Journal of Chromatography, 112 (1975) 595–604

CHROM. 8472

IDENTIFICATION OF MICROORGANISMS BY GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF CELLULAR FATTY ACIDS

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SUMMARY

The cellular fatty acid compositions of strains of *Pseudomonas aeruginosa*, *P. cepacia*, and *P. maltophilia* were determined by gas–liquid chromatography. A variety of acids was detected in these organisms, including branched and straight-chain acids, cyclopropane, and hydroxy acids. Comparison of the presence and relative amounts of these acids was useful in distinguishing the three species. Three branched-chain hydroxy acids not found in other bacteria were present in *P. maltophilia* and were identified by gas chromatography, mass spectrometry, and infrared spectroscopy.

INTRODUCTION

Identification and classification of microorganisms is presently based on their morphological, biochemical, serological, and toxigenic characteristics. However, it is often difficult to assign some organisms to their proper group on the basis of these characteristics alone. Consequently, newer techniques, such as polyacrylamide-gel electrophoresis[1], deoxyribonucleic acid base composition[2], and nucleic acid hybridization techniques[3] have been applied to the taxonomic study of microorganisms. Although these techniques provide additional valuable information, their application in a routine diagnostic laboratory would be time consuming and impractical for handling a large number of isolates.

Classification of microorganisms on the basis of their chemical composition has been proposed[4]. The feasibility of using this approach has been significantly advanced through the development of sensitive analytical techniques and procedures such as gas–liquid chromatography (GLC) and combined gas–liquid chromatography–mass spectrometry (GLC–MS). We have used these powerful analytical tools to study various chemical components of microorganisms, including cellular fatty acids[5–8]. The results from these studies have provided the microbiologist additional criteria for more rapid identification of bacterial cultures. In recent studies, we have investigated the fatty acid compositions of whole cells of reference or type strains of several *Pseudomonas* species. Data from these studies have shown that one or more hydroxy fatty acids are present in each strain; comparison of the relative amounts of these

and other cellular fatty acids is useful for distinguishing various species of *Pseudomonas*.

EXPERIMENTAL*

Bacterial cultures

Both reference strains and clinical isolates were included in the study. The reference strains were obtained from the American Type Culture Collection (ATCC, Rockville, Md., U.S.A.); the clinical isolates originated from different geographical locations where they were cultured from a variety of clinical materials. All cultures were identified by the extensive cultural and biochemical testing procedures of the Clinical Bacteriology Unit, Center for Disease Control.

Cultural conditions and derivative formation

Bacteria were grown on Trypticase soy agar (Baltimore Biological Laboratory, Baltimore, Md., U.S.A.) plates at 37° for 24 h. The cell mass from the surface of one plate (100 × 15 mm) was carefully removed and transferred to a 16 × 150 mm test tube containing 5 ml of 5% sodium hydroxide in 50% methanol. The tubes were sealed with PTFE-lined caps, and the cells were saponified for 30 min at 100°. The tubes were removed from the bath, cooled to room temperature, and the pH of the saponified material adjusted to approximately pH 2.0 with 6 *N* HCl. The methyl esters of the free fatty acids were formed by adding 5 ml of 10% (w/v) boron trichloride–methanol reagent (Applied Science Labs., State College, Pa., U.S.A.) and heating the mixture for 5 min at 80°. The fatty acid methyl esters were then extracted from the cooled mixture by shaking the samples for about 20 sec with a mixture of chloroform and hexane (1:4). A few drops of a saturated solution of sodium chloride were added to enhance the separation. A second extraction with 10 ml of solvent removed essentially all of the methyl esters. The solvent layers containing the methyl esters were combined in a 50-ml beaker and evaporated to a volume of 0.2 ml under a gentle stream of nitrogen. A small amount of sodium sulfate was added to remove traces of moisture, and the methyl esters were stored for analysis at −20° in 13 × 100-mm screwcapped test tubes. Approximately 3 μl of the methyl ester sample were injected into the gas chromatograph.

Gas–liquid chromatography

Fatty acid composition was determined by GLC analysis of methyl esters. The GLC analysis was done on a Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer, Norwalk, Conn., U.S.A.) equipped with a flame ionization detector and a DISC integrator recorder. The instrument contained a 0.16 in. (4.06 mm, I.D.) × 12 ft. (3.66 m) 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 Labs.). Helium was used as carrier-gas at a flow-rate of 50 or 60 ml/min. The column temperature was 160° or 170° and, after injection of sample, it was programmed to 265° at a rate of 5°/min. Under these conditions, fatty acid

* The use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

methyl esters ranging from 10 to 20 carbons in length were eluted from the column within 20 or 25 min. Samples were also analyzed on a 0.16 in. (4.06 mm) × 8 ft. (2.4 m) column of 15% ethylene glycol adipate (EGA) coated on 80–100 mesh Chromosorb W (Applied Science Labs.). This column was operated isothermally at 230° with a nitrogen flow-rate of 50 ml/min. Fatty acid methyl ester peaks were tentatively identified by comparison of retention times on each column with retention times of methyl ester standards (Applied Science Labs. and National Institutes of Health, Bethesda, Md., U.S.A.). 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. Relative response factors were determined for each acid, and these were used in the calculation.

Mass spectrometry

Combined GLC–MS 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°, and a molecular separator temperature of 250°. The methyl esters were separated on an 18 ft. (approx. 5.48 m) × 0.16 in. (4.06 mm) glass column packed with 3% OV-1. The column was conditioned for 72 h at 250° before use. Chemical ionization spectra were obtained using a DuPont 21-491B instrument (DuPont, Monrovia, Calif., U.S.A.) with methane as reagent gas.

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 nitrogen, redissolved in 0.5 ml of chloroform–methanol (3:1), and hydrogenated for 2 h at room temperature.

Bromination

Bromination was achieved according to the procedure of Brian and Gardner[9].

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 nitrogen and redissolved in 0.2 ml of chloroform. Approximately 25 μl of trifluoroacetic anhydride (Pierce, Rockford, Ill., U.S.A.) were added, and the contents 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 nitrogen 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.

Infrared spectroscopy

Infrared spectra were recorded on a Beckman Model IR-20 spectrophotometer

equipped with a mirror beam condensor. Samples were prepared by evaporating a chloroform solution of the isolated methyl ester (or acid) onto 4 mg of potassium bromide, which was then pressed into a 1.5-mm diameter pellet.

RESULTS AND DISCUSSION

Typical chromatograms showing the cellular fatty acids of several *Pseudomonas* species examined are presented in Figs. 1–3. The three most abundant acids in *Pseudomonas aeruginosa* (Fig. 1b) were hexadecanoic acid (16:0), hexadecenoic acid (16:1), and octadecenoic acid (18:1). The next most abundant acids were dodecanoic acid (12:0), 3-hydroxydecanoic acid (3-OH 10:0), and 2-hydroxydodecanoic acid (2-OH 12:0). Also present were 17- and 19-carbon cyclopropane acids (17:0Δ1 and 19:0Δ1) and small-to-trace amounts of tetradecanoic (14:0), pentadecanoic (15:0), hepta-

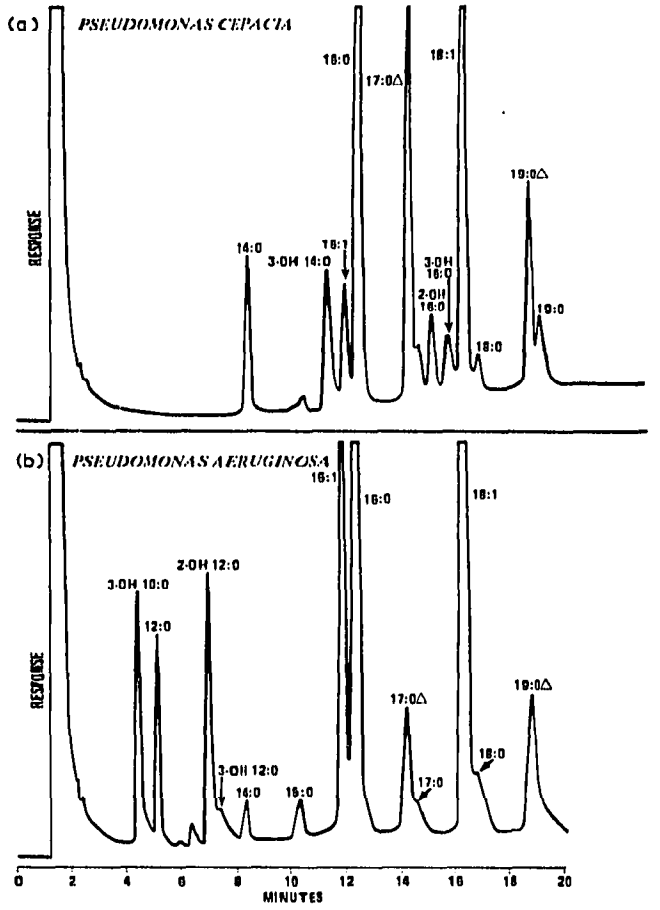


Fig. 1. Gas chromatograms of esterified fatty acids from saponified whole cells of *P. cepacia* and *P. aeruginosa*. Analysis was made on a 3% OV-1 column which was programmed from 170 to 265° at 5°/min. Helium flow was 50 ml/min.

decanoic (17:0), octadecanoic (18:0), and 3-hydroxydodecanoic (3-OH 12:0) acids. Some of the acids present in *P. aeruginosa* were also found in *P. cepacia* (Fig. 1a), but there are major differences between the two species. The most striking difference is the absence of 3-OH 10:0, 12:0, and 2-OH 12:0 acids in *P. cepacia*. In addition this species contained relatively small amounts of three hydroxy acids (3-OH 14:0, 2-OH 16:0, and 3-OH 16:0) which were not detected in *P. aeruginosa*. Moreover, the 17:1 acid was a minor component in *P. aeruginosa* but it was present as a major constituent of *P. cepacia* (Fig. 1).

A chromatogram of the cellular fatty acids of *Pseudomonas pseudoalcaligenes* is presented in Fig. 2a. It is clear that the major differences in the fatty acids of this organism compared with those of *P. cepacia* (Fig. 1a) are: (i) the presence of relatively

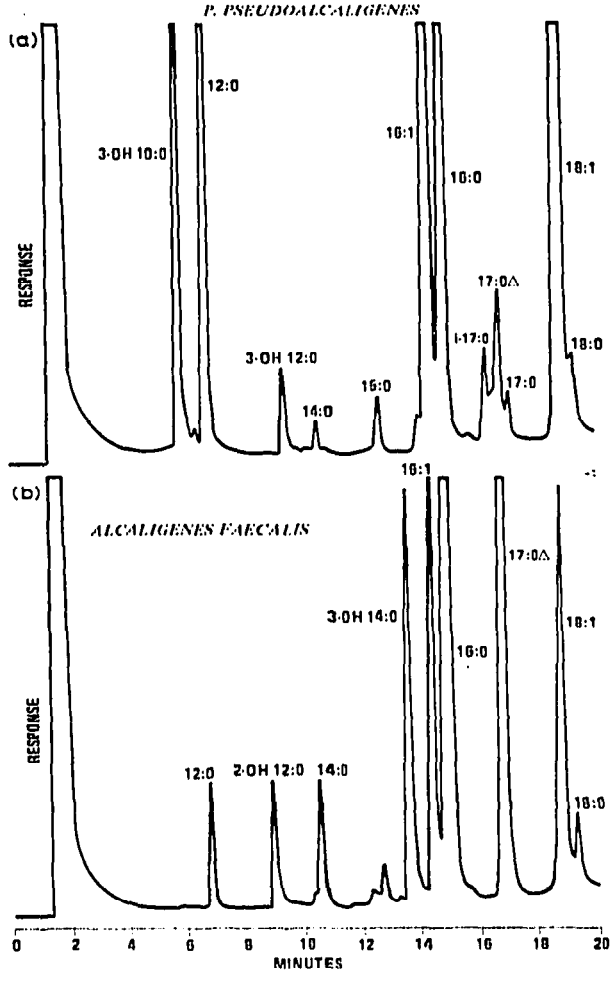


Fig. 2. Gas chromatograms of esterified fatty acids from saponified whole cells of *P. pseudoalcaligenes* and *Alcaligenes faecalis*. Analysis was made on a 3% OV-1 column which was programmed from 160 to 265° at 5°/min. Helium flow was 50 ml/min.

large amounts of 3-OH 10:0 and 12:0 acids, (ii) small amounts of 3-OH 12:0, 19:0Δ, and 19:0 acids, (iii) the absence of 3-OH 14:0, 2-OH 16:0, and 3-OH 16:0 acids, and (iv) the presence of relatively small amounts of 17:0Δ acid compared with significantly larger amounts of 17:0Δ acid in *P. cepacia*. The absence of 2-OH 12:0 and 19:0Δ acids, and the presence of relatively larger amounts of 3-OH 10:0 and 12:0 acids are the principal features which distinguish *P. pseudoalcaligenes* from *P. aeruginosa* (Fig. 1b). Fig. 2b shows the fatty acids of *Alcaligenes faecalis*, an organism which is difficult to distinguish by conventional criteria from non-oxidative pseudomonads such as *P. pseudoalcaligenes*[10]. The presence of 2-OH 12:0 and 3-OH 14:0 acids, the absence of 3-OH 10:0 acid, and a large difference in relative amounts of 16:1, 17:0Δ, and 18:1 acids are major features which distinguish these two closely related bacterial species.

Fig. 3 shows the fatty acid profile of a reference strain of *Pseudomonas maltophilia*. A major difference between the fatty acids of this organism and those discussed above is the presence of relatively large amounts of a branched-chain 15-carbon acid, 13-methyltetradecanoic acid (i-15:0). Other differences include several other branched-chain acids (i-11:0, i-14:0, i-16:0, i-17:1, i-17:0) and three unidentified components designated A, B, and C. Further studies to identify these three components were undertaken since they had not been found in a variety of other bacteria studied in this laboratory[5,7,8,11].

Preliminary data firmly established that components A, B, and C were fatty acids and that they originated from cellular material. The fact that these components

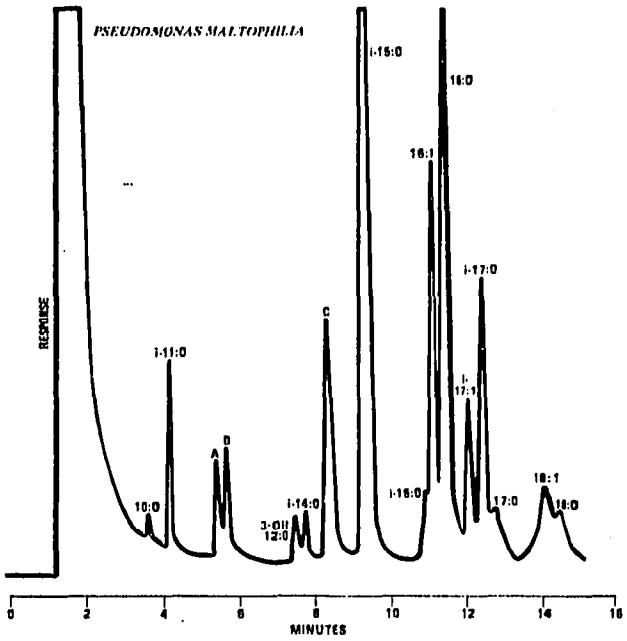
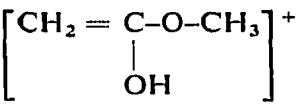


Fig. 3. Gas chromatogram of the methylated fatty acids from saponified whole cells of *P. maltophilia*. Analysis was made on a 3 % OV-1 column which was programmed from 170 to 265° at 5°/min. Helium flow was 60 ml/min.

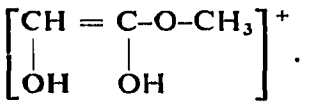
were not affected by hydrogenation or bromination indicated that they were saturated. The possibility that the unknowns contained a cyclopropane structure was ruled out by observing that the sizes of the GLC peaks of A, B, and C were not reduced after bromination[9]. Upon treatment of the methyl ester sample with trifluoroacetic anhydride and subsequent GLC analysis (OV-1), the retention time of each component was reduced by approx. 1 min from that of the methyl ester sample run under identical GLC conditions. A similar reduction in retention time was observed for the methyl ester of 3-OH 12:0.

On the basis of GLC retention data for both methyl esters and trifluoroacetylated-methyl ester derivatives, unknowns A and B appeared to be 11-carbon hydroxy acids and unknown C appeared to be a 13-carbon hydroxy acid. However, when compared with authentic standards of 2-OH 11:0, 2-OH 12:0, and 2-OH 13:0 (Applied Science Labs.), component A eluted from the OV-1 column before 2-OH 11:0 and component C eluted before 2-OH 13:0. Component B eluted between 2-OH 11:0 and 2-OH 12:0. Careful study of these data suggested the possibility of branching in the fatty acid chain of each unknown. Since esters are separated on OV-1 according to their boiling point[4], branching would account for the observed retention characteristics of these components.

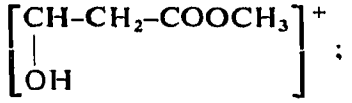
The mass spectra of the methyl ester of unknowns A, B, and C are shown in Fig. 4. These show a molecular ion at 216 for A, 216 for B, and 244 for C. The chemical ionization (CI) spectra showed large M + 1 ions at 217, 217, and 245 for A, B, and C, respectively. These data indicate that both A and B are 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/e* 90 and M − 59 peaks in spectrum A are characteristic fragments of methyl esters of 2-hydroxy acids[12–14]. Additional evidence for substitution at position two in the acid chain is the small *m/e* 74 ion



which is the major ion (base peak) in normal saturated fatty acid methyl esters[13,14]. Substitution at position two eliminates *m/e* 74 as a major ion and, in the case of 2-hydroxy esters, leads to the formation of an *m/e* 90 ion



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



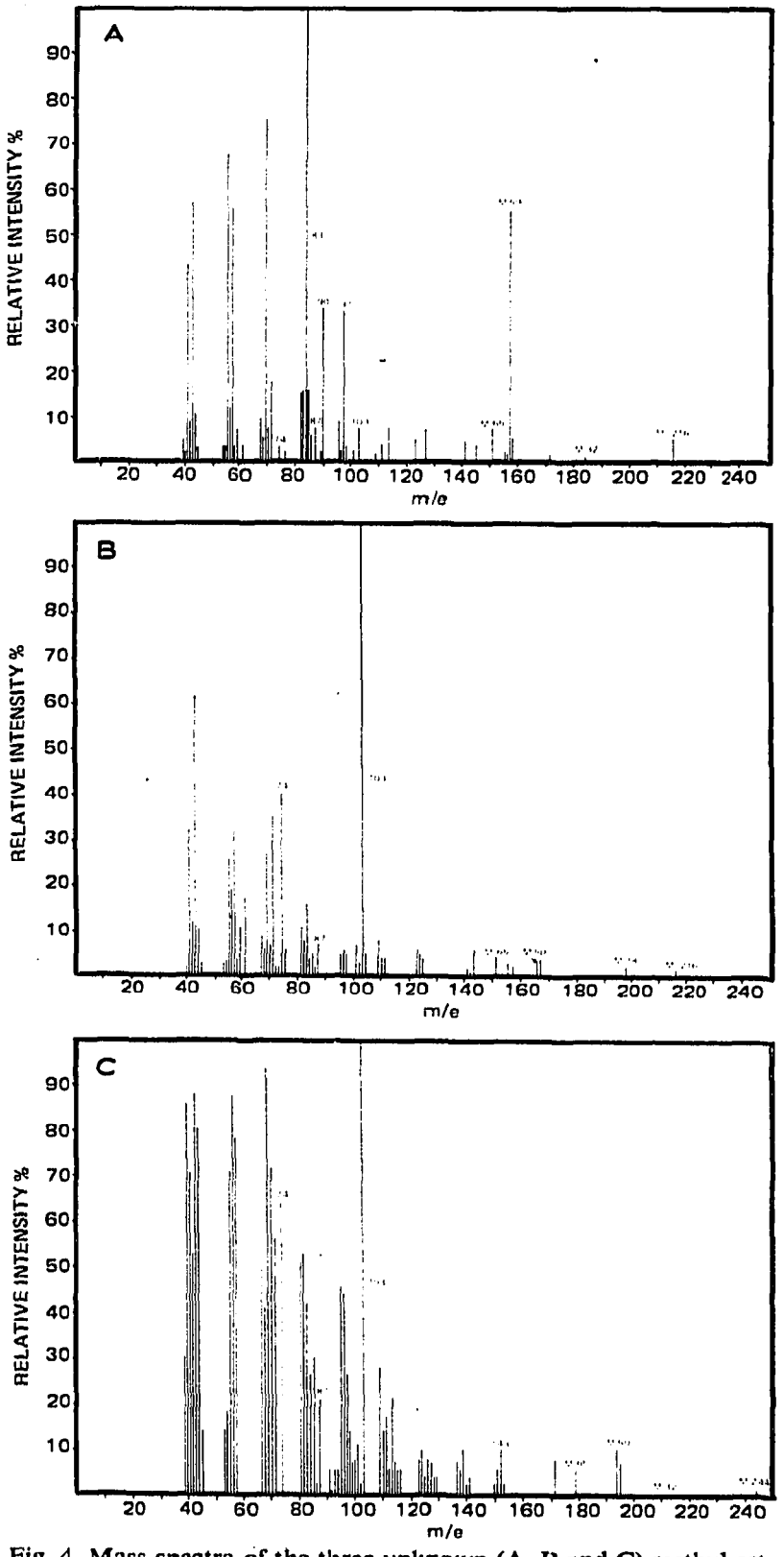


Fig. 4. Mass spectra of the three unknown (A, B and C) methyl esters present in *P. maltophilia* (see Fig. 3).

at m/e 74 due to β-cleavage $\left[CH_2 = \underset{\displaystyle OH}{\overset{\displaystyle |}{C}}-O-CH_3\right]^+$; and at m/e = M − 50

due to the "ketene" ions formed from the loss of HOH and CH_3OH from the molecular ion[12–14]. These data provide conclusive evidence for the presence of a hydroxyl group at position three in unknowns B and C.

The combined data from mass spectrometry and GLC suggested that each of the three unknowns contained a branched methyl group in the fatty acid chain. Close examination of the mass spectra of each compound (Fig. 4, A, B and C) 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[14,15]. Branching was confirmed by infrared spectra which showed that the terminal carbon atoms displayed characteristic absorption in the region 1,380–1,360 cm^{-1}. Unknowns A, B and C were each isolated in pure form by semi-preparative GLC techniques and analyzed by infrared spectroscopy. The spectra of each unknown showed a doublet (1,380 and 1,360 cm^{-1}) with absorption bands of nearly equal intensity which is due to the isopropyl [$(CH_3)_2$–CH–] configuration of the terminal carbon atoms[15,16]. This information and the mass spectra data indicate that unknown A is 2-hydroxy-9-methyldecanoic acid, unknown B is 3-hydroxy-9-methyldecanoic acid, and unknown C is 3-hydroxy-11-

TABLE I

CELLULAR FATTY ACIDS OF *PSEUDOMONAS MALTOPHILIA*

*Fatty acid**	*RYS 67***	*Komagata strains*							
		AJ 2082	*YF-3*	*YF-6*	*YF-8*	*P-1*	*P-7*	*P-7-2*	*P-19*
iso-11:0	5***	4	5	5	3	3	4	3	3
iso-2-OH 11:0	4	4	4	3	3	3	3	3	3
iso-3-OH 11:0	4	4	3	5	3	3	3	3	4
3-OH 12:0	2	2	T	2	T	3	3	3	2
iso-14:0	2	T	3	2	6	T	T	T	2
14:0	6	6	6	7	5	6	6	7	7
iso-3-OH 13:0	4	5	5	4	2	4	4	3	5
iso-15:0	33	30	30	33	35	32	36	32	36
15:0	T	2	3	3	3	4	2	2	2
iso-16:0	T	T	5	3	11	T	T	T	T
16:1	12	14	11	12	7	13	14	14	13
16:0	10	13	14	12	7	14	12	15	14
iso-17:1	6	6	3	3	4	3	4	4	2
iso-17:0	7	8	5	4	6	6	5	5	4
18:1	2	2	3	2	5	4	4	4	3
18:0	3	T	T	T	T	2	T	2	T

* 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.

** Received from R. Y. Stanier, Berkeley, Calif., U.S.A. as the type strain (ATCC 13637).

*** Numbers refer to percentage of total acids; T = less than 2%. Iso-3-OH 13:0 and 14:0 elute together on the OV-1 column but are separated on EGA. Therefore, percentages were calculated from data obtained on EGA.

methyldodecanoic acid. The fact that the point of branching in each of the acids occurs at the iso-position is not surprising since the major acid of this organism (i-15:0) is also branched at this position. It is reasonable to assume that their biosynthesis occurs through the same or similar pathways as that of the i-15:0 acid.

The cellular fatty acid compositions of the type strain of *P. maltophilia* (ATCC 13637) and of eight isolates which were recently assigned to this species, are shown in Table I. The eight isolates were provided by Dr. K. Komagata (University of Tokyo, Tokyo, Japan) who originally identified them as *Pseudomonas melanogena*[17] or *Pseudomonas alcaligenes*[18]. Recently, however, he reported that the morphological and physiological characteristics and deoxyribonucleic acid base compositions of these organisms were not sufficiently different from *P. maltophilia* to justify their separation from this species[19]. The fatty acid data obtained with these organisms strongly support this conclusion. The fatty acid composition of each of the eight isolates was quite similar to the reference strain (Table I). In each case i-15:0 was the major acid (30–36%), and each isolate contained the three branched-chain hydroxy acids (i, 2-OH 11:0; i, 3-OH 11:0; i, 3-OH 13:0) which have been found only in *P. maltophilia*.

The above data indicate that analysis of cellular fatty acids is a useful additional means for identification of various pseudomonads. All strains within a species produced similar fatty acid profiles. Moreover, when each strain was tested two or more times through the entire procedure (growth, extraction, GLC), results were similar. The extraction, derivatization, and GLC steps can be accomplished without difficulty by technical personnel. The speed and simplicity of the saponification procedure[20] are desirable features for application of fatty acid analysis on a routine basis in microbiology laboratories.

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