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Cellular Fatty Acids of *Alcaligenes* and *Pseudomonas* Species Isolated from Clinical Specimens

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The cellular fatty acid composition of 25 clinical isolates of *Alcaligenes* and *Pseudomonas* was determined by gas-liquid chromatography (GLC). The GLC fatty acid profiles of three species of *Pseudomonas* were markedly different from those of *Alcaligenes*. The most significant differences were the presence and relative amounts of hydroxy, branched-chain, and cyclopropane fatty acids. One of the major fatty acids in *A. faecalis* was a 17-carbon cyclopropane (17 Δ) acid, whereas a 15-carbon branched-chain acid (13-methyl tetradecanoate) characterized isolates of *P. putrefaciens*. The determination of these fatty acids by GLC provides a rapid and specific means of distinguishing clinical isolates of *Pseudomonas* and *Alcaligenes*.

In recent studies, we found that species of *Pseudomonas* encountered in clinical specimens can be distinguished by their cellular fatty acids (10, 11, 17; C. Kaltenbach and C. W. Moss, submitted for publication). Both qualitative and quantitative differences were observed in the gas-liquid chromatography (GLC) profiles of seven species. A significant feature of these studies was the differentiation of pseudomonads which are difficult to identify by conventional criteria. The *Pseudomonas* species that do not oxidize glucose (aglucolytic) and are quite unreactive in other diagnostic tests may be easily confused with a number of gram-negative organisms, such as the *Alcaligenes* which give similar reactions (3, 4, 6, 14). In the study reported here, we compared the cellular fatty acids of *Alcaligenes* species and found that they are markedly different from aglucolytic *Pseudomonas*. The data indicate that the GLC procedure would be a useful tool for screening aglucolytic isolates from clinical materials.

MATERIALS AND METHODS

Cultures. Twenty-five clinical isolates of *Alcaligenes* and *Pseudomonas* were analyzed for cellular fatty acids: *A. faecalis*, 11; *A. denitrificans*, two; *A. odorans*, two; *P. alcaligenes*, two; *P. pseudoalcaligenes*, four; and *P. putrefaciens*, four. The identification of these isolates, which were cultured from a variety of clinical materials, was based upon the results of 40 or more biochemical tests routinely used by the Clinical Microbiology Laboratory, Center for Disease Control. A detailed description of each species is given in the Center for Disease Control Syllabus *The identification of unusual pathogenic gram-negative bacteria*. The isolates were transferred each month on semisolid motility medium (Difco, Detroit, Mich.).

Cell preparation and derivative formation. Bacteria were grown on Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.) plates at 37 C for 24 h. Growth from one plate was used for a single determination: cells were carefully removed from the plate and transferred to a test tube (16 by 150 mm) containing 5 ml of 5% NaOH in 50% methanol. The tubes were sealed with Teflon-lined caps, and the cells were saponified for 30 min at 100 C. After the saponificate was cooled, the pH was lowered to 2.0 with 6 N HCl. The methyl esters of the free fatty acids were formed by adding 5 ml of 10% boron trichloride-methanol reagent (wt/vol) (Applied Science, State College, Pa.) and heating the mixture for 5 min at 80 C. The fatty acid methyl esters were then extracted from the cooled mixture with 10 ml of chloroformhexane (1:4). A few drops of saturated NaCl solution were added to enhance the separation. A second extraction with 10 ml of solvent removed all but trace amounts of the methyl esters. The solvent layers containing the fatty acid methyl esters were combined in a 50-ml beaker and evaporated to a volume of 0.2 ml under a gentle stream of N_2. A small amount of Na_2SO_4 (Fisher Scientific, Fair Lawn, N.J.) was added to remove moisture, and the methyl esters were stored at −20 C in screw-capped test tubes (13 by 100 mm). Approximately 3 μl of the methyl ester sample was injected into the gas chromatograph. The acetylation of hydroxy fatty acids was accomplished by reacting the hydroxy acid methyl ester with 50 μl of trifluoroacetic anhydride (Eastman Kodak Co., Rochester, N.Y.) for 30 min at room temperature. The excess trifluoroacetic anhydride was removed from the tube with N_2 and 0.1 ml of hexane was added to replace the evaporated solvent.

GLC. The methyl esters were analyzed on a gas chromatograph (model 990, Perkin-Elmer, Norwalk, Conn.) equipped with a hydrogen flame detector and

a disk integrator recorder. The instrument contained a coiled glass column (3.66 m [12 ft] by 4.03 mm [0.15 in, inside diameter]) that was packed with 3% OV-1 methyl silicone, coated on 80/100 mesh, acid-washed, DMCS-treated, high-performance Chromosorb W (Applied Science, State College, Pa). Highly purified nitrogen was used as carrier gas at a flow rate of 60 me/min. The initial column temperature was 170 C. After injection of the sample, the temperature was increased to 265 C at a rate of 4° per min. Under these conditions, fatty acid methyl esters ranging from 10 to 20 carbons in length eluted from the column within 20 min. For additional GLC identification of fatty acids, samples were also analyzed on a glass column (2.4 m [8 ft] by 4.06 mm [0.16 in]) of 10% Silar (Silicone Apolar-9CP) coated on 100/120 mesh Gas-Chrom Q (Applied Science, State College, Pa.). The column temperature was held at 170 C for 5 min after injection of the sample, and then it was increased to 220 C at 2° per min. Under these conditions, saturated, unsaturated, cyclopropane, and branched-chain methyl esters containing the same number of carbon atoms are resolved from each other. Although excellent separation of methyl esters was achieved with this column, most of the quantitative data was obtained with the OV-1 column because of its temperature stability and low column bleed. Fatty acid methyl ester peaks were tentatively identified by a comparison of retention times on each column (OV-1, Silar) with those of highly purified methyl ester standards (Applied Science Laboratories). Standards of iso branched-chain acids were provided through the courtesy of Toshi Kaneda. Peak areas were determined with the disk 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. GLC response factors for each acid were determined and used in the calculations. Final identification was accomplished by mass spectrometry (10, 16, 19) and by hydrogenation (1, 10).

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 gently 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. This procedure is selective in that unsaturated acids are converted to saturated ones whereas cyclopropane acids are not affected (1).

GLC-mass spectrometry. Combined gas-liquid chromatography-mass spectrometry of the fatty acid methyl esters was performed on an LKB mass spectrometer. The methyl esters were separated on a glass column (5.48 m [18 ft] by 0.64 cm [0.25 in]) packed with 3% OV-1. Operating parameters for this instrument have been described in a previous report (10).

RESULTS AND DISCUSSION

The cellular fatty acids of *Alcaligenes* species isolated from clinical materials were distinct and differed markedly from those of each of the pseudomonads tested. These differences are illustrated in the GLC profiles of *A. faecalis* (Fig. 1), *P. pseudoalcaligenes* (Fig. 1), and *P. putrefaciens* (Fig. 2). The bottom chromatogram (Fig. 1), which shows the cellular fatty acids of *A. faecalis*, is characterized by five major peaks. The two largest peaks, at retention times of 15 and 17 min, were identified as palmitic (16:0) acid and a 17-carbon cyclopropane (17 Δ) acid, respectively. The next most abundant acids were 3-hydroxymyristic (3-OH 14:0), hexadecenoic (16:1), and octadecenoic (18:1) acids. In addition to the major acids, small to trace amounts of lauric (12:0), 2-hydroxylauric (2-OH 12:0), myristic (14:0),

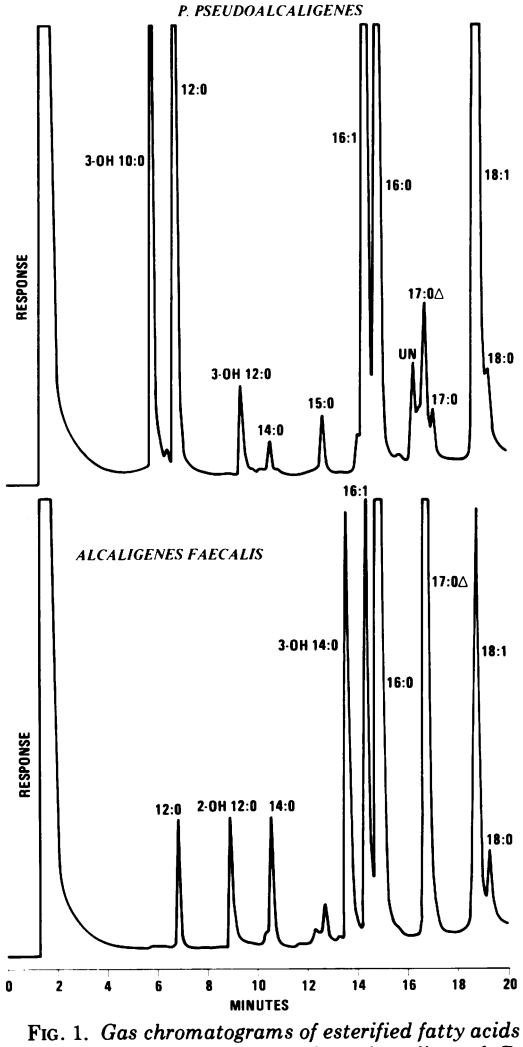

FIG. 1. *Gas chromatograms of esterified fatty acids from saponified whole cells of A. faecalis and P. pseudoalcaligenes. Analysis was made on a 3% OV-1 column. Peak labeled UN in top chromatogram is i-C17:0 acid (15-methyl hexadecanoate).*

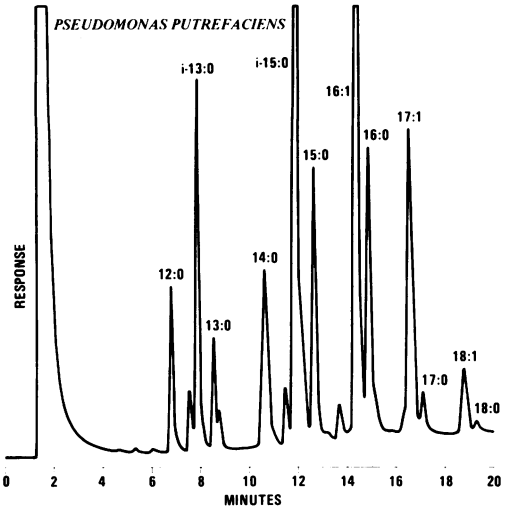

FIG. 2. *Gas chromatogram of esterified fatty acids from saponified whole cells of P. putrefaciens Analysis was made on a 3% OV-1 column.*

and octadecanoic (18:0) acids were detected. Although 3-OH 14:0 is a relatively common fatty acid found in the lipids of the *Enterobacteriaceae* (9), it has been observed in only two species of *Pseudomonas*, *P. cepacia* (11, 17) and *P. pseudomallei* (Kaltenbach, unpublished data).

The top chromatogram (Fig. 1) shows the cellular fatty acids of *P. pseudoalcaligenes*. It is readily apparent that a major difference between this species and *A. faecalis* is the absence of 3-OH 14:0. A second obvious distinction between the two species is that 17 Δ, which is a minor component of *P. pseudoalcaligenes*, is a principal constituent of *A. faecalis*. Although 17 Δ and 19 Δ have been found in the whole cell extracts of other *Pseudomonas* species, the amounts detected are substantially less than that observed for the 17 Δ in *A. faecalis*. The top chromatogram (Fig. 1) also contains relatively large amounts of 3-OH 10:0, 12:0, 16:1, 16:0, and 18:1 acids. In addition, small amounts of 3-OH 12:0, 14:0, pentadecanoic (15:0), heptadecanoic (17:0), 18:0, and an unknown acid were detected. The identity of the unknown methyl ester has recently been established by GLC and mass spectrometry as a 17-carbon branched-chain (i-17:0) fatty acid. The occurrence of 3-OH 10:0 and 2- and 3-OH 12:0 in the lipids of pseudomonads has been previously reported by our laboratory (11) and by other investigators (5, 8). The 3-OH 12:0 acid is one of the major fatty acids of *P. diminuta* and *P. vesiculare* (Kaltenbach and Moss, submitted for publication), whereas both 3-OH 10:0 and 2-OH 12:0 are found in *P. aeruginosa* (5, 11). Moreover, 3-OH 10:0 is a characteristic fatty acid of at least seven clinically important species of *Pseudomonas*, including *P. aeruginosa* (11).

In contrast to other *Pseudomonas* species we have studied (10, 11, 17; Kaltenbach and Moss, submitted for publication), *P. putrefaciens* contained no hydroxy acids. The chromatogram in Fig. 2 shows that 16:1 and two branched-chain acids (i-13:0 and i-15:0) are the major fatty acids of this organism. Although relatively large amounts of i-15:0 acid have been observed in another pseudomonad, *P. maltophilia*, the presence of branched-chain hydroxy acids in this organism readily distinguishes it from *P. putrefaciens* (10). In addition to 16:1, *P. putrefaciens* contained a 17-carbon unsaturated fatty acid (17:1) which, to our knowledge, has not been demonstrated in other species of *Pseudomonas*. The identity of the 17:1 methyl ester was confirmed by mass spectrometry (16, 19) and by hydrogenation experiments (1, 10). After exposure of the unsaturated methyl ester sample to hydrogenation, the methyl ester peak at a retention time of 17 min (Fig. 2) completely disappeared, whereas the size of the 17:0 methyl ester peak was increased proportionately to the size of the 17-min peak. Under the same conditions of hydrogenation, reference standards of 17 Δ and 19 Δ acids as well as the 17 Δ acid present in all of the other cultures were not affected. Other acids detected in *P. putrefaciens* were relatively small amounts of 15:0, 16:0, 17:0, 18:1, and 18:0.

The amounts of cellular fatty acids detected in each of the 25 clinical isolates are presented in Table 1. The data show that each of the species of *Alcaligenes* is characterized by relatively large amounts of straight-chain, hydroxy, and cyclopropane fatty acids. Except in four strains, the 16:0 and 17 Δ acids comprised 40 to 58% of the total cellular fatty acids in each strain, whereas 3-OH 14:0 acid represented about 10% of the total. The fatty acids of four strains (*A. faecalis*, B 4636, B 2566, C 1549; *A. denitrificans*, B 7042) differed from the majority of *Alcaligenes* by a marked decrease in 17 Δ, with concomitant increases in 16:1 and 18:1 acids. As a result, the GLC profiles of these four isolates closely resembled that of *P. cepacia*, a strongly oxidative pseudomonad. However, the presence of 2- and 3-OH 16:0 acids and the ability of *P. capacia* to produce acid in conventional carbohydrate tests was sufficient to distinguish it from the four strains of *Alcaligenes*.

TABLE 1. *Cellular fatty acid composition of Alcaligenes and Pseudomonas species isolated from clinical specimens*

Strain	Straight-chain acids									Hydroxy acids					Cyclo-propane acid	Branched-chain acids		
	12:0[a]	14:0	15:0	16:1	16:0	17:1	17:0	18:1	18:0	3-OH 10:0	2-OH 12:0	3-OH 12:0	2-OH 14:0	3-OH 14:0	17:0	i-13:0	i-15:0	i-17:0
Alcaligenes faecalis																		
C8194	T[b]	8	T	11	27	—	—	11	6	—	5	—	T	9	23	—	—	—
C7859	T	4	T	5	30	—	—	9	6	—	4	—	5	9	28	—	—	—
B5104	2	4	T	14	24	—	—	12	4	—	4	—	T	12	24	—	—	—
B5037	T	7	T	15	25	—	—	14	4	—	3	—	4	7	21	—	—	—
B5069	2	5	T	12	24	—	—	11	5	—	4	—	T	12	25	—	—	—
B2608	T	6	T	16	31	—	—	9	4	—	4	—	T	7	23	—	—	—
B6353	T	6	T	17	22	—	—	16	6	—	4	—	4	7	18	—	—	—
B6288	2	8	T	18	25	—	—	11	5	—	5	—	T	9	17	—	—	—
B4636	—	4	T	24	24	—	—	32	4	—	—	—	T	8	4	—	—	—
B2566	—	6	T	24	25	—	—	24	5	—	—	—	T	11	5	—	—	—
C1549	—	8	T	23	24	—	—	22	6	—	—	—	T	10	7	—	—	—
A. denitrificans																		
KC367	T	7	T	14	27	—	—	7	3	—	8	—	T	12	22	—	—	—
B7042	—	5	T	26	28	—	—	20	3	—	—	—	T	7	11	—	—	—
A. odorans																		
C8486	3	3	T	7	28	—	—	10	6	—	3	—	T	12	28	—	—	—
C8351	2	3	2	8	28	—	—	14	5	—	4	—	T	8	26	—	—	—
Pseudomonas alcaligenes																		
RYS 142	10	4	T	20	21	—	T	20	3	10	—	5	—	—	5	—	—	2
A7708	12	2	T	20	22	—	T	24	3	5	—	4	—	—	6	—	—	2
P. pseudoalcaligenes																		
KC945	14	T	T	20	20	—	T	27	T	9	—	2	—	—	6	—	—	2
B1373	14	T	T	20	24	—	T	22	T	9	—	2	—	—	4	—	—	5
A9863	12	T	T	23	20	—	T	26	3	5	—	2	—	—	2	—	—	7
A2985	11	T	T	21	23	—	T	30	3	5	—	2	—	—	3	—	—	2
P. putrefaciens																		
KC988	3	4	9	21	12	15	4	4	T	—	—	—	—	—	—	7	21	—
B7000	4	3	8	18	11	17	4	6	2	—	—	—	—	—	—	6	21	—
B7120	2	5	9	16	11	19	7	7	2	—	—	—	—	—	—	5	17	—
B7128	3	7	7	18	11	16	6	6	T	—	—	—	—	—	—	7	19	—

[a] Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; 2-OH and 3-OH refer to hydroxy acid; i, iso acid.

[b] Number refers to percentage of total acids; T, less than 2%; —, not detected.

A major point of these findings is that each strain of *Alcaligenes* was easily differentiated from the weakly oxidative and nonoxidative pseudomonads which are similar to *Alcaligenes* in conventional tests. In addition to the complete absence of 3-OH 14:0 acid in *P. alcaligenes* (two strains) and *P. pseudoalcaligenes* (four strains), these strains differed from *Alcaligenes* in that they contained approximately 10% each of 12:0 and 3-OH 10:0 acids and small amounts of 3-OH 12:0 and i-17:0 acids. The similarity of the cellular fatty acids of *P. alcaligenes* and *P. pseudoalcaligenes* is consistent with their close taxonomic relationship established by deoxyribonucleic acid (12) and ribonucleic acid (13) homology tests and by substrate utilization (18). The four strains of *P. putrefaciens* were obviously distinct from other pseudomonads and were unique in that they contained a 17:1 acid (mean, 17%). The presence of 17:1, i-13:0, and i-15:0 and the absence of hydroxy and 17 Δ acids clearly distinguish *P. putrefaciens* from *Alcaligenes*.

The difficulty encountered in the identification of nonoxidative species of *Alcaligenes* and *Pseudomonas* is a well-recognized problem in the diagnostic laboratory (2, 7, 14). Although a large number of biochemical tests are routinely used to delineate these organisms, the ambiguous results obtained in conventional tests often cause errors in speciation (2). Numerous reports dealing with the extensive testing of *Alcaligenes* and *Pseudomonas* species are available in the literature (3, 4, 7, 14, 15, 18). In Table 2 we have listed five diagnostic tests which, from our review of the literature, appear to be major criteria for differentiation of these species. These five tests have been recommended for the minimal characterization of the species included in Table 2 (6). It is obvious from the data presented in the table that the reactions of *A. faecalis* and the three *Pseudomonas* species in these diagnostic tests are not sufficient for adequate identification. The tests which appear to be reliable are the flagellar morphology of *A. faecalis* and the production of ornithine decarboxylase by *P. putrefaciens*. However, it should be noted that the demonstration of flagella in many strains of *A. faecalis* is difficult because of poor growth or improper staining technique. In contrast to the ambiguous reactions observed in conventional tests, the data show that *A. faecalis* and each of the *Pseudomonas* species listed in Table 2 can be readily distinguished by characteristic cellular fatty acids. Some of the fatty acids (3-OH 10:0, 3-OH 14:0, i-13:0, i-15:0, 17:1) represent qualitative differences; others (17 Δ, 16:0, 18:1) reflect large quantitative differences between species. Although all strains within a species gave similar fatty acid profiles, a number of additional isolates of each species must be tested to more thoroughly evaluate strain variability. However, the data presented

TABLE 2. *Identification of Alcaligenes faecalis and Pseudomonas species by conventional diagnostic tests and by cellular fatty acids*

Determinants	*A. faecalis*	*P. pseudo-alcaligenes*	*P. putrefaciens*	*P. diminuta*
Conventional test				
Flagella	Peritrichous	Polar, 1-2	Polar, 1-2	Polar, short wavelength amplitude
Oxidase	+[a]	+	+	+
Acid, glucose OF[b] medium	−	−	+ or −	+ or −
Nitrate reduction	+ or −	+ or −	+	−
Ornithine decarboxylase	−	−	+	−
Characteristic fatty acids				
Hydroxy	3-OH 14:0[c]	3-OH 10:0 3-OH 12:0	−	3-OH 12:0
Cyclopropane	17:0	17:0	−	19:0
Branched-chain	−	i-17:0	i-13:0, i-15:0	−
Straight-chain	14:0, 16:1, 16:0, 18:1, 18:0	12:0, 16:1, 16:0, 18:1	12:0, 14:0, 15:0, 16:1, 16:0, 17:1, 17:0, 18:1	16:0, 18:1

[a] +, Positive; −, negative.
[b] OF, Oxidative-fermentative.
[c] Number to the left of the colon refers to number of carbon atoms; number to the right refers to number of double bonds; -OH refers to hydroxy acid; i, iso acid.

in this report indicate that cellular fatty acid analysis is a useful criterion for the identification of *Alcaligenes* and *Pseudomonas* species.

The GLC procedure is both rapid and reproducible. The test is relatively simple to perform and is recommended for use in laboratories that handle large numbers of isolates. In conjunction with selected conventional tests, the determination of cellular fatty acids serves as a practical screening test for examining a variety of strains from clinical specimens.

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