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Monoalkylether phospholipids in the sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*

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Abstract In this study, cellular lipid compositions of two mesophilic sulfate-reducing bacteria were analyzed by high performance liquid chromatography-mass spectrometry (HPLC-MS). In *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, alkylether-containing phospholipids were detected which had previously only been found in significant amounts in deeply branching hyperthermophilic bacteria and archaea. Combining information from HPLC-MS analysis and chemical degradation experiments, ether lipids were identified as 1-alkyl-2-acyl-phosphatidyl ethanolamines, glycerols and cholines. In *Desulforhabdus amnigenus*, *n*-penta-, *n*-hexa- and *n*-heptadecyl ethers were present (in order of decreasing abundance), whereas *Desulfosarcina variabilis* solely contained *n*-hexadecyl ether side chains.

Keywords Ether lipids · Sulfate-reducing bacteria · Phospholipids · HPLC-ESI-MS

Abbreviations *ESI* Electrospray ionization · *PC* Phosphatidyl choline · *PE* Phosphatidyl ethanolamine · *PG* Phosphatidyl glycerol · *PI* Phosphatidyl inositol · *PLFA* Polar lipid fatty acids · *PXM* Phospholipid of type X with mixed, i.e. alkyl and acyl side chains

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Introduction

For chemotaxonomic classification of microorganisms, whole-cell fatty acid patterns, obtained by analysis of the products of acid or alkaline hydrolysis with subsequent methylation, have been widely applied (e.g. Ueki and Suto 1979; Vainshtein et al. 1992; Vancanneyt et al. 1996). Fatty acid patterns of polar cellular lipids are often used to distinguish different kinds of viable microorganisms in the analysis of in situ microbial community structures (e.g. Guckert et al. 1985; White et al. 1996). Compounds detected include saturated and unsaturated, branched and straight-chain fatty acids as well as cyclopropyl and hydroxyl-substituted fatty acids. The presence of dimethylacetals, derived from vinyl ethers, has also been reported (e.g. Moore et al. 1994). All ester-bound compounds are liberated from lipopolysaccharides and cellular polar lipids by alkaline hydrolysis. In contrast, amide and ether bonds can only be cleaved under acidic conditions, release of the latter requiring more vigorous acidic conditions (Hanahan 1972; Goossens et al. 1986).

In the last few years, direct structural analysis of intact phospholipids has become possible using electrospray ionization-mass spectrometry, which is now widely applied to characterize bacterial isolates (e.g. Black et al. 1997; Fang and Barcelona 1998). Using this technique, information about phospholipid types and their corresponding side chains is obtained. This information allows a better taxonomic differentiation than fatty acid patterns alone (Fang et al. 2000). Additionally, not only easily hydrolyzable ester-linked fatty acids, but also ether-linked side chains can be detected by intact phospholipid analysis (e.g. Han and Gross 1995).

Whereas all described archaea possess lipids with ether-linked isoprenoidal side chains (Kates 1997; Koga et al. 1998), there have been only a few observations of ether linkages in bacterial lipids. Vinyl ether lipids (plasmalogens) were proposed to be common in strict anaerobes as they were detected, e.g., in *Clostridium* spp. and other rumen bacteria, but also in soil bacteria grown under anoxic

conditions and a *Desulfovibrio* sp. (Kamio et al. 1969; Goldfine and Hagen 1972;. Fischer et al. 1994) O-Alkyl-acyl-phospholipids occur in trace amounts in bacteria that also contain plasmalogens (e.g. Paltauf 1994; Goldfine and Hagen 1972). Indications for the presence of non-isoprenoidal glycerol ether lipids were reported for the myxobacterium *Stigmatella aurantiaca* (Asselineau and Asselineau 1990) and the halophilic sulfate reducer *Desulfohalobium retbaense* (Ollivier et al. 1991). In contrast, large proportions (i.e. ≥90% of the core lipids) of saturated, non-isoprenoidal mono- and dialkyl glycerol ether lipids were discovered in thermophilic bacteria such as *Aquifex pyrophilus* (Huber et al. 1992) and the sulfate-reducing *Thermodesulfobacterium commune* (Langworthy et al. 1983). Therefore, among prokaryotes, the presence of substantial amounts of saturated ether lipids has, until now, seemed to be confined to archaea and the deepest phylogenetic branches within the Bacteria (Gambacorta et al. 1994).

Indications for the presence of ether lipids in non-thermophilic organisms come from reports of the ubiquitous distribution of tetraether lipids in cold environments. Some tetraethers combine structural features of archaeal lipids (isoprenoidal branching and cyclopentyl rings) and bacterial lipids (monomethyl branching) within a single alkyl chain (Schouten et al. 2000). Furthermore, in cold methane seep sediments from the Eel River Basin (offshore southern Oregon) and the Santa Barbara Basin (offshore southern California) as well as in carbonate crusts of mud volcanoes of the eastern Mediterranean Sea, the occurrence of ^{13}C-depleted, non-isoprenoidal monoalkyl and dialkyl ethers has recently been reported (Hinrichs et al. 2000; Orphan et al. 2001; Pancost et al. 2001). Anaerobic oxidation of methane in seep sediments and in sediment sections overlying methane hydrates is thought to be mainly carried out by syntrophic consortia of methane-oxidizing archaea and sulfate-reducing bacteria (Boetius et al. 2000; Hinrichs et al. 2000; Pancost et al. 2000). Whereas Pancost et al. (2000, 2001) and Hinrichs et al. (2000) suggested mesophilic sulfate reducers as the source organisms for the monoalkyl and dialkyl ether lipids, they were proposed to originate from yet unknown types of bacteria additionally involved in the anaerobic oxidation of methane by Orphan et al. (2001).

We investigated the lipid composition of mesophilic sulfate-reducing bacteria using HPLC-ESI-MS and -MS/MS. In two strains, ether-containing phospholipids were detected. Here, we report detailed evidence for the presence of alkyl-acyl-phospholipids in *Desulforhabdus amnigenus* and *Desulfosarcina variabilis*.

Materials and methods

Organisms

Pure cultures of the sulfate-reducing bacteria *Desulfosarcina variabilis*^{T} (DSMZ 2060) and *Desulforhabdus amnigenus*^{T} (DSMZ 10338) were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). They were grown at their optimum growth temperatures of 28 and 37 °C, respectively, using growth media recommended by the DSMZ, with propionate (*Desulforhabdus amnigenus*) or benzoate (*Desulfosarcina variabilis*) as electron donor and carbon source. Cells were harvested at the end of the exponential growth phase by centrifugation, washed with phosphate buffer (pH≈7) of appropriate salinity, freeze-dried and stored at –20 °C.

Extraction and clean-up

Freeze-dried samples were ultrasonically extracted ten times by a modified Bligh-Dyer method using a solvent mixture of methanol/dichloromethane/phosphate buffer, pH 7.4 (2:1:0.8 by volume). Dichloromethane and phosphate buffer were added to the combined extracts to achieve a final ratio of methanol/dichloromethane/phosphate buffer of 1:1:0.9 (by volume). After phase separation, the organic phase was removed. The aqueous phase was reextracted five times with dichloromethane. Combined organic phases were dried over anhydrous sodium sulfate, evaporated to dryness and stored at –20 °C. Different lipid classes were separated by column chromatography on silica gel (2 g silica 60, 63–200 µm, Merck, Germany, dried at 110 °C for 16 h). Eight fractions were obtained by elution with the following solvents: (1) 10 ml *n*-hexane, (2) 25 ml *n*-hexane/diethylether (98:2 v/v), (3) 30 ml *n*-hexane/diethylether (8:2 v/v), (4) 20 ml diethylether, (5) 10 ml 0.5 vol% acetic acid in diethylether, (6) 20 ml acetone/dichloromethane (1:1 v/v) and 10 ml acetone/ dichloromethane (8:2 v/v), (7) 10 ml 5 vol% methanol in dichloromethane and 25 ml acetone, (8) 30 ml dichloromethane/methanol/water (5:4:0.4 by volume). Phospholipids eluted in the most polar fraction. All fractions were evaporated to dryness and stored at –20 °C.

Transesterification, gas chromatographic and gas chromatographic-mass spectrometric analysis

An aliquot of the polar lipid fraction, i.e. of fraction 8, was transesterified by mild alkaline hydrolysis as described by White et al. (1979). The methyl esters obtained were analyzed by gas chromatography (GC-FID, (Hewlett Packard HP 5890 series II gas chromatograph, Hewlett Packard, Waldbronn, Germany, equipped with a cold injection system, Gerstel KAS3, Gerstel, Mühlheim a.d. Ruhr, Germany) on a DB-5HT column (30 m×0.25 mm, 0.1 µm film thickness, J & W, Folsom, Calif., USA) and by gas chromatography-mass spectrometry (GC-MS) using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, Calif., USA). For GC-FID and GC-MS analyses, helium was used as the carrier gas (constant column head pressure of 12.5 psi). The following temperature program was applied: 60 °C (1 min), then 3 °C/min to 400 °C (5 min). The flame ionization detector was operated at 300 °C.

Whole-cell hydrolysis

Freeze-dried cells (approximately 20 mg) were saponified under reflux overnight with 5 ml KOH solution (5% $KOH \cdot H_2O$ grade "Suprapur", Merck, Darmstadt, Germany, in methanol/water 4:1 by volume) under a nitrogen atmosphere. Afterwards, the alkaline solution was decanted to a separation funnel and the solution acidified using hydrochloric acid (2 $mol \cdot l^{-1}$). Non-saponifiable, solid residues were extracted three times with 10 ml dichloromethane. These dichloromethane extracts were added to the acidified methanol/water phase in the separation funnel. After phase separation, the organic phase was removed and the aqueous phase was reextracted three times with 10 ml dichloromethane. Combined organic phases were dried over anhydrous sodium sulfate. After removal of the solvent, aliquots of samples were transformed into their trimethylsilyl ester derivatives using *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany). The trimethylsilyl derivatives of fatty acids were ana-

lyzed by GC-FID and GC-MS as described above. Positions of double bonds were confirmed using dimethyldisulfide addition as described by Dunkelblum et al. (1985). As the hydrolysis was originally designed for determination of fatty acid patterns, the organic phase was not checked for the presence of stable glycerol phosphatides.

HPLC-MS and -MS/MS

Phospholipids were analyzed on an HPLC instrument (Thermo Separation Products, San Jose, Calif., USA) coupled to an ion-trap mass spectrometer equipped with an electrospray source (Finnigan LCQ, Thermoquest-Finnigan) and to an evaporative light-scattering detector (ELSD 500, Alltech, Deerfield, Ill., USA) using a flow splitter (split ratio 1:0.88, with the larger flow feeding the ELSD). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5 µ, Merck) using a 2×125 mm column. A flow rate of 0.35 ml·min^{-1} was employed with the following solvent gradient: 1 min 100% A, increasing over 20 min to 35% A, 65% B using a concave curvature, followed by 40 min of reconditioning. Eluent A was a mixture of *n*-hexane/*i*-propanol/formic acid/ammonia (25% solution in water) (79:20:1.2:0.04 by volume), eluent B was *i*-propanol/water/formic acid/ ammonia (25% solution in water) (88:10:1.2:0.04 by volume). The mass spectrometer was set to the negative ion mode with a spray voltage of –4.5 kV and a capillary temperature of 200 °C. MS/MS experiments were done in the dependent-scan mode, i.e. the most intense quasi-molecular-ion species of each full scan was automatically isolated and fragmented. For MS/MS experiments, an octapole voltage of 45 V had to be applied to prevent adduct formation with eluent components; helium was used as collision gas (relative collision energy: 35%). The light-scattering detector was operated at a drift-tube temperature of 75 °C using a nebulizer gas flow rate of 2.25 l N_2/min.

Mass spectra (full scan and MS/MS) were used for compound identification and determination of (fatty acid) substituents. Quantitation was done after external calibration of ELSD signals with different phospholipid standards (synthetic dipalmitoyl phosphatidyl glycerol, ethanolamine, choline, and phosphatidyl inositol and diphosphatidyl glycerol isolated from soy bean and from bovine heart, respectively; all standards were purchased from Sigma Aldrich, Deisenhofen, Germany) taking into account the non-linear response of the ELSD.

Ether cleavage

For ether cleavage (e.g. Panganamala et al. 1971), an aliquot of the polar lipid fraction (fraction 8) was refluxed with 3 ml of hydroiodic acid (57%, Aldrich, Taufkirchen, Germany) and 2 ml of acetic acid (99.8%, Riedel de Haen, Seelze, Germany) for 3 h under a nitrogen atmosphere. The resulting solution was transferred into a separation funnel; *n*-hexane and water were added. The water phase was reextracted with 10 ml of *n*-hexane. Combined organic phases were washed with 5% $NaSO_4$ solution and twice with water. The resulting organic phase was dried over anhydrous sodium sulfate and then reduced to dryness. Crude material was separated by column chromatography on silica gel (silica gel 100, 63–200 µm, Merck, Darmstadt, Germany) into an apolar (elution with 10 ml *n*-hexane) and a polar fraction [elution with 15 ml dichloromethane (with 10% methanol by volume)/50 µl 99% acetic acid]. The polar fraction was silylated as described above. Apolar and polar fractions were analyzed by GC-MS on an HP-1 column [60 m×0.25 mm, 0.25 µm film thickness, Hewlett Packard; GC temperature program: 60 °C (2 min) – heating at 15 °C min^{-1} to 150 °C, then at 2 °C min^{-1} to 300 °C (held for 45 min); other GC conditions as described above] using a Finnigan MAT 95Q mass spectrometer (Finnigan-Thermoquest) set to a resolution of about 1,000. Blanks were run to check for contamination.

Results

HPLC-ELSD and -MS analysis

The HPLC conditions were optimized using phospholipid standards to separate different phospholipid types, which means that the headgroup was the main factor controlling retention times. Nevertheless, differences in fatty acid side chains led to minor shifts in retention times (<0.1 min/C atom), so that compounds with longer side chains eluted earlier than those with shorter substituents. Major changes in the side chains – such as introduction of an ether bond – shifted retention times significantly (approximately 0.6 min). For example, for phosphatidyl choline (PC) standards with ether- and ester-linked side chains, the following order of retention times was observed: di-O-hexadecyl<O-hexadecyl-palmitoyl<dipalmitoyl. The structurally related compounds dipalmitoyl phosphatidyl monomethyl ethanolamine and dipalmitoyl phosphatidyl dimethyl ethanolamine eluted shortly before the dipalmitoyl phosphatidyl ethanolamine (PE) and nearly 1.5 min before the dipalmitoyl phosphatidyl choline standard, so that a coelution of O-alkyl-acyl-PC or -PE and other (isobaric) compounds could be excluded. Therefore, retention times allowed different phospholipid types to be distinguished and to discriminate alkyl-acyl from diacyl or dialkyl side chains.

In the two investigated strains of sulfate-reducing bacteria, PE was the major phospholipid type (Fig. 1a, b). In *Desulfosarcina variabilis* minor amounts of phosphatidyl glycerols (PG) and traces of phosphatidyl cholines were present (Fig. 1b). In *Desulforhabdus amnigenus*, phosphoglycolipids, tentatively identified from the retention time as phosphatidyl inositols (PI), were detected in addition to smaller amounts of PG and PC (Fig. 1a).

Whereas in *Desulfosarcina variabilis* phospholipids with ether-linked alkyl chains accounted for only 38% of all phospholipids, they dominated the phospholipid pattern of *Desulforhabdus amnigenus*, alkyl-acyl-PEs being the most abundant compounds (34% of all phospholipids). Note that due to the non-linear response of the light-scattering detector used for quantitation, relative amounts of different types of phospholipids could not be deduced directly from peak area ratios (Figs. 1a, b).

In HPLC-MS analysis in the negative ion mode, phosphatidyl choline formed adducts with the buffer component formate to "neutralize" its positively charged quarternary ammonia group (see Kerwin et al. 1994; Han and Gross 1995). To prevent this adduct formation and to enable further fragmentation of the phosphatidyl choline quasi-molecular ions, an octapole voltage had to be applied for MS/MS experiments. Under these conditions, the major quasi-molecular-ion species was $[M-15]^-$, resulting from the loss of a methyl group as a means to eliminate the positive charge in the headgroup.

In MS/MS experiments, the diacyl phosphatidyl choline standard exhibited a fragment resulting from the elimination of the acyl side chain, i.e. neutral loss of a ketene,

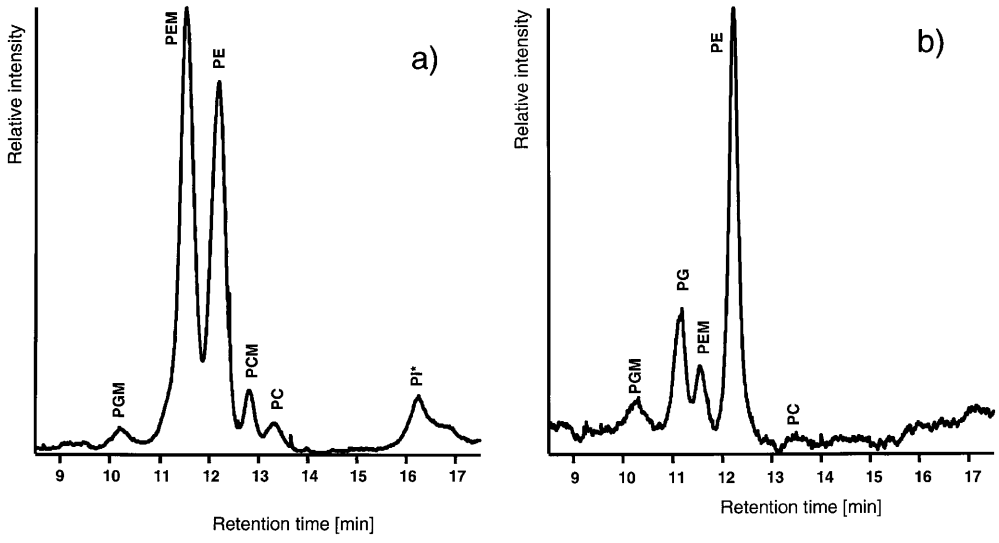

Fig. 1 HPLC-ELSD (evaporative light-scattering detector) chromatogram of phospholipid fraction of **a** *Desulforhabdus amnigenus* and **b** *Desulfosarcina variabilis*. *PG* Diacyl phosphatidyl glycerols, *PE* diacyl phosphatidyl ethanolamines, *PC* diacyl phosphatidyl cholines, *PI** phosphoglycolipid, tentatively identified as diacyl phosphatidyl inositol, *PXM* phospholipid of type X with mixed, i.e. O-alkyl and acyl, side chains

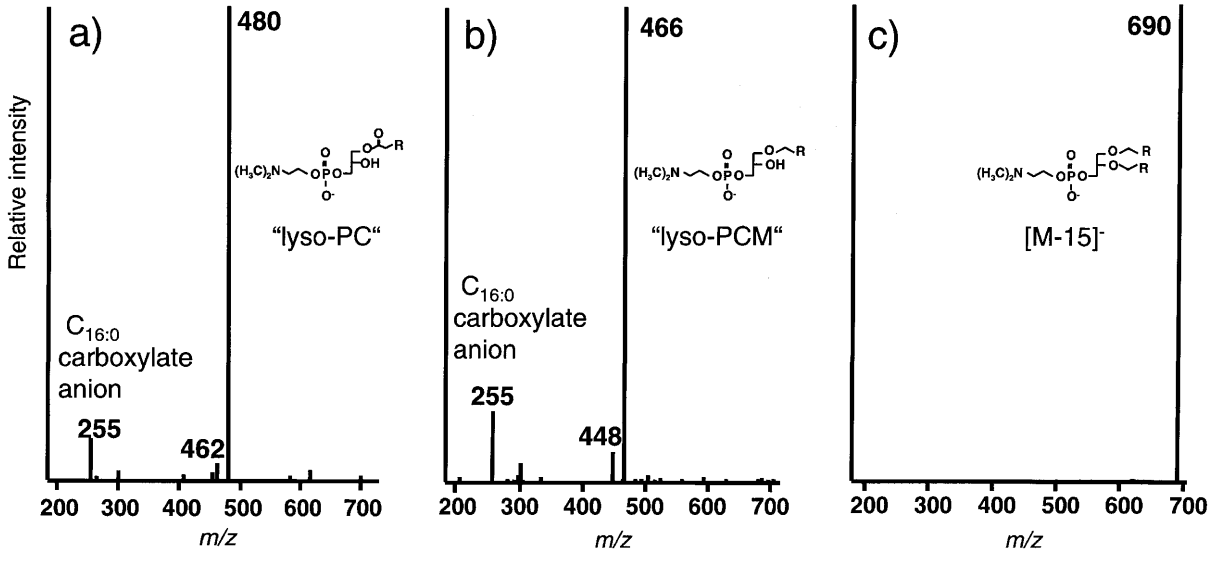

Fig. 2a–c MS/MS spectra of phosphatidyl choline molecular ion species $[M-15]^-$. **a** dipalmitoyl phosphatidyl choline (PC; *m/z* 718), **b** 1-O-hexadecyl-2-palmitoyl phosphatidyl choline (PCM [M for mixed side chains]; *m/z* 704), **c** di-O-hexadecyl phosphatidyl choline (*m/z* 690)

from the central glyceryl carbon atom (Fig. 2a), which is known to be the preferred fragmentation pathway of this compound type (e.g. Lehmann 1996; Hsu and Turk 2000). Di-O-hexadecyl phosphatidyl choline gave no fragments under the MS/MS conditions chosen (Fig. 2c). The mixed 1-O-alkyl-2-acyl choline standard showed an MS/MS spectrum very similar to that obtained from the diacyl phospholipid standard, i.e. loss of the fatty acid from the central carbon atom yielded a prominent fragment (Fig. 2b). Therefore, the fragmentation behavior of phospholipids in MS/MS experiments allowed differentiation between phospholipids with fatty acid side chains and compounds solely substituted with ether-linked side chains. Furthermore, from the mass to charge ratios (*m/z*) of quasi-molecular ions, the total number of carbon atoms present in the two side chains could be calculated when the phospholipid type was known, e.g., from the retention time, and when information about the presence of ether-linked alkyl side chains was available. The fatty acid side chain substituted at the central carbon atom was determined directly through the MS/MS experiment, because the major fragmentation was the loss of this central fatty acid side chain (see Fig. 2). The number of carbon atoms in the second side chain was calculated from the difference between the total number of carbon atoms in the two side chains and that of the fatty acid from the central carbon atom. Where no MS/MS data were available, fatty acid combinations were tentatively assigned using the most abundant compounds of polar lipid fatty acid (PLFA), i.e. fatty acids obtained after transesterification of the "phospholipid fraction." Likely combinations of alkyl side chains and fatty acid substituents of major phospholipids of the two investigated strains of sulfate-reducing bacteria are given in Table 1. In *Desulfosarcina variabilis* only ether phospholipids with hexadecyl substituents were found, whereas HPLC-MS and -MS/MS data indicated the pres-

Table 1 Major phospholipids of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*; fatty acids confirmed in MS/MS experiments are printed in *bold*, other fatty acid combinations were tentatively assigned according to the most abundant fatty acids present in the "phospholipid fraction". – Not detected, *tr* minor component with unknown side chains, *asterisk* phosphoglycolipid tentatively identified as phosphatidyl inositol

	m/z	*Desulforhabdus amnigenus* Side chains	*Desulfosarcina variabilis* Side chains
O-Alkyl-acyl phosphatidyl glycerols	705	**17:1**/O–15:0	–
	693	16:0/O–15:0	**15:0**/O–16:0
	679	15:0/O–15:0	tr
Diacyl phosphatidyl glycerols	747	**17:1**/17:0	tr
	733	tr	16:1/17:0
	721	tr	**16:0**/16:0
	719	tr	**16:1**/16:0
	707	tr	**15:0**/16:0
O-Alkyl-acyl phosphatidyl ethanolamines	688	**17:1**/O–16:0	tr
	674	**17:1**/O–**15:0**	16:0/O–16:0
	662	tr	**15:0**/O–16:0
	660	16:1/O–15:0	tr
Diacyl phosphatidyl ethanolamines	716	**17:1**/17:0	tr
	714	17:1/17:1	tr
	702	**16:1**/17:0	**17:1**/**16:0**
	690	17:0/15:0	tr
	688	**17:1**/**15:0**	**16:1**/**16:0**
	676	tr	**15:0**/16:0
O-Alkyl-acyl phosphatidyl cholines	776	**17:1**/O–16:0	–
	762	**17:1**/O–15:0	–
Diacyl phosphatidyl cholines	804	**17:1**/17:0	–
	802	17:1/17:1	–
	778	tr	16:0/16:0
	776	17:1/15:0	–
Diacyl phosphatidylinositols*	821	**17:1**/16:0	–
	807	**17:1**/15:0 + **16:1**/16:0	–
	793	**17:1**/14:0	–

ence of pentadecyl and hexadecyl ethers in *Desulforhabdus amnigenus*. Plasmalogens, i.e. alkenyl ether phospholipids, were not detected in either one of these bacteria.

Whole-cell hydrolysis and fatty acid patterns

After whole-cell hydrolysis, glycerol monoethers were detected in the products obtained from both sulfate reducers in addition to fatty acids liberated by saponification. These glycerol monoethers are considered to be hydrolysis products of mixed ether/ester phospholipids. By GC-MS analysis, the position of the O-alkyl chains in the monoethers could be determined, because mass spectra of 1-O-alkyl glycerols (Grönneberg and Albone 1976) are different from those of 2-O-alkyl glycerols. In *Desulfosarcina variabilis* only 1-O-hexadecyl glycerol was found (identified by its mass spectrum with a base peak at *m/z* 205 characteristic of 1-O-alkyl glycerols). In *Desulforhabdus amnigenus* additionally 1-O-pentadecyl and 1-O-heptadecyl glycerols were present, which formed a series of homologues indicating analogous branching in all three compounds. 2-O-alkyl ethers were not detected in any of the two sulfate reducers investigated.

PLFA patterns of both investigated strains closely matched the fatty acid patterns obtained after whole-cell hydrolysis (Table 2). Major fatty acids of *Desulforhabdus amnigenus* were straight-chain-saturated and monounsaturated C_{15}, C_{16}, and C_{17} fatty acids. In *Desulfosarcina variabilis* mainly *n*-$C_{16:0}$, *ai*-$C_{15:0}$ and *n*-$C_{16:1\omega7}$ were found.

Ether cleavage

To confirm the presence of ether-bound alkyl side chains in the phospholipids of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, ether bonds were cleaved using hydroiodic acid. Resulting alkyl iodides were directly analysed by GC-MS. The ether cleavage procedure was checked using dipalmitoyl- and di-O-hexadecyl-PC standards and by running blanks. In the case of dipalmitoyl-PC, the $C_{16:0}$ fatty acid was recovered in the polar fraction after "acid hydrolysis" by HI treatment.

In *Desulforhabdus amnigenus*, penta-, hexa- and heptadecyl iodides were detected in the apolar fraction after hydroiodic acid treatment, whereas in *Desulfosarcina variabilis* only hexadecyl iodide was present in amounts well above the background level (Fig. 3). These chain-length distributions obtained after ether cleavage were in good agreement with the results of whole-cell hydrolysis and HPLC-MS/MS analyses of intact phospholipids, although in the latter no significant amounts of O-heptade-

Table 2 Relative amounts of fatty acids in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* – comparison of whole-cell hydrolysis, transesterification of polar lipid fatty acid fraction and ether cleavage. *n.d.* Not detected ++ major component, + minor component

Fatty acids	*Desulforhabdus amnigenus*			*Desulfosarcina variabilis*		
	Whole-cell hydrolysis	PLFA	Polar fraction after HI treatment	Whole-cell hydrolysis	PLFA	Polar fraction after HI treatment
i-14:0	n.d.	n.d.	–	0.6	0.4	–
n-14:0	1.7	1.5	+	2.8	2.2	+
i-15:0	3.3	3.1	+	2.0	2.6	+
ai-15:0	0.1	0.4	–	23	20	++
n-15:1ω6	1.6	1.6	–	n.d.	n.d.	–
n-15:1ω4	5.6	5.4	–	n.d.	n.d.	–
n-15:0	18	18	++	0.9	1.0	+
i-16:1ω6	n.d.	n.d.	–	2.5	1.7	–
i-16:0	n.d.	n.d.	–	1.2	1.3	–
n-16:1ω7	0.8	1.2	–	16	17	–
n-16:1ω5	10	10	–	2.0	1.6	–
n-16:0	7.2	7.2	++	32	35	++
i-17:1ω7	n.d.	n.d.	–	3.1	4.1	–
ai-17:1ω7	n.d.	n.d.	–	6.5	5.6	–
i-17:1ω5	5.9	6.2	–	n.d.	n.d.	–
ai-17:1ω5	0.2	0.2	–	n.d.	n.d.	–
i-17:0	0.3	0.4	–	0.2	0.4	–
ai-17:0	n.d.	n.d.	–	1.6	1.8	–
n-17:1ω8	1.5	1.7	–	n.d.	n.d.	–
n-17:1ω6	24	25	–	n.d.	n.d.	–
cyc-17	4.7	4.2	–	n.d.	n.d.	–
n-17:0	11	10	+	0.7	1.3	+
n-18:1ω9	0.8	0.5	–	1.0	0.7	–
n-18:1ω7	1.1	1.3	–	2.1	0.8	–
n-18:0	1.6	1.4	+	2.8	2.8	–

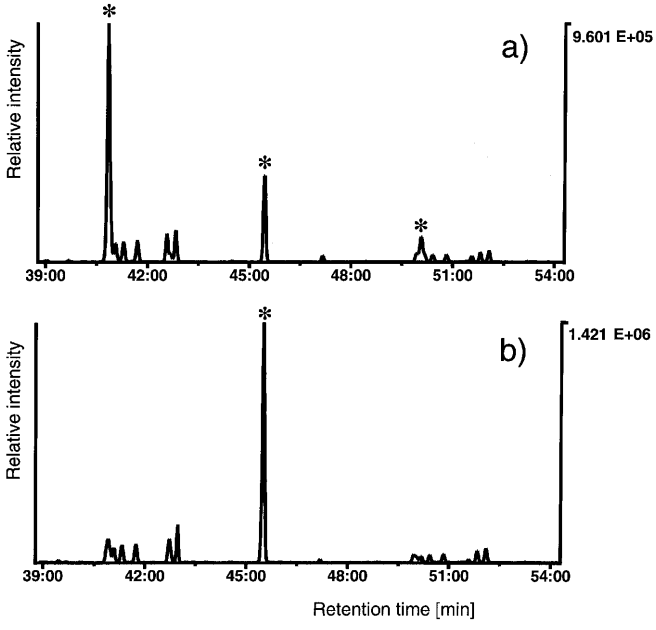

Fig. 3 Mass chromatograms (*m/z* 57+127) of apolar fractions after HI treatment of **a** *Desulforhabdus amnigenus* and **b** *Desulfosarcina variabilis*. Peaks of *n*-penta-, *n*-hexa- and *n*-heptadecyl iodides are marked with *asterisks*

cyl phospholipids were detected in *Desulforhabdus amnigenus* (Table 1). Hexadecyl iodide from the lipids of our bacteria had the same retention time in GC-MS analysis as the *n*-hexadecyl iodide generated by hydroiodic acid treatment of the PC standard. This provided evidence that the two investigated strains of sulfate-reducing bacteria contained solely straight-chain alkyl substituents.

When distributions of fatty acids recovered in the polar fraction after hydroiodic acid treatment were compared to those after whole-cell hydrolysis and those of the polar lipid fraction (Table 2), the lack of unsaturated and cyclopropyl fatty acids in the former was striking, but could be readily explained by the fact that double bonds and cyclopropyl rings are labile towards strong acid treatment (e.g. Vollhardt and Schore 1998). The distribution patterns of all major saturated fatty acids remained unchanged by the ether cleavage procedure.

Combining the results of the analysis of whole-cell hydrolysis products, PLFA and ether cleavage products, and those of HPLC-MS analyses, we provide evidence that *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* contained phosphatidyl glycerols, ethanolamines and, in the case of the latter, also phosphatidyl cholines, with straight-chain O-alkyl substituents and various fatty acid side chains. In these phospholipids, the ether-linked side chains were present at the terminal carbon atom of the

glycerol; the exact sterical configuration at the central carbon atom was not determined. We tentatively assume a levorotary configuration, i.e. *sn*-1-O-alkyl-2-acyl-phospholipids, because this configuration is the one that all described bacteria show, whereas lipids of archaea contain *sn*-2,3-glycerols (Koga et al. 1998).

Discussion

Our fatty acid data for *Desulforhabdus amnigenus* are in good agreement with the PLFA pattern reported by Oude Elferink et al. (1998), although an unknown monounsaturated C_{18} fatty acid accounted for 14.5% of total fatty acids in their analysis, whereas we could only detect 1.3% *n*-$C_{18:1\omega7}$ among the PLFA. Our PLFA pattern of *Desulfosarcina variabilis* was similar to that described by Kohring et al. (1994), although we found a much higher abundance of the *ai*-$C_{15:0}$ fatty acid. The occurrence of monoethers was not reported by either of these authors.

The phospholipid patterns of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, i.e. PE as the most abundant phospholipid type with minor amounts of PG and other phospholipid types, is in agreement with those observed for many sulfate-reducing bacteria (H. Rütters and H. Sass, unpublished data; Knoblauch et al. 1999; Makula and Finnerty 1974) and other gram-negative bacteria (Wilkinson 1988).

In our two strains of sulfate-reducing bacteria, alkyl-acyl-containing phospholipid types occurred together with the corresponding diacyl phospholipids, as was also observed for plasmalogens (Goldfine and Langworthy 1988).

Among the prokaryotes, the biosynthesis of large amounts of alkyl ether lipids seemed to be confined to archaea and the most deeply branching thermophilic Bacteria (Gambacorta et al. 1994). The presence of significant amounts of mono- and dialkyl ethers in environmental samples (Orphan et al. 2001; Pancost et al. 2001) and the detection of alkyl ether phospholipids in mesophilic sulfate-reducing bacteria (this study) show that these ether lipids may be more common membrane lipids of bacteria than previously thought. One reason for the few reports of alkyl ether lipids in bacteria may be related to the analytical procedures commonly employed. To analyze ether moieties from polar lipids by gas chromatography, either the ether bond has to be cleaved by strong acid treatment or the phosphate/sugar headgroup (and the fatty acid side chain) has to be removed by acid or strong alkaline hydrolysis, yielding mono- or dialkyl glycerol ethers. In this way, Langworthy et al. (1983) and Huber et al. (1992) detected alkyl ether lipids in hyperthermophilic bacteria after hydrolysis of extracted lipids with methanol/hydrochloric acid. For investigation of PLFA patterns, polar lipid fatty acids are usually transesterified under mild alkaline conditions, as described by White et al. (1979); however, this method will not remove the phosphate group of most phospholipid types. As the alkyl-acyl-phospholipids form a significant proportion of the total phospholipids of our investigated sulfate-reducing bacteria (38% and 47% in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, respectively) and possibly of other bacteria as well, analysis restricted to polar lipid fatty acids in environmental samples may seriously underestimate viable biomass.

The occurrence of monoalkyl ethers with 14 to 18 carbon atoms in anoxic sediments of methane seeps (Orphan et al. 2001) may partly be explained by the degradation of bacterial ether phospholipids, e.g. from sulfate-reducing bacteria involved in the anaerobic oxidation of methane. Using 16S rRNA clone libraries, Orphan et al. (2001) found that sequences related to *Desulfosarcina* were the most common phylotypes in their methane seep sediments. As the type strain *Desulfosarcina variabilis* only contained hexadecyl ether lipids in our investigation, other (sulfate-reducing) bacteria appear to be involved in the methane oxidation process as well. In the closest relatives of *Desulfosarcina variabilis*, *Desulfonema limicola* and *Desulfococcus multivorans*, no phospholipids with ether-linked side chains were detected (H. Rütters and H. Sass, unpublished results). The second strain of sulfate-reducing bacteria containing ether lipids, the freshwater strain *Desulforhabdus amnigenus*, is not closely related to *Desulfosarcina sp*. Whether its closest relatives, the *Syntrophobacter* spp., or representatives of other phylogenetic groups are capable of biosynthesising ether lipids, remains to be determined.

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