JOURNAL OF BACTERIOLOGY, June 1973, p. 1018-1024

Vol. 114, No. 3
Printed in U.S.A.

Occurrence of Branched-Chain Hydroxy Fatty Acids in *Pseudomonas maltophilia*

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Received for publication 6 February 1973

Three branched-chain hydroxy acids not previously reported in other bacteria were found in extracts from saponified whole cells of *Pseudomonas maltophilia*. On the basis of evidence from mass spectrometry, infrared spectroscopy, and gas chromatographic procedures, they were identified as 2-hydroxy-9-methyldecanoic acid, 3-hydroxy-9-methyldecanoic acid, and 3-hydroxy-11-methyldodecanoic acid. These acids appeared to be tightly bound to other cellular components since they were not extracted from lyophilized cells with a chloroform-methanol (3:1) mixture.

In a previous study from this laboratory, we found that the cellular fatty acid composition of whole cell hydrolysates of *Pseudomonas maltophilia* is quite different from that of other *Pseudomonas* (12). The principal feature of the fatty acids of this organism is the presence of relatively large amounts (22%) of a branched-chain 15-carbon acid (13-methyltetradecanoic acid) which is not found in several other *Pseudomonas* species. In addition, this organism contains three unidentified fatty acids which we have not detected in a variety of other bacteria (10, 11, 12). This report describes the isolation and identification of these three unusual acids of *P. maltophilia*.

MATERIALS AND METHODS

Bacterial strains. *P. maltophilia* was obtained from R. Y. Stanier of the University of California, Berkeley, as RYS 67, and is synonymous with ATCC-13637. Five clinical isolates of *P. maltophilia*, obtained from the Clinical Bacteriology Section of the Center for Disease Control, were also studied.

Culture conditions. Cells for routine fatty acid analysis were obtained from 24-h growth in Trypticase soy broth (Baltimore Biological Laboratory, Baltimore, Md.). The cells were harvested by centrifugation, washed with distilled water, and then saponified with 4% NaOH in equal volumes of methanol and water for 1 h at 100 C. The fatty acids were extracted from the saponified mixture and methylated by methods described earlier (11). Cells used for isolation of the unidentified acids were grown for 24 h at 37 C on Trypticase soy agar (BBL) plates or in Trypticase soy broth. The cells were washed twice with distilled water, lyophilized, and stored at −20 C until needed.

Gas-liquid chromatography. Fatty acid composition was determined by gas-liquid chromatography (GLC) analysis of methyl esters. The GLC analysis was done on a Perkin-Elmer Model 900 Gas Chromatograph (Perkin-Elmer, Norwalk, Conn.) equipped with a hydrogen flame detector and a DISC integrator recorder. The instrument contained a 0.16 inch (4.06 mm, inside diameter) × 12 ft (3.66 meter) coiled glass column packed with 3% OV-1 methyl silicone which was coated on 80/100 mesh, acid-washed, DMCS-treated, high performance Chromosorb W (Applied Science Laboratories, State College, Pa.). Helium was used as carrier gas at a flow rate of 60 ml/min. The column temperature was 150 C and, after injection of sample, it was programmed to 255 C at a rate of 5 C/min. Samples were also analyzed on a 4.06 mm × 8 ft (2.4 meter) column of 15% ethylene glycol adipate (EGA) coated on 80/100 mesh Chromosorb W (Applied Science Laboratories). The column was operated isothermally at 230 with an N_2 flow rate of 50 ml/min. Fatty acid methyl ester peaks were tentatively identified by comparison of retention times on each column (OV-1, EGA) with retention times of methyl ester standards (Applied Science Laboratories and National Institutes of Health, Bethesda, Md.). Final identification was established by a combination of techniques including mass spectrometry and infrared spectroscopy, hydrogenation of unsaturated acids, bromination, and trifluoroacetylation of hydroxy acids. Peak areas from GLC were determined with the DISC integrator, and the percentage of each acid was calculated from the ratio of the area of its peak to the total area of all peaks.

Mass spectrometry. Combined gas-liquid chromatography-mass spectrometry of the fatty acid methyl esters was carried out on an LKB 9000 instrument.

The mass spectra were recorded at an electron energy of 70 eV and a trap current of 60 μA, an ion source temperature of 290 C, and a molecular separator temperature of 250 C. The methyl esters were separated on an 18 ft × ¼ in (approximately 5.48 m × 0.64 cm) glass column packed with 3% OV-1. The column was conditioned for 72 h at 250 C prior to use.

Hydrogenation. Unsaturated fatty acid methyl esters were hydrogenated by exposure to hydrogen gas in the presence of 5% platinum on charcoal as follows: the methyl ester sample was reduced to dryness under N_2, redissolved in 0.5 ml of a 3:1 mixture of chloroform-methanol, and hydrogenated for 2 h at room temperature.

Bromination. Bromination was done according to the procedure of Brian and Gardner (4).

Trifluoroacetylation of hydroxy acids. The presence of hydroxy fatty acids was verified by trifluoroacetylation of the methyl ester sample. The methyl esters were reduced to dryness under N_2 and redissolved in 0.2 ml of chloroform. Approximately 25 μliters of trifluoroacetic anhydride (Pierce Chemical Co., Rockford, Ill.) were added, and the contents were mixed. The reaction vial was sealed and maintained at room temperature for 30 min. After this time, the sample was reduced to dryness under a gentle stream of N_2 and then redissolved in hexane for analysis by GLC. The trifluoroacetylated methyl ester sample was analyzed by GLC on both the OV-1 and EGA columns under the same conditions used for methyl esters.

Isolation of unknown fatty acids. Two grams of lyophilized cells were extracted with chloroform-methanol (70:30, vol/vol) for 72 h in a Soxhlet extraction apparatus. The organic fraction was reduced to near dryness on a rotary evaporator. A sample was removed to a screwcapped tube, reduced to dryness, and saponified. The fatty acids of this fraction were extracted, methylated, and analyzed by GLC.

The chloroform-methanol-extracted cells were removed from the extraction thimble, dried under N_2, saponified, and then the fatty acids were extracted (11). A small portion of the fatty acids of this fraction was methylated and analyzed by GLC; the remaining portion was used for thin-layer chromatography.

Thin-layer chromatography. Thin-layer chromatography (TLC) was performed on precoated chromatogram sheets (Eastman, type K301R2); hexane-ethyl ether (70:30, vol/vol) was used as the developing solvent. Fatty acids from chloroform-methanol-extracted cells were spotted in a thin line across the entire sheet, approximately 1 inch (approximately 2.54 cm) from the bottom edge. The sample was air-dried for 1 h, and the sheet was placed in a chromatography chamber that was saturated with vapors from the developing solvent in the bottom of the chamber. The chromatogram was developed until the solvent front was 0.5 inch (approximately 1.27 cm) from the top, at which time it was removed and air-dried. Fatty acids were visualized by spraying with 0.1% 2',7'-dichlorofluorescein (in isopropanol), and by ultraviolet light at 254 nm and 366 nm. The separated fatty acid bands were marked, scraped from the sheet, and eluted from the silica by a 3-h extraction (Soxhlet) with chloroform-methanol (70:30, vol/vol). The chloroform-methanol was evaporated under N_2 and the purified fatty acids were analyzed by GLC, by mass spectrometry and by infrared spectroscopy.

Infrared spectroscopy. Infrared spectra were recorded on a Beckman Model IR-20 spectrophotometer equipped with a mirror beam condenser. Samples were prepared by evaporating a chloroform solution of the isolated methyl ester (or acid) onto 4 mg of KBr, which was then pressed into a 1.5-mm diameter pellet.

RESULTS AND DISCUSSION

The fatty acid profile of saponified whole cells of *P. maltophilia* strain RYS 67 is shown in Fig. 1. The major components are 13-methyltetradecanoic acid (i-15:0), 9-methyldecanoic acid (i-11:0), and three unidentified compounds designated A, B, and C. The relatively large amounts of i-15:0 and i-11:0 acids are characteristic features which distinguish *P. maltophilia* from other *Pseudomonas* (10). In addition, components A, B, and C have not been found in other bacteria studied in this laboratory (10, 11, 12).

Preliminary data indicated that components A, B, and C were fatty acids because they were not detected by GLC until the acid extract was methylated. Moreover, they appeared to be of cellular origin because they were not detected in spent growth media or from saponified uninoculated control media. The fact that these components were not affected by hydrogenation or bromination indicates that they were saturated. The possibility that the unknowns contained a cyclopropane structure was ruled out by observing that the size of the GLC peaks of A, B, and C were not reduced after bromination (4).

The slight tendency of A, B, and C for tailing when chromatographed as methyl esters on the OV-1 column indicated that they might contain a hydroxyl group. This tailing effect, which was also observed for several hydroxy acid methyl ester standards, was eliminated when the unknowns or standards were treated with trifluoroacetic anhydride. These compounds then formed trifluoroacetylated-methyl ester derivatives which were chromatographed as sharp, symmetrical peaks. The GLC retention data obtained with both methyl esters and trifluoroacetylated-methyl ester derivatives indicated that unknowns A and B were both 11 carbon hydroxy acids and that unknown C was a 13-carbon hydroxy acid. In addition, the observation that 2-OH acids have a shorter retention time than 3-OH acids of the same chain length, i.e., 2-OH 12:0 < 3-OH 12:0 (6),

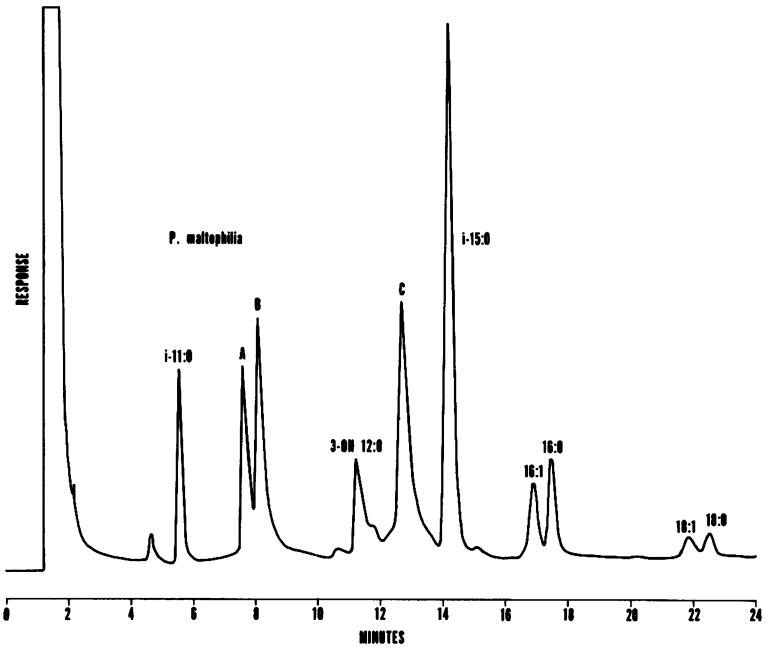

FIG. 1. *Gas-liquid chromatogram of the methylated fatty acids from saponified whole cells of P. maltophilia strain RYS 67. Analysis was made on a 3% OV-1 column.*

suggested that unknown A was possibly 2-OH 11:0, unknown B possibly 3-OH 11:0, and unknown C possibly 3-OH 13:0.

Comparison of retention times with standard hydroxy acids, however, clearly showed that the unknown compounds were not identical to any of these standards. As shown in Table 1, unknown A eluted from the nonpolar column (OV-1) between 3-OH 10:0 and 2-OH 11:0, unknown B between 2-OH 11:0 and 2-OH 12:0, and unknown C between 3-OH 12:0 and 2-OH 13:0. Since esters are separated on OV-1 according to their boiling point (1), the elution sequence was useful in establishing the relationship between the boiling point and the possible structure of the unknowns.

To gain additional information on the identity of the unknowns, we attempted to separate them from other fatty acids. Lyophilized cells were extracted with chloroform-methanol for 72 h in a Soxhlet extraction apparatus to remove "loosely bound" lipids (7). The chloroform-methanol fraction was reduced to dryness and saponified, and the fatty acids were determined by GLC. The acids detected in this fraction were 14:0, i-15:0, 16:1, 16:0, 18:1, and 18:0. The other acids (i-11:0, 3-OH 12:0, and unknowns A, B, and C) in whole cell hydrolysates (Fig. 1) were detected only after saponification of cells that had been extracted with chloroform-methanol. These acids are apparently tightly bound to other cellular components. Separation of i-11:0 from unknowns A, B, and C and 3-OH 12:0 was accomplished by TLC as described above. Two spots were observed on the plate, one at an R_f of 0.5 and the other at an R_f of 0.1. Elution and subsequent GLC analysis of the material at R_f 0.5 showed only i-11:0. The material at R_f 0.1 contained 3-OH 12:0 and

TABLE 1. *Comparison of gas chromatography retention times of standard hydroxy acids with those of unknowns A, B, and C from Pseudomonas maltophilia*

Sample	3% Siloxane (OV-1) column		15% Ethylene glycol adipate column	
	ME[a] derivative (min)	ME-TFA[b] derivative (min)	ME derivative (min)	ME-TFA derivative (min)
3-OH 10:0	5.5	4.4	11.0	3.5
A	7.6	6.5	12.5	4.0
2-OH 11:0	7.9	6.8	13.2	4.2
B	8.2	7.1	17.5	5.2
2-OH 12:0	10.6	9.5	19.0	ND[c]
3-OH 12:0	11.2	10.1	26.8	ND
C	12.8	11.7	31.2	8.6
2-OH 13:0	13.1	12.0	25.5	8.0

[a] ME = methyl ester derivative.
[b] ME-TFA = trifluoroacetylated-methyl ester derivative.
[c] ND = not determined.

unknowns A, B, and C as determined by GLC. These data demonstrate the utility of this simple TLC procedure for isolation of hydroxy acids.

Mass spectra of unknowns A, B, and C are shown in Fig. 2-4, respectively. These show a molecular weight of 216 for A, 216 for B, and 244 for C. These data indicate that both A and B are saturated 11-carbon hydroxy acid methyl esters (empirical formula $C_{12}H_{24}O_3$) and that C is a saturated 13-carbon hydroxy acid methyl ester (empirical formula $C_{14}H_{28}O_3$). The prominent M-59 and *m/e* 90 peaks in A (Fig. 2) are characteristic fragments of methyl esters of 2-hydroxy acids (3, 6, 9, 13). Further evidence for substitution at position two is the small *m/e* 74 ion

$$+\left[\begin{array}{l}CH_2{=}C{-}O{-}CH_3\\ \quad\ |\\ \quad OH\end{array}\right]$$

which is the major ion in normal saturated fatty acid methyl esters (3, 6, 9, 13). Substitution at position two eliminates *m/e* 74 as a major ion and, in the case of 2-hydroxy esters, leads to formation of an *m/e* 90 ion

$$\left[\begin{array}{ll}CH{=}C{-}O{-}CH_3\\ |\quad\ |\\ OH\ \ OH\end{array}\right]+.$$

The mass spectra of unknown B (Fig. 3) and unknown C (Fig. 4) show major peaks characteristic of the methyl esters of 3-hydroxy acids. These peaks occur at *m/e* 103 due to the ion

$$\left[\begin{array}{l}CH{-}CH_2{-}COOCH_3\\ |\\ OH\end{array}\right]+,$$

at *m/e* 74 due to β-cleavage

$$\left[\begin{array}{l}CH_2{=}C{-}O{-}CH_3\\ \quad\ |\\ \quad OH\end{array}\right]+,$$

and at *m/e* M-50 due to the "ketene" ions formed from the loss of water and methanol from the molecular ions (3, 6, 9, 13). These data provide conclusive evidence for the presence of

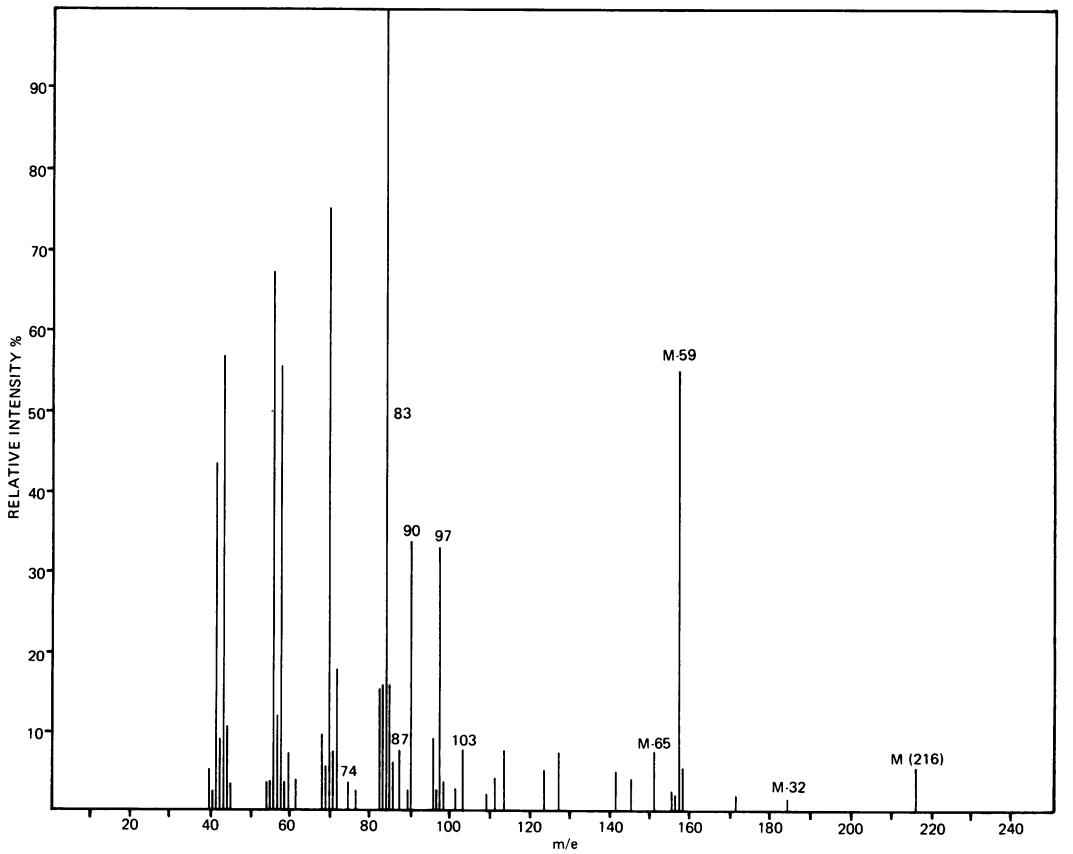

FIG. 2. *Mass spectrum of the methyl ester of unknown A from P. maltophilia.*

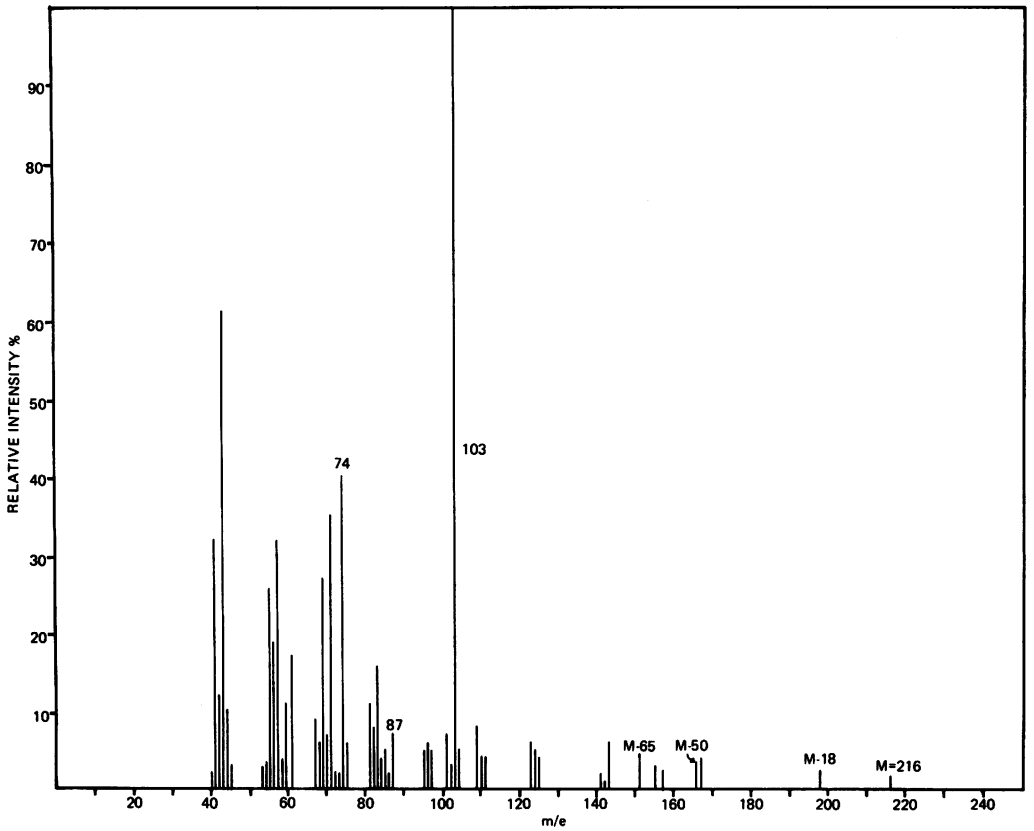

FIG. 3. *Mass spectrum of the methyl ester of unknown B from P. maltophilia.*

an hydroxyl group at position three in unknowns B and C.

The elution characteristics observed on the OV-1 column together with the mass spectra data suggested that the unknown compounds contained a branched-methyl group in the fatty acid chain. Close examination of the mass spectra of each compound showed a small peak at *m/e* = M-65 which is characteristic of a methyl-branch at the iso (penultimate) carbon atom in the fatty acid chain (13, 15). Branching was confirmed by infrared spectra which showed that the terminal carbon atoms displayed characteristic absorption in the region 1,380 to 1,360 cm^{-1}. Unknowns A, B, and C were each isolated in pure form by semipreparative GLC techniques and analyzed by infrared spectroscopy both as methyl esters and as free acids. In each case, the spectra of A, B, and C show a doublet (1, 380 and 1,360 cm^{-1}) with absorption bands of nearly equal intensity. This is directly attributable to the isopropyl $[(CH_3)_2\ CH-]$ configuration of the terminal carbon atoms (14, 15). These data together with mass spectra indicate that (i) unknown A is 2-hydroxy-9-methyldecanoic acid (i, 2-OH 11:0), (ii) unknown B is 3-hydroxy-9-methyldecanoic acid (i, 3-OH 11:0), and (iii) unknown C is 3-hydroxy-11-methyldodecanoic acid (i, 3-OH 13:0).

Hydroxy fatty acids are not common to all bacteria but they have been reported in several species (2, 3, 5, 6, 8, 12). Twelve- and 14-carbon straight-chain acids are found most frequently (2, 3, 10). Straight-chain hydroxy acids of 10, 13, 15, 16, and 18 carbons in length have also been reported (3, 9, 12). In most cases the hydroxyl group is located at position three in the carbon chain; 2-hydroxy acids have also been reported (3, 9, 12). To our knowledge this is the first report of the presence of branched-chain hydroxy acids in bacteria. The fact that the point of branching in each hydroxy acid occurs at the iso-position is not surprising since the major fatty acids of *P. maltophilia* (i-15:0, i-11:0) are also branched at the iso-position. It is reasonable to assume that their synthesis probably occurs through the same pathways as those of the i-15:0 and i-11:0 acids.

The cellular fatty acid composition of the

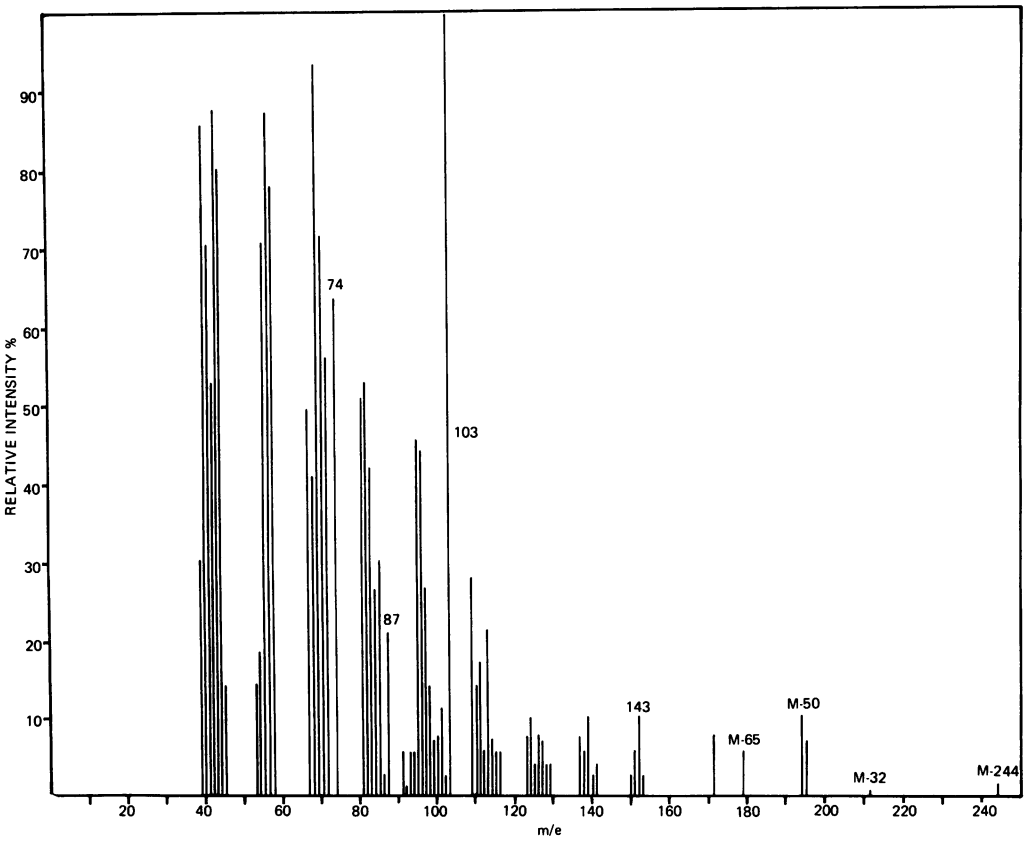

FIG. 4. *Mass spectrum of the methyl ester of unknown C from P. maltophilia.*

TABLE 2. *Cellular fatty acids of Pseudomonas maltophilia*

CDC (no.)	Straight-chain acids					Hydroxy acids					Branched-chain acids	
	14:0[a]	16:1	16:0	18:1	18:0	iso-2-OH 11:0	iso-3-OH 11:0	2-OH 12:0	3-OH 12:0	iso-3-OH 13:0	iso-11:0	iso-15:0
(ATCC-13637)[b]	7[c]	7	9	3	3	8	11	2	9	10	5	26
A-2897	5	6	14	8	8	7	9	T	5	8	8	22
B-3545	7	5	7	5	4	9	10	2	7	11	10	23
B-3588	7	5	9	5	5	8	12	2	9	10	8	20
B-3643	6	5	9	5	4	9	13	2	6	10	9	22
B-3731	6	6	13	4	3	9	13	2	8	8	9	19

[a] Number to the left of the colon refers to the number of carbon atoms; number to the right refers to the number of double bonds; 2-OH and 3-OH refer to the position of the hydroxyl group; iso indicates a methyl group at the penultimate carbon atom.

[b] All cultures except ATCC-13637 are clinical isolates.

[c] Number refers to percentages of total acids; T = less than 2%. Unknown C and 14:0 elutes together on the OV-1 column but are separated on EGA. Therefore, percentages were determined from data obtained on EGA.

type strain of *P. maltophilia* and of five clinical isolates is shown in Table 2. These data clearly show the similarity of fatty acid composition among various *P. maltophilia* isolates. Each culture contained relatively large amounts (19 to 26%) of i-15:0 acid and moderate amounts (5 to 10%) of i-11:0 acid. The presence of these two branched-chain acids as major components of the fatty acids of *P. maltophilia* distinguishes this organism from other *Pseudomonas* we have

tested (12). In addition, each culture contained moderate amounts of the three previously unidentified acids (i, 2-OH 11:0, i, 3-OH 11:0, and i, 3-OH 13:0). These acids have not been found in other bacteria. Thus, the uniqueness of the fatty acid profile of *P. maltophilia* should serve as a useful criterion for taxonomy of this organism. The routine analysis of fatty acids of microorganism by GLC is feasible because of the accuracy, speed, and sensitivity of the technique.

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