. Cultivation of microorganisms

*N. moolapensis* (JCM 14361), *N. pharaonis* (strain Gabara, DSM 2160), and *H. salinarum* (strain R1, DSM 671) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *N. pharaonis* and *H. salinarum* were cultivated as described previously (Falb et al., 2005; Oesterhelt and Krippahl, 1983). *N. moolapensis* was adapted to modified minimal medium DBCM2 (pyruvate replaced by 40 mM sodium acetate) (Dyall-Smith, 2009). Cell mass for preparative lipid extraction was produced in a 10-l New Brunswick bioreactor (airflow 75 l/min, temperature 40 °C). To allow growth to higher cell densities, carbon, nitrogen and phosphorous sources were added during cultivation: at $OD_{600}$ = 0.8–0.9: 4 ml acetic acid and 22 ml 2 M ammonium acetate, at $OD_{600}$ = 1.2 and 2.7–3.0: 10 ml acetic acid, 30 ml 2 M ammonium acetate and 5 ml 0.5 M potassium phosphate (pH 7.4). Cells were harvested after eight days of cultivation by centrifugation for 30 min at 9500 × *g*.

### 2.2. Membrane preparation

25 g of wet cell mass was mixed with 7 ml distilled water and 3 mg DNaseI (Sigma–Aldrich, St. Louis, MO) followed by 2 h stirring at room temperature. To complete cell lysis, the suspension was dialyzed (MWCO: 14 kDa) exhaustively against distilled water. The amount of polyhydroxyalkanoic acid (PHA) bodies in the lysate was reduced by centrifugation for 20 min at 400 × *g* and 10 °C. From the supernatant the whole membrane fraction was collected by centrifugation for 1 h at 113,000 × *g* and 10 °C. The sediments were resuspended in distilled water and pooled for lipid extraction.

### 2.3. Lipid extraction

Polar lipids were isolated by chloroform–methanol–water extraction as described before (Renner et al., 2005; Bligh and Dyer, 1959) and solvent was evaporated under vacuum. PHAs present in the *N. moolapensis* extract interfered strongly with the subsequent preparative TLC. To separate glycolipids from PHA contaminations the solid was extracted four times with 5 ml of dimethylformamide (DMF) each for 3 h and DMF fractions were pooled. After solvent removal DMF extracts were purified by preparative TLC.

### 2.4. Thin-layer chromatography

Lipids were purified and analyzed on silica gel 60A plates (Merck, Darmstadt, Germany; 20 × 20 cm × 0.5 mm or 10 × 10 cm × 0.25 mm, respectively). Elution was performed with solvent A (chloroform–methanol–90% acetic acid; 65:4:35). For detection, TLC plates were charred 10 min at 220 °C after application of 15% sulfuric acid in ethanol. Preparative TLC plates were incubated with iodine vapor and glycolipid was recovered with solvent B (chloroform–methanol; 1:1). For 2D $^{1}$H-$^{31}$P-HSQC and 2D $^{1}$H-$^{31}$P-HSQC–TOCSY NMR experiments the recovered fraction was one time identically re-chromatographed to remove residual PGS. The repurified lipid fraction was subjected to cesium EDTA washing. Cesium EDTA solution was prepared as described before and used as water substitute in chloroform–methanol–water extraction (Meneses and Glonek, 1988; Corcelli et al., 2002). After the first extraction step the aqueous phase was removed and the organic phase was washed three times with 0.3 volumes of pure water to remove excess EDTA.

### 2.5. Enzymatic determination of sugars

To analyze the presence of D-glucose and D-galactose in the glycolipids, a glucose oxidase assay (Sigma–Aldrich, St. Louis, MO) and a galactose oxidase assay (Molecular Probes, Eugene, OR) were performed. Both are specific for the D-isomers. For sample preparation, 0.69–0.75 mg glycolipid was hydrolyzed with 200 μl 2 M trifluoroacetic acid at 120 °C for 1–3 h and the solvent was evaporated subsequently under stream of nitrogen. After 3 times washing with 500 μl methanol, the dried residuals were dissolved in 240 μl water and used for the specific assays. For determination of correction factors due to degradation, D-glucose and D-galactose standards were processed identically. Furthermore samples were analyzed by thin-layer chromatography on silica gel 60A plates (10 × 10 cm × 0.25 mm) using acetone/isopropanol/0.1 M lactic acid as running solvent and anisidine phthalate for staining.

### 2.6. Mass spectrometry

ESI–MS analysis of polar lipid extracts was conducted on a Waters Q-TOF Ultima instrument in negative ionization-mode. The instrumental conditions were as follows: direct infusion flow rate 5 μl/min, nebulizing gas flow 100 l/h, drying gas flow 400 l/h (150 °C), capillary voltage, 1.75 kV, mass range 50–2000 *m*/*z*, calibrant 0.1% phosphoric acid. Data were analyzed using MassLynx 4.1 software (Waters, Eschborn, Germany). High resolution $MS^2$ analysis of purified glycolipid fractions was performed using a Bruker micrOTOF-Q II equipped with an electrospray ionization source set to negative ionization mode. Samples were introduced by direct infusion using a syringe pump at a rate of 3 μl/min. Capillary voltage was set at 2.5 kV and nitrogen gas was used as nebulizing (0.4 bar) and drying gas (4.0 l/min, 180 °C). The instrument was calibrated using a sodium formate calibrant solution (10 mM). $MS^2$ fragmentation of the glycolipids was carried out by collision induced dissociation with a collision energy of 50.0 eV and isolation width of 10 Da. $MS^2$ data were acquired within the mass range *m*/*z* 50–1500 and were analyzed using DataAnalysis 4.0 software (Bruker Daltonik, Bremen, Germany). Calibration of the instrument for $MS^2$ analysis resulted in an RMS of 0.10 ppm for the difference of observed and calculated masses (six reference ions within *m*/*z* 200–2000).

### 2.7. NMR spectroscopy

With one exception, all NMR spectra were recorded at 600.6 MHz ($^{1}$H), 243.1 MHz ($^{31}$P) and 151.0 MHz ($^{13}$C) resonance frequency at a Bruker Avance III spectrometer equipped with a 5 mm QXI probe using TopSpin 2.1. The sample was locked using a 4:3 ratio between $CDCl_3$ and $CD_3OD$ (final lipid concentration about 2 mM). For individual assignment the following experiments were performed: 1D $^{1}$H, 1D $^{13}$C, 1D $^{13}$C DEPT, 1D $^{31}$P, 2D $^{1}$H-$^{13}$C-