Journal of Chromatography, 558 (1991) 215–221
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 446

Capillary gas chromatography–mass spectrometry of unusual and very long-chain fatty acids from soil oligotrophic bacteria

T. ŘEZANKA*
Institute of Microbiology, Vídeňská 1083, 14220 Prague (Czechoslovakia)
I. V. ZLATKIN
Institute of Microbiology, Prosp. 60-letia Oktiabrya 7/2, 117811 Moscow (USSR)
I. VÍDEN
Institute of Chemical Technology, 16628 Prague (Czechoslovakia)
and
O. I. SLABOVA and D. I. NIKITIN
Institute of Microbiology, Prosp. 60-letia Oktiabrya 7/2, 117811 Moscow (USSR)
(First received March 15th, 1991; revised manuscript received May 6th, 1991)

ABSTRACT

More than 60 fatty acids (as oxazolines) were identified in soil oligotrophic bacteria by capillary gas chromatography–mass spectrometry. The fatty acids covered the whole range from enanthic to cerotic acid and included both saturated and, to lesser extent, even monoenoic acids. Linear non-hydroxy and/or 3-hydroxy and branched-chain acids (iso- or anteiso) were also detected. Palmitic and stearic acid were major acids. Some species were found to contain unusual fatty acids; *e.g.*, *Arcocella flava* contained almost 20% of caprinic acid and *Blastobacter novus* more than 6% of 3-hydroxyisopentadecanoic acid (i-3-OH-15:0). Very unusual hydroxy fatty acids, *e.g.*, 3-hydroxy-5-dodecenoic acid, were identified in three strains.

INTRODUCTION

The study of oligotrophic soil bacteria is very complicated, *e.g.*, because of their difficult cultivation. Only a few papers have therefore dealt with these unique bacteria [1,2].

Gram negative bacteria are characterized by a variable content of fatty acids (FAs). In addition to their typical representatives (*e.g.*, *Escherichia coli*), much attention has been paid to the structure of lipopolysaccharides of clinically important pathogens, such as *Salmonella* and *Legionella* [3–5]. This structure is very complicated, the unifying component being the type of fatty acids present. Straight-chain fatty acids are saturated or monoenoic and are often observed. Branched-chain fatty acids may also be found, most frequently iso- and/or anteiso. In both types, the acids are often substituted by a hydroxy group at C-2 or -3.

0021-9673/91/$03.50

Identification of fatty acids in oligotrophic soil bacteria has been carried out several times [6,7]. Unfortunately, in these attempts many peaks remained unidentified, even though capillary gas chromatography–mass spectrometry (GC–MS) was used. In another publication on soil bacteria [8], some FAs were identified by using reversed-phase high-performance liquid chromatography (RP-HPLC) and GC–MS on packed columns.

Based on our previous results, we performed also this identification using capillary GC–MS [9,10]; the number of FAs identified showed a tenfold increase compared with similar published data. The fatty acid analysis was carried out even in unique soil oligotrophic bacteria.

EXPERIMENTAL

Bacterial isolates and growth conditions

All the bacteria used were from the collection of the Institute of Microbiology, Moscow, USSR. *Arcocella aquatica* NO.502, *Hyphobacter diversus* NP-802, *Pedodermatophilus paradoxus* NP-801, *Flectobacillus major* and *Spirosoma linguale* No. 1 were grown on PYG medium [peptone–yeast extract–glucose, 0.1% (w/v) each]. *Geodermatophilus obscurus* NP-800 was grown on yeast extract (0.5%), glycerol (5%) and calcium carbonate solution (0.1%). *Blastobacter novus* NP-141, *Renobacter vacuolatum* strains NP-300-W, NP-300-G and VZ-9, *Methylbacterium* sp. No. 5 were grown on medium [11] No. 337 with 1% or 0.3% (strain NP-141) methanol. All the strains were grown aerobically to the stationary phase (5–8 days at 28°C). The cells were harvested by low-speed centrifugation, washed with 0.1 *M* sodium chloride solution and stored at −25°C.

Derivatization and GC–MS identification

Methyl esters of corresponding fatty acids were prepared by alkaline hydrolysis and reaction of free acids with boron trifluoride–methanol [9]. The oxazolines and/or their trimethylsilyl (TMS) ethers were prepared using a modification of published methods [12,13] (method b, *e.g.*, lower temperature method): 5 mg of dicyclohexylcarbodiimide (Sigma, St. Louis, MO, USA) were added to a solution of 5 mg of FAs (free form) in 1 ml of dichloromethane. After stirring (10 min), 5 mg of 2-amino-2-methylpropanol (Sigma) were added (20°C, 4 h). The evaporated mixture was dissolved in 1 ml of diethyl ether and treated with 0.5 ml of thionyl chloride (20°C, 1 h), washed with ice-cold water and dried (anhydrous sodium sulphate). The eluate was evaporated, dissolved in pyridine (0.5 ml) and heated with trimethylsilyl chloride (50°C, 4 h).

Methyl esters were identified and quantified (by total ion current) in a Shimadzu (Kyoto, Japan) QP-1000 apparatus equipped with a fused-silica capillary column (60 m × 0.32 mm I.D.) coated with a 0.25-μm layer of SPB-1 (Supelco, Gland, Switzerland); splitless injection was used, with helium as carrier gas. Replacement of helium by hydrogen improved the elution and resolution only slightly (*ca.* 5–7%). The oven temperature was programmed from 100 to 320°C at 4°C/min. The mass spectrometer was operated with an ionization energy of 70 eV and an electron multiplier voltage of 2.5 kV.

TMS ethers of oxazolines were identified under similar conditions to the methyl

esters, except that the column temperature was programmed from 150 to 330°C at 5°C/min.

RESULTS AND DISCUSSION

The separation and identification of more than 60 peaks representing FAs typical of oligotrophic bacteria is shown in Table I. About half of the FAs had not previously been described in these microorganisms. Straight-chain FAs, iso-, anteiso- and monoenoic acids were identified.

The amount of straight- and branched-chain FAs (iso- and anteiso-) was large. Fewer than 15 of the total of 32 peaks were identified in a previous study [6] on soil bacteria. Therefore, the presence of almost 35 non-hydroxy saturated FAs is not so striking. The major fatty acids were, as usual, saturated with an even number of carbon atoms in the molecule (*e.g.*, 14:0, 16:0, 18:0). Other FAs were much more variable; for example, the series i, ai and/or n FAs were present, from C_{13} up to C_{20}.

Monounsaturated FAs were separated on a capillary column and, in some instances, two positional isomers were identified. Only one positional isomer has previously been identified [6,8] in oligotrophic soil bacteria. On the basis of the mass spectra of 2-alkenyl-4,4-dimethyloxazolines, two possible structures (n − 9 and n − 7) were detected; the former was markedly prevalent. By using these derivatives [12,14] we were able to identify monoenoic acids even in the complex mixture obtained from oligotrophic bacteria. The derivatization of FAs to their oxazolines has a great advantage because, as reported by Yu *et al.* [15], their elution temperature is only 5°C higher than that for fatty acid methyl esters (FAMEs). Fig. 1 shows one of the most interesting parts of the chromatogram, *i.e.*, that including the range from 12:0 to 14:0. Also, part of the mass spectrum of tentative 3-OH-12:1 is depicted (see caption of Fig. 1).

Another interesting group of FAs found in soil bacteria are hydroxy acids. In some work they have been neglected, probably because of their poor identification by GC (lack of standards). In keeping with literature data [4,6], most hydroxy acids exhibited a chain length of C_9–C_{16}. In this study the majority were C_{11}–C_{15}. Both normal and iso-hydroxy acids have been observed [3,4], albeit in other genera of Gram negative bacteria. We detected a higher content of FAs with an odd number of carbon atoms in the molecule, in contrast to the previous work [4,6].

Also, a 3-hydroxymonoenoic acid was identified, in the molecule of which the position of the double bond was thought to be between C-5 and -6 (see also the caption to Fig. 1). In the only work known [16], the position of the double bond in monoenoic 3-OH-FA was shown to be between the C-5 and -6 (determined by means oxidative splitting with potassium permanganate).

To our knowledge, FAs longer than C_{20} have not been detected in soil oligotrophic bacteria. The presence of very long-chain fatty acids (VLCFAs), *i.e.*, acids having more than 24 carbon atoms in the molecule, is unusual and these acids have not yet been found in oligothrophic bacteria, although they are described in a report on FAs in soil [17]. As mentioned by Nikitin *et al.* [18], oligotrophic bacteria represent a major part of soil biomass and, therefore, the presence of VLCFAs in these microorganisms is not surprising. C_{24} acids dominated; 24:0 was always present with the exception of the genus *Methylobacterium*. In the strains NO-300-G and VZ-9 of the

TABLE I

FATTY ACID COMPOSITION OF SELECTED OLIGOTROPHIC BACTERIA (IN MOL. %)

Fatty acid[a]	*Arcocella aquatica*	*Blastobacter novus*	*Flectobacillus major*	*Geodermatophilus obscurus*	*Hyphobacter diversus*
7:0	1.10	0	0.75	0	0.12
8:0	0.98	0	0.46	0	0.61
9:0	0.28	0	0.31	0.07	0.11
10:0	17.23	0.13	10.58	0.12	0.79
3-OH-9:0	0.56	0.59	0.42	0	0.30
11:0	3.37	2.01	0.98	0.08	1.09
i-5-12:1	0	0	0	0.21	0
3-OH-10:0	0	0	0	0.13	0.24
i-12:0	0	0	0	0.11	0
12:0	6.45	9.44	8.71	0.53	2.49
i-3-OH-11:0	0	0	0	0.12	1.46
3-OH-11:0	0.56	0.15	0.63	0.41	5.64
i-13:0	0	0	0	0.26	0
ai-13:0	0	0	0	0.93	0
13:0	1.54	2.42	2.31	0.25	1.03
i-3-OH-12:0	0.29	0-10	0.15	0	1.44
3-OH-5-12:1	0	0	0	0	1.33
3-OH-12:0	0	0	0.12	0	1.21
i-14:0	0.98	1.17	0.31	3.52	0
7-14:1	0	0	0	0.41	0.31
14:0	4.49	8.57	8.21	5.38	2.61
i-3-OH-13:0	0	0	0	0	1.10
3-OH-13:0	0.30	0.42	0.25	0	0.29
i-15:0	2.52	6.21	3.52	0.57	0.67
ai-15:0	1.26	0.56	0.76	7.31	1.34
8-15:1	0	0.86	0	0.90	0.49
15:0	2.38	8.74	1.91	5.46	10.92
3-OH-14:0	4.35	3.12	3.58	0	0.36
i-7-16:1	0	0.26	0	0.15	0
i-16:0	1.19	3.37	1.39	7.65	1.58
7-16:1	3.09	4.52	3.55	4.18	0.85
16:0	21.04	12.63	17.21	10.31	13.95
i-3-OH-15:0	6.31	6.27	5.68	0	0.55
3-OH-15:0	0.42	0.69	0.59	0	0.30
i-17:0	0.58	0.13	0.63	3.84	0.36
8-17:1	0	0	0	0	3.64
ai-17:0	0	0	0	9.16	0
10-17:1	0	0.17	0	1.28	2.91
17:0	0.84	0.38	0.25	0.54	20.83
3-OH-16:0	0.41	0.42	0.63	0	0
9-18:1	3.92	9.25	8.28	9.56	3.22
i-18:0	0	0	0	1.24	0
11-18:1	0	4.13	3.42	3.50	0
18:0	8.09	11.62	9.39	7.32	8.49
i-10-19:1	0	0	0	0	1.03
i-19:0	0	0	0	3.12	0.28
ai-19:0	0	0	0	6.18	0
10-19:1	0	0	0	0.20	2.90
19:0	0.15	0	0	0.17	0.92
i-20:0	0	0	0	1.46	0

Methylobacterium sp. No. 5	*Pedodermatophilus paradoxus*	*Renobacter vacuolatum*			*Spirosoma linguale*
		NP-300-W	NP-300-G	VZ-9	
0	0	0	0	0.21	0.56
0	0	0	0.17	0.14	0.75
0.42	0.13	0.13	0.24	0	0.31
0.84	0.10	0.29	0.35	1.46	15.46
0.88	0	0.17	0	0.21	0.31
0.42	0.14	2.28	0.17	0.95	3.85
0.09	0.22	0	0	0.37	0
0	0.12	0.16	0	0.24	0
0	0.11	0.24	0	0	0.13
3.10	0.62	3.98	1.62	3.71	6.92
0	0.15	0.34	0	0.22	0.12
3.81	0.11	0	0.08	0.26	0.63
0.89	0.21	0	0	0.58	0
0.22	0.71	0.34	0	0.94	0
1.98	0.31	2.03	2.08	1.39	1.85
0.74	0	0	0	0.36	0.31
0	0	0	0	0.95	0.17
0	0.13	1.28	0	0.55	0
3.48	5.33	1.64	0.63	0.76	1.46
0.98	0.11	1.77	0.17	0.33	0
8.19	3.60	3.67	2.64	2.38	5.15
0	0	1.19	0	0	0
1.41	0	0	0	0.42	0.25
2.67	0.27	3.23	3.11	1.74	4.47
0.90	14.06	3.54	4.37	2.73	3.28
0	0.32	0	0.42	0.31	0.07
7.51	3.46	6.67	5.15	8.44	3.56
0.86	0	0	0.24	1.41	6.17
2.37	0.36	1.24	0	0	0
4.35	8.00	6.82	3.46	2.18	3.74
3.16	1.38	1.96	1.41	0.92	4.07
21.14	6.65	18.62	18.68	15.37	17.42
0	0	0.14	2.47	2.15	3.29
0.35	0	0.67	0.80	0.48	0.24
0.51	5.31	0.18	0.31	0.94	0.84
1.43	0	0.05	0	0	0.15
0.83	11.76	1.46	0	0.43	0
0.17	0.75	0.18	0.39	0.31	0
0.29	3.34	1.25	0.17	0.24	0.56
0	0	0	0.56	0.85	0.93
12.39	1.73	6.57	1.41	0.86	1.48
0.18	2.53	0	0.91	0	0
1.46	0.32	0	0.27	0.30	0
9.43	6.30	17.32	21.23	29.08	8.57
0	0	0	0	0	0
0	4.35	2.89	0.15	0.42	0
0	5.41	3.38	0.91	0.66	0
1.15	0.80	0	0	0	0
0.96	1.02	1.07	1.37	2.28	0
0	1.38	0.58	0	0	0

(Continued on pp. 220 and 221)

Fatty acid[a]	*Arcocella aquatica*	*Blastobacter novus*	*Flectobacillus major*	*Geodermatophilus obscurus*	*Hyphobacter diversus*
11-20:1	0	0	0.58	0.28	0
20:0	1.96	0.67	1.35	1.31	0.97
i-21:0	0	0	0	0.08	0
21:0	0	0	0	0.59	0
i-22:0	1.14	0.46	0.98	0	0
13-22:1	0.15	0	0.12	0	0
22:0	0.30	0.13	0.48	0.47	0.42
i-24:0	0.32	0	0.19	0.12	0.07
15-24:1	0.84	0	0.90	0	0.42
24:0	0.61	0.41	0.34	0.45	0.36
i-26:0	0	0	0	0	0
17-26:1	0	0	0	0	0
26:0[b]	0	0	0.08	0.07	0

[a] First number, number of carbon atoms in the chain; second number, number of double bonds; number before the hyphen, position of double bond or hydroxy group; i = iso-acid; ai = anteiso-acid; OH = hydroxy acid.

[b] By means of SIM (m/z = 55 and 74) some peaks were identified that have retention times corresponding to 28:1 and 28:0.

genus *Renobacter*, the presence of 28:1 and 28:0 acids was suggested on the basis of the occurrence of ions of m/z 55 and 74 (base peaks for saturated and monounsaturated FAMEs). By using single ion monitoring (SIM), we were able to detect the acids up to C_{28} in two strains, which is nine carbon atoms higher than previously reported.

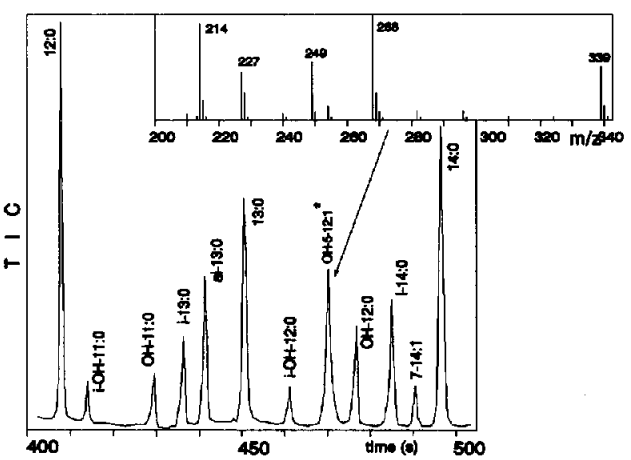

Fig. 1. Partial GC–MS of TMS-oxazolines from *R. vacuolatum* VZ-9. Mass spectrum of tentative FA (3-OH-5-12:1) is shown at the top. Proposed structures of ions from the mass spectrum: m/z 339, M^+; m/z 324, $M-CH_3$; m/z 310, $M-C_2H_5$; m/z 296, $M-C_3H_7$; m/z 282, $M-C_4H_9$; m/z 268, M − chain from C_8 to C_{12}, *e.g.*, C_5H_{11}; m/z 254, M − chain from C_7 to C_{12}; m/z 249, M − TMSOH; m/z 240 M − chain from C_6 to C_{12}; m/z 228, M − chain from C_5 to C_{12}; m/z 227, chain from C_3 to C_{12}; m/z 214, M − chain from C_4 to C_{12}. On the basis of the above fragmentation pattern of the derivatized FA the structure 3-hydroxy-5-dodecenoic acid is proposed.

Methylobacterium sp. No. 5	*Pedodermatophilus paradoxus*	*Renobacter vacuolatum*			*Spirosoma linguale*
		NP-300-W	NP-300-G	VZ-9	
0	0.11	0.07	0.74	0	0
0.37	5.09	0.95	8.85	2.65	1.01
0	0.71	0	0	0	0
0	1.51	0	0	0	0
0	0	0.13	0.55	0.91	0.23
0	0	0.14	0.33	0.58	0.07
0.07	0.53	0.84	6.42	0.52	0.44
0	0.11	0.17	0.91	0.31	0.25
0	0	0	1.14	1.88	0.49
0	0.27	0.37	3.15	3.01	0.35
0	0	0	0.36	0.48	0
0	0	0	0.85	0.20	0
0	0.07	0	0.76	0.83	0.09

In conclusion, the most important feature of this work is the detailed description of new FAs, exceeding several times the number of FAs identified previously in oligotrophic bacteria. No significant differences in either the separation or the proportion of the compounds were observed on comparing the chromatographic properties of FAMEs and oxazolines.

REFERENCES

1 P. Hirsch, *Annu. Rev. Microbiol.*, 28 (1974) 391.
2 J. M. Poindexter, *Microbiol. Rev.*, 76 (1981) 123.
3 E. Fautz, G. Rosenfelder and L. Grotjahn, *J. Bacteriol.*, 140 (1979) 852.
4 W. R. Mayberry, *J. Bacteriol.*, 147 (1981) 373.
5 A. Sonesson, E. Jantzen, K. Bryn, L. Larsson and J. Eng, *Arch. Microbiol.*, 153 (1989) 72.
6 Y. Suwa and T. Hattori, *J. Gen. Appl. Microbiol.*, 32 (1986) 451.
7 Y. Suwa, and T. Hattori, *Soil. Sci. Plant. Nutr.*, 33 (1987) 235.
8 L. V. Andreev, V. N. Akimov and D. I. Nikitin, *Folia Microbiol.*, 31 (1986) 144.
9 T. Řezanka, M. Yu. Sokolov and I. Viden, *FEMS Microbiol. Ecol.*, 73 (1990) 231.
10 T. Řezanka and M. Yu. Sokolov, *J. Chromatogr.*, 508 (1990) 275.
11 P. Hirsch and S. F. Conti, *Arch. Microbiol.*, 48 (1964) 339.
12 Q. T. Yu, B. N. Liu, J. Y. Zhang and Z. H. Huang, *Lipids*, 23 (1988) 804.
13 A. P. Tulloch, *Lipids*, 20 (1985) 652.
14 J. Y. Zhang, Q. T. Yu, Y. M. Yang and Z. H. Huang, *Chem. Scr.*, 28 (1988) 357.
15 Q. T. Yu, B. N. Liu, J. Y. Zhang and Z. H. Huang, *Lipids*, 24 (1989) 79.
16 E. A. Bishop and K. Still, *Biophys. Res. Commun.*, 7 (1962) 337.
17 J. F. Dormaar, *Can. J. Soil Sci.*, 62 (1982) 487.
18 D. I. Nikitin, O. Yu. Vishnewetskaya, K. M. Chumakov and I. V. Zlatkin, *Arch. Microbiol.*, 153 (1990) 123.