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Application of the Gas Chromatography–Fatty Acid Methyl Ester System for the Identification of Environmental and Clinical Isolates of the Family *Micrococcaceae*

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Traditionally, the identification of microorganisms requested by industrial hygienists has been achieved by determining characteristics microscopically and using positive/negative biochemical tests. Specific assays designed to measure growth factor requirements, toxicity to selected animals, hemolysis and coagulase reactions, phage or plasmid typing, odor, colonial morphology (size, shape, pigmentation, etc.), and ability to grow on selective and differential media have had varying degrees of utility and may involve substantial subjectivity and estimation in the interpretation of results. Microbial Identification System (Microbial Identification, Inc., Newark, Delaware) was evaluated with 98 environmental and clinical American Type Culture Collection (ATCC®) strains of the family *Micrococcaceae*. This included 22 species (29 strains) of *Staphylococcus* and 5 species (8 strains) of *Micrococcus*. Overall accuracy of this investigation (correct to species level with 0.3 similarity index) based on single analysis of each sample was 98.0 percent. Cultures obtaining sufficient growth for analysis after a 24-hour incubation period at 28°C exhibited an average similarity index of 0.55. Differences in the gas chromatography–fatty acid methyl ester (GC-FAME) profiles of the *Staphylococcus* and *Micrococcus* species were sufficient to distinguish between closely related members of the family *Micrococcaceae*. The GC-FAME method of bacterial identification may serve as a primary means for identifying staphylococci and micrococci of air and bulk samples collected during indoor environmental quality investigations. PENDERGRASS, S.M.; JENSEN, P.A.: APPLICATION OF THE GAS CHROMATOGRAPHY–FATTY ACID METHYL ESTER SYSTEM FOR THE IDENTIFICATION OF ENVIRONMENTAL AND CLINICAL ISOLATES OF THE FAMILY *MICROCOCCACEAE*. APPL. OCCUP. ENVIRON. HYG. 12(8):543–546; 1997. PUBLISHED 1997 BY AIH.

Interest by National Institute for Occupational Safety and Health (NIOSH) investigators in chemical components as the causative agents for many indoor air quality problems (dizziness, headaches, lethargy, difficulty in breathing) has been accompanied by increased emphasis on the microbial agents. These microbial agents include bacteria, yeasts, and filamentous fungi, and their fragments and metabolic by-products. This interest has created the need for the development of increasingly accurate and rapid means of identifying these biological components.

Traditionally, the identification of microorganisms has been achieved by determining characteristics microscopically and using positive/negative biochemical tests. Specific assays designed to measure growth factor requirements, toxicity to selected animals, hemolysis and coagulase reactions, phage or plasmid typing, odor, colonial morphology (size, shape, pigmentation, etc.), and ability to grow on selective and differential media have had varying degrees of utility and may involve substantial subjectivity and estimation in the interpretation of results.[1] In the past few years, many different instrumental techniques and automated methods have been developed and used to identify microbial agents with varying degrees of success.[2] An alternative to classical microbiological identification techniques is the use of gas chromatography–fatty acid methyl ester (GC-FAME) microbial identification system (MIS) for these analyses.[2–6] Because genomic expressions (i.e., DNA/RNA homology, lipid composition, protein synthesis, etc.) are conserved, they are reliable indicators of species.[7] Cellular fatty acid composition of microorganisms reflects these factors, and their analysis may be performed by chromatographic techniques.

Cellular fatty acids (CFAs) have been used for profiling microorganisms for more than 20 years.[4,6,8] Because of their diversity in microorganisms, CFAs are suitable for use as an identification tool. The CFAs of bacteria are structural in nature, occurring in the cell membrane and/or cell wall of bacteria. When the bacteria are grown under standardized conditions (i.e., temperature, relative humidity, and growth medium), the CFA profiles are reproducible within a taxon (down to a subspecies or strain level in some microorganisms). CFA profiles have been shown to parallel DNA/RNA homology, retaining their genetic integrity in each generation.[6] Haack *et al.*[9] found that community fatty acid profiles can be used to assess the relative similarities and differences of microbial communities that differ in taxonomic composition.

The results of indoor air studies conducted by NIOSH over the past few years suggest that members of the family *Micrococcaceae* and genus *Bacillus* form a major fraction of the culturable microbial populations in buildings—37 and 41 percent, respectively. *Staphylococcus sp.* are mainly associated with the skin and mucous membranes of warm-blooded animals. *Mi-*

crococcus sp. are primarily derived from mammalian skin and soil, and are commonly isolated from food products and the air. Rashes, fever, and open wound infections are common health effects associated with infection by species in these genera.[10,11] The samples from these studies were cultured and the microorganisms were then identified using either GC-FAME or a biochemical multitest kit. Amy *et al.*[12] compared three methods of identifying microorganisms isolated from a mine tunnel system (water and rock). Though many organisms were not identified by any system, the GC-FAME method and one of the two biochemical multitest kits identified two to three times more microorganisms than the second multitest kit.[12] Additionally, Miller *et al*,[13] in an evaluation of the latter multitest kit, reported overall accuracies of 47.7 and 59.3 percent upon initial testing of selected members of the family *Micrococcaceae* (consisting mainly of the genus *Staphylococcus*). Since documentation of the overall accuracy of the GC-FAME system in analyzing members of the family *Micrococcaceae* is limited, this study investigated the efficacy of the GC-FAME system in characterizing staphylococci and related organisms.

Materials and Methods

All bacteria used for this study were obtained from the American Type Culture Collection (ATCC®, Rockville, Maryland) and designated as environmental or clinical based on their origin.[14] Of the 98 isolates processed, 83 percent were originally from environmental isolates, of which 57.1 percent were in the Microbial Identification, Inc. (MIDI, Newark, Delaware) MIS library. Pure cultures (single colony isolates) to be tested were rehydrated from freeze-dried ATCC specimens and streaked on plates or slants of BBL® trypticase soy broth agar (TSBA; Becton Dickinson and Company, Cockeysville, Maryland). The TSBA culture medium, as specified by MIDI, was prepared by dissolving 30 g of BBL trypticase soy broth and 15 g of BBL granulated agar to 1 L of deionized water. The TSBA was sterilized at a temperature of 121°C and a pressure of 1 atmosphere for 21 minutes. After initial growth, the organisms were quadrant-streaked on 15 × 100-mm sterile plastic petri plates containing TSBA. These plates were coded such that the analyst did not know their identities. The cultures were incubated at 28°C for 24 hours. Cultures not achieving suitable growth after 24 hours were allowed to incubate for 48 hours. After 48 hours, plates with no growth were listed as NG (no growth), while cultures with insufficient growth to harvest for GC-FAME analysis were listed as IG (insufficient growth).

Isolation and Preparation of Bacteria

Using a 4-mm loop, approximately 40 mg (this equates to a range of 100,000 to 500,000 area counts on a chromatogram) of the bacterial colonies were collected from the third quadrant (or the quadrant most closely exhibiting colony growth characteristic of the late log phase, the quadrant between confluent growth and individual colonial growth) of each culture plate. Each sample was placed into a 13 × 100-mm screw-capped Kimax test tube (Kimble Glass, Vineland, New Jersey) for the conversion of CFAs to the fatty acid methyl ester (FAME) analytes.

A stock solution of the saponification reagent was prepared by dissolving 45 g of sodium hydroxide (Mallinckrodt AR, Paris, Kentucky) in 150 ml of high performance liquid chromatography (HPLC)-grade methanol (Burdick and Jackson, Muskegon, Michigan) and 150 ml deionized water. This solution was used to lyse the cell wall and convert the CFAs to their Na^+ analogues. One milliliter of the saponification reagent was pipetted into each test tube. Each tube was placed in boiling water (100°C) for 5 minutes, vortexed vigorously for 10 to 20 seconds, and replaced into boiling water for 25 minutes to complete the saponification procedure.[3]

A stock solution of the methylating reagent was prepared by mixing 325 ml of 6 N hydrochloric acid (Ricca Chemical Co., Arlington, Texas) and 275 ml of HPLC-grade methanol (Burdick and Jackson). This solution was used to convert the saponified fatty acids to the FAMEs. After the test tube and sample cooled to room temperature, 2 ml of this methylating reagent was pipetted into each test tube. After a brief vortexing period (10 to 20 seconds), the test tubes were placed in an 80°C water bath for 10 minutes.[3]

A stock solution of the extraction reagent was prepared by mixing 200 ml of HPLC-grade hexane (Burdick and Jackson) and 200 ml of HPLC methyl *t*-butyl ether (Burdick and Jackson). This solution was used to extract the FAME compounds into an organic layer for analysis. A 1.25-ml aliquot of this extraction reagent was added to each test tube. The tubes were rotated end over end for 10 minutes using a hematology mixer (Fisher-Scientific Co., Pittsburgh, Pennsylvania) and the lower liquid phase (aqueous layer) was discarded by pipetting.[3]

The organic phase remaining in each test tube was washed with 3 ml of sodium hydroxide (10.8 g of sodium hydroxide dissolved in 900 ml of deionized water). The tubes were then rotated end over end for 5 minutes.[3] A few drops of saturated sodium chloride solution were added to each tube to break up any emulsions formed during the base washing process. The organic phase (top layer) was then pipetted into 1-ml crimp-top GC autosampler vials (12 × 32 mm; Supelco, Inc., Bellefonte, Pennsylvania) for analysis.[3]

GC Analysis

GC analysis of the FAME solutions was achieved using a Hewlett-Packard 5890 Series II GC (Hewlett-Packard Co., Avondale, Pennsylvania) with a flame ionization detector (FID). Separation of the FAME compounds was achieved using a 25 m × 0.2 mm ID cross-linked Ultra 2 phenyl methyl silicone fused silica capillary column (Hewlett-Packard Co.). The temperature was ramped from 170° to 270°C (5°C/minute).[3] Hydrogen was used as the carrier gas and nitrogen as the make-up gas. Compressed air, purified by a pure air generator (Balston Co., Haverhill, Massachusetts), was supplied to the FID. The GC was controlled by a Dell 386DX computer (Dell Computer Corporation, Austin, Texas) containing MIDI software (version 3.8), which is used to operate the system.

Results and Discussion

In this blind evaluation, 27 species of the family *Micrococcaceae* were analyzed using the GC-FAME system (n = 98 isolates) and are listed in Table 1. Fourteen strains of *S. aureus* were included to determine reproducibility of this system. Analysis of four old cultures of *S. aureus* showed that similarity indices (SIs) fall as cultures age. Some strains exhibited better growth

TABLE 1. Growth Characteristics of Microorganisms Used and Accuracy and Similarity Index Results of the Evaluation of MIDI MIS

Bacterium	ATCC No.	Growth Characteristics[A]	No. Tested	No. Correct	SI Range (24 Hours)	SI Range (48 Hours)
Staphylococcus arlettae	43957		2	2	0.91–0.95	—
Staphylococcus aureus subspecies aureus	29213/12600/25923		14	14	0.76–0.98	0.45–0.58
			4	4	0.29–0.42[E]	
Staphylococcus capitis subspecies capitis	35661/27843	Min growth @ 48 hours	3	3	—	0.26–0.36
Staphylococcus capitis subspecies ureolyticus	49327	Min growth @ 48 hours	2	2	—	0.53–0.69
Staphylococcus caseolyticus	13548	No growth @ 48 hours	NG[B]	NG	—	—
Staphylococcus chromogenes	43764		2	2	0.56–0.71	—
Staphylococcus cohnii subspecies cohnii	29974		5	5	0.60–0.65	0.50–0.53
Staphylococcus delphini	49171	Insufficient growth @ 48 hours	IG[C]	IG	—	—
Staphylococcus epidermidis	14990/35547		5	5	0.84	0.66–0.87
Staphylococcus felis	49168	Insufficient growth @ 48 hours	IG[C]	IG	—	—
Staphylococcus haemolyticus	29970		5	5	—	0.60–0.81
Staphylococcus hominis	27844		4	4	0.64	0.28–0.46
Staphylococcus kloosii	43959	One culture overgrown	3	2	0.33	0.73
Staphylococcus lentus	29070	Min growth @ 48 hours	4	3[D]	0.23–0.34	—
Staphylococcus lugdunensis	43809	48-hour culture	2	2	—	0.54–0.57
Staphylococcus saprophyticus	15305/49453		3	3	0.72–0.81	—
Staphylococcus schleiferi subspecies schleiferi	43808		2	2	0.83–0.88	—
Staphylococcus sciuri subspecies sciuri	29060		2	2	0.91–0.93	—
Staphylococcus simulans	27848		2	2	0.51–0.54	—
Staphylococcus species	12715	48-hour culture	2	2	—	0.53–0.62
Staphylococcus warneri	27836/17917	48-hour culture	4	4	—	0.73–0.83
Staphylococcus xylosus	29971/49148		6	6	0.51–0.65	0.29–0.35
Micrococcus kristinae	27570/27571	Min growth @ 48 hours	2	2	—	0.47–0.55
Micrococcus luteus	4698/381	Min growth @ 48 hours	8	8	0.46–0.67	0.31–0.51
Micrococcus lylae	27566	48-hour culture	2	2	—	0.56–0.62
Micrococcus roseus	186/177	48-hour culture	6	6	0.79–0.87	0.67–0.97
Micrococcus spp	398	48-hour culture	4	4		
			98	96		
				98.0%		

[A]All cultures were harvested after 24 hours unless otherwise noted.
[B]NG refers to no growth observed on TSBA.
[C]Insufficient growth (IG) was observed on TSBA (not enough to harvest for GC-FAME method).
[D]While the correct identification of *S. lentus* was achieved, the SI was below the acceptable cutoff of 0.3.
[E]*S. aureus* culture was several months old.

characteristics than others under the standardized growth conditions specified by MIDI. Three strains, *S. delphini*, *S. felis*, and *S. caseolyticus*, after repeated attempts, failed to produce adequate growth on TSBA after 48 hours. However, these organisms did grow on blood agar medium. *S. capitis subspecies capitis*, *S. capitis subspecies ureolyticus*, *S. hominis*, and *S. lentus* grew poorly on TSBA. Again, blood agar may have been a better growth medium, but for standardization, MIDI MIS used TSBA as the standard medium in the development of their environmental library and blood agar in their clinical library. This study was designed and based upon the environmental library. Many environmental isolates from indoor air are a result of human exfoliation.[15] Perhaps future studies should include both the clinical and the environmental libraries and the appropriate medium for each (i.e., blood agar and TSBA).

When 26 type strains were analyzed, 98.0 percent (96 of 98) were correctly identified to the species level during the initial analysis. Another sample was correctly identified but had an SI below the threshold of 0.3. All cultures producing suitable growth after 24 hours were correctly identified with high SIs (Table 1). Further evidence of the accuracy of the GC-FAME system, which was designed for the analysis of environmental and clinical isolates, is that 17 percent of the ATCC cultures obtained for this investigation were originally clinical isolates and 83 percent were environmental isolates. The fact that most

isolates were cultured on TSBA and correctly identified using the TSBA environmental library supports the efficacy of this system.

The identification of bacteria by SIs using the MIS is based on the average FAME profiles of approximately 100 geographically different isolates for each test strain analyzed. SI is a numerical value that expresses how closely the composition of an unknown isolate compares with the CFA composition of the library match(es).[3] Each MIS library entry is a computer-generated composite average of the reference strains (approximately 100) of each species or subspecies group. In general, the reference strains are ATCC Preceptrol® and type strains. The closest matching species entry or entries are issued on all reports, and are designated as environmental or clinical, based on the type of medium used. Based on historical analysis of more than 300 gram-negative and gram-positive bacteria by the authors, an acceptable SI for reporting identification information was established at 0.3 or greater. As such, reports with SIs greater than 0.3 and exhibiting single identifications are considered to represent acceptable genus and species identifications. When using an SI of 0.5 or greater as the identification criterion, 76 of the 98 (77.6 percent) would have been identified. Of the 76 identified, 74 would have been identified correctly (97.4 percent), not significantly different from 98.0 percent reported using 0.3 as the identification criterion. The major benefit to using the lower SI is the identification of more samples without significant loss of accuracy. In addition, environmental isolates not included in the development of the MIDI MIS library had SIs ranging from 0.23 to 0.98, while those included in the library ranged from 0.45 to 0.98.

Conclusions

The results of this study suggest that the GC-FAME-based MIS adequately identifies the evaluated members of the family *Micrococcaceae*. The differences in the FAME profiles of the *Staphylococcus* and *Micrococcus* species were sufficient to distinguish between closely related species of the members of the family *Micrococcaceae*. In comparing the results of this evaluation with the results presented by others using an automated multitest system,[13] the GC-FAME system exhibits greater accuracy in the identification of the family *Micrococcaceae* when using an SI of either 0.3 or 0.5 as the identification criterion. Therefore, the GC-FAME method of bacterial identification may serve as a primary means for identifying culturable *Staphylococcus* and *Micrococcus* species from air and bulk samples collected during indoor environmental quality investigations. In such cases, identification of the bacteria to the species level may help in the epidemiological and medical aspects of the investigation.

Disclaimer

Mention of commercial names or products does not constitute endorsement by the Centers for Disease Control and Prevention.

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