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Rhamnolipid-producing thermophilic bacteria of species *Thermus* and *Meiothermus*

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Abstract Novel rhamnolipid-producing strains of three thermophilic bacteria, *Thermus* sp., *T. aquaticus* and *Meiothermus ruber* were identified that have not been previously described as rhamnolipid producers. Rhamnolipids were extracted from supernatant and further purified by thin-layer chromatography. Mass spectrometry with negative electrospray ionization revealed 77 rhamnolipid homologues varying in chain length and unsaturation. Tandem mass spectrometry identified mono-rhamnolipid and di-rhamnolipid homologues containing one or two 3-hydroxy-fatty acids, saturated, monounsaturated or diunsaturated, even- or odd-chain, up to unusual long chains with 24 carbon atoms. The stereochemistry of rhamnose was *L* and that of 3-hydroxy-fatty acids was *R*, the position of double bonds in monoenoic acids was *cis* ω-9. All three strains produced a rhamnolipid that differs in structure from *Pseudomonas aeruginosa* rhamnolipids and exhibits excellent surfactant properties. Importantly, in comparison to *P. aeruginosa* both strains, i.e., *Thermus* and *Meiothermus*, are Biosafety level 1 microorganisms and are not pathogenic to humans.

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Introduction

Bacteria are known to produce various types of biosurfactants. These compounds have a very broad range of applications, including household/laundry detergents or environmental applications such as removal of environmental contamination, biosurfactant-enhanced bioremediation, biosurfactant-enhanced soil washing, and oil recovery and processing. Biomedical applications make use of the ability of biosurfactants to disrupt membranes and their antimicrobial, antiviral and antifungal activities. Biosurfactants can also be used as anti-adhesives, etc. (Abdel-Mawgoud et al. 2010; Cameotra and Makkar 1998). Among the best known biosurfactants are rhamnolipids (Soberon-Chavez 2011). Their hydrophilic part is composed of rhamnose(s) (Rha) and the hydrophobic part is typically 3-hydroxy-fatty acid(s) (3-OH-FAs) (also named β-hydroxy, i.e., β-OH-FAs). The rhamnose moieties are linked to each other through 1,2-glycosidic linkage. The rhamnose is in the L-form (Jarvis and Johnson 1949) and anomeric configurations are α on the basis of 2D ^{1}H COSY (COrrelation SpectroscopY) spectra and of the small vicinal $^{3}J_{H1-H2}$ constant and $^{1}J_{C1-H1}$ at 170.5 Hz (Sharma et al. 2007; Price et al. 2009). The chains of fatty acids are most commonly saturated or, less often, mono- or diunsaturated. 3-OH-FAs have an even carbon number from C8 to C16 (Abdel-Mawgoud et al. 2010), exceptionally an odd number of carbon atoms (C9, C17) or very long chain 3-OH-FA (3-hydroxy-fatty acid), either saturated (3-OH-24:0) or monoenoic (3-OH-24:1) (Nie et al. 2010). To date, about

60 different molecular species have been reported (Abdel-Mawgoud et al. 2010). Although Abdel-Mawgoud et al. (2010) and Soberon-Chavez (2011) mention many methods for qualitative and quantitative determination of rhamnolipids, only mass spectrometry, or LC–MS (liquid chromatography–mass spectrometry) or tandem-MS with electrospray ionization (ESI–MS) preferably in negative mode gives satisfactory results in the identification of rhamnolipids, including the determination of their structure(s). In terms of the structure of rhamnolipids, and only MS/MS (tandem mass spectrometry) enables the identification of single molecular species, including positional isomers, e.g., Rha-C8-C10 from the isomeric Rha-C10-C8 (Sharma et al. 2007; Deziel et al. 2000; Heyd et al. 2008; Haba et al. 2003). ^{1}H and ^{13}C NMR, including 2D methods, are absolutely necessary to identify the stereochemistry of rhamnolipids (Sharma et al. 2007; Price et al. 2009).

The main source of these glycolipids has long been *Pseudomonas aeruginosa*. However, many other bacteria have been reported to be also rhamnolipid producers (Abdel-Mawgoud et al. 2010; Cameotra and Makkar 1998). These are other *Pseudomonas* species, or species having taxonomically more distant relationship to *Pseudomonas*, e.g., *Acinetobacter calcoaceticus*, *Pseudoxanthomonas* sp., *Enterobacter* sp., *Pantoea* sp. or *Burkholderia* sp. (Abdel-Mawgoud et al. 2010; Rooney et al. 2009). Mass spectrometry was used to identify predominantly C10–C10 mono- and dirhamnolipids in *A. calcoaceticus*, *Enterobacter asburiae*, *E. hormaechei*, and *Pantoea stewartii* (Rooney et al. 2009). Several *Burkholderia* species are able to produce rhamnolipids that contain a C14–C14 lipid moiety (Abdel-Mawgoud et al. 2010).

Rhamnolipids were also identified in cultivation medium of bacterium *Renibacterium salmoninarum* by TLC (thin-layer chromatography) and IR (infrared) spectra (Abdel-Mawgoud et al. 2010), *Nocardioides* sp. by TLC accompanied by erythrocyte hemolysis tests and growth inhibition of *Bacillus subtilis* (Abdel-Mawgoud et al. 2010), and *Tetragenococcus koreensis* by HPLC (high performance liquid chromatography) using UV (ultraviolet) detection.

By using TLC, IR and LC–MS, Pantazaki et al. (2010) identified mono- and di-rhamnolipids also in the thermophilic bacterium *Thermus thermophilus*. Busscher et al. (1994) isolated biosurfactant-producing *Streptococcus thermophilus* strains from the pasteurizers in the dairy industry. De Trebbau Acevedo and McInerney (1996) managed to isolate bioemulsifiers with special characteristics from *Methanobacterium thermoautotrophicum*. As mentioned above, Pantazaki et al. (2010) studied the strain *T. thermophilus* HB8 as a potential rhamnolipid producer. Cameotra and Makkar (1998) summarized more reports dealing with production of biosurfactants in a thermophilic environment; however, the examined bacteria were thermotolerant rather than thermophilic.

Thermophilic microbes are spread across the whole taxonomic system, in various taxonomic groups and at various phylogenetic distances (Siristova et al. 2009). They are able to survive and grow at temperatures higher than 42°C (Siristova et al. 2009; Cameotra and Makkar 1998). Their use in biotechnological processes has many advantages, such as: faster reaction rates, reduced risk of contamination, reduced cooling costs while large fermentors are operated, reduced viscosity of growth media at higher temperatures and a reduced energy requirement for agitation, increased solubility of molecules at higher temperatures and the fact that thermophilic treatment kills pathogenic bacteria and viruses (Horikoshi 2011; Cameotra and Makkar 1998).

This report is part of our investigation of thermophilic bacteria. We extended our analysis from glycophospholipids (Siristova et al. 2009), unusual acylphosphatidylglycerols (Rezanka et al. 2009a), and *O*-acyl glycosylated cardiolipins (Rezanka et al. 2009b) to rhamnolipids. The structure of rhamnolipids was confirmed not only by ESI–MS, but, after isolation, also by the measurement of ^{1}H- and ^{13}C-NMR, including determination of absolute configuration of all chiral atoms, including the position and geometry of double bond. Our strains produced structurally novel rhamnolipids with very good emulsification activity for aromatic and aliphatic hydrocarbons, and several plants oils.

Materials and methods

Instrumentation

The Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) with electrospray ionization was used. The lipid extracts of different classes of rhamnolipids were each redissolved in 200 μL of dichloromethane–methanol–glacial acetic acid (65:15:2). Typically, ~0.1 mg of rhamnolipids was dissolved in 200 μL of the solvent mixture and then immediately infused into the ESI source at 5–10 μL min^{-1}. The ionization mode was negative, the nebulizing gas (N_2) pressure was 345 kPa and the drying gas (N_2) flow and temperature were 9 L min^{-1} and 300°C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4,000 V. The cone voltage was kept at −45 (−80) V. The mass resolution was 0.1 Da and the peak width was set to 6 s. For an analysis, total ion currents (full scan) were acquired from 100 to 1,000 Da.

CID ion mass spectra were acquired by colliding the Q1-selected precursor ions with Ar gas as a collision target

gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 200–1,600 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization, and CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 μm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program: 5 min at 50°C, increasing at 10°C min^{-1} to 320°C and 15 min at 320°C. Helium was the carrier gas at a flow of 0.52 mL min^{-1}. All spectra were scanned within the *m/z* 50–600 range. The spectra was identified manually, see also Rezanka (1993).

The oven temperature for identification of dimethyl disulfide adducts of acetylated 3-OH FAMEs was programmed from 50 to 275°C at 10°C min^{-1} and further at 5°C min^{-1} to 350°C and further with 8 min isothermal elution. The other conditions of GC–MS apparatus were identical.

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H) and 125.7 MHz (^{13}C). All compounds were purchased from Sigma-Aldrich (Prague, Czech Republic).

Cultivation and extraction

Three strains of thermophilic bacteria *Thermus aquaticus* CCM 3488, *Thermus* sp. CCM 2842, and *Meiothermus ruber* CCM 4212 originating from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) were used in present work. Both 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) with the addition of sunflower seed oil in a concentration of 2 g L^{-1}. Batch cultivation was carried out under aerobic conditions on a temperature-controlled shaking device (HT Inflors AG CH-4103, Hungary). Incubation proceeded for 44 h, at 65°C and 200 rpm. After incubation the biomass was separated from culture broth by centrifugation (Sorvall Evolution RC with SLC-6000 rotor) and 2 L volumes of supernatant for each strain were obtained. The supernatant was concentrated by evaporation under reduced pressure at 70°C. Then pH was adjusted to 2 with 3 M H_2SO_4, an equal volume of dichloromethane:methanol (2:1) was added and the mixture was well mixed by shaking in separating funnel. After a few minutes the lower organic phase was separated from upper aqueous phase. Extraction procedure was repeated twice more for each sample and all three organic parts were mixed together. Then the organic solvent was removed by evaporation under reduced pressure and the lipidic extract was lyophilized to remove any residual amount of water. After centrifugation, the yields (culture volumes 2 L) were 5.58 g (strain 3488), 4.24 g (strain 4212), and 3.01 g (strain 2842), respectively.

TLC separation

First, parts of total lipid extracts from the three strains, i.e. *Thermus* sp. CCM 2842, *T. aquaticus* CCM 3488, and *M. ruber* CCM 4212 (~1 g) were applied to Sep-Pak Vac Silica cartridge 35 cc (Waters; with 10 g of silica) and non-polar lipids (predominantly non-metabolized TAGs) were subsequently eluted from the cartridge with 40 mL of hexane–dichloromethane (9:1), and so were total rhamnolipids with 60 mL of dichloromethane–methanol–glacial acetic acid (49:49:2). The eluate was reduced in volume and subjected to preparative TLC (PLC silica gel 60 F254, 2 mm × 20 cm × 20 cm, Merck; dichloromethane–methanol–glacial acetic acid 65:15:2). Visualization was carried out by dipping the plates in the mixture of 0.1% orcinol and 15% H_2SO_4 in ethanol followed by heating. For preparative TLC, only left and right edges of the TLC plate were dipped into orcinol-sulfuric acid to a depth of 1 cm. In preparative mode, visualized spots were scraped off and the lipid was eluted with 2 mL of dichloromethane–methanol–glacial acetic acid (3:3:1). For quantification the developed chromatogram was scanned (by Shimadzu TLC scanner CS-930) in absorbance mode at 225 nm. The signals from the scanner were collected by Data Recorder DR-2 (Shimadzu) and were integrated and calculated using Statistica 9 software (StatSoft, ČR). It should be noted that using PC software for quantification of the results yields mostly semi-quantitative rather than quantitative results.

Identification of L configuration of rhamnose

The identification and determination of the D or L configuration of rhamnose was performed using GC–MS (Gerwig et al. 1978), with some modifications, and with SPB-1 (Supelco) column (30 m × 0.25 mm I.D.). The Rha-Rha-FA-FAs (1 mg) were subjected to a mild methanolysis in 0.5 mL of 0.1 M methanolic HCl at room temperature overnight. The solvent was removed under Ar gas. To the dried sample was added (+)-2-butanol (300 μL) and acetyl chloride (50 μL) and the ampoule was then sealed. After butanolysis at 80°C for 8 h, the solution was neutralized with Ag_2CO_3. After centrifugation at 2,000 rpm for 10 min the supernatant solution was concentrated under reduced pressure at 45°C. The residue was treated with acetic anhydride (100 μL) in pyridine (200 μL). Heating at 70°C

for 30 min brought about acetylation. The column temperature was programmed to rise 5°C min^{-1} from 170 to 260°C. The flow rate of the helium carrier gas through the column was 1.5 mL min^{-1}. The temperature of the injector was 270°C. Authentic 1-((+)-2-butyl)-2,3,4,6-tetraacetyl-L-rhamnose (α and β anomers) were eluted as peaks with retention times of 10.07 and 10.19 min, respectively. The retention times of α and β anomers were 8.91 min and 10.16 min, respectively.

Enzymatic hydrolysis of rhamnolipids

A solution of Rha-FAs, Rha-Rha-FAs, Rha-FA-FAs and Rha-Rha-FA-FAs (each 1 mg) in acetate buffer (pH 4.4, 10 mL) was treated with hesperidinase for 48 h at 37°C. The reaction solution was evaporated to dryness, and the residue was used for further analysis.

Acid hydrolysis of rhamnolipids

A solution of Rha-FAs, Rha-Rha-FAs, Rha-FA-FAs and Rha-Rha-FA-FAs (each 1 mg) was refluxed in 2 N HCl (0.5 mL) for 2 h. The 3-OH-FAs were extracted three times with EtOAc (10 mL). The free 3-OH-FAs were derivatized by diazomethane and resulting methyl esters were analyzed by GC–MS. After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$ and evaporated.

Dimethyl disulfide adducts of acetylated 3-OH FAMEs

About 1 mg of 3-OH FAMEs was dissolved in acetic anhydride in pyridine (1 mL, 5:1) and was left at room temperature overnight. The mixture was evaporated and the resulting mixture of 3-*O*-acetyl FAMEs was further dissolved in dimethyl disulfide (0.2 mL) and a solution (0.05 mL) of iodine in diethyl ether (60 mg mL^{-1}) was added. The mixture was stirred for 24 h, then hexane (5 mL) was added, and the mixture was washed with dilute sodium thiosulfate solution, dried over sodium sulfate and evaporated to dryness. The product was analyzed by GC–MS.

(*S*)-MTPA esters

To a CH_2Cl_2 solution (100 µL) were added methyl esters of 3-OH-FAs (0.3 mg), DMAP (1.0 mg), and Et_3N (2 µL) (*R*)-(−)-MTPACl (3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl chloride) (2.0 mg) at room temperature, and stirring was continued for 3 h. After evaporation of the solvent, the residue was purified by Si gel TLC (hexane–AcOEt, 2:1) to afford the (*S*)-MTPA ester as colorless oil. The esters of the 3-OH-FAs from the glycosides (Rha-Rha-FAs, Rha-FAs-FAs and Rha-Rha-FAs-FAs) are identical with (*S*)-MTPA ester obtained from Rha-FAs.

(*R*)-MTPA ester

Methyl esters of 3-OH-FAs (0.3 mg) were treated with (*S*)-(+)-MTPACl (2.0 mg) using the same procedure as described above to afford the (*R*)-MTPA ester as colorless oil. The esters of the 3-OH-FAs from the glycosides (Rha-Rha-FAs, Rha-FAs-FAs and Rha-Rha-FAs-FAs) are identical with (*R*)-MTPA ester obtained from Rha-FAs.

Emulsifying activity with different hydrophobic compounds

Emulsification index was measured using the method described by Silva et al. (2010) whereby 1 mL of non-polar compounds (*n*-hexadecane, gasoline, diesel, canola sunflower oil and soybean oils) was added to 1 mL of the supernatant in a graduated screw-cap test tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

Results and discussion

Cultivation and isolation of rhamnolipids

The biomass of the three thermophilic strains (*Thermus* sp., *T. aquaticus*, and *M. ruber*) was harvested in early stationary phase when the optical density of the culture was maximal. The time of cultivation was 44 h. After centrifugation, the supernatants (culture volumes 2 L) were extracted according to Bligh and Dyer (1959). Part of total lipids was fractionated by means of cartridges with silica-based stationary phase, which were rinsed in a hexane-dichloromethane mixture. Rhamnolipids were eluted by a mixture of dichloromethane–methanol–glacial acetic acid and further separated by TLC with the same mixture as mobile phase. Neutral lipids, especially TAGs of sunflower seed oil that were not consumed by the bacteria, showed a TLC band at Rf 0.88. Four additional bands were detected with Rf values 0.24, 0.32, 0.50 and 0.69. The lower band having Rf 0.24 together with the higher band (Rf 0.32) consisted of dirhamnolipids, while the bands at Rf 0.50 and 0.69 consisted of monorhamnolipids (see below). Our results are similar to previously reported results of TLC of rhamnolipids from different strains of *P. aeruginosa* (Abdel-Mawgoud et al. 2010), which differ only in the chain lengths of FAs. Visualization was carried out with orcinol

followed by heating. Quantification was performed by means of a TLC scanner and calculated using Statistica 9 software (Fig. 1).

In the preparative mode, the appropriate four bands were scraped off from the preparative plates, eluted by the mixture dichloromethane–methanol–glacial acetic acid (3:3:1) and evaporated. The mixtures of four distinct rhamnolipid bands were further identified by mass spectrometry. Table 1 shows the yields of individual classes of rhamnolipids (Rha-Rha-FAs, Rha-Rha-FAs-FAs, Rha-FAs, and Rha-FAs-FAs).

Fatty acid analysis

Table 2 shows the composition of FAME of four classes of rhamnolipids after GC–MS analysis. Only fatty acids with abundances above 0.1% of total fatty acids are included in Table 2, which shows the presence of both odd- and even-chain 3-OH-FA, including saturated, monoenoic and dienoic 3-OH-FAs. A total of 23 3-OH-FAs were identified, i.e., more than seven times the number of commonly identified acids (Monteiro et al. 2007; Sharma et al. 2007).

As mentioned in the introduction, the vast majority of rhamnolipids contain even-numbered saturated and, to a much lower extent, monoenoic 3-OH-FAs from C8 to C14 (Abdel-Mawgoud et al. 2010). The major were C10 acids (saturated and monoenoic), followed by C8 and C12 ones, again both saturated and/or monounsaturated. Other acids, very rarely present, were e.g., dienoic 3- such as 12:2 or 14:2. Exceptionally, longer acids were also found, mainly in the rhamnolipids from *Burkholderia thailandensis*, which contained 3-hydroxy-palmitic acid (Dubeau et al. 2009). Only rarely were identified acids with an odd number of carbon atoms (C9) or very long chain 3-OH-FAs such as saturated (3-OH-24:0) and monoenoic (3-OH-24:1) (Nie et al. 2010).

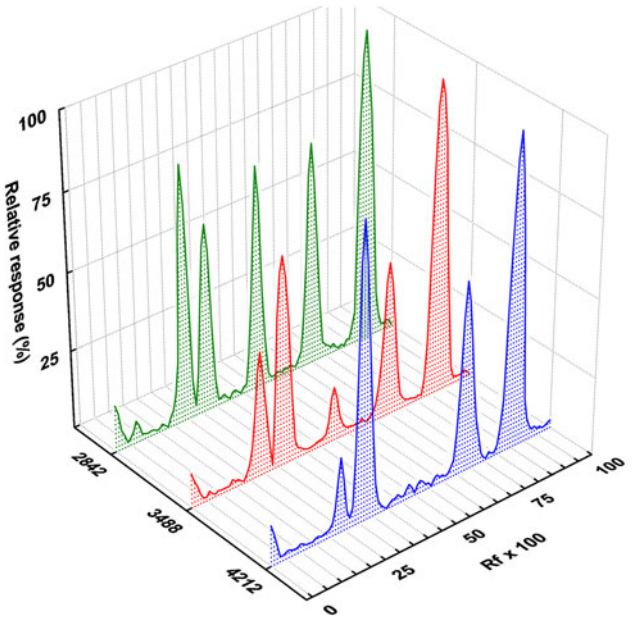


Fig. 1 Quantification of rhamnolipids separated by TLC scanner and calculated using Statistica 9 software

Stereochemistry of lipid moiety

Since the identification of the position of double bonds in 3-OH-FAs is very difficult, if not impossible with commonly used nitrogen derivatives such as picolinyl esters

Table 1 TLC analysis of rhamnolipids extracted from culture broth of *Thermus* sp. CCM 2842, *T. aquaticus* CCM 3488, and *Meiothermus ruber* CCM 4212

Rhamnolipid	Strain 2842	Strain 3488	Strain 4212
Rha-FAs-FAs	24[a]	32	37
Rha-FAs	26	7	0
Rha-Rha-FAs-FAs	21	42	54
Rha-Rha-FAs	29	19	9

[a] The values in the table are in relative percent

Table 2 The composition of FAME of four classes of rhamnolipids determined by GC–MS of strain *Thermus* sp. CCM 2842

Fatty acid	Rha-FA–FA	Rha-FA	Rha–Rha-FA–FA	Rha–Rha-FA
10:0	0[a]	0	0	1.8
11:0	0	0.9	0	0
12:0	2.1	5.3	3.3	8.2
13:0	0	2.4	0	0
14:0	7.8	14.6	12.7	21.5
15:0	0	3.2	3.6	0
16:0	27.5	40.2	29.4	37.6
16:1	6.3	8.4	5.3	4.3
17:0	2.4	2.6	1.2	0.7
18:0	17.4	12.5	20.7	15.4
18:1	3.3	2.4	3.2	3.2
19:0	1.4	0	0	0
20:0	9.6	4.2	9.4	4.7
20:1	6.4	2.1	3.8	1.5
20:2	1.8	1.2	0	0
21:0	0.1	0	0	0
22:0	3.4	0	5.3	1.1
22:1	1.8	0	2.1	0
22:2	0.9	0	0	0
23:0	0.2	0	0	0
24:0	2.7	0	0	0
24:1	3.5	0	0	0
24:2	1.4	0	0	0

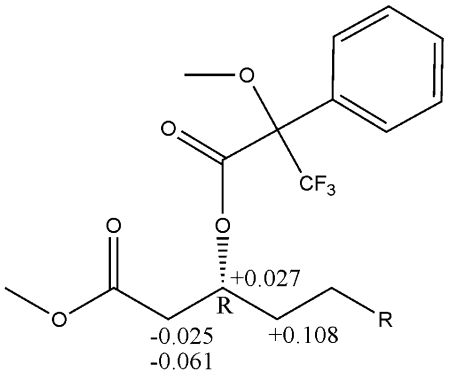
[a] The values in the table are in relative percent

(Christie and Han 2010), we used the method described for the unsaturated 2-OH-FAs (Imai et al. 2000), in which dimethyl disulfide was added on the double bond and the resulting appropriate dimethyl disulfides, i.e., adducts of 3-*O*-acetyl methyl esters, were separated and identified by GC–MS.

Based on mass spectra of five major derivatives, i.e., even-numbered FAs from 16 to 24 carbon atoms, we found that the double bond(s) are in position ω-9. Important ions were identified in the mass spectra of all monoenoic derivatives. The mass spectrum of C16 derivative is described as an example. The dimethyl disulfide adducts give the following characteristic ions: the molecular ion of C16 methyl ester at *m/z* 420, identifies the adducts as methyl 3-*O*-acetyl bis(methylthio)hexadecanoate. Further ion at *m/z* 360 $[M\text{-}60]^+$ (M-AcOH) is present in very low intensity. Other ions include the ions at *m/z* 328 $[M\text{-}92]^+$ (M-AcOH-MeOH) and *m/z* 286 $[M\text{-}134]^+$ (M-AcOH-74 Da, the latter being the McLafferty rearrangement ion of the methyl ester moiety). Further ions indicating the original position of the double bond include two major ions at *m/z* 173 and at *m/z* 247 and one minor ion at *m/z* 215, which arises from the ion at 247 Da by loss of methanol. The further peaks on the chromatogram (homologues) also gave the key major fragment ion at *m/z* 173 and the characteristic fragments as the base peaks at *m/z* 275, 303, 331, and 359, respectively, i.e., ions of type $[M\text{-}173]^+$, indicating the presence of an ω-9 series of monoenoic 3-OH-FAs having C18, C20, C22, and C24 carbons.

Another problem is the stereochemistry of double bond, i.e., geometric isomerism (*cis–trans*, or better *E-Z*) of double bond(s). The allylic groups associated to olefinic carbon atoms have chemical shifts in ^{13}C NMR, which depend on double bond configuration, i.e., the chemical shifts usually decrease by about 2.5 ppm for *cis* (*Z*) double bond (to about 27.2 ppm), while for *trans* (*E*) double bond they increase to 32.5 ppm (Bus et al. 1976) compared to methylene in saturated FA. We found that the δ value of our mixture of acids for allyl carbon ranged from 27.1 to 27.2 ppm. It follows that the configurations of double bonds are *cis* (*Z*).

The absolute configuration of 3-OH group of FAs was identified by using the modified Mosher's method (Ohtani et al. 1991). Treatment of methyl ester hydroxy acids with (*R*)-(−)-MTPACl (3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl chloride) or (*S*)-(+)-MTPACl converted them into the (*S*)- and (*R*)-MTPA esters. The values of $\Delta\delta = [\delta(S\text{-MTPA ester}) - \delta(R\text{-MTPA ester})]$ in the ^{1}H NMR spectra suggested that the absolute configuration at C-3 of 3-OH-FAs was *R* (Fig. 2), see also similar compounds described by Warabi et al. (2005). Our esters had also a $COOCH_3$ signal at $\delta = 3.64$ ppm in the ^{1}H NMR spectrum. The signal at $\delta = 3.56$ of the diastereomeric esters could not be detected, which again confirms the absolute configuration of 3-OH-FAs as *R*, see also Jakob et al. (1996).



Δδ=[δ(S-MTPA ester)-δ(R-MTPA ester)]

Fig. 2 The absolute configuration at C-3 of MTPA esters 3-OH-FA by ^{1}H NMR method

Stereochemistry of sugar moiety

Analysis of rhamnolipids and their exact structure including stereochemistry has so far been attempted only rarely. The structural analysis can be divided into two parts, i.e., complete identification of carbohydrates, including the determination of their stereochemistry (α, β, L or D), and identification of hydroxy fatty acids (*R* or *S*, and/or geometric isomerism, i.e., *cis–trans*).

The form of rhamnose, i.e., L or D, in rhamnolipids has so far been identified only by Jarvis and Johnson (1949). The D form is commonly found in *P. aeruginosa* as a D-rhamnose-rich polysaccharide antigen (Knirel 1990). The presence of L- and D-rhamnose in one microorganism is not common.

Basically, we repeated previously described (Price et al. 2009) 2D NMR experiments and we mention here in detail only the measurements that have not been described or whose results were misinterpreted; the basic chemical shifts of ^{1}H and ^{13}C NMR are described in Table 3.

The exact disposition of the two monosaccharide units and the position of the interglycosidic linkages in compound Rha-Rha-FA-FA were determined using 2D NMR spectroscopy. 2D-COSY experiments established the proton sequence within each sugar fragment starting from the well-resolved anomeric proton signals of both rhamnopyranoses in Rha-Rha-FAs-FAs. Chemical shifts, multiplicity of the proton signals, values of coupling constants, and chemical shifts of carbons indicated that both sugars must be in the α-rhamnopyranosyl form. In Rha-Rha-FAs-FAs, a glycosidation shift at C-2 (i.e., Δ 8.4 ppm) in the first rhamnose and the chemical shifts of H-1′ (δ_H 4.93) and

Table 3 ^{1}H and ^{13}C NMR of Rha-Rha-FA-FA

	No.	^{1}H NMR	^{13}C NMR
Aglycone	1	–	173.9
	2	2.60 m	39.2
	3	5.28 m	72.1
	4	1.64 m	33.9
	Mid chain CH_2	1.35 m	29.1–29.6
	ω-2	–	31.9
	ω-1	–	21.9
	ω-CH_3	0.92 t, J = 6.2	14.2
	1′	–	171.8
	2′	2.55 m	39.6
	3′	4.08 m	73.9
	4′	1.58 m	34.0
	Mid chain CH_2	1.35 m	29.1–29.6
	ω-2	–	31.9
	ω-1	–	21.9
	ω-CH_3	0.92 t, J = 6.2	14.2
Saccharides	1	4.96 brs	97.9
	2	3.75 brs	79.3
	3	3.70 m	71.5
	4	3.50 dd, J = 9.3, 9.3	73.5
	5	3.70 m	69.3
	6	1.28 d, J = 6.1	16.6
	1′	4.93 d, J = 1.2	103.1
	2′	4.00 m	70.9
	3′	3.70 m	69.8
	4′	3.50 dd, J = 9.3, 9.3	73.0
	5′	3.70 m	69.1
	6′	1.28 d, J = 6.1	16.6

C-1′ (δ_C 103.1) of the second rhamnose indicate that the first rhamnose is glycosidated at C-2 and linked at the aglycon, i.e., 3-OH-FAs. The second rhamnose was determined to be terminal by the absence of any glycosylation shift. These deductions were confirmed by a COLOC (correlation through long-range coupling) spectrum, which showed some diagnostic long-range correlations between H-1′ of rhamnose (δ_H 4.96) and C-3 (δ_C 73.9) of the 3-OH-FA, between H-1′ (δ_H 4.93) of the second rhamnose and C-2 (δ_C 79.3) of the first rhamnose. The sugar moieties were shown to be rhamnosyl-rhamnose.

The anomeric proton splitting $^{3}J_{H1-H2}$, which depends on the dihedral angle between H-1 and H-2, shows the anomeric configuration. However, for mannosides (rhamnose is 6-deoxy mannose) having the axial 2-OH group, the chemical shift gives no unequivocal information about the anomeric configuration and the anomeric proton splitting has virtually the same values for both configurations and it is inadvisable to use proton splitting to assign molecular configuration, see the value 1.8 Hz for α and 0.9 Hz for β anomer (Podlasek et al. 1995). Another way to identify which of the anomers is present in the glycoside is the use of the following method. The one-bond coupling constant between the anomeric carbon and its attached hydrogen, i.e., $^{1}J_{C1-H1}$ in α-pyranosides differed from that of β-pyranosides approximately by 10 Hz, i.e., $^{1}J_{C1-H1}$ in α-pyranosides is approximately 170 Hz and in the corresponding β-pyranosides it is about 160 Hz (Kasai et al. 1979). Values of $^{1}J_{C1-H1}$ ~165 Hz for α- and ~155 Hz for β anomer were measured chiefly for mannosides and rhamnosides (Kasai et al. 1979). These data were sometimes misinterpreted, see publications such as Monteiro et al. (2007) who measured a value of 168 Hz ($^{1}J_{C1-H1}$) and identified it wrongly as typical for the β anomer, while Sharma et al. (2007) measured a value of 170.5 Hz and identified it correctly as characteristic of the α anomer. Our measured value at 167 Hz clearly shows the presence of only α-rhamnose anomer.

To determine the absolute configuration of rhamnose in the glycosides, free rhamnoses were prepared from the glycosides by enzymatic hydrolysis. Rha-Rha-FAs-FAs fraction was hydrolyzed by hesperidinase (EC 3.2.1.40) from *Aspergillus niger*. This confirmed that rhamnoses are present in the glycosides in L-form. Another confirmation of the L-form was obtained by GC–MS of diastereoisomers (Gerwig et al. 1978). *O*-Peracetylated-2-butyl derivatives of rhamnose were obtained after derivatization of free sugars, which were obtained by acidic hydrolysis. The acetylated (+)-2-butyl derivatives of rhamnose (see Experimental) were eluted as the peaks with retention times identical with those of tetraacetyl (+)-2-butyl-L-rhamnose, prepared from commercially obtained standard of L-rhamnose (see Experimental). These results revealed that Rha-Rha-FAs-FAs contained only L-rhamnose.

Tandem mass spectrometry of rhamnolipids

Table 2 shows 23 3-OH-FAs identified in the Rha-FAs-FAs of strain *Thermus* sp. CCM 2842. Of them, 74.6% were saturated, 4.1% were odd-chain, 21.3% monoenoic and 4.1% dienoic. We believe that the wide variation in the chain length is caused by the fact that rhamnolipids are produced by thermophilic bacteria.

The splitting of rhamnolipids in the mass spectrometer has described by Denekamp et al. (2000). Positive ESI gives rise to pseudomolecular ions of $[M + H]^+$ and $[M + Na]^+$ and sometimes $[M + K]^+$, which makes identification difficult.

Firstly, we optimized the cone voltage at −45 V. At this voltage the pseudomolecular ions $[M–H]^-$, which could be attributed to deprotonation of the carboxylic group, are sufficiently abundant. The proportions of the different

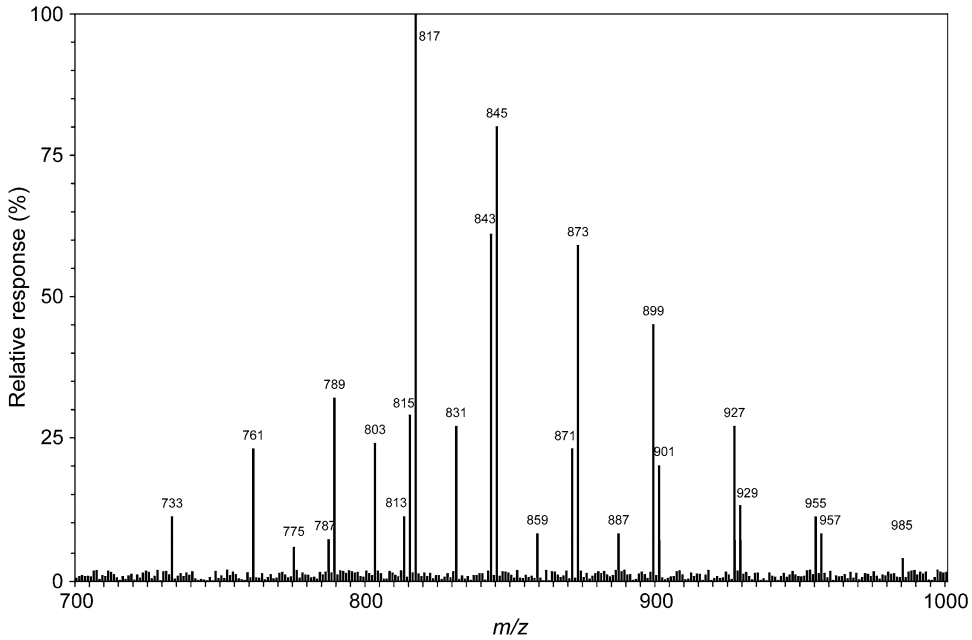


Fig. 3 The mass spectrum of pseudomolecular ions $[M–H]^-$ of the fraction Rha-Rha-FAs-FAs (band at Rf 0.32 from TLC) of the strain *Thermus* sp. CCM 2842

homologues of rhamnolipids were obtained from the relative intensities of their $[M–H]^-$ ions. Unfortunately, isomers of rhamnolipids having a different sequence of FAs could not be resolved and the intensity of $[M–H]^-$ ion could not be used for quantification of these homologues having the same molecular weight.

Since at the cone voltage −45 V sufficiently abundant fragments cannot be distinguished from pseudomolecular ions in the mass spectrum, we increased cone voltage to −80 V. We used tandem mass spectrometry in the collision-induced dissociation (CID) mode, which increased the produced fragments of the $[M–H]^-$ in the source of the mass spectrometer and enabled us to distinguish isomers, because of the *m/z* of fragments based on the variation in the side chains of 3-hydroxy fatty acid.

The literature usually describes only the Rha-FAs-FAs and Rha-Rha-FAs-FAs (Sharma et al. 2007; Monteiro et al. 2007), whereas the Rha-Rha-FAs and Rha-FAs were identified by mass spectrometry only sporadically (Shen et al. 2005; Silva et al. 2010). We discuss here the mass spectra of all four classes of rhamnolipids, which give four bands when analyzed by TLC.

The mixtures of separated rhamnolipids after TLC were analyzed by direct infusion of the sample into the mass spectrometer. Figure 3 presents the mass spectrum of pseudomolecular ions $[M–H]^-$ of the fraction Rha-Rha-FAs-FAs (band at Rf 0.32 from TLC) of the strain CCM 2842. The figure shows that rhamnolipid homologues were identified by means of the pseudomolecular ions in the interval of 733 and 985 Da. Table 4 shows qualitative and quantitative results for this class of rhamnolipids.

Figure 4 shows the molecular structure and mass spectra of the two positional isomers, Rha-Rha-16:1–18:1 and Rha-Rha-18:1–16:1. The $[M–H]^-$ ion at *m/z* 841was the most intense ion in the mass spectrum (cone voltage −80 V). Two pairs of main ions were observed in the mass spectra of rhamnolipids; these ions correspond to the cleavage at the three carbon–oxygen bond of the 3- moiety and the cleavage at the *O*-glycosidic bond in the rhamnose unit (see Fig. 5). Similarly, the ion corresponding to two rhamnose units at *m/z* 309 was also seen in mass spectra.

As with the preceding molecular species, tandem-MS was performed on the further ion at *m/z* 803 corresponding to Rha-Rha-16:0–15:0, Rha-Rha-15:0–16:0, and Rha-Rha-14:0–17:0. It gave daughter ions at *m/z* 563 (Rha-Rha-16:0), at *m/z* 549 (Rha-Rha-15:0), and *m/z* 535 (Rha-Rha-14:0), all representing the cleavage of the second fatty acid, see Fig. 6 and Fig. 1s. Other molecular species were identified in a similar way; the results of qualitative and quantitative analysis are presented in Table 4.

To confirm the structure and the qualitative and quantitative analysis of the FAs, Rha-FAs-FAs were submitted to the tandem mass spectrometry. Fig. 2s presents the mass spectrum of pseudomolecular ions $[M–H]^-$ of the fraction Rha-FAs-FAs (band at Rf 0.24 from TLC) of the strain CCM 2842. The important ion of this fraction at *m/z* 669, Rha-16:0–16:1, showed daughter ions at *m/z* 415, corresponding to the splitting of the ester bond, *m/z* 253, corresponding to the saturated FAs, and *m/z* 163, corresponding to the splitting of the bond rhamnose-FA (Figs. 3s and 4s). Tandem-MS was also performed on the further ions of this fraction. The minor ion at *m/z* 699 gave

Table 4 Qualitative and quantitative results of fraction Rha-Rha-FAs-FAs of strain *Thermus* sp. CCM 2842

Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Total %	ECN
14:0–12:0	1.5	12:0–14:0	0.3													1.8	26:0
16:0–12:0	3.4	12:0–16:0	0.2													3.6	28:0
15:0–14:0	1.0	14:0–15:0														1.0	29:0
16:1–14:0	1.1															1.1	30:1
16:0–14:0	4.3	18:0–12:0	0.2	12:0–18:0	0.5											5.0	30:0
16:0–15:0	2.8	15:0–16:0	0.5	14:0–17:0	0.4											3.7	31:0
16:1–16:1	1.7															1.7	32:2
16:0–16:1	3.9	16:1–16:0	0.6	18:1–14:0	0.1											4.6	32:1
12:0–20:0	0.2	16:0–16:0	13.8	18:0–14:0	0.6	20:0–12:0	0.1									14.7	32:0
15:0–18:0	0.4	16:0–17:0	2.9	17:0–16:0	0.2	18:0–15:0	0.8									4.3	33:0
16:1–18:1	0.9	18:1–16:1	0.1													1.0	34:2
16:0–18:1	5.9	16:1–18:0	0.4	18:0–16:1	2.8	18:1–16:0	0.2	20:1–14:0	0.3							9.6	34:1
16:0–18:0	11.4	18:0–16:0	0.7	20:0–14:0	0.4											12.5	34:0
15:0–20:0	0.1	17:0–18:0	0.2	18:0–17:0	0.7	20:0–15:0	0.3									1.3	35:0
16:0–20:1	2.3	16:1–20:0	0.2	16:1–22:0	0.3	18:0–18:1	0.2	18:1–18:0	0.1	20:0–16:1	0.2	20:1–16:0	0.2	22:1–14:0	0.1	3.6	36:1
16:0–20:0	4.9	18:0–18:0	2.3	20:0–16:0	1.4	22:0–14:0	0.6									9.2	36:0
17:0–20:0	0.6	20:0–17:0	0.6													1.2	37:0
16:0–22:1	3.1	18:0–20:1	0.7	18:1–20:0	0.3	20:0–18:1	0.1	20:1–18:0	1.4	22:0–16:1	0.6	22:1–16:0	0.8			7.0	38:1
16:0–22:0	2.1	18:0–20:0	0.2	20:0–18:0	0.4	22:0–16:0	0.4									3.1	38:0
18:0–22:1	1.4	20:0–20:1	0.4	20:1–20:0	0.2	22:1–18:0	0.1									2.1	40:1
18:0–22:0	2.9	20:0–20:0	1.1	22:0–18:0	0.3											4.3	40:0
20:0–22:1	0.9	22:1–20:0	0.3													1.2	42:1
22:0–20:0	0.7	20:0–22:0	1.1													1.8	42:0
22:0–22:0	0.6															0.6	44:0

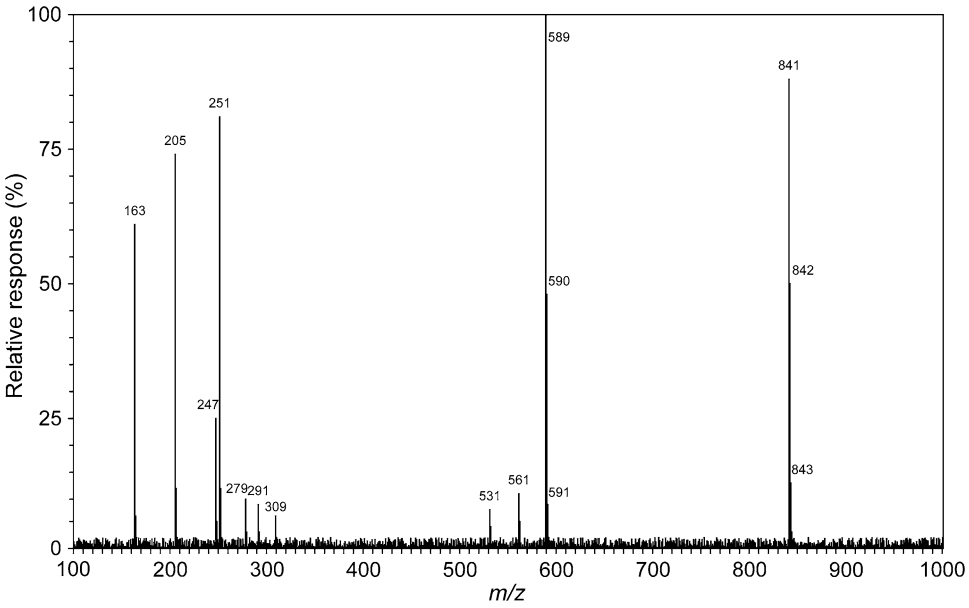


Fig. 4 The molecular structure and mass spectra of the two positional isomers, Rha-Rha-16:1–18:1 and Rha-Rha-18:1–16:1

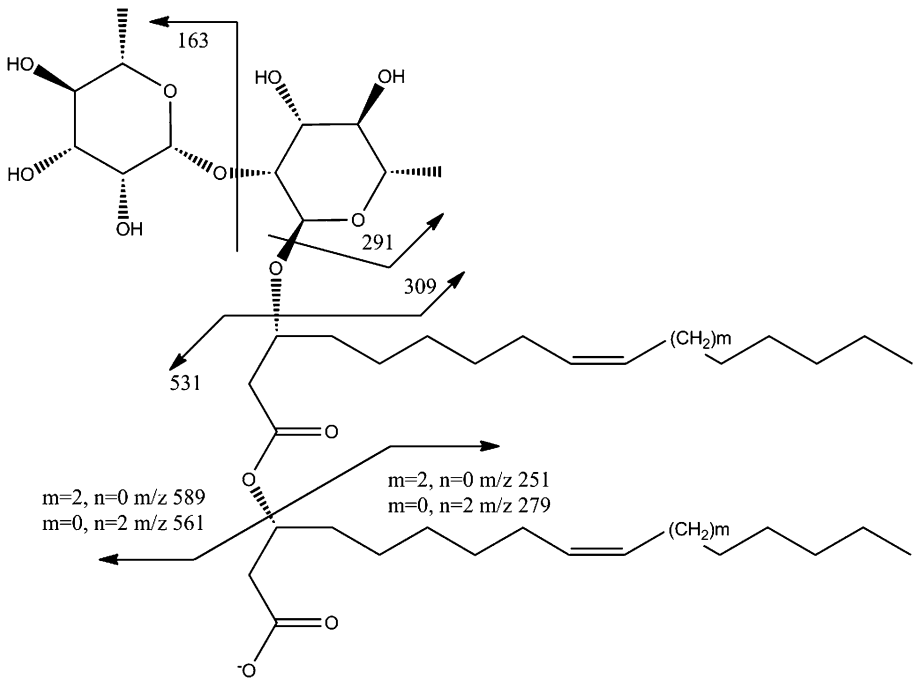


Fig. 5 Structures of the fragment ions from Rha-Rha-16:1-18:1 and Rha-Rha-18:1–16:1, m and n denote the number of CH_2 groups

two series of daughter ions at *m/z* 225, 253 and 281, which are consistent with Rha-16:0, Rha-18:0 and Rha-20:0, respectively. The series of ions at *m/z* 417, 445 and 473 shows that these FAs are not directly linked to the rhamnosyl moiety (see also Figs. 5s and 6s). The results of the qualitative and quantitative analysis of this class of rhamnolipids by MS are shown in Table 5.

Two other rhamnolipids, if present, do not require such conditions of MS analysis as the previous two classes. This is due to the structure which involves no combination of the two FAs. Hence, the mass spectra are very simple.

The spectra of both classes show that the splitting is in accordance with the above-mentioned rules. For example, the mass spectrum of the Rha-FAs (Rha-16:0) features the majority ions at *m/z* 417 ($[M–H]^-$) and two further ions, ion at *m/z* 163 belonging to rhamnose and carboxylate anion at *m/z* 253.

The major ions for further class, i.e. for the Rha-Rha-FAs and, e.g., for molecular species Rha-Rha-18:1 are those at *m/z* 589 ($[M–H]^-$) and a rhamnose-like series of ions, i.e., ions at *m/z* 163, 205, 247, and 309 and anion at *m/z* 289 belonging to carboxylate. Again, the results of

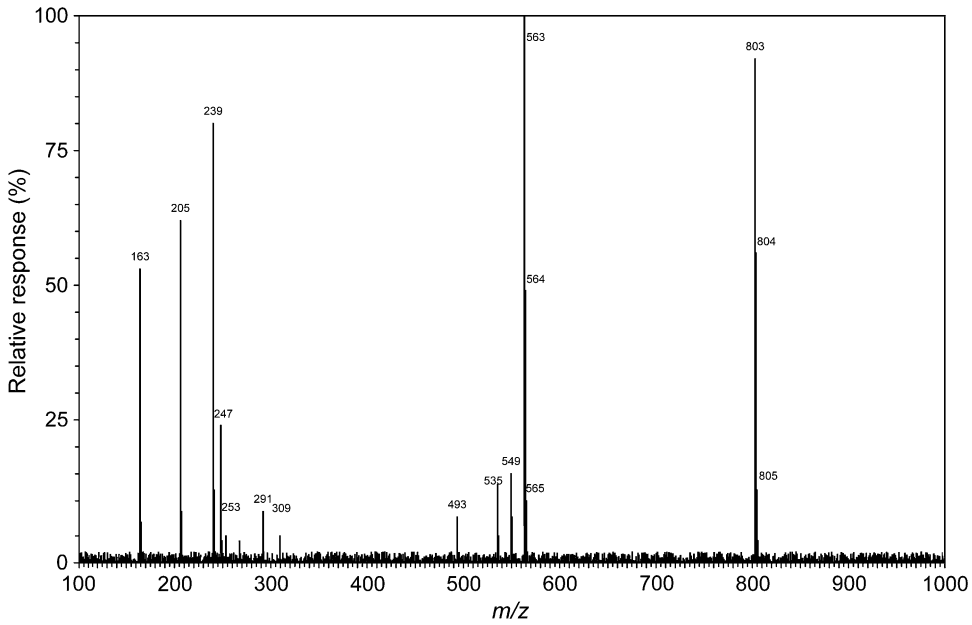


Fig. 6 Tandem-MS of molecular species from the ion at *m/z* 803 corresponding to Rha-Rha-16:0–15:0, Rha-Rha-15:0–16:0, and Rha-Rha-14:0–17:0

Table 5 Qualitative and quantitative results of fraction Rha-FAs-FAs of strain *Thermus* sp. CCM 2842

Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Mol. spec.	%	Total %	ECN
12:0–16:0	0.1	16:0–12:0	1.5									1.6	28:0
16:1–14:0	0.6											0.6	30:1
12:0–18:0	0.1	18:0–12:0	0.9	16:0–14:0	2.9	20:1–14:0	0.6					4.5	30:0
16:1–16:1	0.5											0.5	32:2
16:0–16:1	4.4	16:1–16:0	0.4									4.8	32:1
16:0–16:0	11.4	18:0–14:0	0.9									12.3	32:0
16:0–17:0	1.7	17:0–16:0	0.1									1.8	33:0
16:0–18:1	2.3	18:1–16:0	0.3	16:1–18:0	0.1	18:0–16:1	3.2					5.9	34:1
16:0–18:0	10.7	18:0–16:0	3.5	20:0–14:0	1.0							15.2	34:0
17:0–18:0	0.1	18:0–17:0	1.0	16:0–19:0	1.0	19:0–16:0	0.1					2.2	35:0
16:0–20:2	1.3	16:1–20:1	1.0	20:1–16:1	0.1							2.4	36:2
16:0–20:1	5.0	20:1–16:0	0.3	16:1–20:0	0.5	20:0–16:1	1.1	18:0–18:1	1.5	18:1–18:0	0.1	8.5	36:1
16:0–20:0	7.2	20:0–16:0	0.3	18:0–18:0	5.2							12.7	36:0
16:0–22:1	1.3	22:1–16:0	0.1	18:0–20:1	3.0	20:1–18:0	0.1					4.5	38:1
16:0–22:0	2.7	22:0–16:0	0.1	18:0–20:0	4.1	20:0–18:0	0.9					7.8	38:0
16:0–24:2	1.0	24:2–16:0	0.1	20:1–20:1	0.5							1.6	40:2
16:0–24:1	2.5	24:1–16:0	0.1	20:0–20:1	1.3	20:1–20:0	0.6					4.5	40:1
16:0–24:0	2.0	24:0–16:0	0.1	18:0–22:0	1.5	22:0–18:0	0.4	20:0–20:0	1.3			5.3	40:0
18:0–24:1	1.7	24:1–18:0	0.1									1.8	42:1
18:0–24:0	1.4	24:0-18:0	0.1									1.5	42:0

qualitative and quantitative MS analysis of both classes (Rha-FAs and Rha-Rha-FAs) are described in Table 6. The content FAs of the two other strains is described in the Supplemental (Tables 1s–6s).

The mass spectra of negative ESI of all four classes of rhamnolipids can be used to determine qualitative and quantitative content of individual rhamnolipids including their two-dimensional structure.

Rhamnolipids from *Pseudomonas* exhibit low variability and all four classes of rhamnolipids have been identified by only a few authors (Shen et al. 2005; Silva et al. 2010). The number of molecular species identified by different authors is also low: Price et al. (2009) identified twelve molecular species, Monteiro et al. (2007) only eight. By contrast, the species *Thermus* sp. and *T. aquaticus* contain all four rhamnolipid classes, whereas *Meiothermus ruber* lacks

Table 6 Qualitative and quantitative MS analysis of both classes (Rha-FAs and Rha-Rha-FAs)

Mol. spec.	Total %	Total %
10:0	0	1.6
11:0	0.7	0
12:0	4.8	7.7
13:0	2.2	0
14:0	13.6	20.2
15:0	3.1	0
16:1	8.3	4.2
16:0	41.2	38.1
17:0	2.7	0.7
18:1	2.5	3.3
18:0	12.9	16.5
20:2	1.3	0
20:1	2.3	1.6
20:0	4.4	4.9
22:0	0	1.2

Rha-FAs. The difference is even more striking on comparing a single class of rhamnolipids from our study, e.g. 77 molecular species of Rha-Rha-FAs-FAs produced by strain CCM 2842, with all currently known 58 rhamnolipids of all classes produced by all known producers (Abdel-Mawgoud et al. 2010). Although individual strains exhibit considerable variability in both chain lengths and the unsaturation of FAs, we were able to define several rules determining the proportion of individual FAs in rhamnolipids. We found that, with rhamnolipids containing two FAs, the molecular species having a saturated FAs bound by a glycosidic bond directly to rhamnose is always more abundant. This holds, e.g., for the 11:1 ratio of Rha-Rha-16:0–18:1 to Rha-Rha-18:1–16:0. Further, even-numbered FAs were found to be again preferentially bound by a glycosidic bond to rhamnose, as indicated, e.g., by the 17:1 ratio of Rha-Rha-16:0–17:0 to Rha-Rha-17:0–16:0. The last rule concerns the chain lengths—the acids preferentially bound to rhamnose are those with chain length C16 and C18; this is illustrated, e.g., by the 15:1 ratio of the molecular species Rha-Rha-16:0–12:0 to Rha-Rha-12:0–16:0. This holds also for longer-chain homologues—see the 14:1 ratio of Rha-Rha-18:0–14:0 to Rha-Rha-14:0–18:0.

We arrived at the same conclusions in the other three classes of rhamnolipids. As an example of their great complexity we give here the complete systematic formula of one of the major molecular species, i.e., Rha-Rha-16:0–20:1, which is (*R*,*Z*)-3-((*R*)-3-((2*R*,3*R*,4*R*,5*R*,6*S*)-4,5-dihydroxy-6-methyl-3-((2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yloxy)tetrahydro-2*H*-pyran-2-yloxy)hexadecanoyloxy)icos-11-enoate.

Emulsification index of the rhamnolipids

The percentages of emulsification were checked with three plant oils and with three fuels, see Table 7s. For plant oils, the emulsification indexes were in the range of 40–55% and for hydrocarbon fuels they were higher by about a third. The highest emulsification index was determined with hexadecane. The diesel-emulsifying ability of rhamnolipids produced by all three strains of bacteria is nearly comparable. This fact is very interesting since, e.g., in the decontamination of soil, longer hydrocarbons are less easily degraded than shorter-chain hydrocarbons contained in gasoline. Above all, all the three supernatants after cultivation of thermophilic bacteria were able to form stable emulsions for more than 24 h, which suggests their large potential for industrial applications.

In conclusion, we envisage two focal points in future exploration of rhamnolipids. One is the systematic use of modern analytical techniques for determining the complete structure of rhamnolipids including their complete stereochemistry, which identified a total of 77 molecular species, which far exceeds the number all species as yet described in all producers. The other, much more important tenet is the finding that rhamnolipids are produced also by bacteria taxonomically distant from *Pseudomonas*, which are, moreover, nonpathogenic (Biosafety level 1).

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