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Structural analysis of mycolic acids from phenol-degrading strain of *Rhodococcus erythropolis* by liquid chromatography–tandem mass spectrometry

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Abstract We used reversed phase liquid chromatography–electrospray ionization tandem mass spectrometry for direct analysis of mycolic acids (MAs) from four different cultivations of *Rhodococcus erythropolis*. This technique enabled us to identify and quantify the specific molecular species of MAs directly from lipid extracts of the bacterium, including the determination of their basic characteristics such as retention time and mass spectra. We identified a total of 60 molecular species of MAs by means of LC/MS. In collision-induced dissociation tandem mass spectrometry, the $[M\text{-}H]^-$ ions eliminated two residues, i.e., meroaldehyde and carboxylate anions containing α-alkyl chains. The structural information from these fragment ions affords structural assignment of the mycolic acids, including the lengths and number of double bond(s). Two strains, i.e., *R. erythropolis* CCM 2595 and genetically modified strain CCM 2595 *pSRK 21 phe* were cultivated on two different substrates (phenol and phenol with addition of humic acids as a sole carbon source). The addition of humic acids showed that there is a marked increase of unsaturated mycolic acids, mostly in the range of 20–100 %. This effect is more pronounced in the *R. erythropolis* CCM 2595 strain.

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Introduction

Mycolic acids are 2-alkyl-3-hydroxy (α-alkyl-β-hydroxy) substituted fatty acids, characterized by their occurrence in cell walls of some gram-positive bacteria. They occur in the genera *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and other genera of bacteria of the order Actinomycetales (Actinobacteria) (Goodfellow 1992; Rainey et al. 1995; Lechavalier and Lechavalier 1970). The genus *Rhodococcus* contains mycolic acids with 30 to 54 carbon atoms, while the genus *Corynebacterium* contains acids in the interval of 22 to 38 and genus *Mycobacterium* from 60 to 90 carbon atoms (Goodfellow 1992). Mycolic acids are present in both free and bound form. They are bonded by ester linkage to the arabinogalactan cell wall polysaccharide (Sokolovska et al. 2003). An important characteristic of mycolic acids is their diversity in α-alkyl side chains and the main meromycolate chain. Alkyl part of the molecule is shorter, containing only 10 to 14 carbon atoms, whereas the main chain is longer and can carry up to four unsaturated bonds (Alshamaony et al. 1976; Barton et al. 1989).

Structure determination of mycolic acids has been performed many times; the classical methods are best described in the recent review by Yassin (2011). The analysis mostly included by gas chromatography–mass spectrometry with electron impact (EI) ionization (Larsson 1994). Although this technique has provided important structural information about the fragments that resulted both from the α-branched and also β-hydroxylated chains, the fragmentation is complicated because there exist a series of homologues. Further,

the loss of water and methanol from the native fragments and parent compound considerably complicated identification of appropriate molecular species. Unfortunately, EI ionization has further limitations (Dubnau et al. 1997) including the necessary derivatization of mycolic acids before gas chromatography (GC)/mass spectrometry (MS) analysis to trimethylsilyl derivatives (Nishiuchi et al. 2000). Further, capillary GC does not identify the high molecular species of mycolic acids. The development of high-performance liquid chromatography (HPLC) made it possible to use this method for the analysis of mycolic acids (Butler and Guthertz 2001). Identification of mycolic acids using only HPLC without mass spectrometry brings about a loss of information about their structures. The sensitivity of detection of mycolic acids was increased by derivatization by UV-absorbing esters, and it can be also improved by the use of fluorescent labeling compounds (Butler and Guethertz 2001).

Compounds such as fatty acids can sometimes be separated without any derivatization. Reversed phase HPLC (RP-HPLC) separation of underivatized fatty acids from plant oils was proposed after transesterification. A simple HPLC system allowing the separation of unreacted oil and the products of hydrolysis including free long chain fatty acids has been described (Turkan and Kalay 2006). More sophisticated and precise methods combining HPLC and mass spectrometry were developed to determine compounds occurring during the production of biodiesel from plant oils (Holcapek et al. 1999, 2001).

Identification of mycolic acids requires the LC–MS analysis. Many papers (e.g., Butler and Guthertz 2001 for review) described the separation of mycolic acids, namely in the form of UV-absorbing esters, but unfortunately only as a fingerprint of the individual strains, without any identification of molecular species (Song et al. 2009). The pioneering paper of Hsu et al. (2011) described the use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) to identify tens of molecular species of mycolic acids.

In negative mode, it always showed the presence of $[M\text{-}H]^-$, which was majority-represented. The spectrum also contains peaks of ions corresponding to the cleavage of a single bond between α and β carbon, so it is possible to determine the structure of the branched chain meromycolate and thus the structure of the whole molecule.

Structural changes in cell wall lipids, predominantly in mycolic acids, occur in response to various cultivation conditions, such as different temperature, starvation, change of pH, addition of further organic compounds, etc., and can modify the ratios of saturated to unsaturated mycolic acids (Beney and Gervais 2001; Ramos et al. 2002). It follows that changes in the cultivation conditions can have a great influence on the maintenance of membrane fluidity and permeability (Sokolovska et al. 2003) and thus on the uptake of hydrophobic carbon sources.

Based on our previous experience with the cultivation of *Rhodococcus erythropolis* (Schreiberova et al. 2010) and with LC–MS of lipids (Rezanka et al. 2011b), we used here physiologically adapted/genetically modified strains utilizing phenol as a sole carbon source. An application of soluble humic acids was based on previous findings (Cejkova et al. 2005) confirming their positive impact on the utilization of phenol by *R. erythropolis*.

Materials and methods

Cultivation and extraction of bacterium strain

R. erythropolis CCM 2595 was obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). The strain was subjected to a 6-month physiological adaptation to phenol, indicated in the text as "adapted." *R. erythropolis* CCM 2595 *pSKRK 21 phe* (Vesely et al. 2003; Martinkova et al. 2009) conducted a plasmid construct consisting of the vector pSRK21 and a 3.5-kb DNA fragment from the strain CCM 2595 with the operon coding for subunits of phenol hydroxylase (*pheA2*+*pheA1* and the transcriptional regulator *pheR*), indicated in the text as "modified."

Cells were cultivated in 500-ml Erlenmeyer flasks containing 200 ml of basic salt medium. Cultivation was performed on rotary shaker (100 rpm, 30 °C). Medium composition in milligrams per liter: KH_2PO_4 170; $MnCl_2{\cdot}4H_2O$ 1; K_2HPO_4 130; $CaCl_2{\cdot}2H_2O$ 0.26; $(NH_4)_2SO_4$ 710; $FeSO_4{\cdot}7H_2O$ 0. 6; $MgCl_2{\cdot}6H_2O$ 340; and $Na_2MoO_4{\cdot}2H_2O$ 2; pH was adjusted to 7. The sole carbon source (initial concentration, phenol 300 mg/l) was added after sterilization (120 °C, 20 min). The cells were collected at the end of exponential phase (30 h) by centrifugation (10 min, 9,050×*g*), washed twice with physiological solution, and lyophilized.

Mycolic acids were prepared as described previously (Hsu et al. 2011; Dembitsky et al. 1992, 1993). Briefly, the lyophilized biomass was hydrolyzed with 4 ml of 10 % KOH for 1 h at 80 °C, acidified with 6 N HCl to pH 2.0, and extracted with 1 ml of *n*-hexane. For further purification, the crude lipid extract with mycolic acids in 1 ml of $CHCl_3$/CH_3OH (2:1, *v*/*v*) was applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted nonacidic lipids with 40 mL of chloroform–methanol (7:3), and mycolic acids with 30 mL of chloroform–methanol-concentrated aqueous ammonia (70:30:2) containing 0.4 % (*w*/*v*) ammonium acetate. The eluate was reduced in volume and subjected to LC–MS.

Table 1 Growth (OD at 600 nm) of "adapted" *R. erythropolis* CCM 2595 and "modified" *R. erythropolis* CCM 2595 *pSRK 21 phe* on phenol (300 mg/l) as a sole carbon source without and with humic substances PAB 32

Time (h)	Adapted		Modified	
	Phenol 300 mg/l OD (600 nm)	Phenol 300 mg/l, PAB 32 10 mg/l OD (600 nm)	Phenol 300 mg/l OD (600 nm)	Phenol 300 mg/l, PAB 32 10 mg/l OD (600 nm)
0	0.116	0.156	0.160	0.111
5	0.140	0.178	0.170	0.160
10	0.220	0.230	0.210	0.299
15	0.309	0.283	0.259	0.425
20	0.372	0.338	0.305	0.529
25	0.410	0.379	0.358	0.567
30	0.420	0.420	0.398	0.580
40	0.475	0.475	0.445	0.591
50	0.510	0.507	0.490	0.577

Humic substances

Humic substances (PAB 32) were isolated from the oxihumolites (oxidatively altered lignite analogues) of a brown coal deposit in the northern part of the town Bílina (Czech Republic) by using KOH (0.15 mol/l) and $K_4P_2O_7$ (0.1 mol/l; 80 °C) (Novak et al. 2001; Salati et al. 2011). Humic substances PAB 32 were prepared as 2 % potassium humate at the Research Institute of Inorganic Chemistry (Ústí nad Labem, Czech Republic). Humic acids at a concentration of 10 mg/l were used in the experiments.

Measurement of cell surface properties

Cell surface properties of "adapted" and "modified" *R. erythropolis* strains were determined by means of the microbial adhesion to solvents (MATH) test (Lichtenberg et al. 1985) and determination of permeability of the cells (Mukhopodhyay et al. 1997; D'Aimmo et al. 2007).

Briefly, bacterial cells were washed twice in a 50-mM phosphate buffer solution and resuspended in the same buffer solution to an optical density of A_0 (at 600 nm)=0.4–0.6. Then, 0.5 ml of hexadecane was added to 5 ml of bacterial suspension, and the two-phase system was vortexed for 30 s and allowed to separate for 15 min. The aqueous phase was carefully transferred to a cuvette, and absorbance at 400 nm was measured (A). The latter procedure was repeated six times. The hydrophobicity was assessed by the percentage of cells retained by hydrocarbons calculated using the formula: (percentage)=$100\times(1-A/A_0)$. Experiments were done in triplicate.

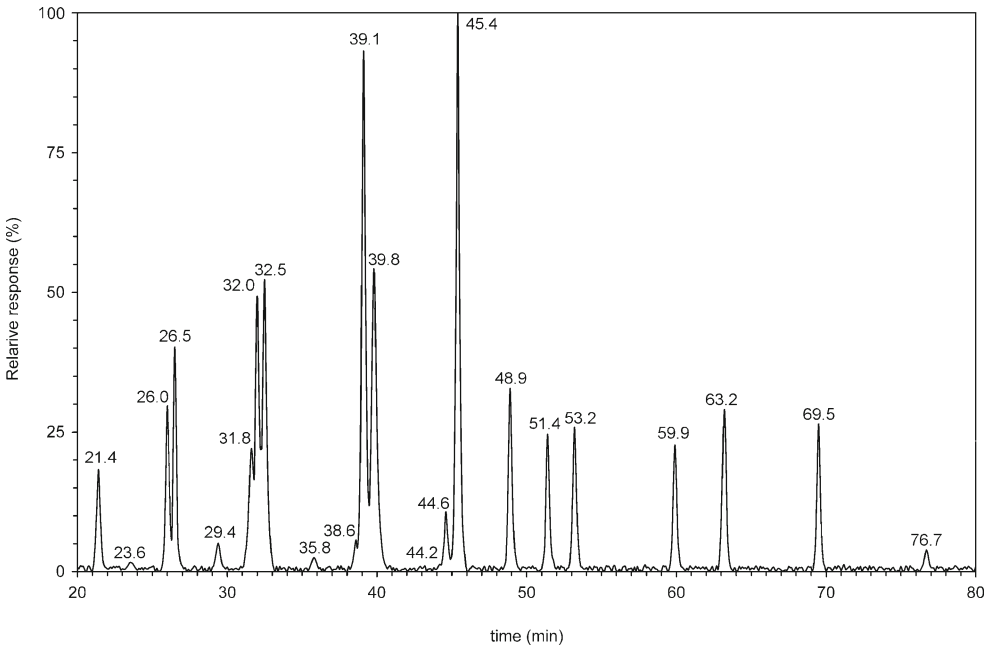

Fig. 1 RP-HPLC/ESI-MS chromatogram (total ion current) of the mycolic acids from the bacterium *R. erythropolis*. Numbers above peaks mark the retention time of appropriate peak in minutes. Minority mycolic acids amounting to up to 0.2 % of total mycolic acids were omitted

Table 2 Relative quantities (perecentage) of MAs identified in *Rhodococcus* cultivated under different conditions, their retention times (RT), the masses of deprotonated molecules $[M-H]^-$, equivalent chain length and number of double bonds (CL:DB), the resulting structure of mycolic acid, and characteristic fragment ions acyl carbon number ($R_\alpha COO^-$)

RT	$[M-H]^-$	CL:DB	Mycolic acids (meromycolate chain/α-branch)	$R_\alpha COO^-$	"Adapted"				"Modified"			
					Phenol		Phenol + PAB 32		Phenol		Phenol + PAB 32	
					LC %[a]	MS %[b]	LC %	MS %	LC %	MS %	LC %	MS %
21.4	495	32:0	22:0/10:0	171	2.78	0.77	1.29	0.29	2.68	2.12	2.37	1.88
	495		20:0/12:0	199		2.01		1.00		0.64		0.49
23.6	509	33:0	21:0/12:0	199	0.24	0.05	0.15	0.04	0.33	0.06	0.28	0.09
	509		20:0/13:0	213		0.19		0.11		0.25		0.19
26.0	521	34:1	22:1/12:0	199	4.51	4.51	4.86	4.86	3.71	3.63	4.42	4.42
26.5	523	34:0	22:0/12:0	199	6.12	3.45	2.44	1.43	7.58	4.84	5.39	3.60
	523		21:0/13:0	213		0.08		0.05		0.27		0.27
	523		20:0/14:0	227		2.59		0.96		2.49		1.52
29.4	537	35:0	23:0/12:0	199	0.77	0.07	0.37	0.02	1.04	0.12	0.82	0.08
	537		22:0/13:0	213		0.58		0.29		0.76		0.67
	537		21:0/14:0	227		0.12		0.06		0.10		0.07
31.8	547	36:2	23:1/13:1	211	3.35	2.97	4.13	3.74	1.89	1.62	3.18	2.24
	547		20:1/16:1	253		0.38		0.39		0.33		0.94
32.0	549	36:1	24:1/12:0	199	7.51	5.37	8.00	5.69	5.61	4.08	7.43	4.32
	549		23:0/13:1	211		0.13		0.14		0.10		1.39
	549		22:1/14:0	227		1.15		1.26		0.87		1.07
	549		20:0/16:1	253		0.86		0.91		0.45		0.65
32.5	551	36:0	24:0/12:0	199	7.95	1.34	3.15	0.41	11.67	2.15	8.26	1.57
	551		23:0/13:0	213		0.19		0.05		0.65		0.48
	551		22:0/14:0	227		6.42		2.69		8.73		6.21
35.8	565	37:0	23:0/14:0	227	0.38	0.38	0.11	0.11	0.69	0.76	0.45	0.45
38.6	575	38:2	26:2/12:0	199	0.86	0.86	1.53	1.53	1.17	1.15	0.78	0.78
39.1	577	38:1	26:1/12:0	199	14.19	2.59	18.98	3.42	12.42	2.09	14.10	2.74
	577		24:1/14:0	227		11.22		14.83		9.89		10.84
	577		22:1/16:0	255		0.38		0.73		0.36		0.52
39.8	579	38:0	26:0/12:0	199	8.25	0.19	2.69	0.05	18.01	0.34	8.91	0.18
	579		24:0/14:0	227		3.36		1.03		6.68		3.41
	579		22:0/16:0	255		4.03		1.37		9.52		4.56
	579		20:0/18:0	283		0.67		0.24		1.39		0.76
44.2	593	39:0	27:0/12:0	199	0.20	0.06	0.05	0.02	0.43	0.08	0.42	0.08
	593		26:0/13:0	213		0.09		0.02		0.22		0.24

Table 2 (continued)

RT	[M-H]$^-$	CL:DB	Mycolic acids (meromycolate chain/α-branch)	$R_\alpha COO^-$	"Adapted"				"Modified"			
					Phenol		Phenol + PAB 32		Phenol		Phenol + PAB 32	
					LC %[a]	MS %[b]	LC %	MS %	LC %	MS %	LC %	MS %
	593		25:0/14:0	227		0.05		0.01		0.11		0.10
44.6	603	40:2	28:2/12:0	199	1.63	1.25	2.29	1.72	2.03	1.49	1.47	1.22
	603		26:2/12:0	199		0.38		0.57		0.48		0.25
45.4	605	40:1	28:1/12:0	199	15.21	2.59	20.76	3.16	11.38	1.37	15.39	1.83
	605		26:1/14:0	227		9.15		12.93		7.99		10.94
	605		24:1/16:0	255		3.47		4.67		2.08		2.62
48.9	607	40:0	26:0/14:0	227	4.99	0.77	0.64	0.12	10.30	1.63	5.07	0.90
	607		24:0/16:0	255		4.22		0.52		8.66		4.17
48.3	617	41:2	29:2/12:0	199	0.12	0.12	0.24	0.24	0.04	0.05	0.14	0.14
49.3	619	41:1	27:1/14:0	227	0.13	0.13	0.34	0.34	0.05	0.04	0.12	0.12
51.4	631	42:2	30:2/12:0	199	3.74	1.73	5.71	2.11	1.08	0.47	3.67	1.08
	631		26:1/16:1	253		2.01		3.60		0.66		2.59
53.2	633	42:1	30:1/12:0	199	3.93	0.86	4.71	0.98	1.27	0.34	4.01	1.28
	633		28:1/14:0	227		3.07		3.73		0.92		2.73
55.6	645	43:2	31:2/12:0	199	0.07	0.07	0.17	0.17	0.02	0.03	0.08	0.08
57.9	647	43:1	29:1/14:0	227	0.19	0.19	0.37	0.37	0.15	0.14	0.17	0.17
59.9	659	44:2	32:2/12:0	199	3.45	0.48	4.73	0.84	1.24	0.29	3.52	0.98
	659		31:1/13:1	211		2.59		3.34		0.82		1.89
	659		30:2/14:0	227		0.38		0.55		0.18		0.65
63.2	661	44:1	33:0/11:1	183	4.42	2.98	5.37	3.47	1.98	1.08	4.39	2.78
	663		32:1/12:0	199		0.19		0.25		0.24		0.42
	661		31:1/13:0	213		0.48		0.70		0.35		0.51
	661		30:1/14:0	227		0.77		0.95		0.42		0.68
71.2	663	44:0	33:0/11:0	185	0.19	0.19	0.00	0.00	0.76	0.81	0.25	0.25
64.4	673	45:2	32:2/13:0	213	0.12	0.05	0.56	0.34	0.02	0.02	0.11	0.05
	673		31:2/14:0	227		0.07		0.22		0.01		0.06
69.2	675	45:1	31:1/14:0	227	0.09	0.09	0.15	0.15	0.09	0.08	0.08	0.08
69.5	687	46:2	32:2/14:0	227	4.02	3.64	5.39	4.58	2.08	1.63	4.11	2.93
	687		30:2/16:0	255		0.38		0.81		0.56		1.18

Table 2 (continued)

RT	[M-H]$^-$	CL:DB	Mycolic acids (meromycolate chain/α-branch)	$R_\alpha COO^-$	"Adapted"				"Modified"			
					Phenol		Phenol + PAB 32		Phenol		Phenol + PAB 32	
					LC %[a]	MS %[b]	LC %	MS %	LC %	MS %	LC %	MS %
76.7	689	46:1	32:1/14:0	227	0.59	0.48	0.82	0.62	0.28	0.21	0.61	0.42
	689		30:1/16:0	255		0.11		0.20		0.10		0.19

[a] Determined from the total ion current

[b] Determined from the ratio of the ions from the mass spectrum

Cell permeability was determined by exposing the cells to two antibiotics—tetracycline (hydrophilic molecule) and rifampicin (hydrophobic molecule). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of tetracycline and rifampicin were determined. The MIC and MBC values of tetracycline against the tested strains were 0.005 to 100 mg/l and of rifampicin 0.01 to 100 mg/l, respectively. The inoculum was adjusted to a turbidity equivalent to 0.5 McFarland standards ($\approx 5 \times 10^5$ cfu/ml) and incubated for 24 h at 30 °C; 10-μl volumes of the inoculum were then used to inoculate each agar plate. The MIC was defined as the lowest concentration of the antibiotic completely inhibiting visible growth as compared with the antibiotic-free control well after a 4-day incubation at 30 °C. The minimum bactericidal concentration was defined as the antibiotic concentration needed to kill 99.9 % (MBC99.9) or 100 % (MBC100) of visible growth in comparison to the antibiotic-free control (well) after a 4-day incubation at 30 °C. The experiments were replicated at least three times to verify reproducibility of the method under the above-mentioned conditions.

LC–MS analysis

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump, and automatic injector (HP 1090 series, Hewlett Packard, USA) and two Hichrom columns HIRPB-250AM 250×2.1 mm ID, 5 μm particle size, in series. This setup provided us with a high-efficiency column—approximately 26,000 plates/250 mm. LC was performed at a flow rate of 300 μL/min with a linear gradient from mobile phase containing acetonitrile with 1 mM acetic acid to isopropanol/acetonitrile with 1 mM acetic acid (70:30; *v/v*) for 90 min, followed by a 15-min isocratic elution at the same final composition. The whole HPLC flow (0.37 mL/min) was introduced into the ESI source without any splitting.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, ON, Canada) operating in negative electrospray ionization mode. The pressure of nebulizing gas (N_2) was 345 kPa, and the drying gas (N_2) flow and temperature were 9 L/min and 300 °C, respectively. The skimmer was at ground potential, and the electrospray needle was set at 4 kV.

The cone voltage was kept at 250 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 ions 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* 100–1,000 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3 %

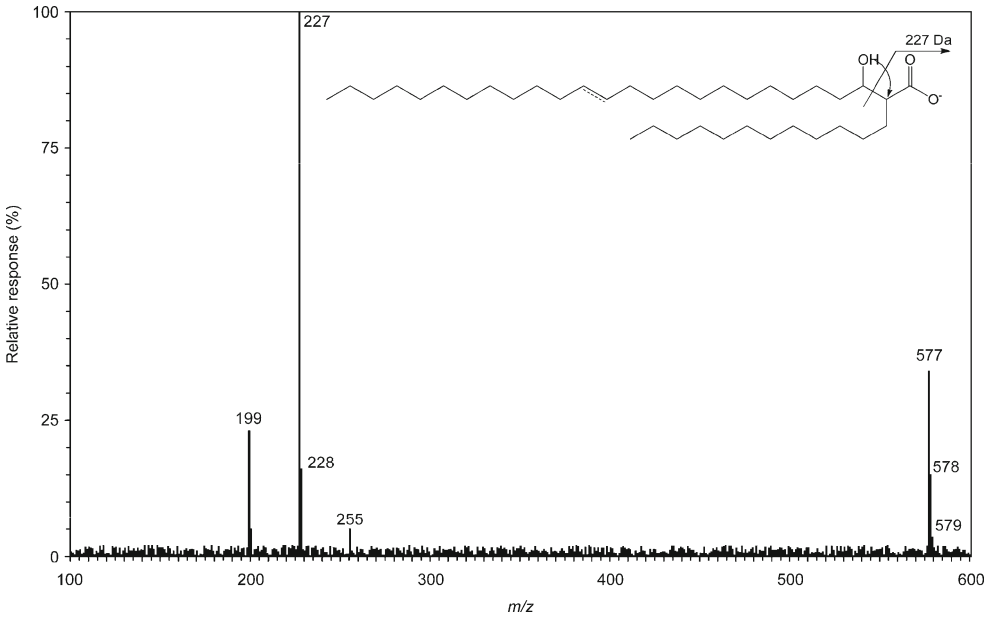

Fig. 2 Negative ESI mass spectrum of mycolic acids (12:0/26:1, 14:0/24:1, and 16:0/22:1). Structure of the majority fragment ion at *m/z* 227 from $[M\text{-}H]^-$ at 577 Da is shown in the upper right corner

intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

Results and discussion

R. erythropolis belongs to the nocardioform actinomycetes. It has a specific composition of cell envelopes with a high content of mycolic acids, which participate in the high cell surface hydrophobicity. Genus *Rhodococcus* has a large genome, which provides a great potential for genetic modification, and on the level of phenotype, it allows considerable variation in metabolism, determined among others by cultivation conditions (Niescher et al. 2006).

R. erythropolis CCM 2595 adapted to utilization of phenol as a sole C-source, and its genetically modified CCM 2595 *pSRK 21 phe* (Vesely et al. 2003) with higher phenol hydroxylase activity (the first enzyme of phenol biodegradation pathway) were used as model microorganisms for the study of structural diversity of mycolic acids. Soluble humic acids (PAB 32) were added to the cultivation media as potential modulators of the transport of organic substances into the cells. Humic acids, due to their surface activity, also intensively interact with components of cell surface envelops (Jirku et al. 2009).

Laboratory cultivation showed that the genetically modified strain *R. erythropolis* CCM 2595 pSRK 21 *phe* growing in the presence of PAB 32 achieved the highest growth rate and total biomass concentration compared with other cultivation variants (Table 1). The different growth characteristics in this case are probably due to a combination of two phenomena, the influence of humic acids on the transport of phenol and the higher level of phenol hydroxylase in the genetically modified strain.

Based on the above-mentioned papers and on our previous experience with the analysis of lipid compounds (Rezanka et al. 2011a, b), we used our method, i.e., RP-HPLC/ESI-MS/MS for analyzing free mycolic acids in bacteria of genus *Rhodococcus*.

Selection of the correct gradient was most important. Based on our previous papers (Rezanka et al. 2011a, b), we selected a linear ternary gradient elution of solvent mixture—from 100 % acetonitrile with 1 mM acetic acid to 30 % acetonitrile+70 % isopropanol over 90 min, followed by a 15-min isocratic elution of the same final composition.

Figure 1 shows the RP-HPLC with ESI detection of total ion current (from 100 to 1,000 Da) of rhodococcal mycolic acids. More than 60 molecular species of mycolic acids were identified; many of them are unsaturated with high carbon numbers, as demonstrated by previously published HPLC and mass spectrometric data (Stratton et al. 1999; Nishiuchi et al. 2000; Sokolovska et al. 2003). Although the major peaks were separated on the baseline, the separation of some peaks was insufficient, in spite of the fact that we used two RP-HPLC columns with a total of 52,000 theoretical plates. The mass spectra suggested that several peaks contained two or more overlapping species, in agreement with the analysis of mycolic acids from similar strains of genus *Rhodococcus* (Hsu et al. 2011; Ioneda and Ishige 1996).

The mycolic acid mixture separated from *R. erythropolis* by RP-HPLC was subjected directly to the ESI in the negative ion mode. Several series of the $[M\text{-}H]^-$ ions were observed, see Table 2. The mycolic acids with saturated chains in both meromycolate and α-alkyl residues constitute a substantial part of total mycolic acids. Unfortunately, as mentioned above, several isomers were observed in each of the peak(s),

as determined by their tandem mass spectra (Table 2). For example, the tandem mass spectrum of the ion at *m/z* 577 (Fig. 2) is dominated by the ions at *m/z* 199 and 227, arising from cleavage of the C2–C3 bond with participation of the 3-hydroxyl group to eliminate two meroaldehyde, i.e., hexacosenal and tetracosenal (see also the fragmentation shown in Fig. 2.) The ions at 199 and 227 Da are then responsible for C12:0 and C14:0-carboxylate anions, respectively. The results indicate that the $[M\text{-}H]^-$ at *m/z* 577 contains C38:1 mycolic acid, and the structures of two major molecular species are 2-decyl-3-hydroxy-octacosenoic and 2-dodecyl-3-hydroxy-hexacosenoic acids. The minor ion at *m/z* 255 suggests that the molecular species containing C16:0 carboxylate and docosenoic meroaldehyde is also present. The resulting structure is then represented by 2-tetradecyl-3-hydroxy-tetracosenoic acid.

Some peaks, for example the peak with retention time of 29.4 min, contained saturated mycolic acids with one odd-numbered carbon chain and another chain with an even number of carbon atoms. It follows that either the α-alkyl or meromycolate contains an odd-numbered carbon chain. The spectrum of the ion at *m/z* 537 contains the major ion at *m/z* 213 representing C13:0-carboxylate anion and, as supplement, an even-numbered C22:0 meromycolate aldehyde. Two further low-intensity ions at *m/z* 199 and at *m/z* 227 belonging to C12:0 and C14:0-carboxylate anions, respectively, were detected. Their *m/z* values showed that the above-mentioned ions originate from the ion at 537 Da representing a C35:0-mycolic acid, which contains one major molecular species, i.e., 2-undecyl-3-hydroxy-tetracosanoic acid (22:0/13:0) and two minor isomers containing odd-numbered meromycolate chains and even-numbered carboxylate chains (23:0/12:0 and 21:0/14:0).

Anions having one double bond were seen in the peak with retention time of 45.4 min containing $[M\text{-}H]^-$ at *m/z* 605. A number of isomeric structures were repeatedly present. The major ion at *m/z* 227 reveals the presence of C14:0 carboxylate anion and two minor ions at *m/z* 199 and 255 represented by C12:0 and 16:0 carboxylate anions. The results indicate that the ion at *m/z* 605 contains C40:1 mycolic acids. The major one is 24:1/16:0 and the two minor ones are 26:1/14:0 and 28:1/12:0. The double bond in these molecular species is always found in meromycolate chains, in contrast to the peak with RT 32.0 min, where the presence of double bond was proved in carboxylates (α-chains). The values of the ions at *m/z* 211 and 253 correspond to C13:1 and C16:1 carboxylate anions, respectively. Both values indicate that the double bond is present in the α-branch and the mycolic acids thus have the following structures—23:0/13:1 and 20:0/16:1.

In the peak with retention time of 51.4 min, the ion at *m/z* 631 demonstrates that the molecule of mycolic acid must include two double bonds. The tandem mass spectrum of this ion contains the major ion at *m/z* 253 (C16:1) including again the major ion at *m/z* 199 (12:0), from which one can assume that two molecular species are present. One species contains the double bond in both meromycolate and

Table 3 Comparison of differences in the ratio of mycolic acids in cell walls of *R. erythropolis* CCM 2595 (ad) and *R. erythropolis* CCM 2595 *pSRK 21 phe* (mod) on phenol (300 mg/l) without and with humic substances PAB 32

CL:DB	Compared representation of mycolic acids			
	Effect of strain		Effect of the presence PAB 32	
	ad/mod	ad + PAB/mod + PAB	ad/ad PAB	mod/mod PAB
32:0	104	54	**215**	**113**
33:0	67	67	**100**	**100**
34:0	80	44	**254**	**141**
35:0	80	50	**200**	**125**
36:0	68	39	**250**	**141**
37:0	57	20	**400**	**140**
38:0	46	30	**307**	**202**
39:0	50	25	**200**	**100**
40:0	49	12	**833**	**202**
44:0	25	0	0	**267**
34:1	**122**	**111**	92	84
36:1	**134**	**108**	94	76
38:1	**115**	**135**	75	88
40:1	**133**	**135**	73	74
41:1	100	**300**	33	100
42:1	**300**	**118**	83	33
43:1	100	**200**	50	100
44:1	**220**	**123**	81	45
45:1	100	**200**	50	100
46:1	**200**	**133**	75	50
36:2	**179**	**128**	83	59
38:2	75	**188**	60	**150**
40:2	80	**153**	70	**133**
41:2	100	**200**	50	0
42:2	**336**	**154**	65	30
43:2	100	**200**	50	0
44:2	**292**	**134**	74	34
45:2	100	**600**	17	0
46:2	**190**	**132**	74	51
Saturated	39	34	**191**	**165**
Unsaturated	**146**	**132**	76	69

The increase or decrease in mycolic acid contents according to chain length and number of double bonds (CL:DB) expressed as ratio of values from Table 1 are illustrated. The bold values indicate a significant increase in mycolic acid content. Values are expressed as a percentage

Table 4 Changes in cell permeability of *R. erythropolis* CCM 2595 (ad) and *R. erythropolis* CCM 2595 *pSRK 21 phe* (mod) on phenol (300 mg/l) and on phenol with humic substances PAB 32 expressed as changes of tetracycline and rifampicin MIC and MBC

R. erythropolis	Substrate	Tetracycline		Rifampicin	
		MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)
"Adapted"	Phenol 300 mg/l	15±2	70±2	5±1	25±2
	Phenol 300 mg/l, PAB 32 10 mg/l	23±2	80±2	2±1	15±2
"Modified"	Phenol 300 mg/l	42±2	62±2	3±1	20±2
	Phenol 300 mg/l, PAB 32 10 mg/l	42±2	70±2	1±0,5	10±2

carboxylate chains (26:1/16:1), while the other has both double bonds in meromycolate aldehyde (30:2/12:0).

This demonstrated that RP-HPLC, although with relatively long elution times, can be used for separation and subsequent identification by ESI–MS of even- and odd-numbered mycolic acids which differ in homologous length of β-meromycolate branch or α-alkyl chain.

The tandem mass spectra obtained by the LC/MS of the mycolic acid mixtures extracted from four different cultivations are readily distinguishable, see Table 2.

The analysis of the proportion of mycolic acids shows that the genetic modification affecting the kinetics of phenol metabolic pathway and the application of humic acids modulating phenol transport into the cell exhibit a clear effect on mycolic acid biosynthesis (Table 3). Compared with the genetically modified strain, the physiologically adapted *R. erythropolis* has a greater relative proportion of long chain mycolic acids. The presence of PAB 32 in the medium caused a rise in the relative proportion of shorter mycolic acids. Even more significant changes were found with respect to the saturation of mycolic acids. The adapted strain contained (except mycolic acids 28:2 and 40:2) a higher or equal proportion of unsaturated mycolic acids. In both strains, the addition of PAB 32 to the media supported production of all determined mycolic acids.

R. erythropolis utilizes phenol as a sole carbon source by catechol metabolic pathway. The resulting activated intermediates oxoadipyl-CoA and succinyl-CoA are subsequently directed into the citric acid cycle. The synthesis of mycolic acids in mycobacteria involves both fatty acid synthases and polyketide synthases (PKSs) (Gokhale et al. 2007), which allows for considerable variability of the resulting products. The presumable involvement of PKSs in the synthesis of atypical fatty acids in *R. erythropolis* has been shown in our previous papers (Schreiberova et al. 2010; Rezanka et al. 2011a, b). It can be assumed that the actual availability of carbon substrate (phenol), modulated by the presence of PAB 32 in the medium, as well as the actual availability of intermediates for the synthesis of mycolic acids affected by the level of phenol hydroxylase activity can participate in the resulting structure of mycolic acids.

Changes in the proportion of mycolic acids caused by genetic modification, or the addition of PAB 32 to the cultivation medium, have a significant effect on physicochemical properties of cell envelopes such as their permeability for hydrophilic/hydrophobic substances (see Table 4) or surface hydrophobicity (Table 5). A larger proportion of mycolic acids with longer chain in the physiologically adapted strain compared with the genetically modified strain resulted in a 10 % higher hydrophobicity of cell surface. The increase of the proportion of unsaturated mycolic acids in cell envelopes of both studied strains caused by humic acids (PAB 32) was reflected in an increase of cell membrane permeability to hydrophobic compounds (represented by rifampicin) and a decrease of cell membrane permeability of hydrophilic compounds (represented by tetracycline).

It is obvious that the presence of humic acids in soil enhances the content of total phospholipid fatty acids (Wu et al. 2009). Based on our previous analyses, see two photos of the electron microscope (in Electronic supplementary material), we assume that humic acids interact with cell surface structures and affect the fluidity of the phospholipid membrane, see e.g. also (Moura et al. 2007). The cells then respond to this disturbance by changing the spectrum of the produced mycolic acids.

To our knowledge, this is the first time when mycolic acids were identified by RP-HPLC/ESI-MS on such a large scale. So far, their identification has been more or less marginal. The complexity of mycolic acids in different strains of genus *Rhodococcus* is characterized not only by a wide range of homologues, which were separated by RP-HPLC, but also by different molecular species having the same molecular weight but different chain lengths (β-meromycolate branch and α-

Table 5 Changes of surface hydrophobicity of "adapted" *R. erythropolis* CCM 2595 and "modified" *R. erythropolis* CCM 2595 *pSRK 21 phe*" on phenol or on phenol with humic substances PAB 32

R. erythropolis	Substrate	Hydrophobicity (%)
"Adapted"	Phenol 300 mg/l	76.5
	Phenol 300 mg/l, PAB 32 10 mg/l	79.3
"Modified"	Phenol 300 mg/l	93.2
	Phenol 300 mg/l, PAB 32 10 mg/l	90.6

alkyl chain) and also of course the number of double bonds. It follows from Table 2 that many detected minor molecular species are often odd numbered. The RP-HPLC/ESI-MS/MS analysis and identification of mycolic acids in mixture from the cultivated bacteria is a very fast and accurate method which can be very useful in the development of biotechnologies requiring technological strains with specific properties of cell envelopes (e.g., biofilm processes). When using different cultivation conditions with phenol as a sole carbon source, we found that a higher intracellular level of phenol hydroxylase and/or application of humic acids in the medium have a significant effect on the content of mycolic acids in *R. erythropolis* CCM 2595 and its mutant strain CCM 2595 *pSRK 21 phe*.

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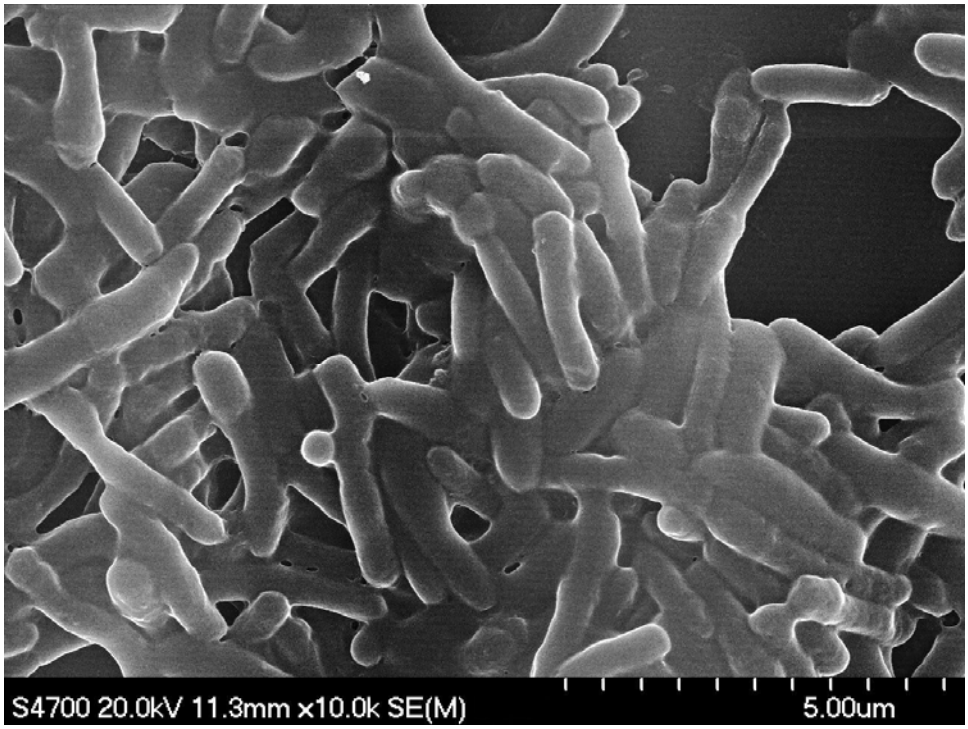
Supplemental material

Fig. 1S. Cells of *Rhodococcus erythropolis* without bound humic acid.



An electron microscope (Hitachi S4700, Japan) was used for the investigation of humic acid-cell surface interaction. The isolated biomass was spread onto cover slides and dried by infrared light. After gold and palladium covering of the slides, the obtained samples were studied. The electron microscopy was performed by Martin Maryska from the Department of Glass and Ceramics of the Institute of Chemical Technology Prague.

Fig. 2S. Cells of *Rhodococcus erythropolis* with bound humic acid.