CLASSIFICATION OF MICROORGANISMS BY ANALYSIS OF CHEMICAL COMPOSITION

I. Feasibility of Utilizing Gas Chromatography

K. ABEL, H. deSCHMERTZING, AND J. I. PETERSON[1]

Research Division, MelPar, Inc., Falls Church, Virginia

Received for publication 13 December 1962

ABSTRACT

ABEL, K. (Melpar, Inc., Falls Church, Va.), H. deSCHMERTZING, AND J. I. PETERSON. Classification of microorganisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. J. Bacteriol. **85:**1039–1044. 1963.—The feasibility of utilizing gas chromatography as a sensitive and rapid method for the analysis of lipids as a natural basis for the classification of microorganisms by chemical composition was investigated. The lipids were extracted and transesterified to component carboxylic acid methyl esters in a single step, after which the methyl esters were resolved by gas chromatography to provide distinctive chromatographic elution patterns. Similarities in the lipid carboxylic acid distribution were noted among selected species of the family *Enterobacteriaceae*, and significant differences were noted among selected families of the class *Schizomycetes*.

Methods for the classification of microorganisms are based upon an analysis of their physical and biochemical characteristics. Although physical characterization has been based largely upon factors which do not change appreciably upon the death of the organism, this is not true of chemical characterization methods. The chemical methods presently in use (with the exception of staining techniques) require living organisms because products of metabolism, enzymatic or antigenic reactions, or chemically induced changes in growth rate or morphology are analyzed. Justifiable criticism has been directed against these methods from many quarters (for example, Baldacci, 1961); yet, in the apparent absence of distinct natural or evolutionary relationships, these methods have provided a workable system of bacterial classification.

Wolochow (1959) suggested that microorganisms (both living and nonliving) could be differentiated from higher forms of life on the basis of chemical compounds which are unique to microorganisms. Extension of this concept to provide for both quantitative and qualitative analysis of several selected compounds could reasonably result in a method capable of differentiating between individual species. The similarities as well as the differences in chemical composition would provide a better theoretical basis for classification than is presently available, because differences in chemical composition would be governed by natural or evolutionary relationships, provided the bacteria are grown under defined conditions.

For characterization of chemical composition to be practical as a method for classification, extremely selective, sensitive, and rapid methods would have to be used. To complement present methods, the analysis should, at a minimum, be of such sensitivity that a single normal-sized colony of microorganisms (such as those grown on Millipore filters) would be sufficient. The method should require no more time than the preparation and examination of a smear from such a colony by optical microscopy and should require no greater skill. It should be sufficiently selective that neither nutrient nor inorganic contaminants would interfere. At present, the only analytical method which appears to have the degree of sensitivity, rapidity, and selectivity required for such analyses is gas chromatography.

In the 10 years since James and Martin (1952) first reported a successful separation by gas-liquid chromatography, this method has developed more rapidly than any other analytical technique. More than 100 different components have been separated on a single chromatographic

[1] Present address: Woodard Research Corp., Herndon, Va.

column (Desty, Goldup, and Swanton, 1961), and detectors capable of sensing as little as 10^{-13} g of component have been developed (Lovelock, 1961) and are in widespread use. Complex mixtures have been separated in minutes, and simpler mixtures have been chromatographed in seconds (Desty et al., 1961). Furthermore, the cost of adequate gas chromatographic equipment need not be greater than the cost of a research microscope.

To test the feasibility of using chemical composition as a basis for classification, use was made of gas chromatography to provide patterns representing the carboxylic acid methyl esters of the lipids from selected bacteria. This chromatographic presentation provides a qualitative array of the different component acids and shows their relative proportions. The gas chromatographic technique alone may be insufficient to provide a complete separation of all the carboxylic acid methyl esters, since unsaturated acid retention times may overlap the retention times of saturated acids. In the present work, the procedure chosen does not provide for additional separatory techniques to enhance resolution of all components. Instead, a simple method developed by the authors for the simultaneous extraction and transesterification of lipids in bacteria was adopted (Peterson, deSchmertzing, and Abel, 1962). This method is rapid and amenable to extremely small quantities of bacteria. The separatory characteristics of the gas chromatographic technique may be varied by choice of chromatographic liquid phase; for example, diethylene glycol succinate may be used to resolve esters according to degree of saturation and silicone rubber may be used to separate esters according to boiling point.

MATERIALS AND METHODS

Eleven bacteria were chosen for this study: *Micrococcus ureae*, *Gaffkya tetragena*, *Escherichia freundii*, *E. coli*, *Aerobacter cloacae*, *Klebsiella aerogenes* (*K. pneumoniae*), *Proteus vulgaris*, *Serratia marcescens*, *Pasteurella tularensis*, *Bacillus subtilis* var. *niger*, and *B. anthracis*. The first seven were grown in our laboratories; the last four were supplied by the U.S. Army Biological Laboratories. *B. subtilis* and *B. anthracis* were in the spore form. The culture media used were BBL Trypticase Soy Broth (TSB) and, for experiments comparing the effects of nutrients, Koser Citrate Medium (KCM). These media were chosen because they are very dissimilar yet both are lipid-free.

Only one strain of each species was analyzed. The strains were grown from lyophilized cultures obtained from the American Type Culture Collection. In all but one set of experiments, the bacteria were harvested in the accelerated death phase. The bacteria were sedimented, washed in isotonic KCl, suspended in distilled water, and lyophilized. Smears obtained during the growth period were stained and examined by optical microscopy to determine the purity and identity of the cultures.

In one set of experiments, *E. coli* was harvested approximately halfway through the phase of logarithmic increase, during the phase of negative growth acceleration, and during the accelerated death phase, to examine briefly the gross effects of active reproduction on the lipid distribution in the bacteria.

The bacteria, in lyophilized form or immediately after centrifugation in certain cases, were treated by the boron trichloride extraction-transesterification technique outlined below. The apparatus shown in Fig. 1 was used.

Extraction-transesterification procedure. Approximately 0.5 g of bacteria was weighed and transferred to a 25-ml volumetric flask; 10 ml of methanol and a glass-covered magnetic stirrer were added. The reflux condensor was attached. The assembly was placed on a hot plate-stirrer, and the stirring rate was adjusted to prevent the bacteria from settling and to eliminate localized overheating. The boron trichloride delivery tube was inserted with the gas already passing through it at a rate delivering about 1 g of BCl_3 in 2 min. In about 1 min, refluxing began from the reaction of BCl_3 with the solution. The hot plate then maintained the required temperature for refluxing. At the end of 2 min of BCl_3 addition, the gas-delivery tube was removed from the solution and the gas shut off. The solution was refluxed for 10 min. The flask contents were transferred to a separatory funnel containing about 75 ml of distilled water. The methyl esters were extracted with diethyl ether, using centrifugation to aid in the extraction. On the chromatographic columns that were used, water trails badly and disturbs the pattern of the chromatograms. Accordingly, the ether solution was dried of water and its volume condensed with nitrogen

over 3 to 5 g of silica gel. This treatment was necessary only when using the thermal conductivity detector, which is sensitive to water, and was not necessary when using the hydrogen flame ionization detector, which is insensitive to water. The solution of esters was then transferred to a 5-ml graduated cylinder with three ether washings of the silica gel. A test showed that no loss of esters occurred by treatment with silica gel. The ether solution was adjusted to the desired volume, usually 2 ml, and was transferred to a 5-ml syringe vial containing about 0.25 g of silica gel. For each chromatogram (Fig. 2 and 3), sufficient ether solution was used to provide samples corresponding to 25 mg of bacteria (dry weight).

The chromatograms shown in Fig. 2 through 4 were obtained with an F & M Scientific Corp. (model 500) linear programmed gas chromatograph equipped with a hot-wire thermal conductivity detector. In all cases SE 30 silicone rubber (General Electric Co., Waterford, N.Y.) on 160/170 mesh Anakrom ABS (Analytical Engineering Laboratories, Inc., Hamden, Conn.) was used as the liquid phase. For the illustrated chromatograms the columns were temperature-programmed from 125 to 300 C at 5.6 C/min. Because the thermal conductivity detector is comparatively insensitive, a Beckman hydrogen flame ionization detector was subsequently used for sensitivity determinations.

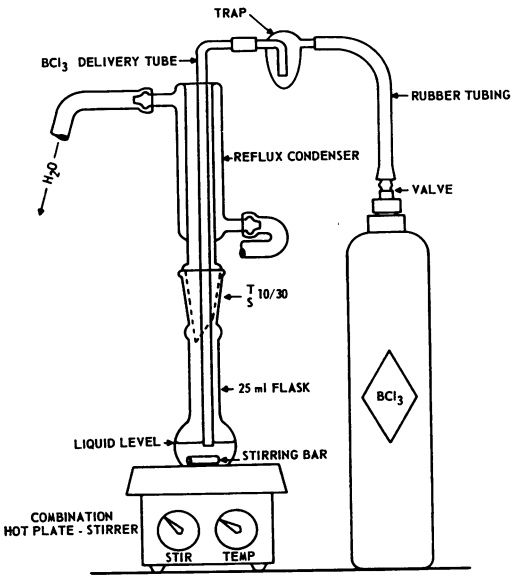

FIG. 1. *Apparatus for direct extraction-transesterification of bacterial lipids.*

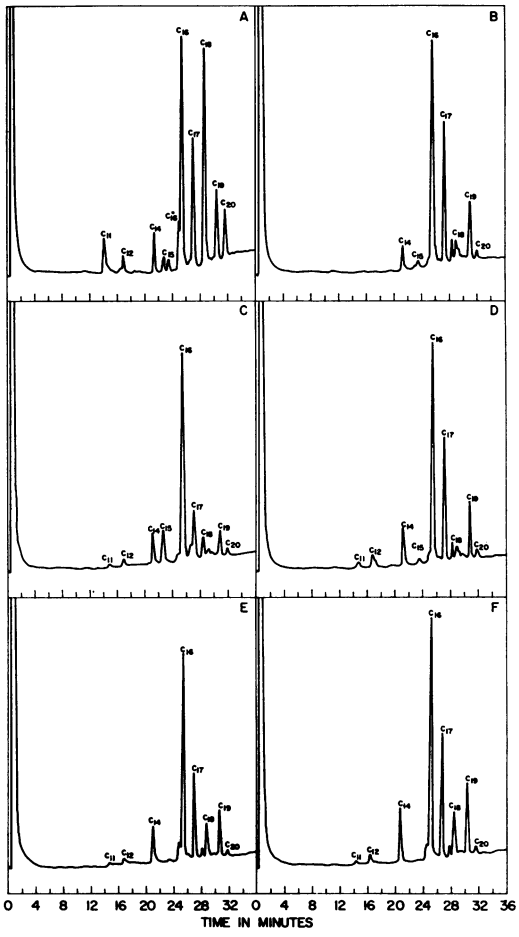

FIG. 2. *Comparison of genus differences in family Enterobacteriaceae. A, Escherichia coli; B, Serratia marcescens; C, Proteus vulgaris; D, E. freundii; E, Aerobacter cloacae; F, Klebsiella aerogenes (K. pneumoniae).*

No attempt was made to identify positively the esters present; the elution times for each component were compared with the elution times of esters of known composition (provided by the Metabolism Study Section, National Institutes of Health), and it is on this basis only that tentative identifications were made to provide a basis for comparison. The subscripts shown adjacent to each peak refer to the number of carbon atoms of the normal carboxylic acid, the methyl ester of which acid has an equivalent elution time; for example: C_{16} signifies a 16-

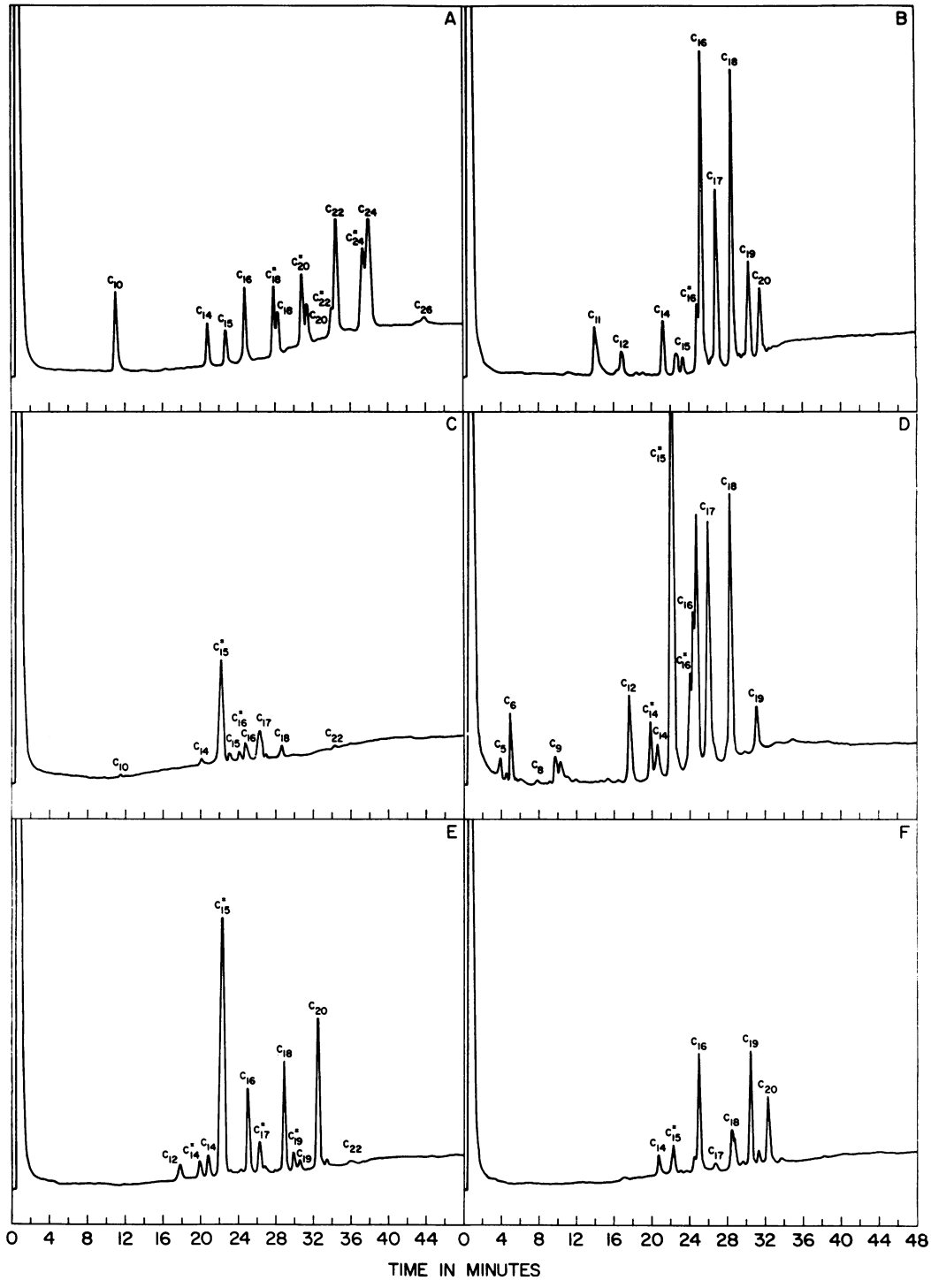

FIG. 3. *Comparison of family differences in class Schizomycetes. (A) Parvobacteriaceae, Pasteurella tularensis; (B) Enterobacteriaceae, Escherichia coli; (C) Bacillaceae, spore form of Bacillus subtilis; (D) Bacillaceae, spore form of Bacillus anthracis; (E) Micrococcaceae, Gaffkya tetragena; (F) Micrococcaceae, Micrococcus ureae.*

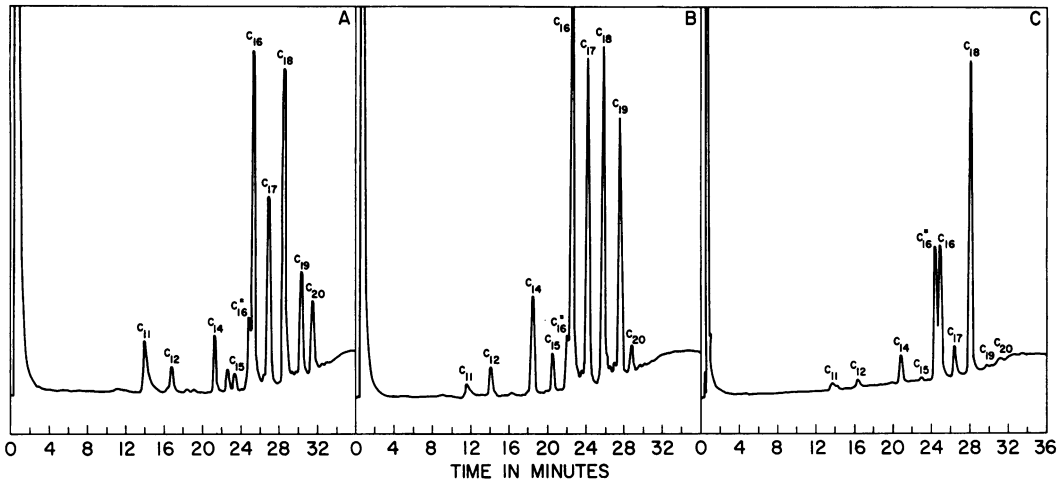

FIG. 4. *Comparison of patterns for different growth periods of Escherichia coli. (A) Accelerated death phase; (B) negative growth acceleration phase; (C) logarithmic increase phase.*

carbon saturated acid while $C_{16}^{=}$ signifies the unsaturated 16-carbon acids.

RESULTS

The chromatograms in Fig. 2 are grouped to show the similarities among six members of the *Enterobacteriaceae*. Although the same carboxylic acids are present, they differ in relative proportions. This is particularly evident for the relative amounts of the C_{16}, C_{17}, C_{18}, and C_{19} carboxylic acids. In Fig. 3 the differences among four families are shown. In chromatograms C and D (*Bacillaceae*) the similarities are not as great as the similarities existing among the various members of the family *Enterobacteriaceae* (Fig. 2). This may be due to the fact that the chromatograms were obtained from the spore forms of these two organisms rather than from the vegatative form.

When *E. freundii* and *A. cloacae* were grown on both TSB and KCM, some changes in the carboxylic acid distribution were evident. These changes were of sufficient magnitude to make species identification difficult, but the pattern characteristics remained distinctive of the family *Enterobacteriaceae*. *K. aerogenes* (*K. pneumoniae*) was induced to grow in both the filamentous form and in its normal encapsulated form. There were no recognizable differences in carboxylic acid distribution.

Figure 4 shows a comparison of the patterns obtained from *E. coli* at different growth stages. Chromatogram A represents the carboxylic acid distribution during the accelerated death phase, B is the distribution during the phase of negative growth acceleration, and C is the distribution during the phase of logarithmic increase. Chromatogram A represents 80 mg for full-scale response, B represents 33 mg under the same chromatographic conditions, and C represents only 7 mg. This demonstrates a much higher concentration of lipid in the actively reproducing bacteria, with the greatest difference appearing in the relative amounts of C_{16}-saturated and C_{16}-unsaturated carboxylic acids. These very significant changes in acid distribution require further research to determine the degree of interference which might be encountered owing to variations in the bacteria during growth.

Lyophilization of the bacteria is not essential. Because of the rapid dehydrating effect of BCl_3, wet, freshly sedimented bacteria have been extracted-transesterified equally as successfully as dry bacteria. This factor, plus the realization that the use of very small bacterial samples would materially reduce the already short time involved in the extraction-transesterification step, indicates that this approach does potentially have the desired speed of analysis.

The hydrogen flame ionization detector is capable of obtaining carboxylic acid ester patterns equivalent to those shown in Fig. 2 to 4 from 10^{-7} to 10^{-9} g of bacteria, which is well within the suggested sensitivity requirements.

DISCUSSION

These studies have demonstrated that gas chromatographic analysis of chemical constituents has potential feasibility as a method for the rapid classification of bacteria. In this study, only lipids were considered. It is possible that amino acid distribution or carbohydrate composition could provide similar or better results. The use of lipids, combined with the rapid extraction-transesterification method described in this report, appears to offer the advantgaes of speed and simplicity which would be desirable in a practical method of classification.

The necessity for further investigation with regard to spore versus vegetative cells and with regard to the degree of consistency of the carboxylic acid distribution of different strains is evident. This additional research into biological reproducibility is required before it can be satisfactorily established that chemical composition is or is not a practical basis for classification.

ACKNOWLEDGMENTS

We are indebted to B. Warshowsky for suggesting the application of this approach to the lipid portion of bacteria.

This work was supported under contract to the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.

LITERATURE CITED

BALDACCI, I. 1961. The classification of actinomycetes in relation to their antibiotic activity. Advan. Appl. Microbiol. **3**:252–278.

DESTY, D. H., A. GOLDUP, AND W. T. SWANTON. 1961. Performance of coated capillary columns. ISA (Instr. Soc. Am.) Proc. **3**:83–95.

JAMES, A. T., AND A. J. P. MARTIN. 1952. Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem. J. **50**:679–690.

LOVELOCK, J. E. 1961. Ionization methods for the analysis of gases and vapors. Anal. Chem. **33**:162–178.

PETERSON, J. I., H. DESCHMERTZING, AND K. ABEL. 1962. Transesterification of lipids with boron trichloride. Abstr. 142nd Meeting, Am. Chem. Soc., p. 21B.

WOLOCHOW, H. 1959. Detection of airborne microorganisms through their unique compounds. Armed Services Technical Information Agency Rept. no. 211170, p. 2–18.