JOURNAL OF CLINICAL MICROBIOLOGY, July 1978, p. 61-66
0095-1137/78/0008-0061$02.00/0

Vol. 8, No. 1
Printed in U.S.A.

Identification of *Achromobacter* Species by Cellular Fatty Acids and by Production of Keto Acids

SALLY B. DEES* AND C. WAYNE MOSS
Center for Disease Control, Atlanta, Georgia 30333

Received for publication 22 May 1978

The cellular fatty acid composition and metabolic products of 12 reference strains of *Achromobacter* sp. and *A. xylosoxidans* were determined by gas-liquid chromatography (GLC). Results showed that the two *Achromobacter* groups are strikingly different and can be readily distinguished on the basis of cellular fatty acids and the short-chain acids produced by *Achromobacter* sp. The major cellular fatty acids of *Achromobacter* sp. were octadecenoic (18:1) and a 19-carbon cyclopropanoic (19:0 Δ) acid, whereas hexadecanoic (16:0) and a 17-carbon cyclopropanoic (17:0 Δ) acid were principal components of the lipids of *A. xylosoxidans*. Hydroxy acids were not found in strains of *Achromobacter* sp. but comprised approximately 20% of the cellular fatty acids of *A. xylosoxidans*. In addition, *Achromobacter* sp. produced relatively large amounts of 2-ketoisocaproic acid, which was detected in only trace amounts from strains of *A. xylosoxidans*. The data show that GLC tests provide additional criteria for differentiating groups which are very closely related when evaluated with conventional tests. The GLC tests can be readily adapted in the clinical laboratory because they are rapid, highly reproducible, relatively inexpensive, and simple to perform.

A detailed description of *Achromobacter xylosoxidans* sp. nov. was published in 1974 (17). Fifty-six strains, including the type strain, ATCC 27061, were uniform in flagellar morphology and guanine-plus-cytosine base composition and demonstrated certain physiological characteristics in several conventional biochemical tests. Five strains had identical cellular fatty acids. These strains and 19 additional isolates (17), which were formerly classified as King's groups IIIa and IIIb, *Alcaligenes faecalis, Alcaligenes denitrificans,* or *Alcaligenes* sp., all fit the criteria defining *A. xylosoxidans*.

A group closely related to *A. xylosoxidans* but not included in the above-mentioned study is *Achromobacter* sp., strains of which have the general characteristics of the genus *Achromobacter* (6) and which at present remain unclassified at the species level. Tatum et al. divided the group into two biotypes which differed only in the number of carbohydrates dissimilated (14). Because of the close similarities between *A. xylosoxidans* and *Achromobacter* sp. in conventional tests and the absence of information on the lipids of clinically isolated strains of *Achromobacter* sp., we decided to compare their cellular fatty acid composition and to determine whether the two groups could be further characterized on the basis of metabolic end products. Using the techniques of gas-liquid chromatography (GLC) and GLC-mass spectrometry, we examined isolates classified as *Achromobacter* sp. and compared them with documented cultures of *A. xylosoxidans*. The results show that *Achromobacter* sp. is a homogeneous group markedly different from *A. xylosoxidans* both in cellular fatty composition and in the short-chain acids produced in common growth media. Application of the GLC tests to the routine identification and taxonomy of *Achromobacter* and related bacteria is discussed.

MATERIALS AND METHODS

Cultures. Seven strains of *Achromobacter* sp., biotypes 1 and 2, and five strains of *A. xylosoxidans* were obtained from the Special Microbiology Section, Center for Disease Control (CDC) and are listed in Table 1 (15, 16) by their CDC accession numbers and by other designations. Procedures used to characterize the isolates were previously published (14). The Japanese strain, KM 543, has been designated the type strain (ATCC 27061) of *A. xylosoxidans* and was the source of KC 1064 (CDC). The remaining four strains (Table 1) were originally part of King's groups IIIa and IIIb (15). King's group Vd, now designated *Achromobacter* sp., contains two biotypes which, according to Tatum et al., are distinguished by the fact that type 2 strains oxidize mannitol, sucrose, and maltose (14).

Growth conditions and derivative formation. Bacteria were processed according to previously published procedures (3). After they were incubated for 24 h on Trypticase soy agar, cells were collected from the plates and placed in test tubes with Teflon-lined

TABLE 1. *Strains of Achromobacter analyzed for cellular fatty acids and metabolic products*

Species	CDC[a] strain	Other designations
Achromobacter xylosoxidans	KC1064	KM543, Yabuuchi and Ohyama (16), ATCC[b] 27061
	D8432	King group IIIa (15)
	D7818	King group IIIa (15)
	D9448	King group IIIb
	D9221	King group IIIb
Achromobacter sp.	C349	King group Vd, type 1 (15)
	C7259	King group Vd, type 1 (15)
	A9112	King group Vd, type 1 (15)
	C675	King group Vd, type 1 (15)
	D8708	King group Vd, type 2
	D9035	King group Vd, type 2
	D9053	King group Vd, type 2

[a] CDC, Center for Disease Control.
[b] ATCC, American Type Culture Collection.

caps. Each step of the procedure—saponification of cells, methylation of free fatty acids, and extraction of esterified fatty acids—was carried out in one test tube (18 by 150 mm). Procedures for extracting and derivatizing short-chain acids obtained from Trypticase soy agar plates after cells are removed were outlined in a previous publication (11). One microliter of the final sample (methyl esters of long-chain fatty acids and butyl esters of short-chain fatty acids) was injected into a gas chromatograph for analysis.

GLC. Using the analytical conditions described in an earlier publication (9), fatty acid methyl esters ranging from 10 to 20 carbons long were determined within 25 min. Analysis time for short-chain acid butyl esters was 30 min. Within this time, the butyl esters of all reference standards including phenylacetic, phenylpyruvic, and glutaric acids eluted. Both long- and short-chain fatty acids were identified by comparing the retention times of the esterified acids in bacterial samples with those of pure standards (Applied Science, Chemical Services, Eastman Organic Chemicals, National Institutes of Health). Quantitative data were obtained on the cellular fatty acids by measuring peak areas with the disk integrator. 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 used in the calculations were determined for each acid. Both short-chain and cellular fatty-acid esters were positively identified with the results of a combination of techniques: hydrogenation (8), acetylation (12), and GLC-mass spectrometry (8, 12). Mass spectra of methyl and butyl esters were obtained with a DuPont instrument type 21-491B equipped with a combination electron impact-chemical ionization source. Isobutane was used as the reagent gas for chemical ionization. The esters were separated on a 3% OV-101 (methyl silicone) column.

RESULTS AND DISCUSSION

The cellular fatty acids of seven isolates of *Achromobacter* sp., including representatives of biotypes 1 and 2, were essentially identical but differed markedly from those present in the type and reference cultures of *A. xylosoxidans*. These acids (Fig. 1) ranged in length from 16 to 20 carbon atoms. The most abundant fatty acids were octadecenoic (18:1) acid and a 19-carbon cyclopropane (19:0 Δ) acid. Less prominent peaks (Fig. 1) were identified as hexadecenoic (16:1), hexadecanoic (16:0), 17-carbon cyclopropanoic (17:0 Δ), octadecanoic (18:0), and eicosanoic (20:0) acids. No hydroxy or branched-chain acids were detected.

Fatty acid analysis of five cultures of *A. xylosoxidans* showed that type and reference strains were essentially identical. Figure 2 contains a typical profile showing the cellular fatty acids of the type strain, KC 1064. In contrast to *Achromobacter* sp., each strain of *A. xylosoxidans* contained moderate amounts (17%) of hydroxy acids. The methyl esters of these acids, present at retention times of 12.0, 15.4, and 15.8 min in the chromatogram (Fig. 2), were identified as 2-hydroxydodecanoate (2-OH 12:0), 2-hydroxytetradecanoate (2-OH 14:0), and 3-hydroxytetradecanoate (3-OH 14:0), respectively. Only trace amounts of 2-hydroxyhexadecanoate (2-OH 16:0), at retention time of 18.8 min, were detected in each strain. In addition to the presence of hydroxy acids, *A. xylosoxidans* differed from *Achromobacter* sp. (Fig. 1) in that the former contained 17:0 Δ and 16:0 fatty acids in major amounts (approximately 50%). Small to trace amounts (<10%) of tetradecanoic (14:0), 16:1, 18:1, 18:0, and 19:0 Δ fatty acids were also characteristic of *A. xylosoxidans*.

The fatty acid composition of saponified whole cells determined by our methods was similar to that reported for *A. xylosoxidans* by Yabuuchi et al. (17). These workers found that the extractable lipids of the type strain, ATCC 27061, and four additional isolates contained 16:0, 16:1, 18:0, 18:1, and 17:0 Δ fatty acids. Three of the four hydroxy acids (2-OH 12:0, 2- and 3-OH 14:0) which we detected in whole cells of these organisms were also found in the bound fatty acid fraction of the strains tested by Yabuuchi et al. (17).

Relative amounts of the fatty acids comprising strains of each *Achromobacter* group are presented in Table 2. Since repeated testings of each strain gave essentially identical results, the percentages given in the table represent averaged values obtained from three separate analyses processed through the entire procedure (growth, saponification, sample preparation, GLC). Obviously, the different ratios of the 17:0 and 19:0 Δ acids provide a means for readily differentiating *Achromobacter* sp. and *A. xylo-*

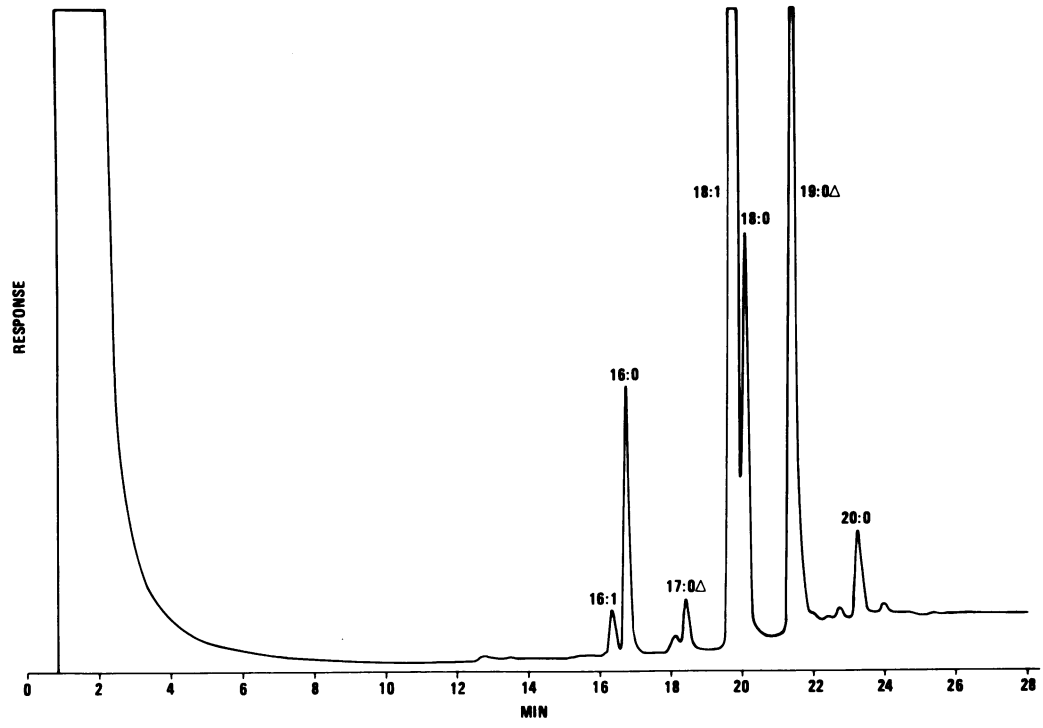

FIG. 1. *Gas chromatogram of esterified fatty acids from saponified whole cells of Achromobacter sp., D8708. Analysis was made on a 3% OV-1 column.*

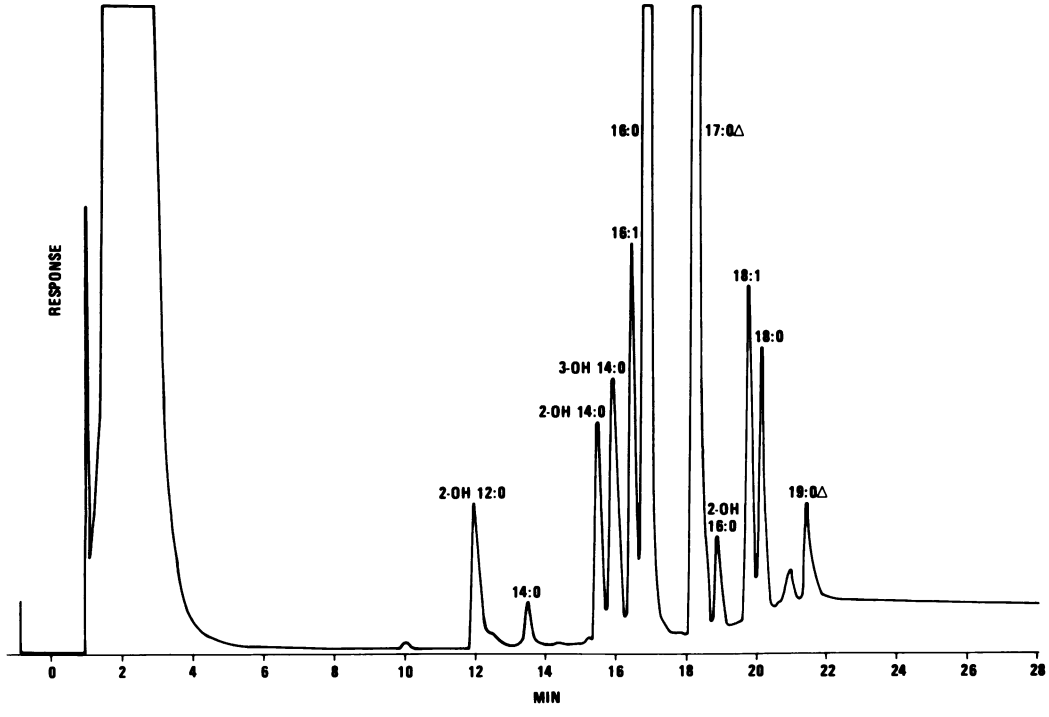

FIG. 2. *Gas chromatogram of esterified fatty acids from saponified whole cells of A. xylosoxidans, KC1064. Analysis was made on a 3% OV-1 column.*

TABLE 2. *Cellular fatty acid composition of A. xylosoxidans and Achromobacter species*

Species	Straight-chain acids									Hydroxy acids				Cyclopropane acids	
	14:0[a]	15:0	16:1	16:0	17:0	18:1	18:0	19:0	20:0	2-OH 12:0	2-OH 14:0	3-OH 14:0	2-OH 16:0	17:0	19:0
Achromobacter sp.															
Biotype 1															
C349	—	—	T[b]	9	3	31	12	T	T	—	—	—	—	T	45
C7259	—	—	2	9	6	32	16	2	T	—	—	—	—	3	31
A9112	—	—	8	18	3	32	12	T	T	—	—	—	—	T	27
C675	—	—	4	12	T	32	16	T	T	—	—	—	—	T	36
Biotype 2															
D8708	—	—	5	16	3	32	14	T	T	—	—	—	—	T	30
D9035	—	—	2	12	4	33	14	T	T	—	—	—	—	2	33
D9053	—	—	3	13	7	29	13	T	T	—	—	—	—	T	35
Mean percentage	Straight-chain: 65										Hydroxy: 0			Cyclopropane: 35	
A. xylosoxidans															
KC1064	T	T	8	33	T	3	3	T	—	3	5	7	2	33	3
D7818	2	T	6	26	T	4	5	T	—	4	6	6	3	33	5
D9221	4	T	11	35	T	4	2	T	—	4	4	5	T	31	T
D8432	T	2	12	21	T	12	6	T	—	5	7	6	2	22	5
D9448	T	T	10	28	T	7	6	T	—	3	5	5	3	30	3
Mean percentage	Straight-chain: 50										Hydroxy: 17			Cyclopropane: 33	

soxidans (Table 2). The ratio of 19:0 Δ to 17:0 Δ is 10:1 or greater in strains of *Achromobacter* sp., whereas in *A. xylosoxidans* this ratio is approximately 1:10. The cyclopropane acids represent approximately 35% of the total cellular fatty acids of isolates from each group. A second distinguishing feature of the two groups is that strains of *Achromobacter* sp. contain relatively large amounts (32%) of 18:1, whereas approximately 30% of the fatty acids of *A. xylosoxidans* is 16:0. The combination of these acids and the cyclopropane fatty acids accounts for greater than 50% of all fatty acids in each group. The presence of hydroxy acids (approximately 17%) in strains of *A. xylosoxidans* constitutes a third major difference between the two groups. Moreover, fatty acids less than 16 carbons long were not found in *Achromobacter* sp., a fact which in itself is sufficient to differentiate *Achromobacter* sp. from closely associated species such as *Pseudomonas* (9) and *Alcaligenes* (3). The relatively large amounts of 17:0 Δ distinguish *A. xylosoxidans* from *Pseudomonas* but not from *Alcaligenes* (3). Although *A. xylosoxidans* is metabolically more active in many conventional tests than *Alcaligenes* sp. (14), these two groups of organisms are quite similar in cellular fatty acid composition (17). Some workers have pointed out similarities among other species of *Achromobacter* and *Alcaligenes* and have recommended that the name *Achromobacter* be abolished and that certain species of this group be reassigned to the genus *Alcaligenes* (4). However, on the basis of the amended description of *Alcaligenes* (4), both *Achromobacter* sp. and *A. xylosoxidans* would be eliminated. The results of this study (4) show that conventional tests have not been adequate in delineating many bacterial groups and that more information such as the GLC data described in this report are needed in order to define taxonomic relationships more accurately.

In addition to analyzing cells for fatty acids, the spent growth medium was also tested for short-chain acid products with a rapid GLC procedure which we developed (7). These compounds, although often present in small amounts, provide valuable information for distinguishing closely related bacterial species (1, 8, 10, 11). Shown in Fig. 3 is a representative chromatogram of the short-chain acid products from strains of *Achromobacter* sp. Peaks labeled 1, 2, 3, and 4 were identified as propionic (C_3) isobutyric (iC_4), isovaleric (iC_5), and isocaproic (iC_6) acids, respectively. On the basis of retention time, the major peak in the chromatogram was thought to be isoheptanoic (iC_7) acid. However, the mass spectrum of a reference standard of this acid was different from that obtained from the bacterial culture. The mass spectrum of peak 4 from the culture also differed from that of the isocaproic acid standard. The mass spectra of peaks 1, 2, and 3 were identical to reference standards of C_3, iC_4, and iC_5 acids, respectively. Close examination of the mass spectra of peak 4 and of the major peak at the retention time of 17 min suggested a possible keto functional group. When the butyl ester sample was treated

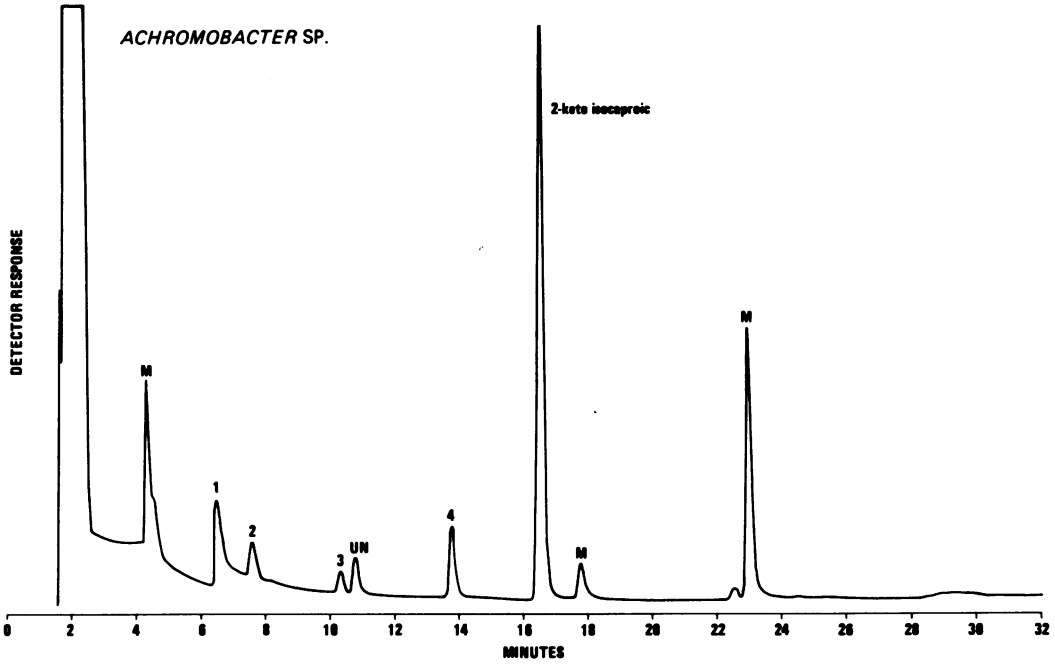

FIG. 3. *Gas chromatogram of esterified short-chain acids produced by Achromobacter sp. Analysis was made on a 15% Dexsil column.*

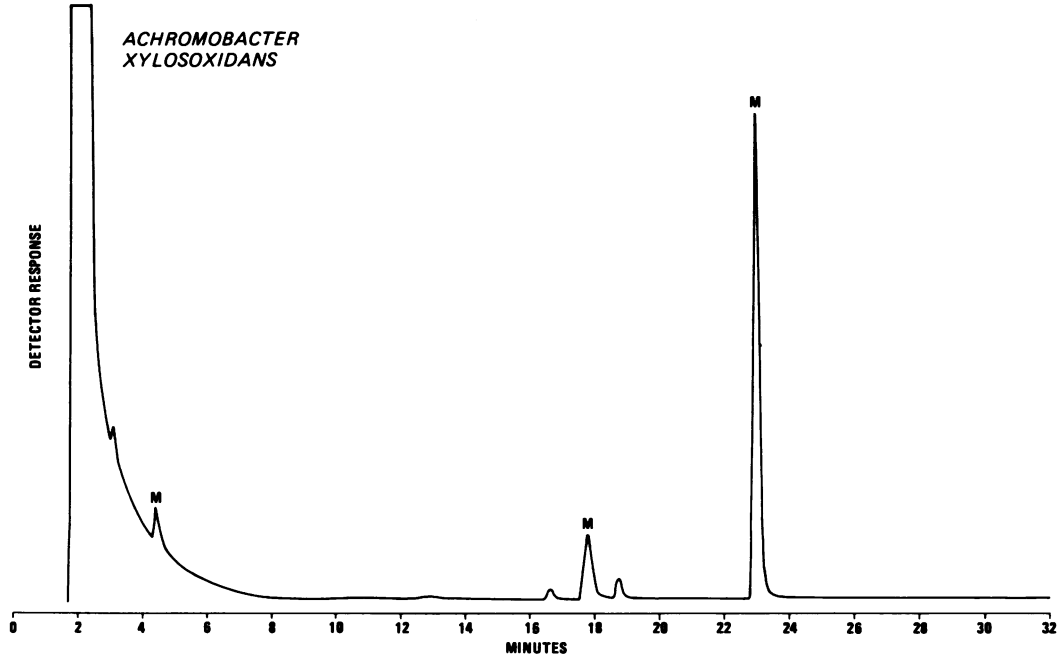

FIG. 4. *Gas chromatogram of esterified short-chain acids produced by A. xylosoxidans. Analysis was made on a 15% Dexsil column.*

with 2,4-dinitrophenylhydrazine, both peak 4 and the 17-min peak disappeared, indicating a carbonyl functional group (14). Subsequent analysis with GLC and GLC-mass spectrometry of reference standards of various keto acids (as butyl esters) established the identity of the acid represented by peak 4 as 2-ketoisovaleric and that of the 17-min peak as 2-ketoisocaproic. GLC retention time and mass spectral data for these two reference acids were identical in all respects to the peaks produced by strains of *Achromobacter* sp. The peak labeled "UN" in Fig. 3 was also removed when the sample was treated with 2,4-dinitrophenylhydrazine, but its identity has not been firmly established. Normal straight-chain acids (C_3, iC_4, iC_5) were not affected by treatment with 2,4-dinitrophenylhydrazine. In contrast to *Achromobacter* sp., strains of *A. xylosoxidans* produced only trace amounts of acids (Fig. 4). Peaks labeled "M" in both chromatograms (Fig. 3 and 4) are media components, whose identification was described in an earlier report (2).

In summary, our data show that two groups which conform to the minimal characteristics of the genus *Achromobacter* (6) can be clearly distinguished from each other and from related bacteria (*Pseudomonas, Alcaligenes*) by differences in their cellular fatty acids and by the short-chain acids produced in Trypticase soy agar medium. The information gained from GLC analysis of these organisms supports the conclusions of Tatum et al., who separated the two groups of *Achromobacter* mainly on the basis of urease production (14). Moreover, our data show that the cellular fatty acid composition of *Achromobacter* sp. is different from that of other gram-negative species. The consistent similarities in their cellular fatty acids among these strains as demonstrated by the GLC procedures strongly suggest that they should be classified under a single, specific epithet. Additional isolates of *Achromobacter* sp. will be tested to confirm our preliminary results. Fatty acid data are also being collected on other gram-negative nonfermenters (agrobacteria) and questionable fermenters (flavobacteria) and will be published at a later date. From the standpoint of increased reproducibility and speed, we recommend that the GLC tests described in this report be used in combination with a minimal number of selected conventional tests (9), thus providing hospital and clinical laboratories with a more accurate and rapid method for identifying new isolates and offering a new approach to the clarification of taxa of "uncertain affiliation" (5).

LITERATURE CITED

1. **Brooks, J. B., R. E. Weaver, H. W. Tatum, and S. A. Billingsley.** 1972. Differentiation between *Pseudomonas testosteroni* and *P. acidovorans* by gas chromatography. Can. J. Microbiol. **18**:1477–1482.
2. **Choudhary, G., and C. W. Moss.** 1976. Gas chromatography-mass spectrometry of some biologically important short chain acid butyl esters. J. Chromatogr. **128**:261–270.
3. **Dees, S. B., and C. W. Moss.** 1975. Cellular fatty acids of *Alcaligenes* and *Pseudomonas* species isolated from clinical specimens. J. Clin. Microbiol. **1**:414–419.
4. **Hendrie, M. S., A. J. Holding, and J. M. Shewan.** 1974. Emended descriptions of the genus *Alcaligenes* and of *Alcaligenes faecalis* and proposal that the generic name *Achromobacter* be rejected: status of the named species of *Alcaligenes* and *Achromobacter*. Int. J. Syst. Bacteriol. **24**:534–550.
5. **Holding, A. J., and J. M. Shewan.** 1974. Genus *Alcaligenes* Castellani and Chalmers 1919, 936, p. 273–275. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology. The Williams and Wilkins Co., Baltimore.
6. **Hugh, R.** 1970. A practical approach to the identification of certain nonfermentative gram-negative rods encountered in clinical specimens. J. Conf. Public Health Lab. Directors **28**:168–187.
7. **Moss, C. W.** 1975. Gas-liquid chromatography as an analytical tool in microbiology. Public Health Lab. **33**:81–103.
8. **Moss, C. W., and S. B. Dees.** 1975. Identification of microorganisms by gas chromatographic-mass spectrometric analysis of cellular fatty acids. J. Chromatogr. **112**:595–604.
9. **Moss, C. W., and S. B. Dees.** 1976. Cellular fatty acids and metabolic products of *Pseudomonas* species obtained from clinical specimens. J. Clin. Microbiol. **4**:492–502.
10. **Moss, C. W., and C. M. Kaltenbach.** 1974. Production of glutaric acid: a useful criterion for differentiating *Pseudomonas diminuta* from *Pseudomonas vesicularis*. Appl. Microbiol. **27**:437–439.
11. **Moss, C. W., and S. B. Samuels.** 1974. Short-chain acids of *Pseudomonas* species encountered in clinical specimens. Appl. Microbiol. **27**:570–574.
12. **Moss, C. W., S. B. Samuels, J. Liddle, and R. M. McKinney.** 1973. Occurrence of branched-chain hydroxy acids in *Pseudomonas maltophilia*. J. Bacteriol. **114**:1018–1024.
13. **Shriner, R. L., R. C. Fuson, and D. Y. Curtin (ed.).** 1956. The systematic identification of organic compounds, 4th ed., p. 111–112. John Wiley and Sons, Inc., New York.
14. **Tatum, H. W., W. H. Ewing, and R. E. Weaver.** 1974. Miscellaneous gram-negative bacteria, p. 270–294. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
15. **Weaver, R. E., H. W. Tatum, and D. G. Hollis.** 1972. The identification of unusual pathogenic gram-negative bacteria (Elizabeth O. King). Center for Disease Control, Atlanta, Ga.
16. **Yabuuchi, E., and A. Ohyama.** 1971. *Achromobacter xylosoxidans* n. sp. from human ear discharge. Jpn. J. Microbiol. **15**:477–480.
17. **Yabuuchi, E., I. Yano, S. Goto, E. Tanimura, T. Ito, and A. Ohyama.** 1974. Description of *Achromobacter xylosoxidans* Yabuuchi and Ohyama 1971. Int. J. Syst. Bacteriol. **24**:470–477.