Journal of Chromatography, 203 (1981) 337–347
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,152

GAS–LIQUID CHROMATOGRAPHY AS AN ANALYTICAL TOOL IN MICROBIOLOGY

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SUMMARY

The use of gas–liquid chromatography (GLC) in clinical and diagnostic bacteriology laboratories has increased significantly in recent years. GLC analysis of metabolic products from bacterial growth and chemical components of bacterial cells has provided useful information for rapid detection and identification of several bacterial groups or species. The use of short-chain acid products and cellular fatty acid composition for identifying and classifying *Pseudomonas* species and other medically important gram-negative non-fermentative bacteria is illustrated. Application of the flexible, fused, silica-glass, capillary column for increased resolution of bacterial fatty acids is also discussed.

INTRODUCTION

In most diagnostic laboratories the identification and classification of microorganisms is based on their biochemical, morphological, serological, and toxigenic characteristics. It is often difficult, however, to assign some organisms to their proper group on the basis of these properties alone. Moreover, many of the conventional tests and procedures used are time-consuming or require extended periods of incubation (7–14 days) before final identification.

In a search for more rapid and specific methods for identification, several workers have used gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and combined GLC–mass spectrometry (MS) to determine the chemical composition and metabolic activity of microorganisms as a basis for their classification[1–6]. In our laboratory, we have used these sensitive tools to study the chemical composition and metabolism of a number of medically important bacteria[7–10]. In recent investigations, we have determined the cellular fatty acid composition and short-chain acid products from various bacterial genera, including *Pseudomonas*, *Flavobacterium*, *Achromobacter* and *Legionella*. Data from these studies show that both short-chain acids and total cellular fatty acids provide useful information for rapidly distinguishing between closely related bacterial species.

EXPERIMENTAL*

Bacterial cultures

Reference or type strains and clinical isolates were included in the study. The reference strains were from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.); the clinical isolates originated from different geographical locations throughout the world and were cultured from a variety of clinical materials. Isolates of *Legionella* were cultured from human lung tissue, pleural fluid, and sputum and from a variety of environmental sources including water from streams, lakes and cooling towers. All cultures were identified by the conventional cultural and biochemical testing procedures of the Clinical Bacteriology Branch, Center for Disease Control. *Legionella* were also identified by the direct fluorescent antibody (FA) technique by using highly specific conjugates[11,12].

Cultures conditions and derivative formation

The *pseudomonads*, *Flavobacteria* and *Achromobacter* were grown on trypticase soy agar (TSA; Baltimore Biological Laboratory, Baltimore, MD, U.S.A.) plates at 37°C for 24 h. The *Legionella* were grown on charcoal–yeast extract (CYE) agar plates for 24–72 h at 37°C. The cell mass from the surface of one plate was carefully removed with sterile distilled water and transferred to an 18 mm or 20 mm × 150 mm test tube containing 5 ml of 5% sodium hydroxide in 50% aqueous methanol. The tubes were sealed with PTFE-lined caps, and the cellular lipids were saponified for 30 min at 100°C. The tubes were removed from the bath and cooled to room temperature; the pH of the saponified material was adjusted to approximately pH 2.0 with 6 *N* hydrochloric acid. The methyl esters of the free fatty acids were formed by adding 4–5 ml of 10% (w/v) boron trichloride–methanol reagent (Applied Science Labs., State College, PA, U.S.A.) and by heating the mixture at 80–85°C. The fatty acid methyl esters were then extracted from the cooled mixture by inverting the tube ten times and shaking the sample for about 10 sec with a mixture of diethyl ether–hexane (1:1). A second extraction with 10 ml of solvent removed essentially all of the methyl esters. The solvent layers were combined in a 50-ml beaker and evaporated to a volume of 0.2–0.5 ml under a gentle stream of nitrogen. A small amount (80–100 mg) of sodium sulfate was added to remove traces of moisture, and the methyl esters were transferred to a 13 × 100-mm screw-capped test tube. The sample was analyzed immediately by GLC or stored at −20°C until analysis could be completed.

For analysis of short-chain acids, approximately 1 ml of 50% (v/v) sulfuric acid was added to the surface of the plate after the removal of cells and was allowed to stand 10 min. The agar was then cut into small pieces, transferred to a test tube with a PTFE cap, and heated for 30 min at 100°C. After the melted agar was cooled to room temperature, the acids were extracted with diethyl ether, concentrated, and converted to butyl esters and/or trifluoroacetyl–butyl esters[13]. An uninoculated plate of TSA which was processed in a manner identical to that used for cultures served as the control.

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* 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 and Human Services.

Gas–liquid chromatography

Short-chain acid products were determined by GLC with a flame ionization detector (FID) and a 3.66 m × 4.03 mm I.D. coiled glass column packed with either 5% OV-101 stationary phase (Applied Science Labs.) or 15% Dexsil 300 GC coated on 80–100 mesh Chromosorb W (Analabs, North Haven, CT, U.S.A.). Details of the instrument settings, conditions of temperature programming, and separating efficiency of the Dexsil column has been published previously[13].

For cellular fatty acids, the methyl esters were analyzed on both packed and capillary columns. The packed column (3.66 m × 4.03 mm I.D.) was placed in a Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a FID and a DISC integrator recorder. The column was packed with 3% OV-1 methyl silicone which was coated on 80–100 mesh, Chromosorb W HP (Applied Science Labs.). Helium was used as carrier gas at a flow-rate of 60 ml/min. The initial column temperature was 160°C and after injection of sample (2–3 μl), it was programmed to 265°C at a rate of 5°C/min. A 50 m × 0.2 mm I.D. fused silica capillary column with SP 2100 as stationary phase was purchased from Hewlett-Packard (Avondale, PA, U.S.A.) and installed in a Varian model 3700 gas chromatograph equipped with an all-glass capillary system (Varian, Palo Alto, CA, U.S.A.). The column was conditioned for 2 days at temperatures from 120°C to a maximum of 260°C and then connected to a glass-lined FID. The injector temperature was maintained at 250°C and the detector temperature at 300°C. Helium was used as carrier gas at a flow-rate of 1.0 ml/min. The sample size was 0.5 μl which was split 50:1 to give 0.01 μl of sample on the column. For analysis of samples, the column temperature was set at 195°C for 5 min and then temperature programmed at 7°C/min to a final temperature of 250°C.

Mass spectrometry

Combined GLC–MS studies were done with a Model 21-491 B instrument (DuPont, Wilmington, DE, U.S.A.) which was equipped for both electron impact and chemical ionization. The reagent gas for chemical ionization was isobutane. The mass spectrometer was coupled to a Varian Model 2700 gas chromatograph through an all-glass system with a jet separator. The 2700 chromatograph contained a glass column (2 m × 2 mm I.D.) packed with 3% OV-101 which was used for separating both butyl and methyl esters for MS studies. In addition to mass spectrometry, both short-chain and cellular fatty acid esters were further identified by a combination of techniques, including retention time comparison to highly purified standards, hydrogenation of unsaturated acids, bromination, and trifluoroacetylation of hydroxy acids[14–16].

RESULTS AND DISCUSSION

Short-chain acids

For several years, our laboratory has examined the short-chain acid products from more than 300 isolates of *pseudomonads* and related bacteria. A number of these were well-characterized reference strains, but most were isolates from clinical sources which were also thoroughly characterized by conventional cultural and biochemical tests[17]. Many of the *Pseudomonas* species including *P. acidovorans*, *P. aeruginosa*, *P.*

TABLE I

SHORT-CHAIN ACIDS PRODUCED BY *PSEUDOMONAS* AND RELATED NON-FERMENTATIVE BACTERIA

Numbers in parentheses indicate number of strains. Numbers refer to relative areas of peaks: T, <10% full-scale deflection; 1, 10–39%; 2, 40–69%; 3, 70–90%; 4, peak with full-scale deflection or greater; 8, peak with twice the area of 4; 12, peak with three times the area of 4; —, acid not detected.

Organism	*Acid**								
	C_2	*C_3*	*iC_4*	*Pyruvic*	*iC_5*	*2-Keto-iC_5*	*2-Keto-iC_6*	*Phenylacetic*	*Glutaric*
Pseudomonas									
testosteroni (9)	2	—	—	—	—	—	—	4	—
alcaligenes (5)	1	—	—	—	—	—	—	1	—
pseudoalcaligenes (8)	1	—	4	—	12	—	—	1	—
maltophilia (17)	8	—	—	—	4	—	—	1	—
diminuta (12)	12	12	12	—	12	1	8	1	4
vesicularis (12)	12	12	12	—	12	1	8	T	—
Achromobacter									
unclassified species (10)	8	2	1	T	1	1	8	1	—
xylosoxidans (10)	T	—	—	—	—	—	—	—	—
Flavobacterium									
meningosepticum (6)	T	T	1	2	3	8	12	T	—
Uninoculated TSA** medium	12	—	—	—	—	—	—	—	—

* Acids: C_2 = acetic; C_3 = propionic; iC_4 = isobutyric; iC_5 = isovaleric or 2-methylbutyric; 2-keto-iC_5 = 2-keto-isovaleric; 2-keto-iC_6 = 2-keto-isocaproic.

** TSA = trypticase soy agar.

cepacia, *P. fluorescens*, *P. mendocina*, *P. pickettii*, *P. pseudomallei*, *P. putida*, *P. putrefaciens* and *P. stutzeri* produced no short-chain acids or only trace amounts. However, other species produced one or more acids which served as useful markers for distinguishing among the species. The data in Table I shows that only phenylacetic acid was produced by *P. alcaligenes* and *P. testosteroni*. However, these two species could be distinguished because all strains of *P. testosteroni* produced relatively large amounts of this acid compared to only small amounts for *P. alcaligenes*. A distinguishing feature of *P. maltophilia* is the presence of relatively large amounts of isovaleric acid (iC_5, see Table I) and no other acids except small amounts of phenylacetate. *P. pseudoalcaligenes*, *P. diminuta* and *P. vesicularis* each produced relatively large amounts of isobutyric and isovaleric acids, but *P. pseudoalcalignes* was different from the other two species because it did not produce propionic acid. *P. diminuta* was easily distinguished from *P. vesicularis* and from other species because it produced glutaric acid. No acids were detected from *A. xylosoxidans* but several acids, including relatively large amounts of 2-keto-isocaproic acid, were produced by each of ten strains of *Achromobacter*-like organisms[18]. This acid was not produced by most species of *Pseudomonas* or by either of the three species of *Alcaligenes*. Major amounts of 2-keto-isocaproic acid were also produced by *F. meningosepticum*, but this organism also produced relatively large amounts of 2-ketoisovaleric acid which was not detected from *Pseudomonas* or *Achromobacter*[18,19].

It is apparent from the above data that analysis of short-chain acid products is of value in the identification of *pseudomonads* and related bacteria in which energy is derived from oxidative reactions with oxygen as the terminal electron acceptor. Although fewer compounds are generally produced by these organisms compared to anaerobic and facultative bacteria[20,21], often unexpected and apparently characteristic acids are produced which can be used as useful markers of a particular group or species. With continued improvements in GLC instrumentation, column technology and methodology, additional metabolites will be found which will aid in rapid identification of other bacterial groups as illustrated in our recent studies[7,22].

Cellular fatty acids

In addition to metabolic products, determination of the cellular fatty acid composition of microorganisms has provided valuable additional information for identification of several bacterial groups or species. Fourteen of the fifteen species of *Pseudomonas* most frequently isolated from clinical materials were placed into one of nine groups on the basis of qualitative or large quantitative differences in their cellular fatty acid compositions[1]. Five of the groups contained only one species, two groups contained two species, and two groups contained three species. On the basis of this cellular fatty acid data in combination with a small number of conventional tests and the short-chain acid products discussed above, a rapid and reliable scheme for identifying medically important *Pseudomonas* species was proposed[1]. Moreover, these species can be distinguished from *A. xylosoxidans*, *F. meningosepticum* and *Alcaligenes* species on the basis of fatty acid composition alone.[18,23]

The use of cellular fatty acid data has played a significant role in the identification and classification of the etiological agents of Legionnaires' disease[24–27]. The first six isolates of this new bacterium were studied in this laboratory in 1977 and were found to differ from other gram-negative bacteria by their content of large

amounts of branched-chain cellular fatty acids[24]. The fatty acid profile of a representative strain of this organism (which was subsequently named *Legionella pneumophila*[28]) analyzed on a packed 3% OV-101 column is shown in the top chromatogram in Fig. 1. The single most abundant acid was a saturated, branched-chain 16-carbon acid (i-16:0) with the methyl branch at the *iso* (penultimate) carbon atom. The next most abundant were a mono-unsaturated 16-carbon straight-chain acid (16:1), a 15-carbon branched-chain acid (a-15:0) with the methyl branch at the *anteiso* (*anti*-penultimate) carbon atom, a saturated 17-carbon branched-chain acid (a-17:0), a saturated 14-carbon branched-chain acid (i-14:0), and a normal saturated straight-chain 16-carbon acid (16:0). Other acids present in relatively small amounts were a mono-unsaturated 16-carbon *iso* branched-chain acid (i-16:1) and normal

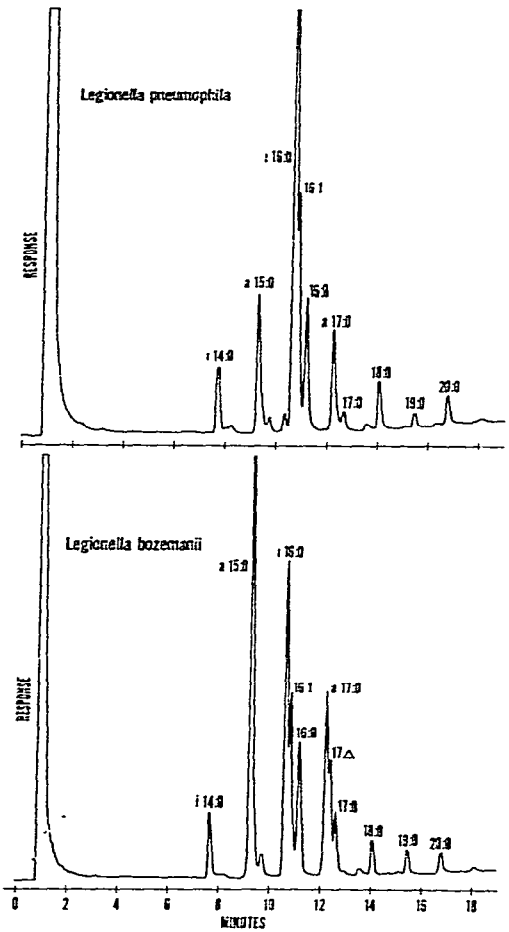

Fig. 1. Gas chromatograms of esterified fatty acids (as methyl esters) of saponified whole cells of *L. pneumophila* (top) and *L. bozemanii* (bottom). Analysis was made on a packed (3.66 m × 4.06 mm) 3% OV-101 column. Peak designation: number before the colon refers to the number of carbon atoms and the number after it to the number of double bonds. i = branched-chain acid with the methyl group at the penultimate carbon atom; a = branched-chain acid with the methyl group at the antipenultimate carbon; *Δ* or cyc = cyclopropane acid.

saturated straight chain acids (15:0, 17:0, 19:0, 20:0). To date, more than 150 strains of *L. pneumophila* have shown similar fatty acid profiles with characteristically high concentrations of i-16:0 acid and a large percentage (approximately 65%) of the total acids as branched-chained. The data have proved invaluable in discovering and defining the serogroups of *L. pneumophila* and for rapid screening of suspect isolates[11,12].

Shown in the bottom chromatogram of Fig. 1 is the fatty acid profile of a second species of *Legionella* which was recently designated *L. bozemanii*[29]. In contrast to *L. pneumophila*, the single most abundant acid of *L. bozemanii* was a-15:0 which constituted approximately 27% of the total acids. The i-16:0 acid, the major component in *L. pneumophila*, constituted only approximately 15%. The other fatty acids of *L. bozemanii* shown in the chromatogram are also present in *L. pneumophila* but in different relative concentrations. The 17-carbon cyclopropane acid (17Δ) at a retention time of 12.5 min was also present in some strains of *L. pneumophila*. Thus, even though *L. pneumophila* and *L. bozemanii* contain the same cellular fatty acids, these two species are readily distinguished on the basis of large quantitative differences in the acids present in them.

A third species of *Legionella* has been described in recent reports from this laboratory[27,30]. This species has been designated *L. micdadei*[31] but another group has proposed the name *L. pittsburgensis*[32]. The fatty acid composition of *L. micdadei* was essentially identical to that of *L. bozemanii* except for a small amount (approximately 4%) of an *anteiso*-17-carbon monoenoic acid[26,27]. Although this acid was present in only relatively small amounts in each of the four strains of *L. micdadei*, it was consistently absent in each of the three strains of *L. bozemanii* and in all strains of *L. pneumophila*.

Fused silica capillary columns

On packed columns with non-polar stationary phase (*i.e.*, OV-101, OV-1, SE-30) positional isomers of acids with the same carbon-chain length are not resolved and thus appear as one peak in the chromatogram. Moreover, other acids are not completely resolved and appear as shoulders on the leading or trailing edge of other peaks in the chromatogram (Fig. 1). Thus, for the identification of the acids of *Legionella* it was necessary to obtain MS data and to analyze the acids on a second column packed with polar or moderately polar materials[33].

Increased separation of poorly resolved components in a complex fatty acid mixture should be possible with a capillary GLC column such as the flexible, fused, silica-glass column which was recently introduced by several commercial companies. Therefore, such a column was used to analyze the cellular fatty acids of representative strains of various bacteria, including the three species of *Legionella*. Shown in Fig. 2 is the fatty acid profile of *L. pneumophila* (top chromatogram) and *L. bozemanii* (bottom chromatogram) run on a 50 m $\times$ 0.2 mm I.D. capillary column with SP 2100 as stationary phase. The increased resolution of the capillary over the packed column is apparent by comparing the chromatograms of Fig. 1 to those in Fig. 2. Baseline separation of the 16-carbon methyl ester series (i-16:1, i-16:0, 16:1, 16:0) and 17-carbon esters (a-17:0, 17:0 cyclopropane, 17:0) with the capillary column is particularly striking. Shown in the top chromatogram of Fig. 3 is the cellular fatty acid profile of *L. micdadei* run on the capillary column. As discussed above, this profile is similar to that

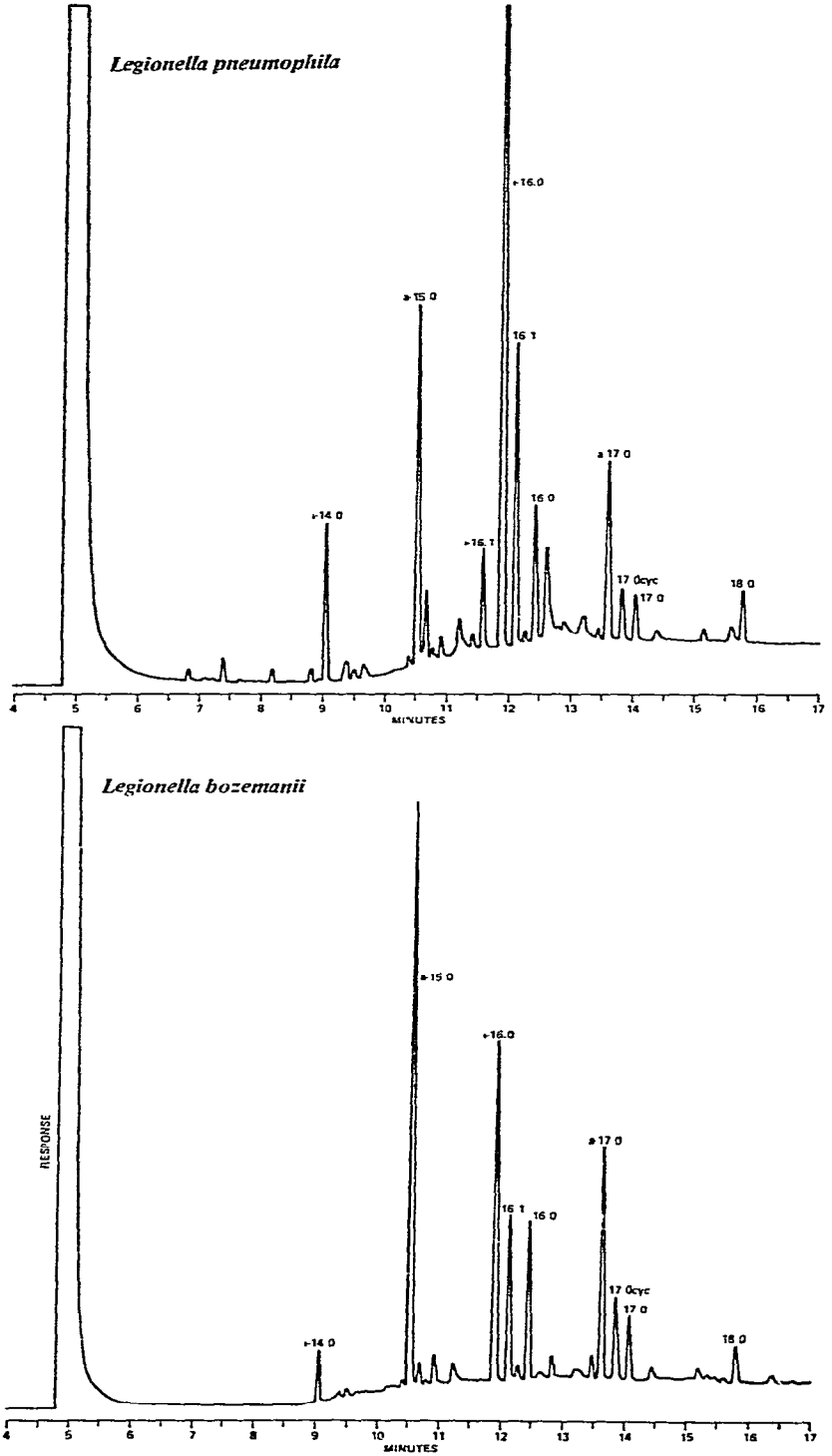

Fig. 2. Gas chromatograms of esterified fatty acids from saponified whole cells of *L. pneumophila* (top) and *L. bozemanii* (bottom) analyzed on a 50 m × 0.2 mm fused silica SP-2100 capillary column. See Fig. 1 for peak designation.

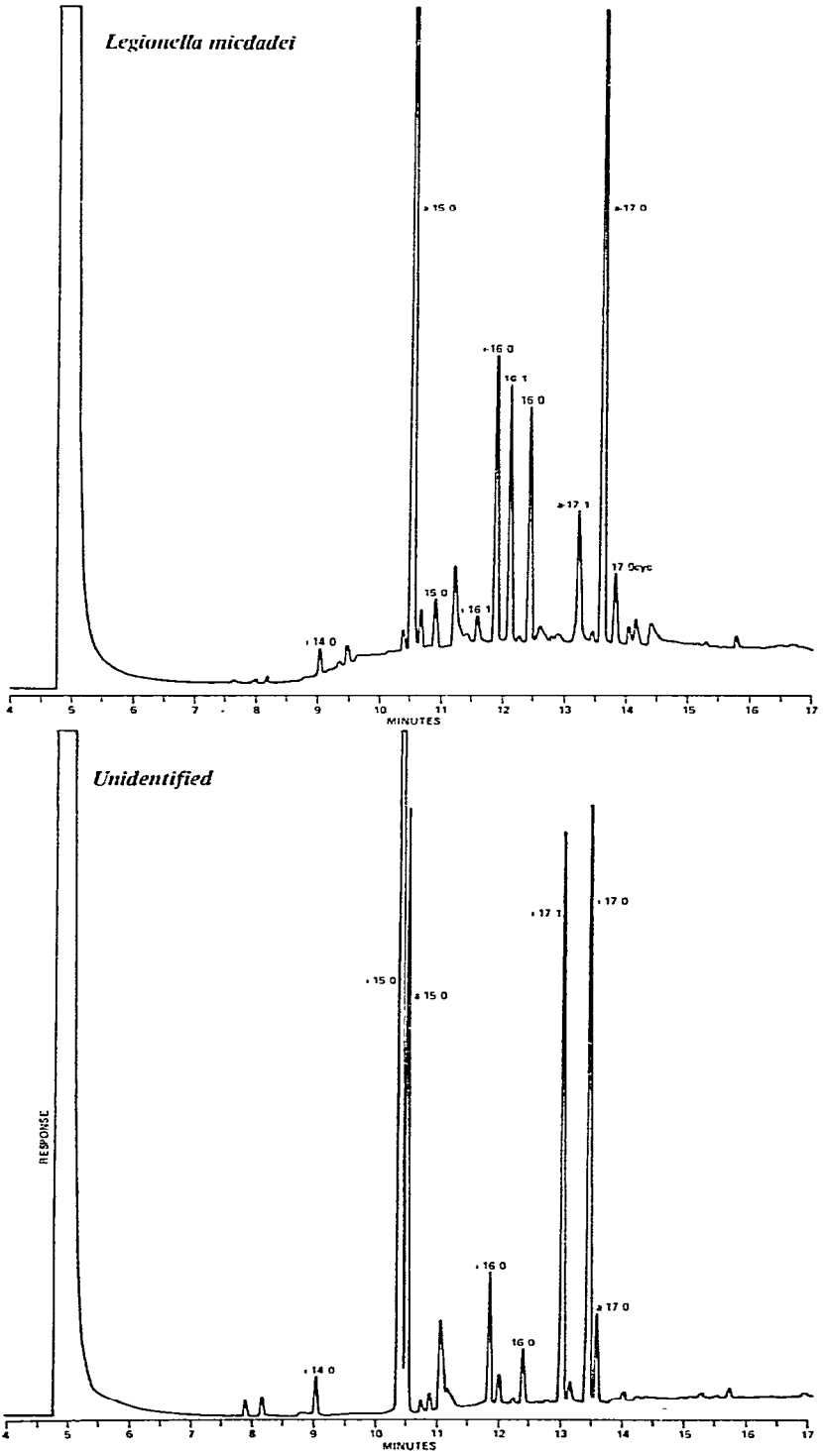

Fig. 3. Gas chromatograms of esterified fatty acids from saponified whole cells of *L. micdadei* (top) and an unidentified spore-forming bacillus (bottom) run on a 50 m × 0.2 mm fused silica SP-2100 capillary column. See Fig. 1 for peak designation.

of *L. bozemanii* with the exception of the presence of the a-17:1 acid which eluted from the capillary column at a retention time of 13.25 min.

Shown in the bottom chromatogram of Fig. 3 is an unidentified bacterial isolate which by conventional microbiological tests was suspected of being a *Legionella*-like organism. The cellular fatty acid profile of this isolate run on the packed OV-101 column appeared to be essentially identical to *L. micdadei* in that it contained major amounts of branched-chain 15:0, 17:0, and 17:1-carbon acids. However, the data from the capillary column clearly show that this isolate contained major amounts of *iso*-17:1 and *iso*-17:0 acids in contrast to *L. micdadei* (top chromatogram) in which these acids were present as the *anteiso* isomer. Additional microbiological studies which will be reported elsewhere firmly established that this isolate was indeed not *Legionella* but rather a thermophilic, spore-forming bacillus.

CONCLUSION

The above data illustrate the role of GLC in determining short-chain acid products and cellular fatty acids as additional means for rapid identification of bacteria. All strains within a group or species produce similar metabolic or cellular component profiles which are reproducible when each strain is retested through the entire procedure (growth, extraction, GLC). The extraction, derivatization, and GLC procedures can be accomplished without difficulty with technical personnel. The availability of high resolution, fused, silica-glass capillary columns which are flexible enough to be used in normal GLC work without fear of breakage should add new dimensions to microbiological studies. The increased resolution and separating capacity of these columns will significantly improve the accuracy and precision of analysis of bacterial cellular fatty acids and metabolic products.

REFERENCES

1 C. W. Moss, in G. L. Gilardi (Editor), *Glucose Non-fermenting Gram-negative Bacteria in Clinical Microbiology*, CRC Press, Boca Raton, FL, 1978, p. 171.
2 E. Jantzen, B. Berdal and T. Omland, *J. Clin. Microbiol.*, 10 (1979) 928.
3 T. Kaneda, *J. Bacteriol.*, 93 (1967) 894.
4 E. Kondo and N. Ueta, *J. Bacteriol.*, 110 (1972) 459.
5 N. Shaw, *Advan. Appl. Microbiol.*, 17 (1974) 63.
6 G. Prefontaine and F. Jackson, *Int. J. Syst. Bacteriol.*, 22 (1972) 210.
7 C. W. Moss, C. Hatheway, M. Lambert and L. McCroskey, *J. Clin. Microbiol.*, 11 (1980) 743.
8 L. Raines, C. W. Moss, D. Farshtchi and B. Pittman, *J. Bacteriol.*, 96 (1968) 2175.
9 K. Lewallen, R. McKinney, D. Brenner, C. W. Moss, D. Dail, B. Thomason and R. Bright, *Ann. Int. Med.*, 91 (1979) 831.
10 B. Thomason, P. Harris, M. Hicklin, J. Blackmon, C. W. Moss and F. Matthews, *Ann. Int. Med.*, 91 (1979) 673.
11 R. McKinney, L. Thacker, P. Harris, K. Lewallen, G. Hebert and P. Edelstein, *Ann. Int. Med.*, 90 (1978) 621.
12 A. England, R. McKinney, P. Skaliy and G. Gorman, *Ann. Int. Med.*, 93 (1980) 58.
13 M. Lambert and C. W. Moss, *J. Chromatogr.*, 74 (1972) 335.
14 S. B. Dees and C. W. Moss, *J. Clin. Microbiol.*, 1 (1975) 414.
15 C. W. Moss and S. B. Dees, *J. Chromatogr.*, 112 (1975) 595.
16 C. W. Moss, S. Samuels, J. Liddle and R. McKinney, *J. Bacteriol.*, 114 (1973) 1018.
17 G. Gilardi, in G. L. Gilardi (Editor), *Glucose Non-fermenting Gram-negative Bacteria in Clinical Microbiology*, CRC Press, Boca Raton, FL, 1978, p. 15 and p. 45.

18 S. B. Dees and C. W. Moss, *J. Clin. Microbiol.*, 8 (1978) 61.
19 S. B. Dees and C. W. Moss, *J. Chromatogr.*, 171 (1979) 466.
20 V. Lewis, C. W. Moss and W. Jones, *Can. J. Microbiol.*, 13 (1967) 1033.
21 J. Brooks, D. Kellogg, L. Thacker and E. Turner, *Can. J. Microbiol.*, 18 (1972) 157.
22 M. Lambert and C. W. Moss, *J. Clin. Microbiol.*, 12 (1980) 291.
23 C. W. Moss and S. B. Dees *J. Clin. Microbiol.*, 8 (1978) 772.
24 C. W. Moss, R Weaver, S. B. Dees and W. Cherry, *J. Clin. Microbiol.*, 6 (1977) 140.
25 C. W. Moss and S. B. Dees, *J. Clin. Microbiol.*, 9 (1979) 648.
26 C. W. Moss and S. B. Dees, *J. Clin. Microbiol.*, 10 (1979) 390.
27 G. Hebert, C. W. Moss, L. McDougal, M. Bozeman, R. McKinney and D. Brenner, *Ann. Int. Med.*, 92 (1980) 45.
28 D. Brenner, A. Steigerwalt and J. McDade, *Ann. Int. Med.*, 90 (1979) 656.
29 D. Brenner, A. Steigerwalt, G. Gorman, R. Weaver, J. Feeley, L. Cordes, H. Wilkinson, C. Patton, B. Thomason and K. Sasseville, *Curr. Microbiol.*, 4 (1980) 111.
30 G. Hebert, B. Thomason, P. Harris, M. Hicklin and R. McKinney, *Ann. Int. Med.*, 92 (1980) 53.
31 G. Hebert, A. Steigerwalt and D. Brenner, *Curr. Microbiol.*, 3 (1980) 225.
32 W. Pasculle, J. Feeley, R. Gibson, L. Cordes, R. Myerowitz, C. Patton, G. Gorman, C. Carmack, J. Ezzell and J. Dowling, *J. Inf. Dis.*, 141 (1980) 727.
33 C. W. Moss and W. Cherry, *J. Bacteriol.*, 95 (1968) 241.