Microorganism Gram-Type Differentiation Based on Pyrolysis-Mass Spectrometry of Bacterial Fatty Acid Methyl Ester Extracts

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Curie-point pyrolysis (Py)–mass spectrometry has been used to differentiate 19 microorganisms by Gram type on the basis of the methyl esters of their fatty acid distribution. The mass spectra of gram-negative microorganisms were characterized by the presence of palmitoleic acid (C_{16:1}) and oleic acid (C_{18:1}), as well as a higher abundance of palmitic acid (C_{16:0}) than pentadecanoic acid (C_{15:0}). For gram-positive microorganisms, a signal of branched C_{15:0} (isoC_{15:0} and/or anteisoC_{15:0}) more intense than that of palmitic acid was observed in the mass spectra. Principal components analysis of these mass spectral data segregated the microorganisms investigated in this study into three discrete clusters that correlated to their gram reactions and pathogenicities. Further tandem mass spectrometric analysis demonstrated that the nature of the C_{15:0} fatty acid isomer (branched or normal) present in the mass spectrum of each microorganism was important for achieving the classification into three clusters.

Application of instrumentation-based techniques in microbiology to perform conventional procedures for the identification and differentiation of microorganisms has increased during the last decade. Gas chromatography (GC) (12, 24), fast atom bombardment mass spectrometry (MS) (17), Curie-point pyrolysis (Py)–MS (8), Py–GC–MS (31), Fourier transform infrared spectroscopy (27), UV resonance Raman spectroscopy (7), flow cytometry (10), and laser-based enzyme profiling (3, 20) have all been used to differentiate microorganisms. These techniques apply established analytical methods to measure a distinct biochemical property and/or chemical distribution of taxonomic significance. Py–MS has been used widely for the rapid identification and differentiation of whole bacteria. Moreover, Py–MS has identified useful chemical constituents in bacteria or biomarkers for the differentiation and identification of microorganisms in the presence of an environmental background (34).

The correlation between bacterial lipid composition and taxonomic classification has been extensively used for the identification of bacteria. The bacterial classifications based on fatty acid profiles have compared well with classifications based on nucleic acid homology (5). Improvements in lipid analysis methodology have extended this correlation to many bacteria. GC of lipid extracts is commonly employed to determine the cellular fatty acids in microorganisms (24, 25). This commercialized method (22) is based on the hydrolysis and extraction of bacterial lipids, methylation of the carboxylic group to produce a fatty acid methyl ester (FAME), and subsequent GC analysis (25). Overall sample preparation time is about 60 to 70 min. The chromatographic analysis of the FAME extracts takes about 20 min per sample, making the total turnaround time for this assay about 1.5 h per sample. In addition, a standard FAME mixture is needed prior to and at intervals during analysis along with the samples, increasing the total analysis time. The success of the procedure in identifying individual fatty acid components relies on the judicious selection of this standard FAME mixture.

In Py–MS the sample is rapidly heated and vaporized in

vacuo, and then the gas-phase molecules are ionized and mass analysis is performed. A histogram of the signal intensity versus the mass-to-charge ratio (m/z) represents the mass spectrum of the compound or mixture of compounds. When Py–MS is performed on a microorganism, the resulting mass spectrum is a profile or chemical signature characteristic of the sample. Sample preparation and data collection (not including FAME extract preparation) is fast, taking about 5 min per sample. The instrument is calibrated once at the beginning of the day with a standard compound. Replacing the chromatographic analysis of the FAME extracts with Py–MS provides a faster assay for the identification and differentiation of bacteria, with the FAME extract preparation as the limiting step. Simple staining techniques can yield Gram-type information; however, automated, instrumentation-based techniques to perform *in situ* bacterial analysis in hostile environments must be developed. Toward this goal, the use of Py as a rapid sample processing and preparation step has proven very successful (6, 18, 31). Before replacing time-consuming extraction and separation steps with Py–MS, the feasibility of Py–MS to provide information useful for the differentiation and identification of microorganisms in a manner comparable to that of the GC–FAME approach must be proven. Py–MS (single-quadrupole instruments) distinguishes compounds on the basis of their mass-to-charge ratios, and hence, isomer differentiation is not always possible with a mixture containing iso-anteiso, *cis-trans* unsaturation and unsaturation position isomers. Therefore, the information content in a FAME extract mass spectrum will be always lower than that in a FAME extract gas chromatogram (when only the FAME's molecular ion region is considered). The lower information content of the mass spectrum, however, does not imply that a minimum amount of information required for successful differentiation and identification of microorganisms at the species level is not present.

In this study, Curie-point Py–MS of bacterial FAME extracts and pattern recognition have been used to differentiate between gram-positive and gram-negative microorganisms. This differentiation was based solely on fatty acid distributions. With the aid of pattern recognition (principal components analysis [PCA]), these differences were attributed to the presence of fragment ions of palmitoleic acid, oleic acid, 3-hydroxy-

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TABLE 1. Microorganisms used in this study

Index	Gram type	Microorganism
A	+	<i>Bacillus cereus</i>
B	+	<i>Bacillus circulans</i>
C	+	<i>Bacillus licheniformis</i>
E	+	<i>Bacillus subtilis</i>
F	+	<i>Bacillus thuringiensis</i>
G	+	<i>Enterococcus (Streptococcus) faecalis</i>
H	+	<i>Listeria monocytogenes</i>
I	+	<i>Staphylococcus aureus</i> subsp. <i>aureus</i>
J	+	<i>Staphylococcus epidermidis</i>
K	+	<i>Streptococcus pyogenes</i>
L	—	<i>Enterobacter aerogenes</i>
N	—	<i>Proteus mirabilis</i>
O	—	<i>Providencia stuartii</i>
P	—	<i>Pseudomonas aeruginosa</i>
Q	—	<i>Pseudomonas cepacia</i>
R	—	<i>Pseudomonas fluorescens</i>
S	—	<i>Pseudomonas putrefaciens</i>
T	—	<i>Pseudomonas stutzeri</i>
U	—	<i>Serratia marcescens</i>

myristic acid, and branched pentadecanoic fatty acids and a greater signal intensity of palmitic acid relative to pentadecanoic acid in the gram-negative microorganisms studied. The presence of these ions was confirmed either by Py-tandem MS (Py-MS/MS) or by comparison with the mass spectrum of a standard compound.

MATERIALS AND METHODS

Instrumentation. Measurements were performed with an Extrel triple-quadrupole mass spectrometer (ELO-400) fitted with a Curie-point Py inlet. A more detailed description of the instrument has been published elsewhere (8). Py was conducted at 510°C for 10 s. All spectra were obtained by using 70-eV electron ionization and a mass scan range of 180 to 350 atomic mass units. This mass range was selected to match the range of FAMES detected by GC-FAME analysis. Py-MS/MS (8) was performed with argon at a pressure of 0.01 millitorr (1 torr = 1 mm Hg) in the collision cell (quadrupole 2).

Chemicals. All standard straight-chain saturated, unsaturated, and hydroxy fatty acids (Aldrich) and branched iso and anteiso fatty acids (Ultra Scientific) were used without further purification. All fatty acid standards were dissolved in glass-distilled methanol (10 mg/ml). Lipid A, monophosphorylated, from *Escherichia coli* F583 Rd-Mutant (Fluka Chemika) was also dissolved in glass-distilled methanol before analysis. A 10- μ l aliquot of each solution was loaded onto the Py wire and air dried at room temperature to form a uniform coating. In situ methylation of the standard fatty acids (18) was performed by adding 10 μ l of 0.1 M trimethylphenylammonium hydroxide in methanol (Fluka Chemika) to the dry, fatty acid-coated wire and allowing it to air dry.

Biological samples. The microorganisms used in this study were chosen at random and are listed in Table 1. All microorganisms were grown on tryptic soy agar at 37°C for 18 h. Cells were isolated by washing with distilled water, and the fatty acids were extracted and methylated as described by Miller (23). FAME samples were redissolved in 1:1 ether-hexane as needed because of solvent evaporation. A 10- μ l aliquot of this solution was applied to the Curie-point wire and air dried at room temperature. The total mass of FAMES deposited on the wire was estimated to be in the low microgram range.

Pattern recognition. PCAs were carried out with the RESOLVE software package developed at the Colorado School of Mines (14, 15). Each mass spectrum was collected as a set of raw intensities. The data were normalized to total intensity and mean centered. Both score and loading plots were generated. The loadings or eigenvector coefficients indicated the magnitude of the contribution of a variable (in this case the mass spectral peaks) to the composition of the projected score (29).

RESULTS AND DISCUSSION

FAME mass spectra of gram-negative and gram-positive bacteria. The spectra of FAME extracts of the following three gram-negative microorganisms are shown in Fig. 1: *Pseudomonas fluorescens*, *Proteus mirabilis*, and *Enterobacter aerogenes*.

FIG. 1. Mass spectra of gram-negative bacterial FAME extracts.

These mass spectra contain a pattern of straight-chain FAME molecular ions varying by one carbon, from $C_{14:0}$ methyl ester ($C_{14:0}$ ME) to $C_{21:0}$ ME, with the palmitic ME signal ($C_{16:0}$ ME; M^{+} m/z 270) being the most intense. Along with the molecular ions, fragment ions due to electron ionization-fragmentation are present. These include the homolytic cleavage of the methoxy bond of $C_{16:0}$ ME, giving rise to the $M-31$ fragment ion at m/z 239, and the carbomethoxy series at m/z 185, 199, 213, 227, etc., with the general formula $CH_3OCO(CH_2)_n^{+}$ (26). In the mass spectra shown in Fig. 1, additional peaks arising from unsaturated FAME are observed. For example, in the mass spectrum of *P. fluorescens*, several other peaks corresponding to monounsaturated (even and odd chain length) FAME molecular ions and their fragment ions were identified by comparison with mass spectra of pure standards or by Py-MS/MS studies.

Most monounsaturated FAMES studied in our laboratory ($C_{16:1}$ to $C_{20:1}$) yielded, along with their molecular ions, fragment ions at $M-32$ (loss of CH_3OH) and $M-74$. Oleic ME ($C_{18:1}$ ME; M^{+} m/z 296) fragment ions appeared at m/z 264 and m/z 222, and palmitoleic ME ($C_{16:1}$ ME; M^{+} m/z 268) fragment ions were found at m/z 236 and m/z 194. The $M-32$ and $M-74$ fragment ions of the unsaturated FAME $C_{17:1}$ ME (M^{+} m/z 282) and/or cyclopropane $C_{17:0}$ ME (cy $C_{17:0}$ ME; M^{+} m/z 282) appeared at m/z 250 and 208, respectively. The ion at m/z 208 may also correspond to the $M-50$, possibly $[M-(CH_3OH + H_2O)]^{+}$, fragment ion of 3-hydroxymyristic ME (3OH $C_{14:0}$ ME) which is observed in the mass spectrum of a known standard. Molecular ions from the hydroxy FAMES, 2- and 3-hydroxylauric ME (2OH and 3OH $C_{12:0}$ ME; M^{+} m/z 230) and 3-hydroxymyristic ME (3OH $C_{14:0}$ ME; M^{+} m/z 258), were not observed. Branched fatty acids were not detected by Py-MS/MS scans in any of the gram-negative bacteria investigated in this work. These observations agree with the known fact that most gram-negative microorganisms contain mostly straight-chain saturated and unsaturated fatty acids (9, 30, 34).

Figure 2 shows the mass spectra of FAME extracts of three gram-positive bacteria: *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The $C_{15:0}$ ME to $C_{21:0}$ ME series of molecular ions is observed in these spectra. These mass spectra are generally less complex than mass spectra of gram-negative bacteria. A smaller relative palmitic ME signal intensity (M^{+} m/z 270) was observed in the gram-positive mass spectra com-

FIG. 2. Mass spectra of gram-positive bacterial FAME extracts.

pared with the mass spectra of gram-negative bacteria (Fig. 1). In the *B. cereus* mass spectrum shown in Fig. 2, the presence of monounsaturated FAMES $C_{16:1}$ ME and $C_{17:1}$ ME (and/or $cyC_{17:0}$ ME) is indicated by fragment ions corresponding to m/z 32 and m/z 74 (m/z 236 and 194 and m/z 250 and 208, respectively). The m/z 31 fragment ion for the molecular ion at m/z 256 ($C_{15:0}$ ME) is observed at m/z 225.

The presence of branched isomers at m/z 256 ($C_{15:0}$ ME) and 284 ($C_{17:0}$ ME) in the mass spectra of the gram-positive microorganisms *Bacillus thuringiensis* and *S. aureus* was further studied by Py-MS/MS. For *B. thuringiensis*, the Py product ion spectrum of the ion at m/z 256 (Fig. 3, top spectrum) was characteristic of $isoC_{15:0}$ ME (m/z 43 base peak, m/z 213). In the case of *S. aureus*, the m/z 256 ion was identified as $anteisoC_{15:0}$ ME on the basis of the Py product ion spectrum shown in Fig. 3. This spectrum is very similar to that of the $anteisoC_{15:0}$ ME (m/z 57 base peak, m/z 199) standard, also shown in Fig. 3. This similarity suggests that this branched isomer is more abundant than either the straight chain or the iso-branched isomer $C_{15:0}$ ME, even though mixtures of $antei-$

FIG. 3. FAME Py product ion mass spectra of the ion at m/z 256. Std., standard.

FIG. 4. Score plot of mass spectra of FAME extracts with principal components 1 and 3.

$soC_{15:0}$ and $isoC_{15:0}$ ME (about 4:1 $anteisoC_{15:0}$ - $isoC_{15:0}$) are known to be present in *Staphylococcus* strains (28). The Py product ion mass spectrum of $C_{15:0}$ ME is also shown in Fig. 3 for comparison. Measurements in our laboratory have shown that mixtures of branched and normal FAME isomers at various ratios from 1:1 to 4:1 can be differentiated from either pure isomer by Py-MS and/or Py-MS/MS. The ion at m/z 284 was determined from Py-MS/MS experiments to be a mixture of $isoC_{17:0}$ ME and its straight-chain $C_{17:0}$ ME isomer (21). These findings are in agreement with previous studies that showed branched fatty acids to be widely present in gram-positive bacteria (9, 28, 30). The presence of branched fatty acids, however, is not in itself indicative of a gram-positive-type bacterium, since there are exceptions, as gram-negative microorganisms in the genera *Xanthomonas*, *Legionella*, *Thermus*, and *Desulfovibrio* and some *Pseudomonas* species (i.e., *P. maltophilia* and *P. pictorum*), as well as many other gram-negative bacteria, have significant amounts of branched and hydroxy-branched fatty acids (32–34).

Multivariate analysis of FAME mass spectra. Differentiation of gram-positive and gram-negative microorganisms can be accomplished by visual inspection of their mass spectra as shown in Fig. 1 and 2. The prominent $C_{16:0}$ ME peak at m/z 270 relative to $C_{15:0}$ ME at m/z 256 is a clear indication of a gram-negative-type bacterium. Conversely, the mass spectra of gram-positive microorganisms are characterized by a larger branched $C_{15:0}$ ME (iso and/or $anteiso$) peak relative to $C_{16:0}$ ME. Visual analysis of large data sets (comprising several replicates) can be tedious, and computer-based data analysis simplifies this task (4, 16, 27). PCA was performed with all the peaks of the mass spectrum. Figure 4 shows the score plot of principal components 1 and 3. Each similar letter or index corresponds to a replicate Py-MS analysis of that microorganism's FAME extract (Table 1). A total of three groups or clusters are observed in the data plot.

Two main clusters are differentiated along the component 1 axis and have been related to bacterial Gram type. Microorganisms with positive values for the component 1 axis are gram positive (clusters II and III), and microorganisms with negative values are mostly gram negative (cluster I). Note that only three gram-positive microorganisms gave a false Gram type in this study (C, K, and G), that is, they were differentiated as gram-negative bacteria. The mass spectra of these microorganisms showed higher abundances of $C_{16:0}$ ME than of branched or normal $C_{15:0}$ ME, and they also showed characteristic un-

FIG. 5. Loading 1 (a) and loading 3 (b) for the score plot shown in Fig. 4.

saturated FAME molecular ions and fragment ions (e.g., $C_{18:1}$ ME).

Figure 5a shows the loading 1 plot or the mass spectral peaks responsible for differentiation along the component 1 axis in Fig. 4. Peaks in the positive direction are characteristic of gram-positive microorganisms, and the negative peaks are characteristic of gram-negative microorganisms. The peaks at m/z 256 and m/z 270 were again responsible for differentiating the two groups along the component 1 axis. The peaks in the positive direction in Fig. 5a correspond to molecular ions and fragment ions of odd-numbered straight-chain and branched fatty acids (iso and anteiso). Peaks from anteiso- $C_{15:0}$ ME are at m/z 256 (M^+), and its major fragment M-57 is at m/z 199; for iso- $C_{15:0}$ ME, the M-43 peak is at m/z 213. The ion at m/z 284 corresponds to a mixture of $C_{17:0}$ ME and iso- $C_{17:0}$ ME, as established by Py-MS/MS measurements. The signal at m/z 241 is also the M-43 fragment ion of iso- $C_{17:0}$ ME. Also, higher-molecular-weight FAMES $C_{19:0}$ ME (M^+ m/z 312) and $C_{20:0}$ ME (M^+ m/z 326) were observed in all mass spectra of gram-positive microorganisms. Peaks in the negative direction of the loading 1 spectrum correlate with molecular ions and fragment ions of even-numbered straight-chain, 3-hydroxy, and unsaturated FAMES. All mass spectra of gram-negative bacteria contained a large $C_{16:0}$ ME (M^+ m/z 270) signal. The ions at m/z 296 and 268 were $C_{18:1}$ ME and $C_{16:1}$ ME, respectively, with their fragments at M-74 and M-32. Also, the fragment ion at

TABLE 2. FAME ions useful for Py-MS bacterial identification

FAME and/or fragment(s)	Ion m/z	FAME and/or fragment	Ion m/z
$C_{14:0}$ ME	242	$C_{16:1}$ ME	268
M-31	211	M-32	236
		M-74	194
3OH $C_{14:0}$ ME			
M-50	208		
anteiso- $C_{15:0}$ ME	256	$C_{17:0}$ ME	284
M-31	225	iso- $C_{17:0}$ ME	284
M-57	199	M-43	241
iso- $C_{15:0}$ ME	256		
M-31	225	anteiso- $C_{17:0}$ ME	284
M-43	213	M-57	199
$C_{16:0}$ ME	270	$C_{17:1}$ ME ^b	282
M-31	239	M-32	250
M-29 ^a	241	M-74	208
-14	227		
-14	213	$C_{18:1}$ ME	296
-14	199	M-32	264
-14	185	M-74	222

^a Fragments of M-29 represent the carbomethoxy series.

^b Also cyclopropyl $C_{17:0}$ ME.

m/z 208, characteristic of 3-hydroxymyristic acid, indicates that this compound might have been present.

Figure 5b shows the loading 3 plot or mass spectral peaks responsible for differentiation along the component 3 axis in Fig. 4. These include high-mass FAMES ($C_{18:0}$ ME and above) and unsaturated, hydroxy, isobranched, and anteisobranched FAMES. In the positive direction, peaks corresponding to $C_{16:0}$ ME, $C_{17:1}$ ME, iso- $C_{15:0}$ ME, and $C_{14:0}$ ME were observed. All these ions are summarized in Table 2.

A better differentiation at the species level (i.e., a larger separation between replicates of *Pseudomonas putrefaciens* [S] and *P. mirabilis* [N]) was observed for gram-negative microorganisms than for gram-positive microorganisms, probably because of the presence of both unsaturated and hydroxy fatty acids (cluster I in Fig. 4). Most of the microorganisms in the genus *Pseudomonas* (P, Q, R, S, and T in Fig. 4) were differentiated at the species level along the component 3 axis. The genus *Proteus* (N) was distinguished from other genera; however, the genera *Serratia* (U), *Providencia* (O), and *Enterobacter* (L) could not be differentiated. Further PCA of mass spectra from only gram-negative microorganisms listed in Table 1 differentiated all microorganisms at the species level, with the exceptions of *Pseudomonas aeruginosa* (P) and *P. fluorescens* (R). On the basis of results from GC-FAME analysis, improved differentiation of gram-negative microorganisms at the species level could be achieved for this data set if the molecular ions of 2- and 3-hydroxy fatty acids, as well as isobranched 3-hydroxy fatty acids, were detected (5).

On the positive side along the component 1 axis of Fig. 4, two clusters (II and III) containing only gram-positive microorganisms are observed. Bacteria classified in cluster II (A, E, and F [Table 1]) are characterized by the presence of iso- $C_{15:0}$ ME (base peak at m/z 213, M-43) and $C_{14:0}$ ME (m/z 242) in their mass spectra. The mass spectra of microorganisms in this cluster also show the presence of small amounts of $C_{17:1}$ ME (or cy- $C_{17:0}$ ME, m/z 282) and corresponding fragments at m/z 250 (M-32) and m/z 208 (M-74). It is important to note that

FIG. 6. Low-mass-scan mass spectra of FAMES from a gram-positive bacterium, *S. pyogenes* (a), and a gram-negative bacterium, *P. mirabilis* (b).

the microorganism *B. cereus* (A) in cluster II is genetically and pathogenically very similar to *Bacillus anthracis*. Cluster III (H, I, and J) is differentiated from cluster II in that the m/z 256 ion is anteiso $C_{15:0}$ ME, yielding a base peak at m/z 199 (M-57). Moreover, segregation of microorganisms listed in Table 1 into three clusters can be accomplished mainly by obtaining the product ion spectra of their m/z 256 ion (Fig. 3).

Reproducibility of the FAME bacterial extract mass spectra was examined by comparing replicate Py-MS analyses for two different bacteria. The similarity between normalized mass spectrum replicates, defined as the inverse of the Euclidian distance (32), was determined for *B. cereus* (A) and *Streptococcus pyogenes* (K). For two identical mass spectra the similarity equals unity. Moreover, the average similarity for an ideal group of identical mass spectra is also unity. Hence, the difference in average similarity between an ideal group and the group of replicates for *B. cereus* (A) was calculated to be 0.007 (0.7%). For *S. pyogenes* (K) this value was 0.06 (6%). These values correlate well with the spread of the letter indices (A and K) on the score plot in Fig. 4. Reproducibility of FAME profiles obtained by MS varies, in part, because of the relative amounts of each FAME present in the mixture. The average similarity differences observed in this study ranged from 0.007 (0.7%) to an upper limit of about 0.1 (10%).

Exceptions to the Gram-type separation shown in Fig. 4, *Bacillus licheniformis* (C), *Enterococcus faecalis* (G), and *S. pyogenes* (K), were further analyzed in order to classify correctly their Gram types. It is well known that a lipid moiety called Lipid A is exclusively present in gram-negative bacterial lipopolysaccharide (13). Lipid A is a complex molecule composed of glucosamine, phosphate, and fatty acids, of which there is a large proportion of 3-hydroxymyristic acid (3OH $C_{14:0}$) (19). The mass spectrum of *E. coli* lipid A in the mass range of 50 to 400 atomic mass units shows a strong ion signal at m/z 89. This ion corresponds to a stable fragment of 3-hydroxy fatty acids (11), as confirmed by Py-MS/MS studies and comparison with a mass spectrum of a 3OH $C_{14:0}$ standard. In 3-hydroxy FAMES, this ion shifts 14 atomic mass units and it appears at m/z 103. Since only gram-negative bacteria are known to have a lipid A moiety and most gram-positive bacteria lack hydroxy fatty acids (30, 35), the presence of this

FIG. 7. Score plot with principal components 1 and 3 obtained by using only masses listed in Table 2.

stable fragment ion is indicative of a gram-negative microorganism. For example, Fig. 6 shows the low-mass-scan mass spectra of *S. pyogenes* (K) and a microorganism known to be gram-negative, *P. mirabilis* (N). The lack of this ion suggests that *S. pyogenes* is not a gram-negative bacterium (Fig. 6a), and the presence of the m/z 103 ion correctly assigns *P. mirabilis* as gram negative (Fig. 6b). The microorganism *B. licheniformis* could not be identified as a gram-positive bacterium by this approach (its mass spectrum showed the m/z 103 fragment ion), and it remains the only exception encountered in this study.

A PCA of the same data set was performed with only peaks corresponding to FAME molecular and fragment ions identified in this study (Table 2). Figure 7 shows the normalized and mean-centered score plot obtained with principal components 1 and 3. This plot is similar to that obtained by PCA using all peaks in each mass spectrum (compare Fig. 7 with Fig. 4). The same number of clusters were obtained by using only the 21 mass spectral peaks listed in Table 2. In addition, the degree of differentiation or distance between the clusters is larger on the basis of the magnitude of the components 1 and 3. This is due to elimination of redundant information (signals common to all samples) and chemical noise (8). This approach can be useful in the analysis of mass spectral data obtained in the presence of an environmental background.

Conclusions. The results presented in this study correlate differences in the fatty acid distributions, observed by Py-MS, in microorganisms with their Gram types. Gram-negative microorganisms are characterized mainly by a high abundance of $C_{16:0}$ in their mass spectra. On the other hand, gram-positive microorganisms contained a high abundance of branched $C_{15:0}$ (iso or anteiso) in their mass spectra. Three PCA clusters were identified in this study. Cluster I contained all of the gram-negative microorganisms and some gram-positive microorganisms. These spectra were characterized by straight-chain and unsaturated fatty acids and were rich in $C_{16:0}$. Cluster II contained gram-positive microorganisms only. The spectra in this group were characterized by straight-chain fatty acids and small amounts of unsaturated and cyclopropyl fatty acids and were rich in iso $C_{15:0}$. Pathogenic *Bacillus* species were grouped within this cluster. Cluster III was composed of gram-positive microorganisms only. Their mass spectra had high levels of anteiso $C_{15:0}$. Furthermore, microorganisms investigated in this study can be assigned correctly to each cluster by obtaining only their Py product ion spectra of the ion at m/z 256.

Differentiation and identification of gram-positive microor-

ganisms should take advantage of the higher abundance of branched, as well as unsaturated, fatty acids present in these microorganisms. Similarly, gram-negative microorganisms can be differentiated mainly by their relative amounts of unsaturated fatty acids.

Future work will be aimed at the development of a whole-bacterium, in situ lipid methylation, Py-MS protocol without the need of time-consuming FAME extraction procedures and chromatographic separations (18). Preliminary work in our laboratory has shown that Py of whole cells and in situ methylation of their lipids can be accomplished with a total turnaround time of 10 min per sample (sample preparation and mass spectral analysis) (1, 2). This will yield a bacterial lipid-based technique for the rapid and accurate differentiation and identification of microorganisms with simple Py-MS instrumentation.

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