New Gas Chromatographic Characterization Procedure: Preliminary Studies on Some *Pseudomonas* Species

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Strains of saccharolytic and nonsaccharolytic *Pseudomonas* species were examined by a new single-step gas chromatographic characterization procedure. Cells were digested in a methanolic solution of tetramethylammonium hydroxide pentahydrate, and the digestates were subjected to gas-liquid chromatographic analysis. The chromatograms were examined for similarities and differences in their overall patterns. A single component was defined for use as an internal qualitative and quantitative standardizing component in order to develop relative retention time-versus-relative peak height profiles of each organism. Comparison of these profiles enabled the characterization of strains of *Pseudomonas aeruginosa*, *P. putida*, *P. cepacia*, *P. pseudomallei*, *P. putrefaciens*, *P. pseudoalcaligenes*, *P. alcaligenes*, *P. diminuta*, *P. denitrificans*, and *P. acidovorans*. The *P. maltophilia* and *P. putrefaciens* digestates showed chromatograms which were superficially similar yet easily distinguished as belonging to different species. The chromatograms of these two organisms were very different from those of other pseudomonads.

Gas-liquid chromatography has been recognized as a potential means of assisting in the characterization of microorganisms (4). These techniques, plus those in reports of more recent origin, show one or another of four major analytic trends. One approach involves the gas chromatographic detection of specific metabolites produced by different microorganisms (2, 3, 6, 14). The three other approaches involve a chromatographic characterization of structural components. They use either derivatives of extracted cellular components (1, 7, 21, 22), derivatives of whole cell hydrolysates (8, 9, 12, 13), or the separation of pyrolytically induced molecular fragments (5, 18). The gas chromatograph can make the characterization of a variety of microorganisms both rapid and simple. In the past, however, the relatively long total time necessary for sample preparation, together with the amount of sample manipulation required, meant that many of the procedures noted above might lose much of their potential as a truly rapid and simple means for the rapid identification of clinical isolates.

It has been shown that tetramethylammonium hydroxide (TMAH) is capable of forming thermally unstable tetramethylammonium salts. These salts can act as methyl ester precursors which will decompose in the high-temperature injector port of a gas chromatograph and result in the release of methyl esters into the carrier gas stream (11, 19). This reagent has the added property of forming highly alkaline solutions capable of bringing about the lysis of bacterial cells. These properties enabled MacGee to first use TMAH to prepare derivatives of bacterial components (12). His procedure was applied by Meyer and Balzevic to the analysis of several strains of mycoplasma (13). We have briefly reported the use of a modified TMAH procedure by which a number of gram-negative and gram-positive bacteria were examined (T. J. Wade, R. J. Mandle, and R. W. Schaedler, Bacteriol. Proc., p. 75, 1970). This paper is a report on more recent modifications of our bacterial characterization procedure using methanolic TMAH pentahydrate as a single-step preparation reagent. Much of the time necessary for sample preparation and handling which is inherent in some other gas chromatographic characterization procedures has been eliminated. Some non-fermentative bacteria that pose a troublesome identification problem for the clinical microbiologist have been chosen for study to evaluate the efficacy of the procedure.

**MATERIALS AND METHODS**

**Organisms.** The following strains of bacteria were used in this study: 12 *Escherichia coli*, strains 1D, 2D,
strains GC56, P. 2872 and Pseudomonas number isolates; methanolic scraping them collected glass TMAH accomplished vial sediment capacity) a sample preparation was harvested 152 ml (0.4-ml ampoule). The cell of the neck while broken a slight vacuum was washed. The ampoule was then washed into the neck of the ampoule. Bacteria were collected in the open portion of the hook by lightly scraping them off of the agar surface. Care was taken not to cut into the agar or otherwise pick up extraneous materials. The cell mass was not washed. A Pasteur pipette was used to place 2 drops (approximately 0.05 ml) of a saturated methanolic solution of TMAH pentahydrate into the bottom of the ampoule. The excess capillary tubing was broken off against the opening of the ampoule neck. The ampoule was sealed by flaying the neck while a slight vacuum was drawn and was then heated for 15 min at 100 C. The vial was opened, and the contents were transferred to a capped polyethylene microcentrifuge tube (0.4-ml capacity) which was then centrifuged in a Beckman Spinco model 152 microfuge for 60 s in order to sediment any cellular material not solubilized by the sample preparation procedure. The sample was generally analyzed within 12 h unless otherwise noted. If the sample vial or microfuge tube was to be kept for any extended period, it was stored at −10 C.

Gas chromatographic analysis. The samples were analyzed with a model 900 Perkin-Elmer gas chromatograph equipped with dual channels and dual hydrogen flame ionization detectors. All samples, however, were analyzed in an uncompensated mode on a single channel. The flame detector system was used in conjunction with pure oxygen. The carrier gas was helium flowing at approximately 35 ml/min at the start of the temperature program. The injector port temperature was set at 320 C. All analyses were performed on a stainless steel column (12 ft by 0.12 inch, about 3.8 m by 0.03 cm) packed with a 10% diethylene glycol succinate (DEGS) on 80-100 mesh chromosorb W-AWDMS (prepacked by Supelco, Inc., Bellefonte, Pa.). The oven temperature was held at 80 C for 4 min and then raised at a rate of 4 C per min to 195 C and held at this temperature for 20 min. A 1- to 3-mliter portion of each sample was used for analysis. The gas chromatograph electrometer was set at a range of 100 and an attenuation of 4. The recorder chart speed was 12 inches (about 30 cm) per h.

The raw column retention value for each component peak in the chromatograms was measured as the perpendicular distance from the upward slope of the solvent peak to the apex of the component peak. Because of the temperature-induced base-line drift, raw peak heights were measured from the apex of each peak to a point directly below it on a manually approximated floating base line (see Fig. 2A).

Treatment of the gas chromatographic data. A reference peak was chosen for the determination of relative retention times (RRT) and relative peak heights (RPH). The sample component which we chose to represent the reference peak was situated at the same position in the chromatogram as that assumed by a methyl palmitate standard (Applied Science Laboratories, Inc., State College, Pa.). This reference peak position can be initially located by mixing a methyl palmitate standard with the sample and noting which peak has an increased detector response proportionate to the amount of standard used. Any subsequent location of the reference peak was easily done either visually, by comparing the raw retention distance of the suspected peak with that of the methyl palmitate standard (sample mixture), or by observing overlapped chromatograms.

Once the reference peak had been located, RRT and RPH values were determined for each peak in the gas chromatogram. This was done by dividing the raw retention distance and the raw height value for each peak by the respective raw value from the reference peak. In this way, the reference peak assumed an RRT of 1.00 and an RPH of 1.00. The RRT and RPH values derived for the other peaks in the chromatogram thereby represented retention time and peak height values proportionate to those of the reference peak. Bar graphs were then set up to represent normalized profiles of the RRT-versus-RPH values of the peaks in the chromatograms.

The chromatograms and the chromatographic profiles produced for each organism were examined in an attempt to locate and define similarities and differences between the organisms. No attempt was made in this study to determine the exact chemical nature of the TMAH-derived cellular components.
RESULTS

Figure 1 shows three general types of chromatograms obtained by our procedure. They are partially overlapping and have been aligned by using the reference peak as an index peak (indicated by arrow). Often, more than 40 to 50 peaks could be detected, and it is evident that many components have not been fully resolved. Although the chromatograms show the presence of a number of components, usually only 5 to 10 peaks need be considered of major importance for the characterization of an organism. For most organisms, these important peaks seemed to be located in the second half of the chromatogram, as is exemplified by the chromatogram of *P. cepacia* (Fig. 1A). This, however, was not always the case. In the chromatogram of *P. pseudomallei* (Fig. 1B), characteristically large peaks were found toward the beginning of the chromatogram. This was the only organism examined in this study which showed such large peaks in the first half of the chromatogram, although strains of some other organisms, such

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**Fig. 1.** Partially overlapping gas chromatograms of three TMAH-digested pseudomonads. (A) *Pseudomonas cepacia*, strain GC171; (B) *P. pseudomallei*, strain GC94C; (C) *P. putrefaciens*, strain PSL-B426. Arrows indicate reference peaks.
as *P. cepacia* (Fig. 1A), sometimes showed a moderate-size peak near RRT position 0.16. The *P. putrefaciens* chromatogram (Fig. 1C) represents a third general type of chromatographic pattern. This organism has a chromatogram which is more complex in the central portion than most of the other gram-negative organisms we have examined. Most of the larger peaks clustered between RRT positions 0.89 and 1.13, with some of these peaks incompletely resolved. The dominant peak occurred at an RRT position of 0.89.

Comparison of the chromatograms of some organisms, such as those shown in Fig. 1, show such gross differences that a visual examination of them will give a good indication of the nature of the organism. Chromatograms of other organisms, however, are similar enough to require a closer examination of the RRT and RPH values derived from the chromatograms in order to accurately define qualitative and quantitative differences between the organisms. This was most efficiently done by drawing normalized profiles of RRT-versus-RPH values and comparing these profiles rather than the actual chromatograms. Figure 2A shows a chromatogram of *P. aeruginosa* strain GC56. Located below the chromatogram (Fig. 2B) is a normalized profile representing the RRT-versus-RPH values of each peak in the chromatogram. The use of RRT values eliminated most of the variations in retention values, and the use of RPH values eliminated the necessity of harvesting the exact amount of cell mass each time a sample was prepared.

As a result of having determined peak positions for more than 500 bacterial samples both visually and as a function of relative retention time, it appears that RRT values for a given peak generally do not vary more than ±0.01 units for those peaks located toward the center of the chromatogram or more than ±0.02 units for those located toward the extreme ends of the chromatogram. These values represent more or less maximal variations which we have come to expect when comparing chromatograms produced 8 to 10 months apart and correlate more with column age than with the nature of the organism examined. Peaks located around a few RRT positions often do not resolve completely enough for accurate determination of individual peak positions and sizes. An example of this is the peak cluster found between peaks 1.04 and 1.13. This cluster occurred in the chromatograms of many of the organisms we examined. For characterization purposes, the whole cluster was treated as a single peak and assigned an RRT value of 1.09C. The RPH value for this position was derived from the height of the highest peak in the cluster.

Generally, the actual presence or absence of a peak at specific RRT positions will give only a partial characterization of most organisms. A more complete characterization requires an examination of the height of selected peaks in relation to that of the reference peak. One or more of these RPH values will often vary significantly between different species of organisms. Table 1 shows a comparison of RPH values obtained for selected peaks from chromatograms of strains of *E. coli* and *P. aeruginosa*. In general, most members of the *Enterobacteriaceae* examined in other phases of our research showed RRT and RPH patterns similar to those of the *E. coli* strains represented. However, the RPH values obtained for *P. aeruginosa* strains showed distinctive differences in overall pattern when compared with those from the *E. coli* strains. The 0.86 and 1.13 peaks became much reduced in size, and the peak at the 0.90 position assumed sufficient size to be considered discriminating. The 1.23 peak played a much more dominant role in the *P. aeruginosa* chromatograms than it did in the *E. coli* chromatograms. It can be seen that there is no overlap in the variations of the RPH values of the two species at RRT positions 0.86, 0.90, 1.13, and 1.23. In addition, the 1.04 peak from the *P. aeruginosa* strains does not show any of the lower RPH values (<0.21) that were observed in the *E. coli* chromatograms.

Studies of the reproducibility of data from repeated chromatograms of individual samples, or of repeated samples of the same strain, show the data for RPH values to be essentially reproducible for all of the RRT positions shown in Table 1 except those for position 0.92. The RPH values obtained for this peak ranged quite erratically, even when the same sample was repeatedly analyzed. As a result of this unpredictable variation, we feel that differences in the size of this peak are unreliable for the characterization of different organisms. This component often did not completely elute from the column. It was, therefore, necessary to do a 10-min "dry run" between samples at a temperature program of 32°C per min in order to minimize any build-up of this component on the columns.

Figures 3 and 4 represent normalized profiles of gas chromatograms obtained from a survey study of some polarly flagellated, nonfermentative, gram-negative rods. The profiles show the RPH values for what are presently considered to be sufficient numbers of RRT positions for the characterization of each species. Variations in
RPH values were noted for peaks with RRT values less than 0.86, but the significance of these variations has not been fully investigated. Profiles of four saccharolytic *Pseudomonas* species are shown in Fig. 3. The profiles produced from *P. maltophilia* strains (Fig. 3A and B) were superficially similar to those which could be derived from *P. putrefaciens* chromatograms (Fig. 1C). Close inspection of our chromatograms, however, consistently shows that our strains of *P. putrefaciens* had relatively high RPH values for the 0.93 peak, and the 0.91 peak was either absent or seen only as a poorly resolved shoulder peak. In *P. maltophilia*, it is the 0.91 peak which has a relatively high RPH value, and it is the 0.93 peak which is seen as a poorly resolved shoulder peak. In addition, the 1.13 peak was virtually absent from the *P. maltophilia* strains we examined, and it always had a high RPH value in our *P. putrefaciens* strains. These two organisms were also critically evaluated for the presence of significant RRT
positions with values less than 0.86. We have noted that *P. putrefaciens* has a moderately large peak at RRT position 0.76. This peak is virtually absent from the *P. maltophilia* chromatograms.

The other saccharolytic pseudomonads in Fig. 3 can be easily distinguished by comparing their overall patterns. The *P. putida* strains (profiles C and D) are conspicuous in the low level of their 1.23 peaks. They also show a tendency toward somewhat higher 1.13 peaks. The *P. stutzeri* strains (profiles E and F) are not easily distinguishable from *P. aeruginosa* strains (Fig. 2B; Table 1) except that the *P. aeruginosa* strains tend to have lower 1.04 peaks (<0.40). The *P. pseudoalcaligenes* strains (profiles G and H) are conspicuous in the high level of the 1.23 peaks. In addition, they show somewhat higher 1.04 peaks.

Figure 4 represents normalized profiles of some weakly or non saccharolytic pseudomonads. *P. acidovorans* (*Comamonas terrigena*) (profiles A and B) and *P. denitrificans* (profiles C and D) show essentially similar profiles except that, in the *P. denitrificans* strains, the 1.04 peaks tend to be higher. The *P. diminuta* strains (profiles E and F) have a somewhat variant 1.23 peak, but this peak was always high and is always associated with a low 1.04 peak. The *P. alcaligenes* strains (profiles G and H) are easily distinguishable from the other nonsaccharolytic pseudomonads by the presence of intermediate-size 1.04 peaks and relatively high 1.23 peaks. In addition, the 0.86 component is significantly higher, and a component is usually present at RRT position 0.93.

**DISCUSSION**

The use of the gas chromatograph as a broad-scale chemotaxonomic tool has been discussed by Ueta et al. (23) and by Okami et al. (16). It appears, however, that until recently there has been very little application of gas chromatographic methods to members of the *Pseudomonadaceae*. Such application might prove fruitful as evidenced by the results obtained in this study and those obtained by various investigators at the Center for Disease Control (3, 15, 20). Because of the small numbers of strains evaluated in this survey, no serious attempt can be made to correlate our profiles with the present taxonomic positions of the organisms studied. However, a few points of interest do seem worth noting. *Pseudomonas maltophilia* appears to be a metabolically atypical pseudomonad (21). This atypical nature is also evident in the chromatographic profiles we have obtained for this species. The superficial similarity of chromatograms of *P. maltophilia* and *P. putrefaciens* might indicate a closer relationship of these two organisms than is evident by routine characterization procedures. The chromatograms of these two organisms are quite distinct from those of the other pseudomonads we have examined. Recent work in our laboratory has shown that some "nonsaccharolytic" pseudomonads (*P. acidovorans*, *P. denitrificans*, and *P. testosteroni*) and some *Alcaligenes* strains have chromatographic profiles similar to those of *Vibrio* and *Aeromonas* strains. It also appears that digestates of *Herellea* strains have chromatographic profiles quite distinct from those of *Mima* or *Moraxella* digestates (unpublished data).

The speed and simplicity of using methanolic TMAH as an agent to prepare microorganisms for gas chromatographic analysis permit a practical application of these methods toward problems in the clinical laboratory. Accurate identification of many nonfermentative gram-negative rods necessitates the use of a relatively large battery of biochemical tests (10, 17). Because of the time and effort involved, many laboratories are forced into the precarious position of having to report the nature of these organisms on the basis of a sometimes meager number of presumptive tests. At times, we have detected misidentified clinical isolates solely on the basis of their gas chromatographic profiles. When these isolates were subsequently processed through a larger number of biochemical tests (10), the new identities suggested by gas chromatography were confirmed.

Gas chromatograms of TMAH-digested microorganisms show the presence of a relatively large number of components. It appears that there will, in time, be a need to determine the exact chemical nature of the components being separated. Studies, at present incomplete, com-
paring the behavior of these components on both polar and nonpolar stationary phases suggest that a number of the components may represent methyl esters of cellular fatty acids. This seems especially true for those components located in the last two-thirds of each chromatogram. The analytical conditions presented in this paper do not represent a set of rigidly defined parameters, but merely a compromise between a number of alternative choices. The use of a temperature-programable oven appears necessary to keep the analysis within reasonable time limits and also to produce peaks narrow enough for the use of a peak-height quantitation procedure. Doubling of the rate of the oven temperature program has shortened the total analysis time and has still enabled the identification of the bacteria. However, the extreme base-line rise resulting from the use of temperature programming limits the useful lower gas chromatograph sensitivity settings which can be used when one uses chromatographic columns containing low-boiling or heat-labile polar stationary phases such as DEGS (Fig. 2A). Such a base-line drift also does not represent the most effective base for the determination of peak heights. Fortunately, this problem of baseline drift can be minimized by the use of a dual
(matched) column gas chromatographic system operating in a compensated mode. Base-line drift can also be minimized by the use of nonpolar, higher-boiling stationary phases such as methyl or methyl phenyl silicones. A temperature-programed, nonpolar stationary phase (SE 30) will effectively separate many of the components present in the sample. The increased base-line stability also enables analyses to be performed at lower gas chromatograph sensitivity settings. An additional advantage is that higher-boiling components which have been noted in digests of mycobacteria are more rapidly eluted. Despite some advantages inherent in using heat-stable, nonpolar columns, we presently are using DEGS columns for our routine analysis of TMAH digestates. There seem to be some components in fungal digestates which are not resolved on an SE 30 column. More importantly, DEGS columns appear to bring about a more complete separation of the reference peak from other peaks in the chromatogram. We have found this to be an advantage when using electronic area integration devices as a basis for characterizing organisms as a function of relative peak areas rather than relative heights.

By using suitably scaled-down procedures, we managed to produce chromatograms from single, 2- to 3-mm bacterial colonies. We also feel that with some organisms it may be possible to bypass the heating stage in sample preparation. We obtained representative chromatograms after bacterial cells were merely suspended in

Fig. 4. Normalized gas chromatographic profiles of some weakly saccharolytic and nonsaccharolytic pseudomonads. (A, B) Pseudomonas acidovorans, strain GC8 and 47H, respectively; (C, D) P. denitrificans, strains PD1 and 41H; (E, F) P. diminuta, strains GC12 and 44H; (G, H) P. alcaligenes, strains OS1 and GC19.
methanolic TMAH pentahydrate solution and then injected directly into the 320 C gas chromatograph injection port. This technique seemed to work well with those organisms which do not form an unworkable viscous mass when exposed to TMAH.

The use of a single-step preparative reagent such as methanolic TMAH may serve as a basis for an easy and rapid evaluation of a large number of clinical isolates. Overall, it appears that it may be possible to characterize an organism within 45 to 90 min after harvesting some of the growth from a primary isolation plate. Harvesting of cells from a differential medium such as triple sugar-iron agar should present few additional problems. These procedures should lend themselves to automation (T. J. Wade, R. J. Mandle, and R. W. Schaedler, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 112, 1973). These premises are currently under investigation along with an on-going analysis, using our present technique, of additional strains of nonfermentative gram-negative bacteria. Application of TMAH procedures to yeasts and molds, as well as mycobacteria, has been implemented.

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