Characterization of Ten Species of Mycobacteria by Reaction-Gas-Liquid Chromatography

DAVID K. OHASHI,[†]* THOMAS J. WADE,^{††} AND ROBERT J. MANDLE

Department of Microbiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received for publication 21 June 1977

The methanolic tetramethylammonium hydroxide whole-cell lysates of nine species of mycobacteria and the "rhodochrous complex" were examined by gas-liquid chromatography. The gas chromatographic patterns produced 10 characteristic chromatographic groups that corresponded to the 10 species studied. The gas chromatograms of *Mycobacterium tuberculosis* were very easily distinguished from the other mycobacterial strains by high levels of a component that eluted much later than the other components. The remaining nine species could be distinguished on the basis of characteristic components and by different amounts of components common to more than one species. This study demonstrated that direct gas-liquid chromatographic characterization of *M. tuberculosis* and other mycobacterial species was not only feasible but practical in the clinical laboratory.

The classical laboratory confirmation of mycobacterial cultures entails the most time-consuming procedures presently employed in the diagnostic microbiology laboratory. Several techniques have been suggested that might identify mycobacteria more rapidly. Gas-liquid chromatography represents an analytic tool by which some microbial species can be more completely classified and more rapidly identified on the basis of their chemical composition (1, 5). Thoen and his associates (11, 13) suggested that *Mycobacterium kansasii* could be differentiated from *M. avium*, *M. marinum*, *M. tuberculosis*, and certain scotochromogens on the basis of greater amounts of a saturated branched-chain fatty acid. No consistent differences were found between *M. avium* and Battey bacilli (12). Larsen and Mårdh (3) were able to differentiate strains of *M. avium*, *M. bovis*, *M. kansasii*, and *M. tuberculosis* on the basis of the relative sizes of seven component peaks representing partially characterized trifluoroacetylated monosaccharides and fatty acids. Reiner and his associates have successfully applied pyrolysis-gas-liquid chromatographic techniques to the classification and differentiation of a large number of strains of mycobacteria (8-10).

In earlier reports from this laboratory, it was suggested that microorganisms could be pre-

pared by direct gas-liquid chromatographic analysis by using a methanolic solution of tetramethylammonium hydroxide (TMAH) as a "single-step" preparation reagent (4, 15). This report presents studies on the gas-liquid chromatographic differentiation of whole-cell TMAH lysates derived from a variety of mycobacterial species.

MATERIALS AND METHODS

Organisms. A total of 44 strains of mycobacteria were used in this study. They were obtained as clinical isolates from Thomas Jefferson University Hospital and Valley Forge General Hospital (U.S. Army) and as cultures from Hugo David (Center for Disease Control, Atlanta, Ga.). All of the strains were characterized by standard mycobacteriological procedures and included the following mycobacterial species: *M. flavescens* (three strains), *M. fortuitum* (four strains), *M. intracellulare* (three strains), *M. kansasii* (five strains), *M. marinum* (three strains), *M. phlei* (five strains), *M. triviale* (five strains), *M. tuberculosis* (eight strains), and *M. vaccae* (four strains). Also included were "rhodochrous complex" (four strains).

Media and growth conditions. All organisms were maintained on Lowenstein-Jensen media. For gas-liquid chromatographic studies, a suspension of each strain was diluted in distilled water to a concentration range of 10^5 to 10^7 colony-forming units (CFU) per ml. By using a 7-inch (ca. 17.78-cm) Pasteur pipette, 5 drops of the suspension was inoculated to the surface of Middlebrook 7H10 agar supplemented with oleic acid-dextrose-catalase enrichment (Baltimore Biological Laboratory, Cockeysville, Md.) in 100-mm petri dishes. The plates were enclosed in CO₂-permeable bags and incubated at 35°C in 5 to 10% CO₂. The cultures were grown shielded from light, and all

[†] Present address: Box 193, Tripler Army Medical Center, Honolulu, HI 96859.

^{††} Present address: Department of Microbiology, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642.

plates were incubated until mature growth was evident. The incubation period ranged from 7 to 10 days for the rapidly growing species and from 25 to 32 days for the slowly growing species.

Preparation of gas chromatographic samples.

The organisms were prepared for gas chromatographic analysis by a modification of the method reported by Wade and Mandle (15). Approximately 50 to 75% of the isolated colonies growing on the supplemented 7H10 agar were transferred to a 5-ml glass ampoule by using a glass capillary tube with a 1-mm collecting hook formed at one end. Care was taken not to pick up any of the agar medium. The cells were not washed. Approximately 0.05 ml of a saturated methanolic solution of TMAH pentahydrate was transferred to the bottom of the ampoule. The ampoule was sealed by flaming the neck while a slight vacuum was being drawn. All ampoules were heated for 20 min at 100°C and then stored at -10°C until the samples were analyzed. A 1- to 4- μ l portion of each sample was used for analysis. The sample size and the sensitivity settings of the gas chromatograph were adjusted so that the response of the tallest peak in the chromatogram was from 60 to 90% of the full-scale recorder response.

Gas chromatographic analysis. A Perkin-Elmer model 900 gas chromatograph equipped with dual flame ionization detectors and glass-lined flash vaporization injector ports was used for the analysis of the samples. The gas chromatographic system was used in a dual-column compensated mode, and all analyses were performed by using stainless-steel columns (12 feet by 0.125 inch [about 3.8 m by 0.32 cm]) packed with 10% diethylene glycol succinate on 80- to 100-mesh Chromasorb W-AWDMCS (Supelco, Inc., Bellefonte, Pa.). The injector port was maintained at 320 to 340°C. The column oven temperature was held at 80°C for 4 min after injection and then raised at a rate of 4°C per min to 195°C and held at this temperature for 65 min. The signal from the gas chromatograph was connected to the Autolab System IV (Spectra Physics, Santa Clara, Calif.), which calculated the relative retention time of each peak. The recorder was connected to an Autolab System IV, and the chart speed was 12 inches (about 30 cm)/h.

Treatment of the gas chromatographic data.

An internal component, naturally present in all the samples examined, was used as an internal reference component. This component was the tallest peak in all of the chromatograms and was situated in the same position in the chromatogram as that assumed by a methyl palmitate standard. The height of this peak varied with extracts of different organisms. To make comparisons between different chromatograms, this peak was used as an internal standard. The retention time and peak height of this component in each chromatogram were each arbitrarily assigned values of 1.0. The retention time and peak height value of the reference component were then used to determine the relative retention time (RRT) and the relative peak height (RPH) values of the remaining peaks in the chromatogram. These normalized values were used to define similarities and differences in the gas chromatograms of each species.

RESULTS

The chromatogram of *M. tuberculosis* shown in Fig. 1 represents the general gas chromatographic pattern obtained from TMAH-digested mycobacteria. The chromatograms of the mycobacterial species used in this study could be differentiated by peaks at selected RRT positions and by the RPH values of selected components. In some instances the chromatograms proved so characteristic that differentiation was possible without a detailed analysis of the whole chromatogram.

Digests of Middlebrook 7H10 medium were prepared and chromatographed. No evidence was found that the medium contained a level of any component used in the analysis.

The sample size and the analytic conditions were adjusted so that the height of the reference component peak was from 60 to 90% of the full-scale recorder response. Under these conditions no peaks were found prior to an RRT value of 0.83 which were to be of any value in differentiating the various species. Some components

FIG. 1. Gas-liquid chromatographic profile of TMAH-digested *M. tuberculosis* strain H37Rv.

were characteristically present or absent in some species. *M. kansasii* was the only species to show the presence of components at relative time positions of 0.83 and 2.46. *M. fortuitum* showed low levels of components at positions 1.42 and 1.70, which were not seen in the other species. The "rhodochrous complex" had a component present at position 2.35, *M. triviale* at position 2.60, and *M. vaccae* at position 1.32. The "rhodochrous complex" conspicuously lacked a component at RRT position 2.08. *M. marinum* lacked the component that was present at low levels for all the other species at position 1.80. The most striking of the potentially characteristic peaks was the presence of high levels of a component at RRT positions 2.89 in all eight of the *M. tuberculosis* strains examined (Peak I, Fig. 1). Even at increased sensitivity settings, no trace of this component was evident in chromatograms of the other 36 mycobacterial species.

An examination of the RPH values of the various peaks in the chromatograms of each species enabled further differentiation of the mycobacteria (Table 1). Four species had ranges of RPH values for one or more peaks, which differed significantly from the range of values observed for the same peaks in the chromatograms of the other species. The *M. fortuitum* strains showed RPH values for peak A that were consistently higher than those of the other nine mycobacteria. *M. marinum* showed consistently higher values for peak B and consistently lower values for peak C. *M. vaccae* always had the highest values for peak C.

Peak E is located on the center of the triad of three incompletely resolved components spanning RRT positions 1.13 to 1.17. For the majority of mycobacteria strains studied, the overall appearance of this triad is similar to that

seen for *M. tuberculosis* (Fig. 1; peaks D-F). In some instances, strains of the "rhodochrous complex" had a very low level of component D; in other instances this same species showed relatively high levels of component D. All in all, peak D proved to be more poorly resolved from peak E than did peak F. Peak E was absent or barely discernible in the chromatograms of *M. marinum* digests. In all of the other strains, peak D was never observed to be higher than peak E. Peak F was constantly higher than peak E in the chromatograms of *M. kansasii*, *M. marinum*, and *M. triviale*. The combination of a relatively low peak D, an absent or barely discernible peak E, and a high peak F was very characteristic of *M. marinum* chromatograms. The presence of comparatively low levels of peaks D and F and high levels of peak E was often seen in the chromatograms of the "rhodochrous complex."

DISCUSSION

The reaction gas chromatographic procedure used in this study produces chromatographic patterns that are somewhat more complex than those obtained by the use of more time-consuming extraction-derivatization procedures (3, 11-13). On the other hand, our chromatograms appear to be simpler than the complex patterns of thermal fragmentation products obtained through the use of techniques involving pyrolysis-gas-liquid chromatography (8-10). The results of the above studies and the results of our study clearly show that various mycobacterial species have inherent differences in their chemical composition, which would make more rapid laboratory identification by gas-liquid chromatographic methods possible. Although some similarities are seen in our chromatograms and those obtained by investigators using other methods

TABLE 1. Comparison of the range of relative peak height values observed for selected gas chromatographic peaks useful in the characterization of TMAH-digested mycobacteria

Strain (no. tested)	Peaks ^a								
	A	B	C	D	E	F	G	H	I
<i>M. flavescens</i> (3)	0.02-0.05 ^b	0.02-0.03	0.10-0.17	0.02-0.08	0.33-0.61	0.14-0.26	0.04-0.11	0.16-0.21	ND
<i>M. fortuitum</i> (4)	0.15-0.21	0.01-0.02	0.17-0.21	0.02-0.03	0.68-0.93	0.17-0.40	0.01-0.08	0.25-0.31	ND
<i>M. intracellulare</i> (3)	0.08-0.09	ND ^c	0.14-0.16	0.07-0.09	0.47-0.58	0.41-0.56	0.01-0.01	0.27-0.45	ND
<i>M. kansasii</i> (5)	0.00-0.05	0.00-0.03	0.07-0.13	0.05-0.07	0.31-0.50	0.33-0.62	0.01-0.03	0.14-0.20	ND
<i>M. marinum</i> (3)	0.04-0.06	0.31-0.39	0.02-0.04	0.11-0.17	0.00-0.03	0.85-0.93	ND	0.26-0.28	ND
<i>M. phlei</i> (5)	0.02-0.08	ND	0.17-0.28	0.02-0.14	0.57-0.74	0.02-0.24	0.14-0.21	0.17-0.20	ND
"Rhodochrous complex" (4)	0.02-0.03	ND	0.22-0.24	0.03-0.37	0.55-0.97	0.03-0.11	ND	ND	ND
<i>M. triviale</i> (5)	0.02-0.06	0.02-0.04	0.21-0.32	0.09-0.14	0.39-0.47	0.50-0.93	0.03-0.07	0.26-0.46	ND
<i>M. tuberculosis</i> (8)	0.01-0.03	ND	0.06-0.18	0.07-0.20	0.38-0.55	0.27-0.49	0.01-0.02	0.02-0.08	0.11-0.20
<i>M. vaccae</i> (4)	0.01-0.06	0.04-0.15	0.34-0.40	0.15-0.30	0.47-0.70	0.37-0.90	0.21-0.53	0.14-0.23	ND

^a See Fig. 1; the relative retention times of peaks A through I were: 0.86, 0.97, 1.04, 1.13, 1.15, 1.17, 1.60, 2.08, and 2.89, respectively.

^b Range of minimum and maximum relative peak height values observed for each peak. Encompasses all data for each species and includes a minimum of two analyses for each strain.

^c ND, Not detected.

(3, 11-13), we have not identified any of the components in our chromatograms; thus data from other laboratories cannot be accurately compared with ours. Whereas the application of gas chromatographic procedures to clinical laboratory identification schemes could also be assisted by a knowledge of colony morphology, chromogenicity, growth rate, and optimum temperature for growth of the mycobacteria under consideration (14), we were able to assign the 44 mycobacterial strains used in this study into groups that reflect their proper species designation. The relative area under the 2.89 peak in chromatograms of the eight *M. tuberculosis* strains indicates that this component will perhaps be present uniquely or in such high concentration in additional strains of *M. tuberculosis* that it might be extremely useful for the rapid diagnosis of *M. tuberculosis* infections. Other mycobacterial infections might also be more rapidly diagnosed, as indicated by the various species-specific chromatographic patterns seen for other mycobacteria. Verification of this hypothesis awaits the study of additional species of acid-fast bacteria, which are represented by a much larger number of strains than was used in this survey.

The strains of the "rhodochrous complex" were included in this study because they must occasionally be differentiated from mycobacteria isolated from clinical sources. Recently, these organisms have been examined by an extensive numerical taxonomic study, and there is evidence to raise this complex to generic status (2, 6). The chromatograms of this new taxon were distinguishable from the rest of the strains, but they did not produce chromatograms as uniquely different from some of the other species than did chromatograms of some of the additional species of mycobacteria.

The various uses of methanolic TMAH as an agent for the direct gas-liquid chromatographic characterization of microorganisms has been discussed by Mandle and Wade (4). It is evident from this study that hot methanolic TMAH will rapidly digest portions of mycobacterial growth. This digestion can be performed in a sealed air-evacuated ampoule containing an essentially indeterminate quantity of mycobacteria and a small volume of methanolic TMAH. Simultaneously with the TMAH digestion process, heat-labile quaternary ammonium salts are formed from many of the organism's structural and intracellular components. A small portion of the TMAH lysate can be directly injected into the high-temperature (320°C) flash vaporization port of the gas chromatograph. The heat-labile quaternary ammonium salts will decompose, releasing methyl esters into the carrier gas stream.

A partial chemical characterization of some of the individual components represented in the chromatograms of TMAH-digested microorganisms shows that many of the major peaks are methyl esters of fatty acids (R. J. Wade and T. J. Mandle, manuscript in preparation). It is not unlikely that thermal breakdown products from the samples can be seen in the chromatograms, especially when a detailed analysis of some portions of the chromatograms are done at sensitivity settings higher than those required to maintain the recorder response for the reference component on scale. It is also felt that the carbohydrate composition of organisms is at least partially degraded or destroyed by the high alkalinity and temperatures used for the sample preparation and analysis. Whether or not differences and similarities in the carbohydrate composition are reflected in the chromatograms of TMAH-digested microorganisms is not known at this time. It is possible that the 2.89 peak seen only in *M. tuberculosis* strains may be a high-molecular-weight fatty acid or a carbohydrate component.

Moss et al. (7) have evaluated the use of methanolic TMAH as an agent for the direct gas-liquid chromatographic analysis of fatty acids in bacterial whole-cell lysates. They have reported that TMAH cannot be relied upon as an agent to accurately examine the fatty acid composition of microorganisms. We concur with their conclusion and would caution any laboratory against using TMAH techniques as anything other than a rapid guide to potential differences in the chemical composition of microorganisms. We have not, however, been handicapped in our studies by an excessively large solvent peak (Fig. 1) when a reasonable ratio of the cell mass to be digested and amount of digesting reagent has been maintained. We have demonstrated that under the proper conditions a very small quantity of cells can be directly reacted with a very small quantity of TMAH inside the flash vaporization port of the gas chromatograph (4). This technique even has the potential to allow the "single-step" examination of the chemical composition of individual colonies of bacteria (Wade and Mandle, manuscript in preparation). We are presently in favor of the use of anhydrous TMAH (2 M methanolic TMAH, Southwestern Analytic Chemicals, Inc., Austin, Tex.) rather than saturated methanolic TMAH pentahydrate. Although no deleterious effects were evident in this study, the unavoidable incorporation of excess water into samples may ultimately shorten the longevity and analytic efficiency of some gas chromatographic stationary phases.

Additional studies are being implemented us-

ing a wide variety of additional species and strains of mycobacteria to determine how rapidly and how accurately *M. tuberculosis* and other mycobacteria can be differentiated by gas-liquid chromatography by using methanolic TMAH techniques.

ACKNOWLEDGMENTS

This study was supported by the U.S. Army, Project Themis contract no. N00014-68-0516, by Electro-Nucleonics, Inc., and by the Percival E. and Ethel Brown Foerderer Foundation.

LITERATURE CITED

1. Drucker, D. B. 1976. Gas-liquid chromatographic chemotaxonomy, p. 51-125. In J. R. Norris (ed.), *Methods in microbiology*, vol. 9. Academic Press Inc., London.
2. Goodfellow, M., and G. Alderson. 1977. The Actinomycete-genus *Rhodococcus*: a home for the "rhodochrous" complex. *J. Gen. Microbiol.* **100**:99-122.
3. Larsson, L., and P. Mårdh. 1976. Gas chromatographic characterization of mycobacteria: analysis of fatty acids and trifluoroacetylated whole-cell methanolysates. *J. Clin. Microbiol.* **3**:81-85.
4. Mandle, R. J., and T. J. Wade. 1975. The use of gas chromatography for the identification of microorganisms, p. 163-180. In J. E. Prier, J. T. Bartola, and H. Friedman (ed.), *Modern methods in medical microbiology; systems and trends*. University Park Press, Baltimore.
5. Mitruka, B. M. (ed.). 1975. *Gas chromatographic applications in microbiology and medicine*. John Wiley and Sons, New York.
6. Mordarski, M., M. Goodfellow, K. Szyba, G. Pulverer, and A. Tkacz. 1977. Classification of the "rhodochrous" complex and allied taxa based upon deoxyribonucleic acid reassociation. *Int. J. Syst. Bacteriol.* **27**:31-37.
7. Moss, C. W., M. A. Lambert, and W. H. Merwin. 1974. Comparison of rapid methods for analysis of bacterial fatty acids. *Appl. Microbiol.* **28**:80-85.
8. Reiner, E., R. E. Beam, and G. P. Kubica. 1969. Pyrolysis-gas-liquid chromatography studies for the classification of mycobacteria. *Am. Rev. Respir. Dis.* **99**:750-759.
9. Reiner, E., J. J. Hicks, R. E. Beam, and H. L. David. 1971. Recent studies on mycobacterial differentiation by means of pyrolysis-gas-liquid chromatography. *Am. Rev. Respir. Dis.* **104**:656-660.
10. Reiner, E., and G. P. Kubica. 1969. Predictive value of pyrolysis-gas-liquid chromatography in the differentiation of mycobacteria. *Am. Rev. Respir. Dis.* **99**:42-49.
11. Thoen, C. O., A. G. Karlson, and R. D. Ellefson. 1971. Fatty acids of *Mycobacterium kansasii*. *Appl. Microbiol.* **21**:628-632.
12. Thoen, C. O., A. G. Karlson, and R. D. Ellefson. 1971. Comparison by gas-liquid chromatography of the fatty acids of *Mycobacterium avium* and some other nonphotochromogenic mycobacteria. *Appl. Microbiol.* **22**:560-563.
13. Thoen, C. O., A. G. Karlson, and R. D. Ellefson. 1972. Differentiation between *Mycobacterium kansasii* and *Mycobacterium marinum* by gas-liquid chromatographic analysis of cellular fatty acids. *Appl. Microbiol.* **24**:1009-1010.
14. Vestal, A. L. 1973. Procedures for the isolation and identification of mycobacteria. Public Health Service Publication. Center for Disease Control, Atlanta, Georgia.
15. Wade, T. J., and R. J. Mandle. 1974. New gas chromatographic characterization procedure: preliminary studies on some pseudomonas species. *Appl. Microbiol.* **27**:303-311.