Extremophiles (2009) 13:101–109
DOI 10.1007/s00792-008-0202-6

ORIGINAL PAPER

Fatty acids, unusual glycophospholipids and DNA analyses of thermophilic bacteria isolated from hot springs

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Received: 18 July 2008 / Accepted: 30 September 2008 / Published online: 7 November 2008

Abstract The composition of fatty acids in 12 strains of the genera *Thermus, Meiothermus, Geobacillus* and *Alicyclobacillus* was analyzed by gas chromatography–mass spectrometry. Major FAs found in the profiles included *i*-15:0, *i*-17:0, *ai*-15:0, *i*-16:0, 16:0, *ai*-17:0, together with some minor components. Branched FAs were predominant, forming more than 80% of all FAs measured. Fast atom bombardment-mass spectrometry was used for analysis of unusual glycophospholipids, i.e., acylglycosyl-cardiolipins from genera *Geobacillus* and *Alicyclobacillus* and 1-(hydroxy(2-(*O*-acylglycosyl-oxy)hexadecyloxy) phosphoryloxy) hexadecan-2-yl esters of C15–C17 acids from genera *Thermus* and *Meiothermus.* Cloning and preliminary sequence analysis of 16S rDNA showed that these isolates belong to the genera *Thermus, Meiothermus, Geobacillus* and *Alicyclobacillus*.

Keywords *Thermus* · *Meiothermus* · *Geobacillus* · *Alicyclobacillus* · Fatty acids · Glycophospholipids · FAB-MS · 16S rDNA analysis · Phylogenetic tree · Hot springs

Communicated by F.Robb.

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Introduction

Thermophilic microorganisms are not grouped into a separate taxonomic unit, but they are spread across the whole taxonomic system, in various taxonomic groups and at various phylogenetic distances. Nevertheless, they have an important common characteristic, i.e., optimal growth temperature that ranges from 50 to 80°C (Charlier and Droogmans 2005; Wiegel and Ljungdahl 1986) and can be even higher in the case of hyperthermophilic bacteria.

On the basis of cell wall structure, genera *Thermus* and *Meiothermus* fall among Gram-negative bacteria and genera *Geobacillus* and *Alicyclobacillus* among Gram-positive bacteria. The selected Gram-negative bacteria are heterotrophic, non-motile, aerobic or obligately aerobic, cells do not have flagella and form no spores (Sharp and Williams 1995; Kristjansson 1992).

In these species, polar lipids form a large proportion of the cellular membrane fractions and usually include phospholipids and glycolipids. The glycolipids usually contain hexoses, *N*-hexosamine, and glycerol. The hydrophobic parts are predominantly formed by *iso*- and *anteiso*-branched fatty acids, while straight-chain fatty acids are minor components (Albers and Driessen 2008; Driessen and Albers 2007). *Anteiso*-branched fatty acids have the branch point on the *ante*-penultimate carbon atom (two from the end), while *iso*-branched fatty acids have the branch point on the penultimate carbon (one from the end as illustrated in Fig. 1. The high proportion of glycolipids in the cell membranes could possibly contribute to the ability of the bacteria to grow at high temperatures, because the relative proportions of the major glycolipids increase with growth temperature (Yang et al. 2006). *Thermus* and *Meiothermus* species have been reported to contain glycophospholipids,

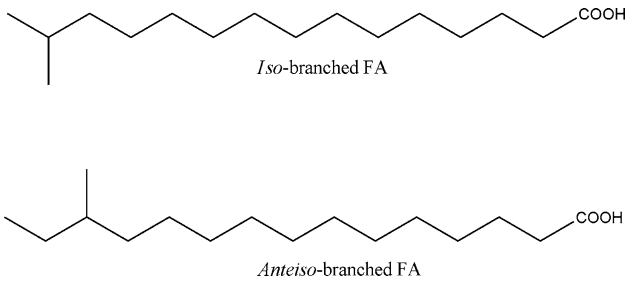

Fig. 1 General structure of *iso*- and *anteiso*-branched fatty acids

although the structures have not yet been fully elucidated (Luzzati et al. 1987; da Costa et al. 2006).

Genus *Geobacillus* forms motile rod-shaped cells occurring either individually or in short chains and produces one endospore per cell. From the metabolic point of view, this genus is chemo-organotrophic, aerobic or facultatively aerobic (oxygen as the electron acceptor is in some species replaceable by nitrate) (Nazina et al. 2001; Zeigler 2001). Different strains of *Geobacillus* were investigated and different polar lipids, mainly phospholipid species such as phosphatidylethanolamines, phosphatidylglycerols and cardiolipins were isolated (Schaffer et al. 2002; Peter-Katalinic and Fischer 1998; Varki et al. 2001; Dembitsky 2004). Cardiolipin (CL, bisphosphatidylglycerol) is an anionic tetraacylphospholipid which has been identified in the cytoplasmic membrane of Gram-positive as well as Gram-negative bacteria. In Gram-positive organisms, several modified cardiolipin species have been detected, such as glucopyranosylcardiolipin, alanylcardiolipin or lysylcardiolipin. The structural elements of different cardiolipins were determined by positive and negative ion fast atom bombardment-mass spectrometry (FAB-MS). The major cellular fatty acids are *i*-15:0, *i*-16:0 and *i*-17:0, which make up more than 60% of the total fatty acids (Zeigler 2001).

The members of genus *Alicyclobacillus* are thermoacidophilic, strictly aerobic, heterotrophic, endospore-forming bacteria. The main lipids found in this genus were glycolipids and phosphatidyl glycerol and cardiolipin (Nicolaus et al. 2001), and the major membrane lipid components are ω-alicyclic fatty acids (Goto et al. 2003), which have bonded a ring with 4–7 carbons (usually C6) on the ultimate carbon atom (Fig. 2). The objectives of this study were to determine the phylogenetic positions of 12 closely related thermophilic bacteria, and to determine

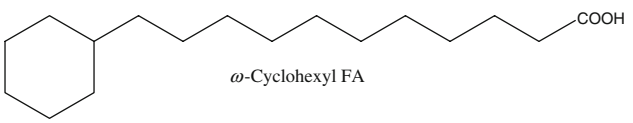

Fig. 2 General structure of the ω-alicyclic fatty acid with hexyl ring

their phylogenetic significances. We compared the biochemical and phylogenetic characteristics of four genera: *Thermus, Meiothermus, Geobacillus* and *Alicyclobacillus*. We also showed that glycophospholipids having a very complex structure were determined in *Thermus* and *Geobacillus*. The structures were determined by FAB-MS and they are shown in Fig. 3 (glycophospholipid from *Geobacillus*) and Fig. 6. (glycophospholipid from *Thermus*). Further, branched long chain fatty acids including ω-alicyclic FA were detected in different genera.

Materials and methods

Microorganisms

Twelve strains of thermophilic bacteria from the Czech Collection of Microorganisms (CCM) were used:

Thermus aquaticus CCM 3488 (source: thermally polluted river near Brussels, Belgium).
Thermus aquaticus CCM 3485 (received from R. A. D. Williams collection, strain DI).
Thermus sp. CCM 4167 (hot spring "Vridlo", Karlovy Vary, Czech Republic)
Thermus sp. CCM 2842 (hot spring of Kamchatka, Kamchatka, Russia)
Meiothermus ruber CCM 4211 (thermal pools, Hveragherti, Iceland)
Meiothermus ruber CCM 4212 (thermal pools, Hveragherti, Iceland)
Geobacillus stearothermophilus CCM 2062 (no information about source)
Geobacillus stearothermophilus CCM 5965 (evaporated milk)
Geobacillus thermoglucosidasius CCM 3731 (soil, Japan, Shimogamo, Kyoto)
Geobacillus thermoglucosidasius CCM 3732 (soil, Japan, Shimogamo, Kyoto)
Alicyclobacillus acidoterrestris CCM 4659 (apple-grape-raspberry juice)
Alicyclobacillus acidoterrestris CCM 4660 (apple-grape-raspberry juice)

Cultivation conditions. Genera *Thermus* and *Meiothermus* were cultivated on medium B39: yeast extract 4 g L^{-1}, tryptone 8 g L^{-1}, NaCl 2 g L^{-1}, pH 7.5. Genera *Geobacillus* and *Alicyclobacillus* were cultivated on medium B10: peptone 5 g L^{-1}, meat extract 3 g L^{-1}, $MnSO_4 \cdot H_2O$ 0.005 g L^{-1}, pH was 7.0 and 5.5, respectively.

Batch cultivation was carried out under aerobic conditions in 500-mL Erlenmeyer flasks filled with 200 mL of medium. Erlenmeyer flasks were placed into a temperature-

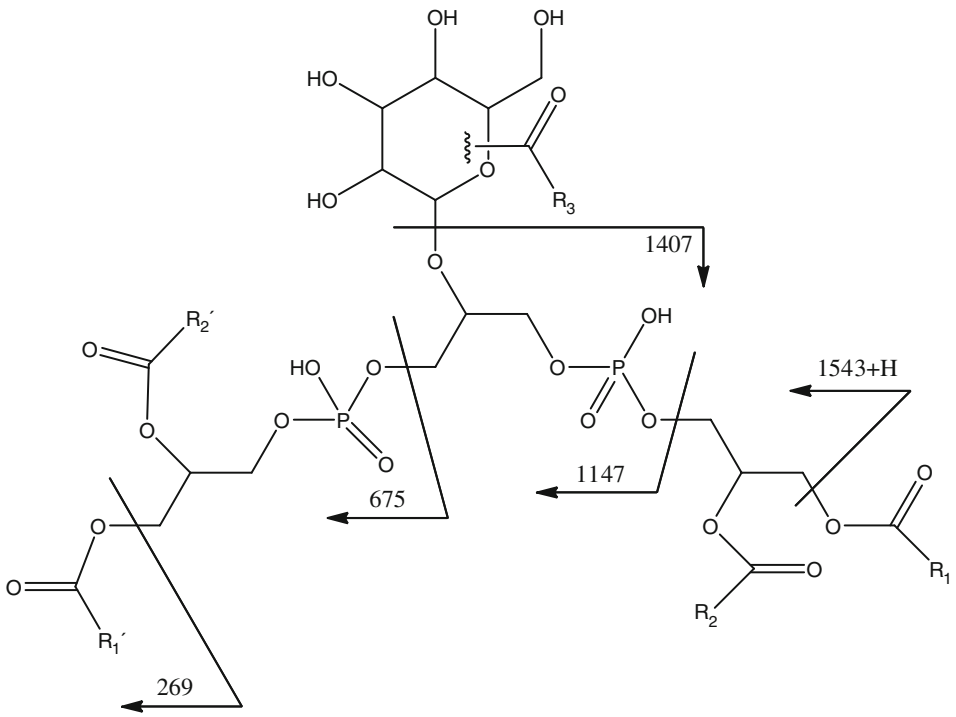

Fig. 3 General structure of the acylhexosylcardiolipin; R_{1-3}-fatty acid residues

controlled shaking device (HT Inflors AG CH-4103, Hungary). Incubation proceeded for 48 h, at 70°C and 200 rpm. After incubation, the biomass was concentrated by centrifugation (10,000 rpm; MEDIFRIGER-BL, Spain) and lyophilized.

Lipid analysis

Total lipids were extracted by the method of Bligh and Dyer (1959) and fractionated by thin-layer chromatography (TLC, Silica Gel 60; Merck AG, Darmstadt, Germany). The mixture of chloroform–methanol–acetic acid–water, 65:25:6:4, v/v/v/v was used as a solvent system for the initial TLC experiment. Fractions of neutral lipids, glycolipids, phospholipids and glycophospholipids were obtained, their R_f values were 0.95–1.00, 0.65–0.80, 0.30–0.65 and 0.25, respectively. The glycophospholipids were identified and isolated by using high performance two-dimensional TLC (2D-HPTLC). One corner of a plate was spotted with 10 mg of total PL. The plate was developed in the first dimension in 65:25:4 (v/v/v) $CHCl_3$–MeOH–H_2O to 9.9 cm and in the second dimension in 8:1:1 (v/v/v) 1-propanol–H_2O–acetic acid also to 9.9 cm. Compounds were analyzed with the following reagents: 5% primulin in 4:1 acetone–H_2O (for detection of lipids); molybdenum blue (for detection of phosphorus); 3.4% *N*-(1-naphthyl)-ethylenediamine in 97:3 MeOH–H_2SO_4 (for detection of carbohydrates). Plates were developed for 5 min at 110°C, except for components reacting with primulin, which were visualized under UV light at 366 nm (Kates 1986).

Mass spectra were recorded using a VG 7070E-HF spectrometer (70 eV, xenon) in positive and negative ion mode. Glycophospholipid extracts (after 2D-HPTLC) were dissolved in 0.1 mL of chloroform and suspended in an equal amount of PEG-400 matrix on a mass spectrometer probe. Data were obtained for ion intensities over a range of *m*/*z* values of 200–2000. Repeat scans were averaged and also printed out for data analysis. Each spectrum was averaged from ten scans. In the spectra, nominal masses were obtained by truncating accurate *m*/*z* values to the preceding integer.

Fatty acids analysis

A volume of 2 mL 15% NaOH in a 1:1 (v/v) methanol–water mixture was added to cca 10 mg of lyophilized bacterial biomass. This mixture was heated at 100°C in a closed test-tube for 30 min. Esterification was carried out by adding 4 mL HCl in methanol [1:3 (v/v)] and by heating the mixture at 80°C for 15 min. The mixture was then diluted with 20 mL of distilled water, and FAMEs (fatty acid methyl esters) were extracted into 2 mL hexane. Extraction proceeded for 1 h**.** After extraction, the organic and aqueous layers were separated. The lower aqueous layer was removed, and the organic layer was dried using anhydrous Na_2SO_4.

FAMEs were analyzed by GC HP 5890 using a flame-ionization detector (FID) under the following conditions: column HP 5 (30 m × 0.25 mm × 0.25 μm), mobile phase nitrogen, pressure 120 kPa (16 psi), temperature rate

150°C (4 min)–4°C per min–280°C (2 min), injector temperature 250°C, detector temperature 300°C, injection volume 1 μL.

FAME identification was carried out either by comparison with a commercial bacterial FAMEs standard solution (BAME, Supelco) and by using a mass spectrometric detector (MS) (Rezanka et al. 1991; Rezanka 1993). GC–MS was used for the identification and confirmation of analyzed structures. At least one sample of each strain was analyzed by GC–MS.

The glycophospholipid from the genus *Thermus* was hydrolyzed with 2 N HCl for 3 h at 100°C, and nonpolar products were extracted twice with hexane. The resulting mixture of fatty acids and diols was separated by TLC on Silica G plates developed with hexane–diethyl ether–acetic acid (70:30:1, v/v/v). Long chain diols were converted to *O*-trimethylsilyl ethers. Trimethysilylation was achieved by treating of the dry samples with 100 mL of bis(trimethylsilyl) trifluoroacetamide for 30 min at 60°C and TMS ethers were analyzed by GC–MS under the above conditions.

DNA isolation, PCR, sequencing of the 16S rDNA genes

Genomic DNA was isolated using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA). Microbial suspension was added to a bead beating tube containing beads, bead solution and lysis solution. The cellular components were lysed by mechanical action using a vortex. The released DNA from the lysed cells was bound to a silica spin filter. The filter was washed, and the DNA was recovered in a buffer in the amount of cca 5 ng.

The amplification of 16S rDNA fragments was performed in the T-Gradient thermocycler (Biometra) using two modified universal amplification primers according to the European ribosomal RNA database (Gent, Belgium):

16S0028F 5′-AGAGTTTGATCCTGGCTCAG-3′
16S0514R 5′-TTACCGCGGCTGCTGGCAC-3′

Fifty microliters of the PCR reaction contain: 1 μL diluted genomic DNA (~5 ng), 2 μL of each amplification primer (concentration was 25 pmol μL^{-1}), 25 μL PPP Master Mix (Top-Bio; containing 2 × PCR buffer, dNTPs and Taq-Purple DNA polymerase) and 20 μL H_2O. The amplification involved the following steps:

1. Initial denaturation for 1 min at 94°C
2. Denaturation for 30 s at 94°C (35 cycles)
3. Annealing for 30 s at 60°C (35 cycles)
4. Elongation for 1 min at 72°C (35 cycles)
5. Final elongation for 5 min at 72°C
6. Cooling down to 4°C

The amplification products were purified using MinElute™ PCR purification columns (Qiagen). Quality and quantity of the purified PCR fragments were checked by agarose gel electrophoresis.

All PCR fragments were sequenced with the ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The chain termination reaction (Sanger et al. 1977) was performed by the cycle sequencing technique (Murray 1989) according to the manufacturer's protocol in both directions. The amplification primers were used as sequencing primers. To remove unincorporated dye terminators, the sequencing products were purified by gel filtration using DyeEx spin columns (Qiagen). The sequences were determined with an ABI PRISM 3130*xl* DNA sequencer (Applied Biosystems).

Statistical analysis

The data obtained after analysis of FAMEs were processed by Cluster Analysis (STATISTICA Cz, version 6, StatSoft, Inc. 2001).

Evolutionary distance matrices (expressed as estimated changes per 100 nucleotides) were calculated with the algorithm developed by Jukes and Cantor (1969). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with the bootstrap analysis with 1,000 replicates by the ClustalX2 software (Larkin et al. 2007).

Results and discussion

Composition of fatty acids

A summarized view of FA content of 12 strains of thermophilic bacteria is shown in Table 1. FAs constituted between 3.9–6.2% of the dry weight of cells growing at 60°C. FA content divided tested strains into two groups: the first one included genera *Thermus, Meiothermus* and *Geobacillus* and the second one consisted of the genus *Alicyclobacillus.*

The members of the first group were characterized by very high content of the branched-chain FAs in their membrane lipids, which were found in the range of 67.7–86.5% of total FAs. *Iso*-branched FAs constituted 52.2–63.9% of total FAs and greatly predominated over *anteiso*-branched FAs, which constituted 13.8–23.6% of total FAs in the case of genera *Thermus* and *Meiothermus* and less than 10% of total FAs in the case of genus *Geobacillus*. The presence of branched FAs is considered to be a means of maintaining membrane fluidity; *iso*-branched FAs generally have higher melting points, while *anteiso*-branched FAs typically have lower melting points.

Table 1 FA content of individually cultivated thermophilic bacterial strains

Fatty acid[a]	3488[b,c]	3485	4167	2842	4211	4212	2062	5965	3731	3732	4659	4660
i-13:0	0.6	–	0.7	1.1	0.2	–	–	–	–	–	–	–
ai-13:0	0.4	–	–	–	–	–	–	–	–	–	–	–
13:0	–	–	–	–	–	–	–	–	–	0.1	–	–
i-14:0	0.6	3.1	1.9	0.8	0.8	0.5	0.2	0.2	0.3	0.5	–	–
14:0	1.6	0.3	0.4	0.4	0.6	0.5	0.8	0.2	1.1	1.4	–	–
i-15:0	26.4	25.5	35.2	23.8	27.9	34.5	17.9	22.9	16.9	22.0	0.9	0.6
ai-15:0	9.9	5.2	6.5	12.2	8.2	12.1	3.7	5.1	2.4	3.2	1.2	0.5
15:0	5.5	2.2	7.2	2.9	2.1	4.2	3.1	1.7	2.9	5.8	0.9	0.6
i-16:0	7.5	14.2	1.2	9.1	3.8	5.6	7.8	7.3	15.2	13.6	1.2	1.0
16:1	–	–	0.3	0.1	–	0.1	–	–	–	–	–	–
16:0	5.5	6.7	6.3	7.5	8.2	3.4	13.9	14.0	18.4	16.8	9.2	8.4
3-OH-15:0	2.2	3.3	–	–	–	–	–	–	–	–	–	–
ai-17:1	–	–	–	–	–	–	–	–	0.2	0.1	–	–
i-17:0	26.4	18.1	17.9	24.9	28.7	21.4	25.4	25.4	29.9	22.5	1.7	1.4
ai-17:0	3.0	9.9	13.6	10.5	12.9	10.4	18.9	8.1	6.4	5.0	4.0	3.0
17:0	2.1	2.3	2.4	4.3	2.9	1.7	5.5	6.3	2.5	6.8	1.1	1.2
3-OH-i-16:0	0.4	5.5	–	–	0.3	0.4	–	–	–	–	–	–
2-OH-16:0	–	–	–	–	0.3	0.4	–	–	–	–	–	–
ω-cyc-17:0[d]	–	–	–	–	–	–	–	–	–	–	64.5	59.4
i-18:0	0.9	1.0	1.5	0.7	0.3	0.4	0.5	0.7	1.3	0.5	–	–
9-18:1	–	–	0.4	0.4	0.1	0.2	–	–	0.1	0.2	–	–
18:0	0.6	0.3	1.1	0.2	0.7	1.2	1.5	2.2	1.4	1.1	1.2	0.9
3-OH-17:0	5.5	1.3	–	0.1	0.3	1.3	–	–	–	–	4.8	4.8
i-19:0	0.4	0.5	1.1	0.5	0.1	0.4	0.4	0.3	0.3	0.1	–	–
ai-19:0	0.5	0.2	2.3	0.3	1.6	1.2	0.4	5.0	0.6	0.2	–	–
19:0	–	–	–	0.1	–	0.1	–	–	0.1	0.1	–	–
ω-cyc-19:0[d]	–	–	–	–	–	–	–	–	–	–	9.3	18.2
20:0	–	0.4	–	0.1	–	–	–	0.8	–	–	–	–
Branched	76.7	77.7	81.9	83.6	84.5	86.5	75.2	74.9	73.5	67.7	9.0	6.4
Iso	62.9	62.4	59.5	60.6	61.8	62.9	52.2	56.7	63.9	59.3	3.8	2.9
Anteiso	13.8	15.2	22.4	23.0	22.8	23.6	22.9	18.2	9.6	8.4	5.2	3.5
Straight	15.3	12.2	18.1	15.8	14.6	11.4	24.8	25.1	26.5	32.3	12.4	11.1
Hydroxy	8.0	10.2	–	0.5	0.6	1.8	–	–	–	–	4.8	4.8
ω-cyc[d]	–	–	–	–	–	–	–	–	–	–	73.8	77.6
Unsaturated	–	–	0.7	0.5	0.1	0.3	–	–	0.3	0.3	–	–
Sum of FA (mg g^{-1})	53.3	55.0	62.1	60.2	57.5	59.7	39.1	41.5	43.3	44.1	40.0	39.3

[a] First number, number of carbon atoms in the chain; second number, number of double bonds; number before hyphen, position of double bond; position of double bonds not localized, only degree of unsaturation is given; *i iso*acid; *ai anteiso*acid

[b] The explanation of numbers (strains), see "Materials and methods"

[c] Each value represents the mean (SD) from six independent chromatographic analyses; three samples were taken from each bacterial strain that was separately processed and evaluated

[d] The ring has six carbon atoms

By contrast, both strains of the genus *Alicyclobacillus* contained a very small amount of branched-chain FAs, only less than 9% of total FAs. The *anteiso*-branched FAs slightly predominated over the *iso*-branched FAs (Table 1). This can be explained by optimal growth temperature, which is the lowest among all tested strains, i.e., only 45°C for the both strains. At low growth temperature the *anteiso*-branched FAs are those that participate in membrane fluidity regulation (Nordstrom and Laakso 1992).

The genus *Alicyclobacillus* was distinguished from the other tested genera by the occurrence of ω-cyclic FAs. These acids clearly predominated in FAs profiles of this

genus, since they were found in amounts higher than 70% of total FAs. These FAs represent a highly unique component of cell envelopes. They were found only in genera *Alicyclobacillus* and *Sulphobacillus* and are therefore used as a chemotaxonomic feature (Matsubara et al. 2002).

Straight FAs occurred rather evenly, 11.1–18.1% of total FAs in the case of genera *Thermus, Meiothermus* and *Alicyclobacillus*; their content at 24.8–32.3% of total FAs in genus *Geobacillus* slightly differed from the others.

The occurrence of hydroxy FAs was found in seven tested strains and the amount was significant only in the following strains: CCM 3488 and CCM 3485 (ca. 10% of total FAs) and CCM 4659 and CCM 4660 (ca. 5% of total FAs).

Unsaturated FAs were found only in small amounts. These results were in accordance with the literature, because it is well known that unsaturated FAs do not have appropriate properties suitable for maintaining membrane fluidity at higher temperatures and they are not widespread in thermophilic microorganisms, especially in the genus *Thermus* (Ray et al. 1971; Kristjansson 1992).

Detection and determination of the molecular glycophospholipid species by FAB-MS

Both Geobacilli contain low concentrations of unusual glycophospholipids. Upon partial purification by a 2D TLC, acylglycosylcardiolipins were identified as molecular species from the presence of pseudomolecular $[M–H]^-$ ions in the negative ion mode.

Cardiolipin is a phosphoglyceride that contains a phosphatidylglycerol linked to a phosphoglyceride unit to make it a diphosphatidylglycerol. CL possesses two phosphate charge sites and forms both $[M–H]^-$ and $[M–2H]^{2-}$ ions when subjected to FAB-MS. The major species of our glycophospholipid found in bacterial cells is 17:0-Hex-(17:0/17:0)(17:0/17:0)-CL, the simplest form of CL. It contains five identical 17:0-acyl substituents residing at *sn*-1, -2, -1′, -2′ and hexose, respectively. The compound gives an $[M–H]^-$ ion at *m/z* 1,824 and an $[M–2H]^{2-}$ ion at *m/z* 912. The splitting of the molecule yields the 17:0 carboxylate anion at *m/z* 269, reflecting the uniform fatty acyl moiety of the molecule. The spectrum also contains the *m/z* 786 ion, a doubly-charged fragment ion arising from the loss of a 17:0-fatty acyl group as a ketene (912-252/2). The R_xCOO^- ion at *m/z* 269 arises from nucleophilic attack of an anionic phosphate charge site onto C2 or C2′ of a glycerol backbone (Fig. 3). This fragmentation process also gives rise to *m/z* 1,555 (912x2-269). In contrast, the $[M–H]^-$ ion at *m/z* 1,824 (Fig. 3) is an acidic ion, which yields *m/z* 1,543 + H by the loss of a 17:0 fatty acid, and *m/z* 1,407 (loss of acyl-hexose) by cleavage of the O–C bond, along with *m/z* 675 (loss of phosphodiacylglycerol) and *m/z* 1,147 (supplementary ion to ion at *m/z* 675). Part of the total spectrum is shown in Fig. 4. As seen in the figure, a total of 11 clusters were identified, which corresponds to all combinations of FA with 15, 16 and 17 carbons atoms.

The data confirmed the indications derived from the MS experiments that isobaric molecular species are present (see also Fig. 3), in which different patterns of fatty acid chain length are represented on four possible substitution sites in two glycerol and one hexosyl moiety. In all investigated $[M–H]^-$ acylglycocardiolipin species, the saturated fatty acid moieties were of C15, C16 and C17.

An unusual glycophospholipid was also isolated from the three strains of *Thermus*. The five major clusters at *m/z* 1,190, 1,204, 1,218, 1,232 and 1,246 and the minor peaks with an *m/z* difference of 14 Daltons in the FAB mass spectrum (Fig. 5) indicated that the aliphatic chains on 1,2-alkyldiols and fatty acids were of different lengths.

The most important ions confirming the proposed structure seem to be, apart from the pseudomolecular ion $[M + H]^+$, ions *m/z* 561 (*m/z* 723), and *m/z* 403 (*m/z* 801). The FA ion at *m/z* 241 shows that the lipid with molecular weight 1,189 ($[M + H]^+ = 1,190$) contains two C15 acids and two C16 long chain 1,2-diols, which was confirmed by

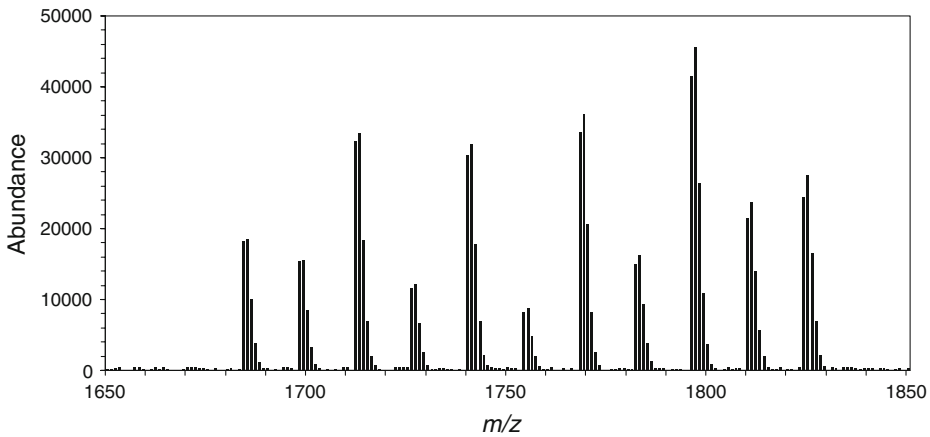

Fig. 4 Negative ion FAB-MS spectrum of acylhexosylcardiolipins from *Geobacillus*

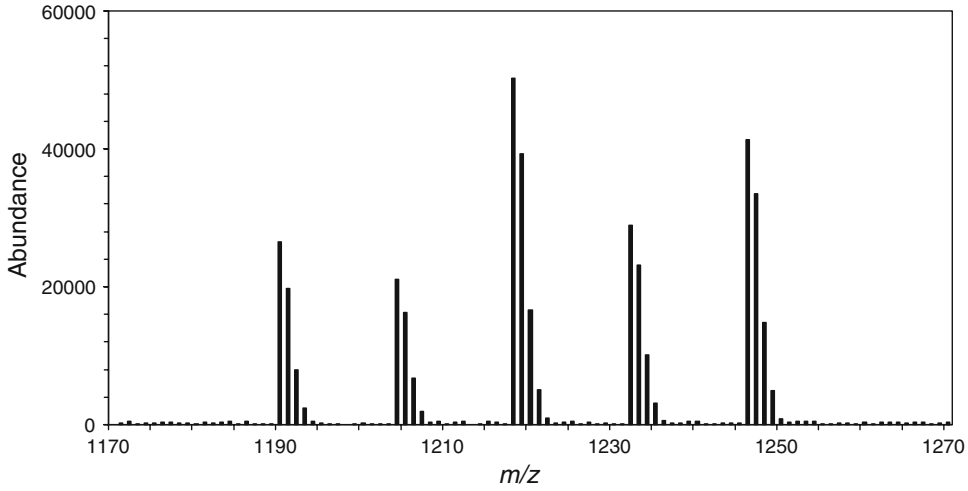

Fig. 5 Positive ion mode FAB-MS spectrum of a phospholipid from *Thermus*

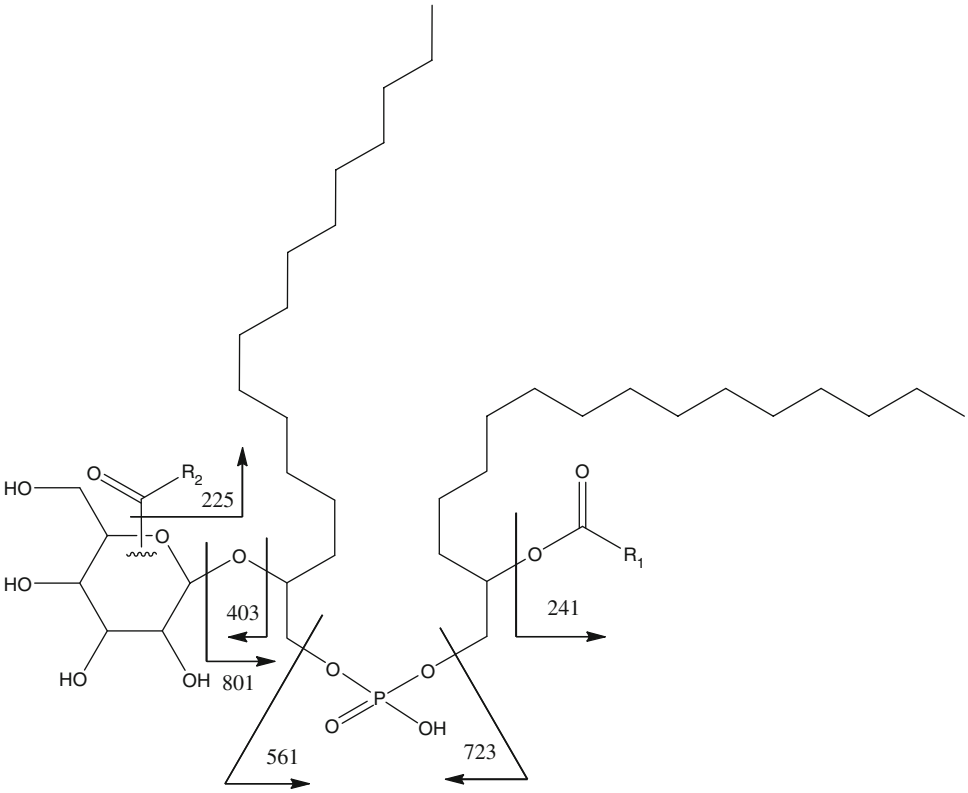

Fig. 6 General structure of the glycophospholipid; $R_{1,2}$-fatty acid residues

GC–MS of trimethylsilyl derivatives after hydrolysis. We assume that the ion at *m/z* 241 arises by splitting of acyls from $[M + H]^+$; this was confirmed by the presence of a weak but significant ion, see Fig. 6. The fragmentation and appearance of ions *m/z* 403 and/or *m/z* 801 further showed that the molecule of the lipid contains one hexose bound to the secondary hydroxyl of one of the two long chain diols. Finally, ions *m/z* 561 and/or *m/z* 723 confirm the presence of phosphoric acid as a connecting bridge between the two asymmetric parts of the lipid.

Based on the above data, we propose the structure shown below (Fig. 6). However, like with the complex cardiolipin mentioned above there are many problems that still await clarification. In order to obtain detailed information about the fatty acid distribution, further measurements with larger samples have to be performed to show that this unusual lipid can be used as a possible chemotaxonomical marker.

Cluster analysis

16S rDNA sequences comprising 413–486 nucleotides of 12 selected strains of thermophilic bacteria from the Czech Collection of Microorganisms were determined, sequence

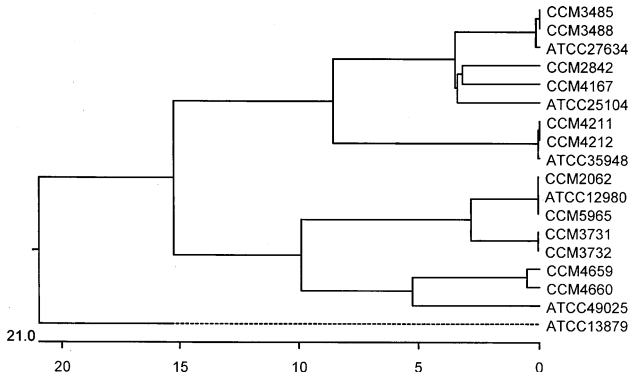

Fig. 7 Phylogenetic tree of 12 tested thermophilic strains from the Czech Collection of Microorganisms (CCM; full names of individual strains are in "Materials and methods") and six strains as controls coming from American Type Culture Collection (ATCC): ATCC 27634 *Thermus thermophilus*, ATCC 25104 *Thermus aquaticus,* ATCC 35948 *Meiothermus ruber,* ATCC 12980 *Geobacillus stearothermophilus*, ATCC 49025 *Alicyclobacillus acidoterrestris*, ATCC 13879 *Streptomyces bambergiensis* (serves as an outgroup). The phylogenetic tree is based on the weighted residue method and is derived from an alignment comprising 16S rDNA gene sequences

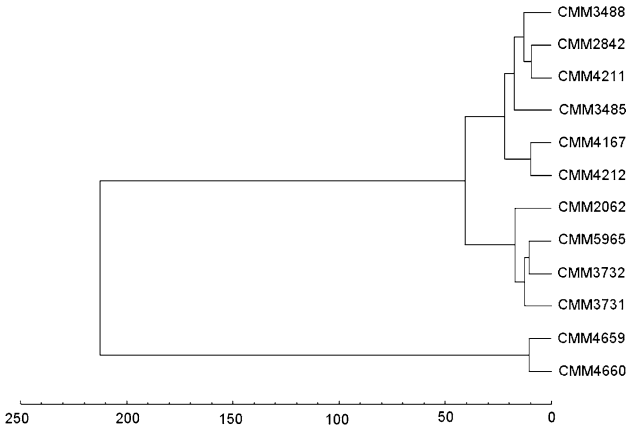

Fig. 8 Hierarchical tree of 12 tested thermophilic strains from the Czech Collection of Microorganisms (CCM, full names of individual strains are in "Materials and methods"), based on Ward's method and Euclidean distance, derived from FA composition

data were compared and the phylogenetic positions of these strains were inferred by constructing a phylogenetic tree by the neighbor-joining method (Fig. 7). The phylogenetic tree shows quite long distances among the three clusters, i.e., two Gram-negative genera *Thermus* and *Meiothermus*, genus *Geobacillus* and genus *Alicyclobacillus* (Fig. 7). Two tested strains CCM 3485 and CCM 3488 were clustered with *T. thermophilus* ATCC 27634 and had 99.8% sequence similarity; however, according to the CCM they should have belonged to *T. aquaticus*. The relatedness of these strains with *T. aquaticus* ATCC 25104 was more distant.

The location of *T. aquaticus* ATCC 25104 was closer to strains CCM 2842 and CCM 4167. Sequence comparison confirmed that they belong to *T. species*.

The other strains were subdivided into clusters in accordance with the classification of the quoted Czech Collection of Microorganisms. Each cluster was complemented with reference sequence of the particular species originating from ATCC collection.

Also the data related to FA composition were processed by cluster analysis (using Ward's method) in order to find any statistical relation between FA content and phylogenetic position. Contrary to the phylogenetic tree, the FA composition divided tested strains into two main clusters, the first includes genera *Thermus, Meiothermus* and *Geobacillus,* the second being formed by two strains of genus *Alicyclobacillus* (Fig. 8). This shows that ω-cyclic FAs are indeed a significant chemotaxonomic marker that can be useful in *Alicyclobacillus* identification.

Figure 8 shows further subdivision of the large cluster into two smaller clusters, the first cluster comprising all the strains of genera *Thermus* and *Meiothermus*. The position of these strains in a dendrogram constructed on the basis of FA content does not correspond with their phylogenetic classification. On the other hand, the second cluster includes four strains of genus *Geobacillus* and their position is approximately in accordance with their phylogenetic classification.

This study determined the structure of the glycophospholipids in thermophilic *Thermus* (Fig. 3) and *Geobacillus* (Fig. 6) strains, which, to our current knowledge, had not been published before. The structural information will be very useful for further investigations of the mechanisms of glycophospholipid biosynthesis in vivo and the determination of the physiological roles of the glycophospholipids. To the best of our knowledge, this is the first description of unusual glycophospholipid structures from thermophilic bacteria of the genus *Thermus* and *Geobacillus*.

Acknowledgments The research was supported by Institutional Research Concept AV 0Z 502 0910 and by the project No. MSM6046137305 of the Ministry of Education, Youth and Sport of the Czech Republic.

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