Applications of Cellular Fatty Acid Analysis

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INTRODUCTION

Medically important microorganisms can be identified in many ways. Conventional methods rely on the expression of certain properties that are usually mediated directly by enzyme activity. Extension of this approach to include numerical identification (120) or automated systems to analyze results often strengthens conclusions. Immunodiagnostic and nucleotide hybridization techniques have improved sensitivity, specificity, precision, and ease of testing. Chemotaxonomy is also precise and can result in the definition of highly discriminatory properties. Cellular fatty acid (CFA) analysis falls into this category (146). In contrast to antigen detection and hybridization, this technique does not require a probe. The main requirement of CFA analysis is proper instrumentation. Technological development by industry involved in the manufacture of chromatographs and capillary columns has made it possible for microbiologists to use gas-liquid chromatography (GLC) more easily. Improved microelectronics and computer-aided interpretation of data have also facilitated numerous applications of GLC in clinical microbiology.

In practice, whole cells of bacteria or yeasts are treated to cause release of their fatty acids, which are converted to a methyl ester derivative and then analyzed by GLC. Early attempts to apply CFA analysis to bacterial identification were made in the 1950s (62). In 1963, Abel et al. (1) were the first to present evidence suggesting that CFA analysis by
GLC could successfully identify bacteria. Other early studies were aimed at CFA analysis from the standpoint of bacterial virulence factors. It was recognized that rough (avirulent) strains of *Vibrio cholerae* lacked branched-chain CFAs but that such chains were present in smooth (virulent) strains (14). An alteration of the lipid A composition of *Salmonella* spp. was reported to correlate with a change in virulence (97). Abel et al. (1) identified different CFA patterns within various members of the family *Enterobacteriaceae* and in some gram-positive bacteria, but no attempt was made to identify specifically the CFAs that characterized each genus or species that they tested. Moreover, the analyses required numerous steps, and the apparatus used for extraction and esterification was cumbersome and technically complicated by today’s standards. Nevertheless, their report established the potential usefulness of CFA analysis and established the foundation of further investigation.

This review will discuss the development of CFA analysis pertaining to microbial identification from the mid-1960s to the present. For the purpose of this review, CFAs will be defined as the components of any cellular lipid that have a carbon chain length of 9 to 20 atoms. This includes the majority of fatty acids located in the cell membrane as glycolipid and phospholipid. It includes the fatty acid constituents of lipopolysaccharides (LPS) but does not include the long-chain (24 to 90 carbon atoms) mycolic acids or the isoprenoid quinones. Understanding the molecular basis and development of the analytic instrumentation is central to an appreciation of the potential use and limitations of this technology. The CFA composition of most microbes has been studied, but data to predict the success of applications in diagnostic microbiology are incomplete in many cases because of the limited scope of the studies or use of older instrumentation. Thus, a fair degree of caution must be exercised in examining the earlier literature and comparing the results of different studies. In addition to changes in technique with respect to chromatographic equipment, the CFA composition of cells varies, at least quantitatively, according to culture conditions (46, 72, 90). Nonetheless, the existing information from these older studies interpreted in light of what has been reported recently can be used to form reasonable conclusions about the role of CFA analysis in clinical microbiology.

**MOLECULAR BASIS OF CFA ANALYSIS**

The source of fatty acids in microbial cells is lipid, primarily that of the cell membranes (e.g., phospholipid) or the lipid A component of LPS in gram-negative bacteria and lipoteichoic acid in gram-positive bacteria. In addition to phospholipid, fungi synthesize sterols (e.g., ergosterol) as a major lipid. The fatty acid content of all lipids is determined by the particular type of biosynthetic pathway of a given species. The process is initiated by synthesis of the coenzyme A ester of a fatty acid, with a molecule of acetyl coenzyme A used as primer. Most bacteria synthesize fatty acids with chain lengths of 10 to 19 carbon atoms, and the most prevalent fatty acids are those with 16 or 18 carbon atoms. The 16-carbon saturated CFA hexadecanoic acid, in particular, is highly conserved among prokaryotes. The variable properties that make an organism’s CFA composition distinctive include quantitative differences in CFA content and the presence of other CFAs, of which more than 100 have been identified (132). The usual profile features 5 to 15 CFAs in significant amounts. Organisms in genera with smaller genomes, such as *Rochalimaea* spp., tend to have few fatty acids, while other eubacteria, such as *Xanthomonas* spp., have more than 20 fatty acids. Bacteria contain some CFAs that are unique, i.e., not generally found in eukaryotic cells. Branched-chain and cyclopropane-containing CFAs characterize many gram-positive and gram-negative bacteria, respectively, but are not found in fungi (or in humans). Conversely, the polyunsaturated fatty acids found in higher organisms tend to be absent in aerobic bacteria. Gram-negative bacteria generally have a higher proportion of saturated and monounsaturated CFAs with an even-numbered chain of carbon atoms than gram-positive bacteria. The latter, represented by *Bacillus* spp. and staphylococci, tend to have saturated, branched-chain CFAs with an odd-numbered chain of carbon atoms and lower amounts of straight-chain, saturated CFAs. Coryneforms and streptococci have straight-chain and unsaturated CFAs.

**Nomenclature of Fatty Acids**

Fatty acids are properly named according to the number of carbon atoms, the type of functional groups, and the double-bond location(s). The systematic name can be simplified by writing C followed by the number of carbon atoms to the left of a colon and the number of double bonds on the right (Table 1). Different conventions are encountered for locating the double-bond positions and cis or trans isomers. w, the lowercase letter omega of the Greek alphabet, indicates the double-bond position from the hydrocarbon end of the chain, and c and t indicate the cis and trans configurations of the hydrogen atoms. It is important to note that, as with other conventions, numbering of branched-chain, cyclopropane-containing, and hydroxy fatty acids proceeds from the carboxyl end of the molecule. To illustrate these principles, Fig. 1 shows the long form of two fatty acids commonly found in bacteria. Some fatty acids were at one time given a common name to reflect the source from which they were originally identified, e.g., “lactobacillic” acid from *Lactobacillus* spp. Since not all fatty acids have been given common names, the use of such terminology can be confusing, and the systematic or simplified form is usually preferred.

**Lipid Content of Microbes**

Figure 2 illustrates typical cell membrane structures and hypothetical examples of commonly found fatty acid moieties in two phospholipids. Figure 3 illustrates the lipid A molecule of *Escherichia coli*. The fatty acids constituting *E. coli* lipid A are qualitatively and quantitatively reflected in the results of whole-cell CFA analysis with respect to the relative proportions of C14:0, C16:0, and 3-OH-C14:0. While 3-hydroxy fatty acids in general are a marker for gram-negative bacteria, 3-OH-C14:0 is indicative of the type of lipid A found in *E. coli* (53). The fatty acid composition of lipid A in other gram-negative bacteria may be different from that of *E. coli* lipid A and similarly is reflected in the CFA analysis of whole cells. For example, the lipid A of *Bacteroides fragilis* contains five fatty acids instead of the six found in *E. coli* (161). The CFAs of *B. fragilis* found in LPS are iso-C14:0, 3-OH-C14:0, iso-3-OH-C16:0, 3-OH-C16:0, and 3-OH-C17:0. Besides the lipids and fatty acids typical of other bacteria, many anaerobes also contain unique lipids called plasmalogens (56). These are phospholipid analogs with an ether linkage instead of the usual ester linkage of a fatty acid.
to a glycerol carbon. The derivative formed is a dimethylacetal instead of the typical methyl ester derivative.

INSTRUMENTATION AND DATA PROCESSING

The only commercially available GLC system dedicated to the identification of bacteria and yeasts by CFA analysis is the Microbial Identification System. It was developed by the Hewlett-Packard Co. and Microbial ID, Inc., Newark, Del., and is now marketed by Microbial ID. The system consists of a gas chromatograph equipped with a flame ionization detector, 5% methylphenyl silicone fused-silica capillary column (25 m by 0.2 mm), automatic sampler, integrator, computer, and printer (163). The original data base for identification of aerobic bacteria was developed by Sasser (132). Software libraries for the identification of a large number of aerobes, anaerobes, mycobacteria, and yeasts have subsequently been developed and updated. Equipment designed and dedicated for the purpose of microbiologic identification is not a necessity, but the Microbial Identification System greatly facilitates the control of conditions during analysis and the interpretation of results. An element of automation is added by the automatic sampler, which lets the operator run up to 100 samples without intervention. Upon injection of a sample into the column containing a specified flow of hydrogen (carrier) gas, the fatty acids are separated because of different retention times under conditions of increasing temperature. A computer-controlled temperature program begins at 170°C and is gradually increased to 270°C at 5°C/min. When the methyl ester derivatives reach the end of the column, a signal from the flame ionization detector is recorded as a peak by the integrator. The area under the peak reflects the relative amount of individual fatty acids. The retention time of a mixture of known fatty acids is used by the computer, or by the individual if done manually, to calculate an equivalent chain length for the molecule. The equivalent chain length is equal to the number of carbon atoms of a straight-chain saturated fatty acid or to a number that can be calculated by interpolation with a mathematical formula for other fatty acids. The accuracy of naming fatty acid peaks by comparing retention times with those of a known mixture is high when the computer is used, but definitive identification can be made only by mass spectrometry. For most applications, mass spectrometry is unnecessary. The amounts of CFAs detected are calculated as a percentage of the total amount, and a summary can be printed at the end of each run to show the names and amounts of the CFAs and, optionally, the most likely identification according to similarity to entries in the database (132, 163). The multivariate statistical method of principal-component analysis is used by the computer as the basis for interpreting data and matching an unknown with data base entries. Each CFA quantitatively represents the objects of the principal-component analysis, resulting in pattern recognition as the basic means of identifying an isolate (89). Numerical analysis of CFA data can also be performed, resulting in a computer-generated dendrogram. An unweighted pair-matching method may be used to show similarities at the genus, species, and subspecies levels (128).

METHODS

The usual preparation of samples for CFA analysis consists of hydrolysis of the whole-cell fatty acids to form sodium salts and then methylation of the CFA esters to make them volatile in the gas chromatograph. Various procedures involving acid or base hydrolysis followed by esterification with methanol have been described before (78, 95, 99, 111, 134). Recent improvements in methods have optimized the recovery of CFAs that formerly were difficult to identify.

TABLE 1. Nomenclature of some fatty acids commonly found in bacteria

<table>
<thead>
<tr>
<th>Type of fatty acid</th>
<th>Systematic</th>
<th>Simplified</th>
<th>Common</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dodecanoic</td>
<td>C12:0</td>
<td>Lauric acid</td>
<td></td>
</tr>
<tr>
<td>Tetradecanoic</td>
<td>C14:0</td>
<td>Myristic acid</td>
<td></td>
</tr>
<tr>
<td>Hexadecanoic</td>
<td>C16:0</td>
<td>Palmitic acid</td>
<td></td>
</tr>
<tr>
<td>Octadecanoic</td>
<td>C18:0</td>
<td>Stearic acid</td>
<td></td>
</tr>
<tr>
<td>Eicosanoic</td>
<td>C20:0</td>
<td>Arachidic acid</td>
<td></td>
</tr>
<tr>
<td>Unsaturated</td>
<td>cis-9-Hexadecenoic</td>
<td>C16:3a,7c</td>
<td>Palmitoleic acid</td>
</tr>
<tr>
<td></td>
<td>cis-9-Octadecenoic</td>
<td>C18:1a,9c</td>
<td>Oleic acid</td>
</tr>
<tr>
<td></td>
<td>cis-11-Octadecenoic</td>
<td>C18:1a,7c</td>
<td>Vaccenic acid</td>
</tr>
<tr>
<td>Branched chain</td>
<td>13-Methyltetradecanoic</td>
<td>Iso-C15:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-Methyltetradecanoic</td>
<td>Anteiso-C15:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-Methyloctadecanoic</td>
<td>10-Me-C19:0</td>
<td>Tuberculostearic acid</td>
</tr>
<tr>
<td>Hydroxy</td>
<td>3-Hydroxytetradecanoic</td>
<td>3-OH-C14:0</td>
<td>β-Hydroxymyristic acid</td>
</tr>
<tr>
<td>Cyclopane</td>
<td>cis-11,12-Methylene-octadecanoic</td>
<td>C19:0 cyc11,12</td>
<td>Lactobacillic acid</td>
</tr>
</tbody>
</table>

* Adapted from reference 134.
reliably. Miller (96) described a simple washing procedure with NaOH that removes free acids and secondarily prevents the tailing of hydroxy acid peaks in fused-silica capillary columns. This and other refinements in the procedures developed by Lambert and Moss (78, 99) have led to a relatively simple four-step process for preparation of samples (132, 163).

First, after cells (approximately 50 mg, wet weight) are harvested from culture plates incubated for 24 to 48 h, saponification is conducted in a sodium hydroxide-methanol solution for 30 min at 100°C. This liberates the fatty acid from cellular lipid. The second step is methylation with HCl in methanol at 80°C for 10 min. Third, the fatty acid methyl esters are extracted into a solution of hexane and methyl tertiary butyl ether (10 min), and finally, the extract is washed in aqueous NaOH for 5 min and then transferred to a GLC vial which is capped for injection into the GLC. Thus, the entire process of sample preparation takes roughly 1 h. Multiple samples can be processed simultaneously, with the incremental increase in the time required generally proportional to the number of samples.

Derivatives of CFAs other than methyl esters can be analyzed. For direct analysis of clinical material in which increased sensitivity may be required, i.e., requiring electron capture rather than flame ionization detection, halogenated CFAs are prepared. Many of the methods pertaining to the preparation of trichloroethanol esters have been developed by Brooks and co-workers (20, 21, 33) and have been most successfully applied to cerebrospinal fluid (CSF) (21) and diarrheal stool specimens (19). A drawback of these applications is that the sample preparation steps are more involved and time-consuming than those for the preparation of fatty acid methyl esters. In addition, a reversed-phase column chromatography step is recommended to partition CFAs of interest from interfering constituents (34).

**FEASIBILITY OF WIDESPREAD USE OF GLC IN CLINICAL LABORATORIES**

The prospects for using GLC of bacterial CFAs in diagnostic microbiology have been appreciated for several years. The idea was ahead of its time, however, because the technology for making it practical and dependable in the clinical laboratory was lacking. The developmental status of most applications reached a plateau in the 1970s and did not justify widespread use of the method until recently. The situation was aptly summed up by O'Leary in a 1975 review (123): "We are still convinced that fast analyses and proper interpretations of cellular lipid contents can be used to identify pathogens, but so far it seems that this is an idea whose time has not yet come, but is close." Interesting foresight was also contained in the title of a contribution by Moss to the proceedings of the Third International Symposium on Rapid Methods and Automation in Microbiology: "Chromatographic Analysis: a New Future for Clinical Microbiology" (98).

Two major advances that have brought the technology forward in terms of making it appropriate for use in the clinical laboratory can be cited. One is the development and implementation of fused-silica capillary columns (99, 104). In contrast to packed columns and those of greater width, these columns allow reproducible recovery of hydroxy fatty acids and the ability to distinguish several isomers of fatty acids with the same carbon chain length (99). The second advance is the efficient data processing afforded by modern microcomputer systems (5, 48). Of considerable importance also are the contributions of Miller (96) and of Moss and others (78, 95, 99, 111), who defined practical steps for sample preparation in the clinical laboratory. A natural extension of current technology will be further automation and thus reduced technologist involvement in the saponification, methylation, and extraction procedures. It also seems possible to concomitantly scale down the process, since the actual sample requirement is only a few microliters. Thus, fewer cells or even single colonies from primary cultures could be analyzed instead of large inocula from pure subcultures.

**Practical Considerations**

From a practical standpoint, the chief drawback of CFA analysis mainly involves the preparation of samples. Al-
though the complexity and number of steps have been minimized by recent methodologic improvements (78, 99, 132), sample preparation is relatively laborious compared with preparation for other identification methods used in clinical microbiology. While the process is amenable to handling one to many samples at a time, it is impractical to handle very few and laborious to handle very many (≥40 to 50) at once. Sufficient technical expertise is necessary to ensure proper preparation of samples. Complete saponification by proper exposure of cells in a boiling water bath and careful control of the time and temperature during methylation are critical steps. The quantity of cells that must be harvested may be achieved easily with a loopful for some strains but with great difficulty for certain strains that have different growth characteristics or colonial properties.

Properly functioning instruments require attention to maintenance. Except for the regular replacement of column liners and injection port septa, the demands of GLC maintenance are not generally very great. High-purity gases are required, but their acquisition and storage should not be a problem for laboratories that maintain CO₂ incubators or anaerobic chambers. The use of gases is economical, as is the use of other reagents, which can be prepared in large quantities and stored at room temperature. Before analysis, samples can be stored in a freezer if desired. The risk of chemical contamination of samples during preparation must be controlled by using clean glassware and avoiding the introduction of interfering substances, especially those such as particulate rubber or oil matter. In contrast to sample preparation, the performance of GLC, including data entry, storage, and retrieval, is economical in terms of manual labor.

**Comparison with DNA Probes**

There are similarities as well as differences between the two approaches of CFA analysis and use of DNA probes (4, 138) in clinical microbiology. Both are widely applicable, since most pathogens contain fatty acids and all contain DNA (or RNA). In this context, CFA analysis requires sophisticated instrumentation, whereas hybridization technology requires a battery of probes. Both methods potentially offer a high level of sensitivity and specificity, although the ultimate sensitivity and specificity are theoretically achievable with probes combined with DNA amplification. The sensitivity of GLC, however, can be made to exceed that of DNA probes without amplification. By using an electron capture detector, femtomole (10⁻¹²) quantities of a *Mycobacterium tuberculosis* CFA can be detected (20). Both methods can provide results the same day that tests are begun, both have similar requirements of sample preparation, and both potentially can be adapted to direct detection of pathogens in clinical material.

Both techniques also share limitations to their widespread use in clinical laboratories. They are generally unwarranted for common pathogens such as *Staphylococcus aureus* that are easily identified by one or two simple tests. Moreover, although CFA and DNA probe analyses are relatively rapid, many simpler tests are more rapid and less expensive. Though not as significant a problem as that associated with the use of radiolabeled probes, the disposal of waste in the form of organic solvents is a minor drawback of GLC. The use of either approach in smaller laboratories may not be cost-effective because of a relatively high cost for low-volume testing. Conversely, laboratories that provide refer-
ence services, mycobacteriology, or high-volume testing may find either technology equally applicable. Characterization of unusual isolates that may be frequently encountered in a reference laboratory can be more readily achieved by GLC than by DNA hybridization because of the limited array of specific probes available. Many laboratories are beginning to address and evaluate the judicious use of DNA probe technology and should consider doing the same for GLC to advance capabilities in the laboratory diagnosis of infectious diseases.

CHARACTERIZATION OF BACTERIA

Analysis of CFA composition can be done in conjunction with other tests or as the main determinant in identification. In general, the greater the dependence on CFA analysis, the greater the need to control variables such as culture media and growth conditions. These must be controlled as carefully as possible to arrive at valid conclusions from CFA analysis as the basis for differentiation at the subspecies level. On the other hand, medium, length and atmosphere of incubation, and temperature generally have little impact on the qualitative detection of CFAs. The influence of temperature, for example, is seen mainly in the relative proportions of CFAs rather than in loss or gain of major components. In E. coli, an increase in temperature results in decreased unsaturated relative to saturated fatty acids (90). For bacteria that form cyclopropane CFAs from monoenoic acids, cells harvested during the stationary phase of growth contain larger amounts of these CFAs than cells harvested during exponential growth (72, 90).

Identification to Genus or Species

Staphylococcus spp. S. aureus and coagulase-negative species have qualitatively similar CFA compositions (47, 76, 122). They consist mainly of the branched-chain iso-C_{15:0}, anteiso-C_{15:0}, and anteiso-C_{17:0} CFAs and C_{18:0} and C_{20:0} CFAs. From the results of early investigation (65), it was concluded that S. aureus could not be distinguished from S. epidermidis by CFA patterns but that there was a difference between Micrococcus and Staphylococcus spp. The latter species contain iso-C_{19:0}, anteiso-C_{19:0}, and C_{20:0} whereas C_{15:0} is present in Micrococcus spp. but is either absent (65, 139) or present in only very low amounts (47) in Staphylococcus species. With the exception of S. saprophyticus and S. warneri, other species of coagulase-negative staphylococci are not readily distinguished by CFA analysis. S. warneri is characterized by a C_{17} CFA, and S. saprophyticus contains larger amounts of C_{16:0} and C_{20:0} than of other chains (47). It is interesting that the species of Micrococcus studied by Jantzen et al. (65) included Micrococcus mucilaginosus (Stomatococcus mucilaginosus), now recognized with increasing frequency in vascular-catheter-related infections. Their study of five strains revealed the absence of C_{16:1}, which characterized other micrococcal species, and the presence of relatively high concentrations of iso-C_{14:0} and C_{16:0} CFAs.

Notwithstanding the reported similarity between the CFAs of S. aureus and other species, which have probably been studied in some cases as a heterogeneous group, it seems that well-characterized species of the coagulase-negative staphylococci deserve further study to determine the usefulness of CFA analysis in identifying them. The results of one such study have been reported recently. Kotilainen et al. (76) found that the CFA compositions of blood isolates could be used to distinguish S. epidermidis, S. haemolyticus, S. warneri, S. capitis, S. lugdunensis, S. simulans, and S. hominis, provided that results were interpreted by cluster analysis. Quantitative differences in the amounts of individual CFAs were insufficient without computer analysis to distinguish species. These results are not entirely consistent with those of O'Donnell et al. (122), who showed that numerical analysis of CFA data separated only 50% of the strains studied into homogeneous clusters. In view of increased rates of nosocomial infections caused by coagulase-negative staphylococci, this technique may be helpful in readily characterizing (i) isolates from a series of blood cultures in a patient or (ii) paired isolates from indwelling line and venipuncture sites, thus guiding interpretation as to the clinical significance of positive blood culture isolates.

Streptococcus and Enterococcus spp. CFA analysis has been used as an aid in classification of the streptococci. Bosley et al. (12) compared the CFAs of streptococci (175 cultures) with those of related genera. Streptococcus spp. contain C_{16:1}a or, which the genera Aerococcus, Enterococcus, Pediococcus, and Lactococcus do not. Those investigators also reported the presence of significant quantities of two unusual unsaturated CFAs, C_{16:1}a9c and C_{16:1}a7c, in Aerococcus spp. Clinically important enterococci contain the cyclopropane-containing CFA C_{19:0} cyc12, which was noted in an earlier study to be present in reproducibly different amounts among four species of enterococci (2). The lowest amount, an average of 1%, occurs in Enterococcus casseliflavus; intermediate amounts are found in E. faecalis and E. faecium; and the greatest amount, an average of 17%, occurs in E. durans (2). Difficulties involving phenotypic variability at the species level may be overcome to a certain extent, but CFA profiles also indicated heterogeneity among certain species. The profile of "S. mitis" (S. anginosus) differs qualitatively by source of isolates. For example, vaginal strains reportedly contain C_{17:0}, which is absent in oral strains (45).

Attempts to identify Lancefield groups by CFA were made by Drucker (44), who determined that representative strains of different groups had profiles that could be distinguished only by computer analysis. Determining the CFA composition may be more helpful among streptococcal species not identifiable by group carbohydrate antigen. For example, Streptococcus mutans and S. salivarius are distinguished from related species by the presence of C_{20:0} and C_{20:1} (80, 130). A relatively large amount of C_{20:1} can be used to distinguish S. mutans or S. salivarius from a physiologically similar species such as S. bovis (130). In summary, the applicability of CFA analysis for Streptococcus spp. is minimal for those species that can be identified by immunodiagnostic reagents and seems to be greatest for other species and for differentiation among related genera.

Gram-positive bacilli. The coryneforms are probably distinguishable on the basis of CFAs, but many have been difficult to identify to the species level by classical means. Therefore, few studies have applied CFA analysis to this group. In general, these organisms contain large amounts of C_{16:0}, C_{17:0}, and C_{18:1}, and some species contain tuberculo-lesauric acid (TBSA), the 10-methyl octadecanoic acid typical of mycobacteria (6). Corynebacterium diphtheriae does not contain TBSA, but other species of Corynebacterium contain small amounts and Rhodococcus species contain larger amounts of it. GLC with well-characterized strains may aid in overcoming the problems of classification that persist with members of this group. Athalye et al. (6)
compared profiles of unidentified pathogenic coryneforms with those of several reference strains and found similarities that may provide aids to identification.

*Listeria monocytogenes* is characterized by branched-chain CFAs 15 and 17 carbons (both iso and anteiso), C<sub>16:0</sub> and C<sub>14:0</sub> (126). The composition of *Bacillus* species is generally the most complex in terms of branched-chain CFAs, with typically seven or more iso or anteiso forms (70). In addition to large amounts of C<sub>16:0</sub>, *Bacillus cereus* is characterized by iso-C<sub>15:0</sub>, iso-C<sub>13:0</sub>, iso-C<sub>17:0</sub>, iso-C<sub>14:0</sub> and C<sub>17:0</sub> anteiso (119). A large study recently examined 56 isolates encompassing the genera *Corynebacterium*, *Arcanobacterium*, *Actinomyces*, *Brevibacterium*, *Erysipelothrix*, *Oerskovia*, *Propionibacterium*, *Rothia*, *Listeria*, *Kurthia*, and *Jonesia* (7). Strains were assigned to one of two broad groups on the basis of overall amounts of branched-chain versus straight-chain CFA. Several but not all species within the two groups could be precisely identified by further quantitative analysis of the CFA profiles, resulting in the investigators' conclusion that conventional tests in conjunction with GLC should be performed for identification of coryneforms and similar bacteria.

**Mycobacterium and Nocardia spp.** Mycobacteria have been chosen frequently for study by GLC analysis because of their high content of cellular lipid. Because of the abundance of mycolic acids in mycobacterial species, several studies have addressed the separation of compounds of both types, i.e., those with chains of >20 carbons (mycolic acid cleavage products) and those with chains of <20 carbons (constitutive fatty acids) (58, 87, 150). Quantitative analysis of fatty acids belonging to these two groups results in accurate identification to the species level (58, 69, 82, 150, 151). Among isolates encountered over a 2-year period, Jantzen et al. (69) determined that *M. tuberculosis* could be identified without exception, largely on the basis of C<sub>26:0</sub> at concentrations of 1 to 13%. Other studies suggest that differentiation of species may be achieved without establishing special chromatographic parameters to detect mycolic acid cleavage products as well as constitutive CFAs (82, 93, 124, 143). Tisdall et al. (144) concluded that GLC was equally accurate as and more rapid than conventional identification. All isolates of *M. gordonae* and *M. kansaii* and 85% of isolates of *M. tuberculosis* were correctly identified in a prospective clinical study. In a subsequent follow-up study, there were no discrepancies in the identification of 42 isolates of *M. tuberculosis*. Of 325 strains overall, 530 matching identifications were obtained by GLC and biochemical profiles (143). The most frequent discrepancies occurred with *M. avium*, *M. scrofulaceum*, and *M. gastri*.

All species of mycobacteria are characterized by an abundance of C<sub>18:0</sub> and C<sub>16:0</sub> and, with the exception of *M. gordonae*, the single-methyl branched constituent TBSA (69, 82, 144). *M. gordonae* may also be recognized by the presence of iso-C<sub>14:0</sub> (82, 144). However, the closely related species *M. avium*, *M. intracellulare*, and *M. scrofulaceum* cannot be easily differentiated, even on the basis of mycolic acid cleavage products (69). Differentiation of species belonging to Runyon group I (*M. kansaii* and *M. marinum*) is possible (69), as is differentiation of the less commonly encountered species *M. terrae*, *M. xenopi*, *M. flavescens* (93), and *M. malmoense* (69, 150). Unusual branched-chain CFAs, i.e., a 14-carbon chain in *M. kansaii* and a 15-carbon chain in *M. marinum*, distinguish these species (141, 144).

Analysis of CFAs may also be useful in differentiating the group IV rapid growers *M. fortuitum* and *M. chelonae* from *Nocardia* spp. (49). A C<sub>12:0</sub> CFA absent in mycobacteria was identified in nine strains of *Nocardia asteroides* and a CFA identified by mass spectra as 2-methyl-2-octadecenoic acid was found in all strains of *M. fortuitum*, but neither of these CFAs was detected in *M. chelonae* or in *Nocardia* spp. In addition, *M. fortuitum* and *M. chelonae*, but not *N. asteroides*, reportedly contain C<sub>14:0</sub> (49).

**Enterobacteriaceae.** Typical CFAs are primarily saturated C<sub>17:0</sub> anteiso, C<sub>19:0</sub> anteiso, and 3-OH-C<sub>14:0</sub>. Most also contain C<sub>16:1</sub> in variable amounts (9, 88). Clear separation of members on the basis of CFA composition has not consistently been established, particularly by earlier investigations. Differences between strains of the same species have been noted, and similarities may be observed from one genus to the next (88). As with other groups, the explanation is largely the uncertainty of taxonomic relationships. More recent studies, however, suggest that the CFA composition of members of the family *Enterobacteriaceae* is at least genus specific and in some cases specific enough for identification to species (9, 154, 163). For example, *Morganella* spp. can be distinguished from *Proteus* and *Providencia* spp. by the presence of a significant amount of C<sub>12:0</sub> (154). Other minor quantitative differences distinguish *Proteus vulgaris* and *Providencia alcalifaciens* from the other species of the *Proteus-Providencia* complex.

Algorithms based on cluster analysis of CFA data were established by Boe and Gjerde (9) to separate members of the families *Vibrionaceae* and *Enterobacteriaceae*. Within the latter family, Boe and Gjeide were able to identify two species of *Salmonella*, *E. coli*, *Enterobacter cloacae*, *Morganella* spp., and *Klebsiella pneumoniae* on the basis of quantitative differences in 17 CFAs. As taxonomic relationship among members of this group continue to become validated, it will be possible to apply CFA analysis for comparison with phenotypic characterization. It is not expected, however, that certain species or even genera such as *Escherichia* (E. coli) and *Shigella* that have high DNA relatedness could ever be wholly distinguished by CFAs, given the presently accepted classification.

**Vibrionaceae.** The *Vibrionaceae* may be distinguished from the *Enterobacteriaceae* by the presence of an array of branched-chain CFAs of C<sub>16:0</sub> and C<sub>18:0</sub> in addition to greater amounts of C<sub>16:1</sub> (9). Various species of *Vibrio* and *Aeromonas* can be distinguished by CFAs. Urdacl et al. (149) studied *Vibrio* isolates belonging to 22 species and found that satisfactory separation of most of them could be achieved on the basis of principal-component analysis of the CFA data. Isolates identified to species level included *V. vulnificus*, *V. fluvialis*, and *V. damsela*. However, *V. cholerae* could not be distinguished from *V. mimicus*, and *V. parahaemolyticus* clustered with *V. alginolyticus* and *V. natriegens*. Among *Aeromonas* species, the ratio of C<sub>16:0</sub> to C<sub>17:0</sub> was used to separate clinical isolates into the species designated *Aeromonas hydrophila* (mean ratio, 8.6), *A. sobria* (mean ratio, 22.7), and *A. caviae* (mean ratio, 42.3) (26). Low amounts of iso-C<sub>15:1</sub> and iso-C<sub>17:1</sub> may serve to distinguish *Aeromonas* from *Vibrio* species (149), but these CFAs were not reported in *Aeromonas* spp. by other investigators (26).

**Neisseria and Moraxella spp.** CFA analysis was first used with members of the genus *Neisseria* to address problems pertaining to classification. Consistent with findings from DNA studies, results of GLC showed a marked difference between *Neisseria* (*Moraxella*) *catarrhalis* and other species (86). *M. catarrhalis* contains significant amounts of C<sub>14:0</sub> and C<sub>18:0</sub>, but less C<sub>14:0</sub> than is found in the other species (66, 86). The "true" *Neisseria* spp. are qualitatively similar, with the
exceptions of *N. flavaescens* and *N. elongata*, which contain 3-OH-C\(_{16:0}\). The pathogenic *Neisseria* spp. contain large amounts of C\(_{16:0}\), C\(_{14:1}\), C\(_{12:0}\), 3-OH-C\(_{12:0}\), and C\(_{14:0}\) (107). In contrast to Moss et al. (107), who reported essentially identical patterns of CFAs in *N. gonorrhoeae* and *N. meningitidis*, Jantzen et al. (66) presented a numerical analysis of data that suggests that minor quantitative differences may be sufficient to reliably distinguish *N. gonorrhoeae* and *N. meningitidis*.

**Campylobacter and Helicobacter spp.** With the recognition of new species of *Campylobacter*-like organisms, CFA composition has become regarded as an important parameter in characterizing true members of this genus. Human isolates of various other species such as *"Campylobacter cinaedi"* and *"C. fennelliae"* have CFA profiles that distinguish them from other campylobacters. The CFAs individually unique to these organisms suggest that these species may not be members of the genus *Campylobacter*. Their assignment to the genus *Helicobacter* has been proposed (152). Blaser et al. (10) first demonstrated that *C. jejuni* could be differentiated from *C. fetus* or *C. intestinalis* by the presence of C\(_{19:0} \omega 7c\). Three isolates of another species, *C. laridis*, in which C\(_{19:0} \omega 7c\) is absent, were recognized by CFA analysis among isolates that were from patients with gastroenteritis and were initially identified as *C. jejuni* (29). Major differences in the CFA composition of species were further documented by Lambert et al. (83). In addition to the association of C\(_{19:0} \omega 7c\) with *C. jejuni*, they found that a group including *C. fetus* was characterized by the presence of 3-OH-C\(_{14:0}\) and 3-OH-C\(_{16:0}\). This observation has been confirmed recently by Brondz and Olsen (17). Unlike *Campylobacter* spp., *Helicobacter pylori* (formerly *C. pyloridis*) is characterized by unusually low amounts of C\(_{16:0}\) (57). The major CFAs of *Helicobacter* spp. are C\(_{14:0}\), C\(_{19:0} \omega 7c\), C\(_{18:1}\), and C\(_{18:0}\).

**Pseudomonas spp. and nonfermenters.** The genus *Pseudomonas* contains a large number of species that can be characterized by CFA composition, which greatly facilitates identification. This is in part due to the fact that typical CFA profiles of pseudomonads have several CFAs which, when quantitatively analyzed, are distinctive. Examples of nearly all major CFA types, i.e., saturated, hydroxy, branched chain, cyclopropane, and unsaturated, are found in this group (37, 39, 40, 42, 101, 113, 131). The division of *Pseudomonas* species into eight GLC groups (101) parallels RNA homology grouping. The GLC groups I and II described by Moss and Dees (101) in 1976 still correspond to the respective RNA groups consisting of *Pseudomonas aeruginosa* and others (group I) and *P. cepacia* and others (group II). CFA analysis is rarely needed to identify *P. aeruginosa*, of course, because much simpler and more reliable techniques exist. However, Vees et al. (156) proposed the use of GLC to identify nonfermenters routinely, including other pseudomonads belonging to a total of 35 species. They identified 19 distinct GLC groups. Members belonging to these groups differed primarily in the types of hydroxy CFAs (Table 2).

Nonfermenters associated with rarely encountered but important infections can be readily identified by GLC. For example, *Eikenella corrodens* and two other pathogens, a *Kingella* sp. and *Cardiobacterium hominis*, with which it shares certain phenotypic characteristics, are distinguishable by CFAs (157). *Eikenella corrodens* contains less C\(_{14:0}\) than the other two and has C\(_{8:1} \omega 7c\) and C\(_{16:0}\) as the major CFAs and C\(_{16:1}\), C\(_{12:0}\), C\(_{14:0}\), and C\(_{18:0}\) in smaller amounts (125, 157). *Cardiobacterium hominis* is the only one of the

### Table 2. Certain CFAs that distinguish nonfermenters

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<th>CFAs (as described on page 429)</th>
<th>Group I</th>
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<td><strong>C(_{8:1} \omega 7c)</strong></td>
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### Table 1. Certain CFAs that distinguish nonfermenters

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three characterized by the absence of 3-OH-C_{12:0}. Another example is in cystic fibrosis patients, from whom the important pathogen *P. cepacia* can usually be isolated and identified from respiratory specimens with little difficulty by using selective media. However, when a closely related species such as *P. gladioli* is present, differentiation of *P. cepacia* may be problematic. Furthermore, the distinction is likely to be important, since *P. cepacia* is a known prognostic factor in pulmonary disease of cystic fibrosis patients but *P. gladioli* is not. The CFA profile of *P. gladioli* differs from that of *P. cepacia* by the presence of 3-OH-C_{10:0} and substantially smaller amounts of C_{16:1} and C_{17:0 cyc} (27).

**Legionella** spp. **Legionella** species contain large amounts of branched-chain CFAs (81, 100, 103, 115). The major component, iso-C_{16:0}, typically accounts for 25 to 50% of the total CFA and has been used as the basis for dividing isolates into major CFA groups (81). Quantitative differences in conjunction with ubiquinone content allow the differentiation of all Legionella species and may permit more rapid identification than any other means. Certain species may be identified by CFA composition alone. **Legionella longbeachae**, for example, is quantitatively different from the relative amounts of iso-C_{16:0} and C_{18:0} it contains (106). In addition to the branched-chain CFAs, **Legionella** spp. also contain small amounts of hydroxy and cyclopropane CFAs, although the amounts are considerably smaller than in other gram-negative bacteria (81, 103). Through generation of the data for entry in a computer data base such as the Microbial Identification System (81), performance of GLC for identification of Legionella spp. becomes a practical approach for laboratories handling these isolates. Unrelated pathogens that may be confused with Legionella spp. because of growth on charcoal-yeast extract agar (e.g., Francisella tularensis) could also be readily differentiated by virtue of CFA content.

**Miscellaneous bacteria.** Members of the family Pasteurel-laceae possess similar CFAs (15, 64) and Haemophilus influenzae (64) or *H. aphrophilus* (13) closely resemble *Actinobacillus actinomycetemcomitans* in CFA composition, but *Pasteurella multocida* can be separated by its greater amount of saturated and unsaturated C_{18} CFAs. Quantitative differences may also permit differentiation of *H. ducreyi*, *H. paraaemolyticus*, *H. aphrophilus*, and *H. suis* from other species (64). Gardnerella (formerly *Haemophilus* vaginalis) does not resemble Haemophilus species in CFA content. It lacks 3-OH-C_{14:0} and other hydroxy CFAs commonly found in gram-negative bacteria (31, 64). Major CFA constituents of *G. vaginalis* are C_{18:1} and C_{18:0}, with smaller amounts of C_{14:0}, C_{16:0}, and C_{16:1}. Jantz et al. (64) reported the absence of C_{18:2} in *G. vaginalis*, whereas this CFA was identified in amounts from 4 to 20% in the study by Csango et al. (31). This discrepancy illustrates the limitation in drawing conclusions from separate studies in which preparation and analysis of cells for CFA may have been performed by different methods.

Differentiation of the unusual from the more frequently encountered organisms at certain sites of infection can be facilitated by CFA analysis. Four organisms that occur along with *Pasteurella multocida* in wound infections resulting from dog bites were studied by Dees et al. (43). *Pasteurella multocida* was the only one that contained 3-OH-C_{14:0}. *Flavobacterium* spp. (group II-j) and *Capnocytophaga can- imorsus* (DF-2) were similar in possessing large amounts of iso-C_{15:0} but could be differentiated from each other by the presence of iso-C_{17:1} in *Flavobacterium* spp. Group DF-3 organisms contain substantially more anteiso-C_{15:0} than iso-

C_{15:0} (158). Group EF-4 and M-5 organisms contain 3-OH-

C_{12:0}, and they can be separated on the basis of 2-OH-C_{16:0},

C_{anteiso-17:1}, and C_{17:0 cyc}, which are all present in group EF-4 organisms. *Capnocytophaga* spp. have been further characterized and found to contain the uncommon CFAs 3-OH-iso-

C_{14:0} and 3-OH-iso-C_{16:0} (38). These serve to distinguish *Capnocytophaga* spp. from other gliding bacteria, but they also are found in *Flavobacterium* spp. (102).

The application of CFA analysis in studying another formerly unclassified group (1Ve) proved useful in establishing proper classification of the urinary tract pathogen *Oli- gella urealytica* (35). The phenotypic characteristics of this organism resemble those of *Alcaligenes*, *Brucella*, and *Bor- detella* spp., but the CFA compositions of these organisms are sharply different. *O. urealytica* (1Ve) contains C_{18:1} in large amounts; *Brucella* spp., except *Brucella canis* (36), are characterized by large amounts of C_{9:0 cyc} and *Alcaligenes* spp. and *Bordetella bronchiseptica* contain C_{17:0 cyc} (35). Cyclo CFAs were not detected by Jantz et al. (68) in *Bordetella pertussis*, whereas significant amounts were found in *Bordetella parapertussis* and, along with 2-OH-

C_{12:0}, in *Bordetella bronchiseptica*. Additional investigations of the CFA compositions of *Brucella* spp. have shown specific patterns for *Brucella abortus*, *Brucella melitensis*, *Brucella canis*, and *Brucella ovis* (28, 43, 140).

Phenotypically similar members of the *Achromobacter* group can be separated by CFA composition. *Achromobacter* xylosoxidans primarily consists of C_{16:0}, C_{17:0 cyc}, and hydroxy CFAs, whereas other *Achromobacter* species contain large amounts of C_{18:1} and C_{9:0 cyc} CFAs (40).

*F. tularensis* has one of the more distinctive patterns, being characterized by long-chain CFAs (63, 118) and the hydroxy CFAs 2-OH-C_{10:0}, 3-OH-C_{16:0}, and 3-OH-C_{18:0} (63). Because there is little similarity between the CFAs of *F. tularensis* and those of other gram-negative bacteria, GLC is highly specific for the identification of *F. tularensis* (61). *Yersinia pestis* and *Y. pseudotuberculosis* have been reported to contain 16- and 18-carbon hydroxy CFAs, but *Yersinia* spp. are different in containing C_{17:0 cyc} (145).

**Spirochetes.** *Treponema pallidum* purified from rabbit testicular tissue has been inconclusively analyzed in regard to CFA content. The CFAs found (mainly C_{16:0}, C_{18:1}, C_{19:0}, and C_{19:1}) were present in the bacteria but were also found in smaller amounts in uninfected tissue (92). Up to 50% of the total CFAs in *Leptospira* spp. are in the form C_{16:0} (74). The rest are mainly unsaturated C_{16:1}, C_{18:1}, C_{18:2}, and C_{14:2} CFAs.

**Anaerobes.** The most common application of GLC in identification of anaerobes has been the detection of metabolic end products of glucose fermentation. Analysis of whole cells also is potentially useful and has been proposed as offering a practical approach, at least to identification of *Bacteroides* spp. (153). The key in a proposed scheme is the relative amount of the 15-carbon CFAs that predominate in clinically encountered species (94, 95). The species within three GLC groups delineated by Mayberry et al. (95) were identified on the basis of ratios of one 15-carbon CFA to another. The clinically important *Fusobacterium* species are clearly separated from *Bacteroides* spp. by CFAs. *Fusobac- teria* are generally characterized by the presence of 3-OH-

C_{14:0}, C_{14:0}, C_{16:1}, 3-OH-C_{16:0}, C_{18:0}, and C_{18:1} (25, 60, 67). In contrast to *Bacteroides* spp., *Fusobacterium* spp. contain no methyl branched CFAs. The amounts of 3-OH-

C_{14:0} vary according to species, and these differences and the presence of 3-OH-C_{16:0} aid in the identification of *Fuso-
Peptostreptococcus micros to significant quantities of iso-
P. micros. Similarly, with the gens (112), those investigators suggested that quantitative Vol.
characterized group I, which was represented by Clostrid-
tive and quantitative differences in CFAs, but 10 additional too complicated. Toxigenic types A, from C. sporogenes phenotypically by commonly used tests, identification, there may be useful applications in special
anaerobes in general. Beyond providing an aid to routine
botulinum. Additional information is needed to validate the
results of CFA analysis with toxigenic types of C. perfrin-
um were studied by Fugate et al. (52). In contrast to the
Microbial Identification System method was thought to be
other propionibacteria. Significant amounts of branched-
amounts of C15:0 and C16:0, CFAs are also found in these species.
The anaerobic gram-positive cocci have not been suffi-
ciently studied in the context of a clear understanding of their taxonomic status (79). Variable amounts of 14- and
15-carbon branched-chain CFAs serve to differentiate sev-
eral of the species (79, 110). They range from none in
Peptostreptococcus micros to significant quantities of iso- C14:0 in Peptostreptococcus anaerobius and iso-C15:0 in
Peptostreptococcus saccharolyticus (79). Similarly, with the
clostridia, studies demonstrating the utility of CFA analysis in
diagnostic microbiology are lacking. Of 41 isolates studied by
Moss and Lewis (112), all were categorized in four
groups. A large amount of C12:0, C14:0, C16:0, and C16:1 characterized group I, which was represented by Clostrid-
ium perfringens. Group II lacked C12:0. Groups II, III, and
IV, represented respectively by Clostridium sporogenes, C. bifermantans, and C. histolyticum, possessed other qualita-
tive and quantitative differences in CFAs, but 10 additional species were assigned to group IV. Cundy et al. (32) reported
recently that 51 of 52 isolates of C. difficile were accurately
identified by CFA analysis with the Microbial Identification System. However, in comparison with headspace GLC, the
Microbial Identification System method was thought to be
too complicated. Toxigenic types A, B, and E of C. botuli-
um were studied by Fugate et al. (52). In contrast to the
results of CFA analysis with toxigenic types of C. perfrin-
gens (112), those investigators suggested that quantitative
analysis of the CFAs could serve to distinguish types of C. botulinum. Additional information is needed to validate the
usefulness of CFA analysis for Clostridium spp. and the anaerobes in general. Beyond providing an aid to routine
identification, there may be useful applications in special
circumstances, such as the isolation of a Clostridium sp. suspected to be C. botulinum from a wound infection.
Because it is not possible to distinguish C. botulinum from C. sporogenes phenotypically by commonly used tests, GLC could potentially provide a rapid means of distinguishing
the two.

Identification to Subspecies and Epidemiologic Typing

In general, the discriminatory power of CFA analysis is
great enough to provide useful typing systems. Signifi-
cant advantages of this analysis, however, include ease,
reproducibility, storage of information when computerized, and universal typeability. The applicability of GLC for
subgrouping (typing) also depends on the species in ques-
tion. Those with more complex profiles, i.e., the gram-
negative bacilli and especially Pseudomonas, species are
more amenable to GLC typing than are those with simpler
CFA patterns. At least three studies (29, 76, 116) have
attempted to use GLC as a tool for identification to the
subspecies level, and they suggest its use in epidemiologic
typing.
Campylobacter jejuni. Coloe et al. (29) proposed GLC as an
easy method of epidemiologic typing based on CFA analysis of
several human and animal isolates of C. jejuni. The ratio of
C15:1 to C18:0 o-cyc was used to place isolates into one of
three groups, resulting in 85% of the isolates of human origin
and 56% of the isolates of other animal origin being assigned
to separate groups. It is uncertain whether variations in the
amounts of cyclopropene-containing CFAs and the corre-
sponding precursor (C16:1 9 9 or C18:1) can be relied on as true
indicators of difference in CFA composition. If the amounts
of cyclopropene-containing CFA and its unsaturated precu-
sor are combined, there is actually very little difference in
the groups identified by Coloe et al. (29). For example,
instead of a distribution of 14, 21, and 37% for the amounts of
C18:1 in groups I, II, and III, respectively, of human isolates,
those amounts when added to the amounts of C19:0 cyc
become 41, 43, and 48%, representing questionable
differences. A stronger case could be made for distinction
of types by analyzing differences, if they existed, among other
features.

Pseudomonas cepacia and Staphylococcus epidermidis. Not
only use of the overall CFA composition but also computer-
assisted numerical analysis greatly enhance our ability to
recognize valid subgroups (116). In a study of P. cepacia
isolates collected from five cystic fibrosis centers, 42 strains
were divided into five subgroups on the basis of overall CFA
compositions. The distribution in subgroups varied from
center to center, but most centers had a predominant sub-
group, which suggests that this subgroup was acquired in all
cases from a common source. Furthermore, the subgrouping
was corroborated in part by biotype distribution in that a
relatively unusual biotype of lysine decarboxylase-negative
strains all came from the same center, and six of the seven
strains also fell within the same GLC subgroup.
Correlation analysis has been applied recently to the CFA
data on blood culture isolates from 60 patients (76). Of
Staphylococcus strains serially isolated from the same pa-
tient, 97.6% gave a correlation value of >95. The correlation
values of nonidentical strains (isolates from different pa-
tients) of S. epidermidis varied from 34.2 to 94.9, and among
different species, the correlation value ranged from 59 to 77.
Consideration of the use of GLC with numerical or correla-
tion analysis for subgrouping strains collected for epidemi-
ologic studies is warranted when an efficient but not highly
discriminating typing method is desired.

CHARACTERIZATION OF OTHER MICROBIAL SPECIES

A limited number of studies have addressed identification of
microbial pathogens other than bacteria by GLC of CFAs.
Purification of a sufficient quantity of the pathogen in ques-
tion is the primary challenge for those organisms that are not
cultivable on artificial media. GLC can potentially be used
as an aid to identification of those organisms that can be
prepared in sufficient quantity and purity. Rickettsia and
Chlamydia spp. tend to have CFAs similar to those of other
bacteria, whereas of the various yeasts, several are charac-
terized by large amounts of di- and triunsaturated 18-carbon
CFAs.

Rickettsia spp.
The CFAs of five Rickettsia spp. and Rochalimaea quintana
were determined by Tzionabos et al. (148). Rickettsia
rickettsii, R. akari, R. typhi, R. prowazekii, and R. cana-
d were purified by a standard method from yolk sacs, and all
had similar CFA profiles. Large amounts of C16:1 3 16:1, and
C16:0 characterize these species. Quantitative but not quali-
tative differences in CFA composition were found. The

bacterium nucleatum (67). Calhoon et al. (25) used the ratio
of C16:0 to 3-OH-C16:0 to differentiate Fusobacterium nucle-
atum from other species isolated from the oral cavity.
Propionibacterium species contain anteiso-C15:0 or iso-
C15:0 as the most abundant CFA (105). However, Propioni-
bacterium acnes is characterized by relatively minor
amounts of anteiso-C15:0 compared with the amounts in
other propionibacteria. Significant amounts of branched-
chain C17:0 and C16:0, CFAs are also found in these species.

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CFAs of *Rochalimaea quinata* grown on blood agar medium resemble the other rickettsial CFAs except that they lack the 3-OH-C_{14:0} found in the others. Additional studies have examined *Rochalimaea quinata* and revealed similar findings (135, 162). That C_{18:1} accounts for such a large percentage of the total CFAs (40 to 60%) is a distinctive feature of *Rochalimaea quinata*. In contrast to other researchers, Westfall et al. (162) analyzed *Rochalimaea quinata* and related species by electron capture GLC and reported that it contained C_{16:0}. However, they noted that complex media may have been the source of this unusual CFA. Neither of the studies by Tsianabos et al. (148) or Slater et al. (135) found C_{20:0} in *Rochalimaea quinata*.

*Coxiella burnetii* has a CFA profile markedly different from that of other rickettsiae (148, 165). Significant amounts of branched-chain CFAs (anteiso-C_{15:0}, anteiso-C_{17:0}, and anteiso-C_{14:0}) are found, along with C_{16:0}, C_{18:0}, C_{20:0}, and C_{16:1}. The similarity between the CFA patterns of *Coxiella* and *Legionella* spp. is interesting, although DNA hybridization has revealed no relatedness between the two (148).

**Chlamydia spp.**

The lipid elementary bodies of *Chlamydia trachomatis* purified from McCoy cells consist largely of C_{16:0}, iso-C_{15:0}, anteiso-C_{15:0}, and C_{18:0} (8). Serotypes D and L3 can be distinguished from serotypes C and G by quantitative differences in the amounts of the branched-chain C_{15:0} CFAs. *C. psittaci* is identifiable and can be similarly differentiated from the *C. trachomatis* serotypes (8). The CFAs of *Chlamydia* spp. were absent in uninfected McCoy cells except for a minor amount of C_{18:1}, which was common to uninfected McCoy cells as well as to the *Chlamydia* serotype.

**Fungi**

The CFA compositions of various yeasts and a few filamentous fungi have been investigated. Initial studies of yeasts by Gangopadhyay et al. (54), Gunasekaran and Hughes (59), and Moss et al. (114) resulted in somewhat conflicting data with respect to the CFAs detected. Their results and those from more recent studies indicate that at least group-specific and, for some, species-specific CFA profiles can be identified. Substantial quantitative differences among *Saccharomyces* spp. (large amounts of C_{16:1}) and *Candida* spp. (large amounts of C_{18:2} and C_{16:1}) separate these genera. Candida spp. are also characterized by the presence of C_{16:0}, C_{18:0}, C_{18:1}, and C_{18:3} (73). *Torulopsis glabrata* is characterized by a high ratio of C_{16:0} to C_{18:0}, and *Cryptococcus* species can be identified by the absence or only trace amounts of C_{16:1} relative to the amounts in other yeasts (91, 114). The results of a study recently reported by Marumo and Aoki (91) show a 95% overall agreement with standard methods in the identification of *Candida* spp., *T. glabrata*, and *Cryptococcus neoformans*. These results, analyzed by discriminate analysis, yielded rates of correct identification ranging from slightly less than 90% for *Candida parapsilosis* and *Candida albicans* to 100% for *T. glabrata*, *Cryptococcus neoformans*, and other *Candida* spp.

Brondz and Olsen (16) examined both cellular carbohydrates and CFAs and found that multivariate analyses distinguished *Candida albicans* from *T. glabrata* and from *Saccharomyces cerevisiae*. In contrast to other investigators, Brondz et al. (18) reported in another study the finding of significant amounts of C_{12:0} and C_{14:0} in *Candida*, Torulo-

lopsis, and *Saccharomyces* spp. A methodologic variable in the form of alcoholysis with ethanol, propanol, or butanol was reported to influence the recovery of these more volatile CFAs.

Approaches to the identification of filamentous fungi on the basis of CFA composition have not been described. Notwithstanding the greater technical difficulty in working with molds rather than yeasts, CFA analysis may in the future become a useful aid as a supplement to morphologic features for identification. *Fusarium* spp. (137) and *Trichophyton rubrum* (75, 164) are examples of fungi that have been analyzed for CFA content. The mycelial mass of growth from fluid cultures was harvested and subjected to fatty acid methyl esterification by these investigators. At various stages of fungal growth, C_{16:0}, C_{18:0}, C_{18:1}, and C_{19:0} were also found. The principal CFAs (C_{16:0}, C_{18:0}, C_{18:1}, and C_{18:2}) of *Fusarium oxysporum* were similar to those of *T. rubrum*, raising the question of whether there is less diversity of CFAs among molds than among other microorganisms. Additional studies need to be performed with related species and genera to develop more experience before GLC can be applied to the identification of filamentous fungi.

**Blood Parasites**

Schistosomes, trypanosomes, *Leishmania* spp., and plasmodia have been analyzed for CFA composition. *Schistosoma mansoni* purified from infected mice (adult stage of the parasite) has a complex fatty acid composition (136), including saturated, unsaturated, and branched-chain components with carbon-chain lengths of 12 to 24 atoms. The fatty acids in the total lipid fractions in *Trypanosoma cruzi* consist mainly of C_{18:2}, C_{18:1}, C_{18:0}, and C_{16:0} (142). Polyunsaturated acids are also found.

Promastigotes of *Leishmania* spp. have been studied to determine whether CFA composition can be used to differentiate various species. Means other than host specificity are generally unable to separate members of this genus. Vessel et al. (155) concluded that distinctive features of the CFA profiles of *Leishmania donovani*, *L. tropica*, and *L. enriettii* were helpful in identification. A significant amount of C_{18:3} in *L. donovani* and its complete absence in the other two species, along with the absence of all unsaturated 18-carbon CFAs in *L. enriettii*, were the major differences.

Using *Plasmodium lophurae* and *P. berghei* released from erythrocytes, Wallace (160) determined the CFA composition of lipid fractions. Both species have complex profiles consisting mainly of C_{16:0}, C_{18:0}, and C_{18:1}. *P. berghei* was reported to contain greater amounts of saturated CFAs than *P. lophurae*; it was noted that these parasites and the blood of the hosts share similar compositions.

**Mycoplasmas and Viruses**

Because of their requirement for exogenous sources of growth factor from either complex medium or host cells, mycoplasmas and viruses can have widely variable fatty acid compositions. The incorporation of several or as few as one fatty acid into membranes will allow mycoplasmas to grow, while only *Mycoplasma (Acholeplasma) laidlawii* is known to synthesize its own CFAs (127).

Lipid constitutes the envelope of viruses such as influenza virus. Variable amounts of several fatty acids with chains of 12 to >20 carbons have been shown to occur, suggesting that
there may be strain differences in the influenza virus (11). Other investigators have analyzed the fatty acid contents of uninfected and virus-infected cells and found modification in the fatty acid compositions. For example, cells persistently infected by measles virus have more C_{16:0} and less C_{18:1} compared with uninfected or lytically infected monkey kidney cells (3).

Unclassified Organisms

CFA characterization is useful in initially placing unclassified species into groups. Establishing the similarity of a CFA profile to that of a recognized species, especially among the nonfermenters, steers definitive taxonomic studies based on genetic analysis. Examples include determination of the CFA composition of "Flavobacterium-like" (41) and "Moraxella-like" (108) organisms. Many belonging to these groups are unusual opportunistic pathogens that may grow slowly and need to be distinguished from traditional fastidious pathogens. In characterizing a collection of strains that resembled *Methylobacterium extorquens*, Wallace et al. (159) relied heavily on CFA composition to define groups with similarities and groups with differences. CFA analysis may reveal differences not readily apparent by phenotypic characterization. An unclassified species responsible for serious infection in association with chronic granulomatous disease (147) was confirmed to be distinct from *Pseudomonas cepacia* by virtue of containing C_{12:0}, which did not characterize other members of the pseudomallei group. The isolate was phenotypically similar to *Pseudomonas cepacia* and *Pseudomonas pickettii*. Similarly, distinct CFA profiles have recently been established for the agent of cat scratch disease (109) and for an unidentified fastidious organism causing septicemia (135). Of seven CFAs found in the cat scratch disease bacillus, an unusual component, 11-methyloctadec-12-enolic acid, was detected by GLC, identified by mass spectrometry, and proposed to serve as a marker of the cat scratch disease agent (109).

**DETECTING MARKERS OF INFECTION IN CLINICAL MATERIAL**

The possibility of diagnosing meningitis by GLC of CSF was reported in 1976 (133). Initial studies focused on the differences in patterns of infected and uninfected CSF without a particular derivatized compounds with any given pathogen. In most cases, derivatives of more than one class of compounds (acids, alcohols, and amines) were analyzed. Cryptococcal (30, 133), enteroviral (24, 30, 133), herpesvirus (30), measles virus, varicella-zoster virus, Rocky Mountain spotted fever, meningococcal (24), nocardial (22), and tuberculous (21, 30, 51) meningitis have been studied. Modification of GLC resulting in increased sensitivity is central to the direct analysis of clinical specimens (20, 85). Preparation of samples appropriate for fatty acid analysis by frequency-pulsed electron capture (FPEC-GLC) involves production of pentafluorobenzyl (20, 33) or trichloroethyl (21) derivatives. FPEC-GLC alone (20, 21) or mass spectrometry with selected ion monitoring in addition to FPEC-GLC (50, 51, 117, 121) is then used to analyze samples. The latter approach is thought by some investigators to be necessary for optimal resolution of analytes. The greatly increased sensitivity of FPEC-GLC along with the heavy background of fatty acids of host origin are thereby managed. Another approach has been to use reversed-phase chromatography columns (34), which simplify the removal of certain components that cause interfering peaks. For computer-assisted interpretation of results, methods that ignore certain peaks normally encountered in clinical specimens can be installed.

Another special consideration relative to direct CFA analysis is specificity. In contrast to analysis of cells grown in cultures for which a profile of CFAs is interpreted qualitatively and quantitatively to arrive at an identification, FPEC-GLC depends more on detection of selected CFAs as markers indicating a microbe's presence. Thus, any of the CFAs uniquely found among prokaryotes could serve as a marker of infection. In CSF, for example, TBSA has been studied most extensively in this regard (20, 50, 51, 84, 117). Such markers are useful for diagnosis when detected in normally sterile sites and in the setting of a clinical picture consistent with the agent represented by the CFA marker. TBSA is also a component of *Nocardia* spp. (22) and other coryneforms, however, so detection in specimens such as sputum must be interpreted cautiously. Despite these limitations, FPEC-GLC promises rapid diagnosis of tuberculosis because of its good sensitivity. Increasing promise of applicability to the rapid diagnosis of tuberculosis has been shown by two recent studies (21, 117). Muranishi et al. (117) tested specimens from the respiratory tract of 223 patients with active tuberculosis. All of 61 specimens with positive smears and cultures were found to be positive for TBSA (100% sensitivity), and 84% of culture-positive, smear-negative patients were detected by TBSA measurement. Of 160 controls, 9.4% were TBSA positive. In 75 patients with meningitis, Brooks et al. (21) reported 95% sensitivity and 91% specificity of FPEC-GLC for diagnosis of tuberculous meningitis on the basis of either the presence of TBSA in CSF or the FPEC-GLC profile of CSF. FPEC-GLC has also been applied directly to naturally infected armadillo tissue for detection of *Mycobacterium leprae* (77), and attempts have been made to analyze joint fluid for diagnosing septic arthritis (23). Results of the latter study, which compared profiles of gonococcal, streptococcal, and staphylococcal arthritis with those of traumatic synovitis, were probably based on an analysis of metabolic products.

Perhaps it would be useful to reexamine FPEC-GLC for the rapid diagnosis of opportunistic infections as well as of the rarely encountered, diagnostically difficult infections such as tuberculous meningitis in the light of current epidemiology. All of the published experience with FPEC-GLC and diagnosis of cryptococcal meningitis, for example, appeared before recognition of AIDS. Bacteremia due to *Mycobacterium avium-Mycobacterium intracellulare*, common in patients with this syndrome, is often of higher magnitude than bacteremias in immunocompetent adults (166), suggesting that serum might serve as the source of detectable amounts of CFAs in FPEC-GLC. It would be interesting to determine the applicability of the technique to the diagnosis of other relatively common opportunistic infections such as histoplasmosis and to actual detection of the human immunodeficiency virus.

**MEASURING ANTIMICROBIAL RESISTANCE**

Among the many prospects for future development of CFA analysis in clinical microbiology are methods of assessing antimicrobial susceptibility or resistance. The basis of this possibility lies either in the direct effects of antimicrobial agents on membrane lipids or on quantitative analysis of the rate of synthesis and incorporation of fatty acids into lipid. The latter would be analogous to the adaptation of DNA
probes used for identification purposes to a system for rapid sensitivity testing with mycobacteria. Kawa et al. (71) successfully used the Gen-Probe system for isoniazid susceptibility testing of M. tuberculosis in 3 days on the basis of a comparison of the total amount of nucleotide hybridized in the presence and absence of various concentrations of isoniazid. Similarly, one may be able to predict susceptibility or resistance by comparing the total area of CFA peaks from analysis of a strain grown in the presence and the absence of antimicrobial agents.

Fungi offer an interesting problem in this context because all of the newer antifungal agents target membrane lipids. There is either a direct effect on membranes, causing release of fatty acids, or an inhibition of lipid biosynthesis. A marked effect of imidazole antifungal agents on the CFA composition of Candida albicans is seen as a ratio of unsaturated to saturated CFAs that decreases from 2.3 to 1.1 in correlation with growth inhibition (55). A shift in the relative amounts of C18:0 and C16:0 is also seen, thus suggesting that alterations in the overall CFA composition may predict antifungal susceptibility of Candida albicans. It would be important to study resistant strains to confirm that differences exist. Whereas the targets of most antibacterial agents such as the cell wall, protein synthesis, or DNA are not directly taken advantage of for measuring antibacterial resistance, fungal lipid may serve as a practical and useful indicator to measure the effects of antifungal agents.

CONCLUSION

The technology of CFA analysis using GLC has gained applicability for several purposes. It offers considerable power as a tool for microbial identification, because characteristic patterns of CFAs can be defined for several microbes to the species level and results are achievable rapidly. While many applications remain highly specialized, laboratories engaged in mycobacteriology or frequent identification of unusual isolates should consider GLC as an effective approach to identification of these isolates. Experience with CFA analysis of these and other microbial groups for purposes of identification is rapidly developing, making the versatility of the method more fully realized. GLC is presently applicable on a broad scale in conjunction with conventional tests for identification of bacterial pathogens. Other applications, including characterization at the subspecies level, identification of nonbacterial pathogens, and direct analysis of clinical material, are enticing as a new technology for achieving epidemiologically and diagnosis-ally useful information.

ACKNOWLEDGMENTS

Excellent secretarial support was provided by Rose Stursa. Geoffrey Mukwaya and Denise Pickett provided helpful discussions and review of the manuscript.

REFERENCES


Hollis, D. G., R. E. Weaver, A. G. Steigerwalt, J. D. Wenger, and G. M. Frey.


Moss, C. W., and S. B. Dees. 1979. Further studies of the


