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Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids

INTRODUCTION

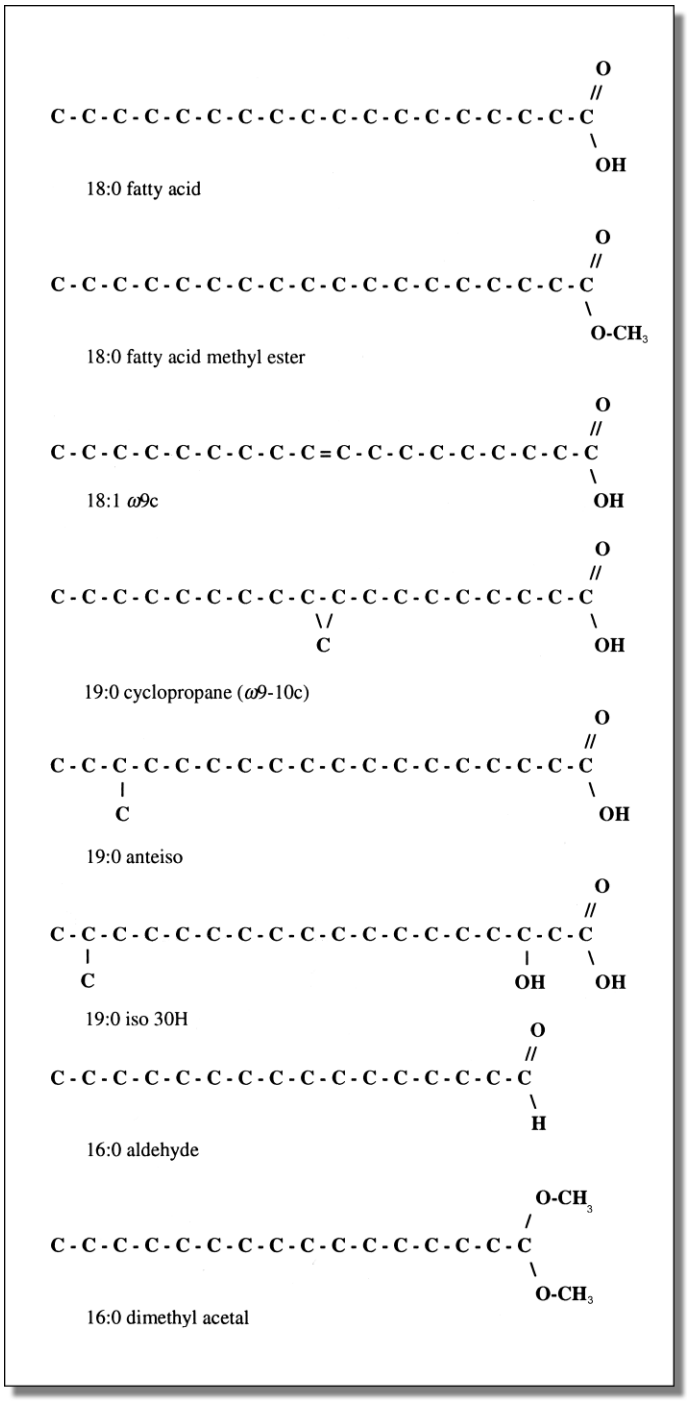
For many years, analysis of short chain fatty acids (volatile fatty acids, VFAs) has been routinely used in identification of anaerobic bacteria. In numerous scientific papers, the fatty acids between 9 and 20 carbons in length have also been used to characterize genera and species of bacteria, especially nonfermentative Gram negative organisms. With the advent of fused silica capillary columns (which allows recovery of hydroxy acids and resolution of many isomers), it has become practical to use gas chromatography of whole cell fatty acid methyl esters to identify a wide range of organisms.

FATTY ACIDS FOUND IN BACTERIA

More than 300 fatty acids and related compounds have been found in bacteria analyzed in the MIDI Research and Development Laboratory. The wealth of information contained in these compounds can be estimated by considering not only the presence or absence of each acid, but also by using the data in quantitative fashion. While the theoretical ability to differentiate among 2^{300} different combinations is not practical due to the nonrandom distribution within groups of bacteria, the huge number of fatty acids creates great "naming" power for the Sherlock MIS.

Sherlock MIS uses fatty acids 9-20 carbons in length. The peaks are automatically named and quantitated by the system. Branched chain acids predominate in some Gram positive bacteria, while short chain hydroxy acids often characterize the lipopolysaccharides of the Gram negative bacteria. The structures of a few of these compounds are shown in Figure 1. In this note, all compounds will be referred to as fatty acids, even though the actual compounds may be aldehydes, hydrocarbons, or dimethyl acetals, and are typically analyzed as the methyl esters. The system of naming used in this note is to count carbons from the "omega" end (i.e. opposite the carboxyl end) and to indicate the other structures where known. The various combinations of features may result in very large numbers of fatty acids. Though most fatty acid identifications have been confirmed by mass spectroscopy, some are still listed as "unknown" or with a letter designating that the double bond position and/or configuration has not been confirmed.

Figure 1. *Structure of Fatty Acids*



CULTURING OF THE BACTERIA

The most stable and reproducible cellular fatty acid profile is achieved by carefully regulating the growth conditions. Several scientific papers have reported the effects of growth temperature and of different growth media on bacterial fatty acid composition. To minimize these variables, a specific temperature and growth medium have been chosen for each library. For example, most aerobic bacteria will grow well on Trypticase Soy Broth Agar (TSBA), which consists of 30g Trypticase Soy Broth and 15g of agar (BBL). Those aerobic bacteria, which will not grow well on TSBA, are grown on the medium which would be most commonly used for their growth in the laboratory (e.g. *Legionella* on buffered charcoal yeast extract, and *Haemophilus* on chocolate agar). The temperature chosen for the TSBA database was 28°C to enable growth of a wide range of organisms. A separate database, CLIN, uses 35°C and blood agar (Trypticase Soy base) as the standards, with specialized media for specific organisms.

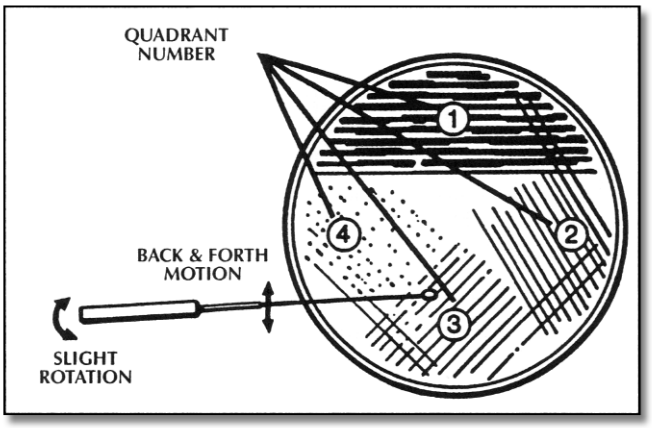
Table 1 *Sherlock Standard Libraries*

Package	Name	Description
Aerobe	TSBA40	Aerobes, 28°C, 24hr, on Trypticase Soy Broth Agar
	CLIN40	Clinical Aerobes, 35°C, 24-48hr, on Blood Agar, Chocolate, etc.
	MI7H10	Mycobacteria, 35°C, on Middlebrook 7H10 Agar with OADC enrichment
Anaerobe	BHIBLA	Anaerobes, 35°C, 48hr, on BHIBLA plates in Gas Paks
	MOORE	VPI Broth-grown Anaerobe Library, 35°C, in PYG Broth
Yeast	YST28	Yeasts, 28°C, 24hr, SAB Dextrose Agar
	YSTCLN	Clinical Yeasts, 28°C, 24hr, SAB Dextrose Agar
	FUNGI	Fungi, 28°C, 2-5 days, SAB Dextrose Broth, 150 RPM shake culture
	ACTIN1	Actinomycetes, 28°C, 3-10 days, Trypticase Soy Broth, 150 RPM shake culture

For the anaerobic bacteria, the plate-based dataset uses cultures grown at 35°C on brain-heart infusion with supplements. A database containing more than 800 entries was developed (by the VPI Anaerobe Lab) using overnight cultures from peptone-yeast extract-glucose broth.

The effect of age is minimized in the broth cultures by harvesting at a given turbidity. When using plate cultures, the growth period is 24 hours for aerobes and 48 hours for anaerobes. Standardization of physiological age of culture is obtained by choice of sector from a quadrant streak on the plate (Figure 2). Slow growing organisms may be incubated for the period of time necessary to obtain adequate growth.

Figure 2 *Quadrant Streak*



REAGENTS

Four reagents are required to cleave the fatty acids from lipids:

Reagent 1, Saponification—45g sodium hydroxide, 150ml methanol, and 150ml distilled water. Dispensing through use of an autopipet assures reproducibility and allows for large numbers of assays in a day.

Reagent 2, Methylation—325ml certified 6.0N hydrochloric acid and 275ml methyl alcohol. This drops the pH of the solution below 1.5 and causes methylation (for the increased volatility in a partially polar column) of the fatty acid. The fatty acid methyl ester is poorly soluble in the aqueous phase at this point.

Reagent 3, Extraction—200ml hexane and 200ml methyl tert-butyl ether. This will extract the fatty acid methyl esters into the organic phase for use with the gas chromatograph.

Reagent 4, Sample Cleanup—10.8g sodium hydroxide dissolved in 900ml distilled water. This procedure reduces contamination of the injection port liner, the column, and the detector. More than 10,000 analyses can be performed on a column prior to needing any maintenance.

SAMPLE PROCESSING

The five steps to prepare GC ready extracts are illustrated in Figure 3.

Harvesting—A 4mm loop is used to harvest about 40mg of bacterial cells from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate. The cells are placed in a clean 13x100 culture tube.

Saponification—1.0ml of Reagent 1 is added to each tube containing cells. The tubes are securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for ca. 5 minutes, at which time the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating.

Methylation— The cooled tubes are uncapped, 2ml of Reagent 2 is added. The tubes are capped and briefly vortexed. After vortexing, the tubes are heated for 10 ± 1 minutes at 80° ± 1°C. (This step is critical in time and temperature.)

Extraction— Addition of 1.25ml of Reagent 3 to the cooled tubes is followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous (lower) phase is pipetted out and discarded.

Base Wash— About 3ml of Reagent 4 is added to the organic phase remaining in the tubes, the tubes are recapped, and tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase is pipetted into a GC vial which is capped and ready for analysis.

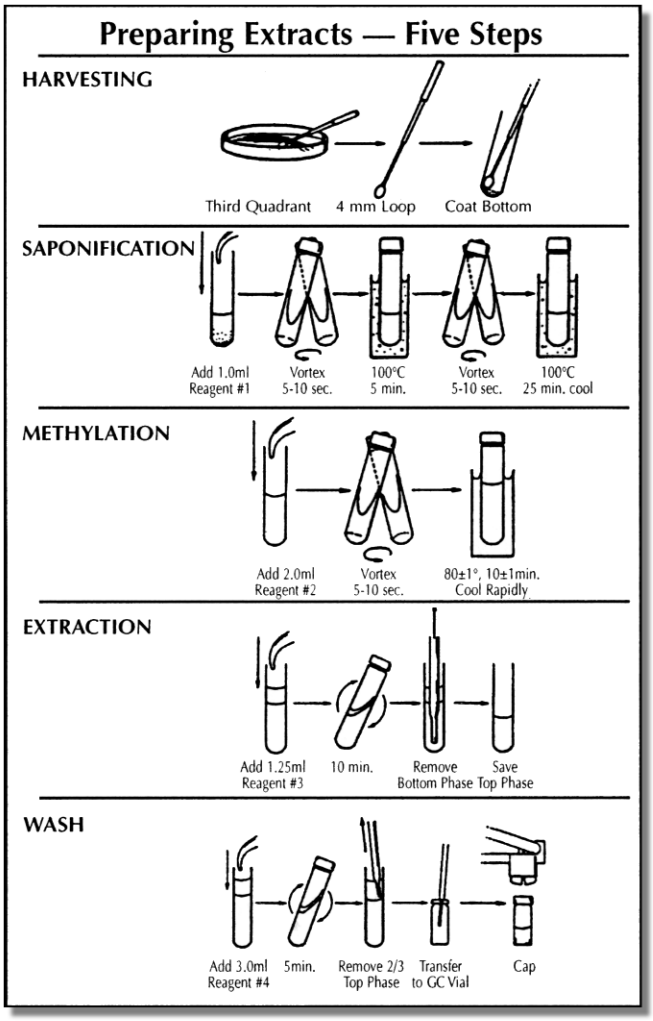


Figure 3 *Sample Preparation*

HARDWARE FACTORS

The Sherlock MIS Software can only be used with the Agilent technologies 5890, 6890 or 6850 gas chromatographs. The Sherlock System's unique configuration is designed for optimal analysis of Fatty Acid Methyl Esters by gas chromatography.

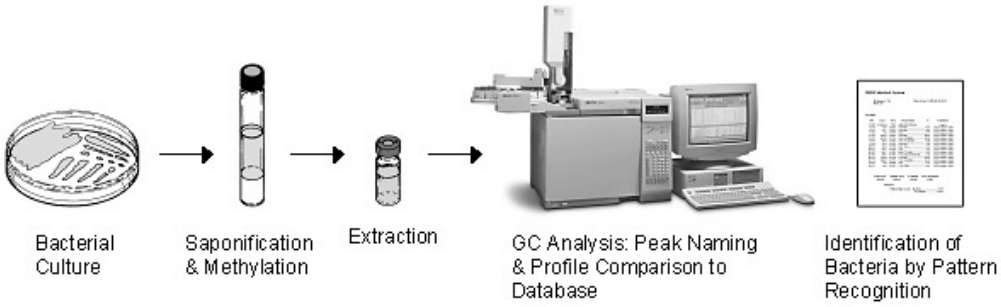
Ultra 2 Column—A 25m x 0.2mm phenyl methyl silicone fused silica capillary column has both the chromatographic performance and the column lifetime desired for routine analysis of bacterial extracts. The column is required to have more than 4,000 theoretical plates per meter for peaks with k = 7 to 9. Since the stationary phase is cross linked to the silica tube, there is less noise and drift during temperature programmed runs.

Gas Chromatograph—The temperature program ramps from 170°C to 270°C at 5°C per minute. Following the analysis, a ballistic increase to 300°C allows cleaning of the column during a hold of 2 minutes. The flame ionization detector allows for a large dynamic range and provides good sensitivity. Hydrogen is the carrier gas, nitrogen is the "makeup" gas, and air is used to support the flame.

Autosampler—Use of an autosampler allows the system to be operated unattended for up to 2 days at a time. Samples are logged into the computerized sample table and all sampling (including STAT samples) is done automatically.

Computer—The electronic signal from the GC detector is passed to the computer where the integration of peaks is performed. The electronic data is stored on the hard disk and the fatty acid methyl ester composition of the sample is compared to a stored database using the Sherlock pattern recognition software.

Figure 4 *Diagram of System*



CALIBRATION AND PEAK NAMING

The Sherlock MIS uses an external calibration standard developed and manufactured by Microbial ID, Inc. The standard is a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy acids. All compounds are added quantitatively so that the gas chromatographic performance may be evaluated by the software each time the calibration mixture is analyzed. The hydroxy compounds are especially sensitive to changes in pressure/ temperature relationships and to contamination of the injection port liner. As a result, theses compounds function as quality control checks for the system

Retention time data obtained from injecting the calibration mixture is converted to Equivalent Chain Length (ECL) data for bacterial fatty acid naming. The ECL value for each fatty acid can be derived as a function of its elution time in relation to the elution times of a known series of straight chain fatty acids.

$$ECL_x = \frac{R_{tx} - R_{tn}}{R_{t\,(n+1)} - R_{tn}} + n$$

Where R_{tx} is the retention time of x; R_{tn} is the retention time of the saturated fatty acid methyl ester preceding x; $R_{t(n+1)}$ is the retention time of the saturated fatty acid methyl ester eluting after x.

Thus, it is possible, by comparison to the external standard, to compute the ECL value for each compound following an analysis. The GC and column allow windows to be set at 0.010 ECL unit wide giving great precision in resolution of isomers. After naming the peaks in an unknown sample, Sherlock compares the ECL values for the most stable series (e.g. saturated straight chain or branched chain acids) to the peak naming table's theoretically perfect values and may recalibrate internally if sufficient differences are detected. This feature allows the system to be run for up to two days unattended without worrying about drift between runs.

LIBRARIES

The Sherlock libraries consist of more than 100,000 analyses of strains obtained from experts and from culture collections. The cultures were collected from around the world to avoid potential geographic bias. Where possible, 20 or more strains of a species or subspecies were analyzed to make the entry. When chromatographic subgroups were found within a taxon, more strains were obtained to delineate each group.

The method of culture and the corresponding library is indicated in the name field of the sample as it is logged into the computer. Analysis of an unknown sample results in an automatic comparison of the composition of the unknown strain to a stored database using a covariance matrix, principal component analysis and pattern recognition software.

The covariance matrix takes into account the mole-for-mole relationship of the conversion of one fatty acid to another (e.g. 16:0 to 16:1 due to action of a desaturase), which might occur in relation to a temperature shift or age difference. The pattern recognition software uses calculations of cross terms (e.g. ratios between fatty acid amounts) in addition to the principal component base. The subtle differences between biovars or subspecies depends upon the power of the pattern recognition software to discriminate at this level.

The libraries are open ended (i.e. not limited by a finite set of biochemical assays) and the number of species in them is large and growing. The libraries are only limited by MIDI's ability to obtain adequate numbers of strains to make the entries. Of course, some groups of bacteria are more amenable to fatty acid composition analysis for identification than others. This relates to whether well characterized strains are available and to whether "species" can be justified on the basis of DNA relatedness (e.g. *Escherichia coli* is related at the species level to *Shigella dysenteriae* [see Brenner, *Int. J. Syst. Bacteriol.* 23: 298-307).

SUMMARY

The Sherlock Microbial Identification System is a fully automated gas chromatographic analytical system, which identifies bacteria based on their unique fatty acid profiles. The system will analyze about 45 samples per day (with a dual tower 90 samples per day can be analyzed), at a cost of about $1.50 per sample in standard laboratory consumables. Since a technician can extract 75 samples per day, the operator time per sample averages about 6 minutes. Because no subjective tests are required, the naming is highly objective and reproducible.

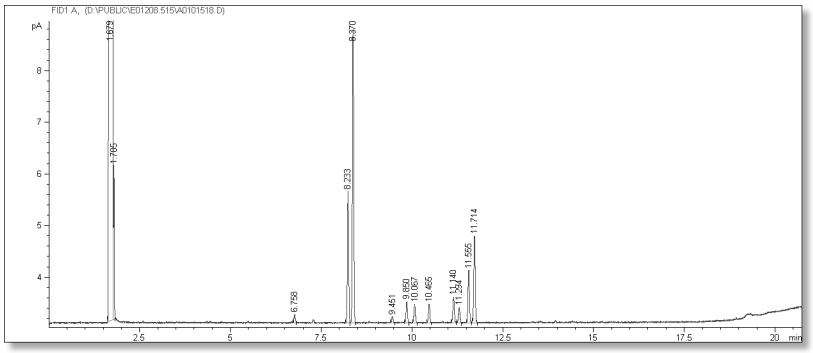


Figure 5. *Sample Chromatographic Run and Composition Report*

Volume: DATA File: E012085.15A Seq Counter: 10 ID Number: 1518
Type: Samp Bottle: 20 Method: TSBA40

Created: 2/8/01 4:07:34 PM

Created By: rredding

Sample ID: UN-Company X-5E(1728-010692B)

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.679	3.453E+8	0.027	—	7.012	SOLVENT PEAK	—	< min rt	
1.785	—	—	—	7.225		—	< min rt	
6.758	771	0.034	1.004	13.619	ISO	1.17	ECL deviates 0.000	Reference 0.009
8.233	12955	0.041	0.973	14.823	15:0 ISO	19.11	ECL deviates 0.000	Reference 0.009
8.370	28869	0.040	0.971	14.713	15:0 ANTEISO	42.48	ECL deviates 0.000	Reference 0.008
9.451	663	0.039	0.954	15.387	16:1 w7c alcohol	0.96	ECL deviates 0.000	
9.850	2118	0.041	0.949	15.627	16:0 ISO	3.05	ECL deviates 0.000	Reference 0.008
10.067	1936	0.040	0.946	15.758	16:1 w11c	2.78	ECL deviates 0.001	
10.465	1913	0.040	0.941	15.999	16:0	2.73	ECL deviates -0.001	Reference 0.007
11.140	2839	0.046	0.934	16.390	ISO 17:1 w10c	4.02	ECL deviates 0.002	
11.294	1631	0.042	0.933	16.479	Sum In Feature 4	2.31	ECL deviates 0.003	17:1 ISO I/ANTEI B
11.555	5794	0.046	0.930	16.631	17:0 ISO	8.17	ECL deviates 0.001	Reference 0.008
11.714	9398	0.043	0.929	16.723	17:0 ANTEISO	13.23	ECL deviates 0.000	Reference 0.007
—	1631	—	—	—	Summed Feature 4	2.31	17:1 ISO I/ANTEI B	17:1 ANTEISO B/i I

ECL Deviation: 0.001 Reference ECL Shift: 0.008 Number Reference Peaks: 7
Total Response: 68887 Total Named: 68887
Percent Named: 100.00% Total Amount: 65965

Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.785	Bacillus-subtilis

KD 4-9-02

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