Identification of Clinical Isolates of Gram-Negative Nonfermentative Bacteria by an Automated Cellular Fatty Acid Identification System

GERARD J. OSTERHOUT, VIRGINIA H. SHULL, AND JAMES D. DICK*

Department of Laboratory Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205

An automated cellular fatty acid (CFA) bacterial identification system, Microbial Identification System (MIS; Microbial ID, Newark, Del.), was compared with a conventional system for the identification of 573 strains of gram-negative nonfermentative bacteria. MIS identifications were based exclusively on the CFA composition following 22 to 26 h of growth at 28°C on Trypticase soy agar. MIS identifications were listed with a confidence measurement (similarity index [SI]) on a scale of 0 to 1.0. A value of ≥0.5 was considered a good match. The MIS correctly listed as the first choice 478 of 532 (90%) strains contained in the data base. However, only 314 (59%) had SI values of ≥0.5. Of the 54 strains in which there was not agreement, 37 belonged to the genera *Acinetobacter*, *Moraxella*, or *Alcaligenes* or were *Pseudomonas pickettii*. Reproducibility studies suggest that SI variation is most likely a function of a difference in culture age at the time of analysis, which is due to the relatively low temperature and time of incubation. Other discrepancies were attributable to insufficiently characterized library entries or an inability to differentiate chemotaxonomically closely related species. The MIS, as the first automated CFA identification system, is an accurate, efficient, and relatively rapid method for the identification of gram-negative nonfermentative bacteria. The development of a CFA library with the media and incubation conditions routinely used for the isolation of clinical pathogens could further decrease the identification time and provide an increase in accuracy.

The nonfermentative gram-negative bacteria are widely distributed in the environment and have become increasingly common isolates in the clinical laboratory. Medically, this group of bacteria has emerged as opportunistic pathogens that cause nosocomial infections, particularly in immunocompromised hosts, and as treatment dilemmas because of antibiotic resistance. In the laboratory, these organisms are frequently difficult to identify since they are usually nonreactive in the conventional systems routinely used for the identification of facultative pathogens. The methods used for the identification of these organisms are in large part cumbersome, requiring a variety of media, reagents, and test conditions, and are time-consuming and susceptible to interpretation error (5, 11, 31, 34, 35). A variety of commercial test systems have been developed for the identification of these organisms (1, 15–17, 20, 30, 32, 37, 40). Although this has resulted in improved efficiency, there is variation in accuracy, cost, and availability among these systems.

Alternatively, the use of chemical analysis of bacterial components, i.e., lipids, polysaccharides, proteins, and nucleic acids, has increasingly been applied to bacterial taxonomy (18, 24, 25). Bacterial lipid determination, particularly cellular fatty acid analysis by gas-liquid chromatography, has long been recognized as a valuable chemotaxonomic tool for the classification and identification of nonfermentative gram-negative bacteria (7, 8, 23, 24, 26, 33, 38, 39). Compared with conventional and other chemical identification methods, other advantages of cellular fatty acid analysis include its relative speed and simplicity and the lack of the need for recultivation of the organism after initial growth or specialized and expensive reagents (22). Bacterial fatty acids, unlike many phenotypic characteristics, are geneti-

cally highly conserved owing to their essential role in cell structure and function. In addition, technical advances in recent years through the development of fused-silica capillary columns, automatic injection systems, digital integrators, and standardized calibrators have increased the applicability of this technology to the clinical microbiology laboratory (27). Despite these improvements, cellular fatty acid analysis and subsequent bacterial identification have relied on manual fatty acid identification and chromatogram interpretation, which require considerable time and expertise on the part of the investigator.

Recently, an automated gas-liquid chromatography system with a computer interface and software for the identification of bacteria on the basis of cellular fatty acid composition has been introduced. Although the library software (version 3.0) for the system has entries for a variety of bacteria, in this study we compared the Microbial Identification System (MIS; Microbial ID, Newark, Del.) with conventional biochemical tests for the identification of nonfermentative gram-negative bacteria exclusively on the basis of their cellular fatty acid content.

MATERIALS AND METHODS

Bacteria. A total of 573 gram-negative nonfermentative bacteria were analyzed in this study. Of this total, 536 were fresh clinical isolates cultured from specimens received by the Microbiology Division, Department of Laboratory Medicine, The Johns Hopkins Hospital. Thirty-seven reference strains were included in the study. *Comamonas acidovorans* ATCC 17438, *Comamonas testosteroni* ATCC 11996, *Moraxella phenylpyruvica* ATCC 23333, *Moraxella osloensis* ATCC 19976, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas alcaligenes* ATCC 14909, *Pseudomonas diminuta* ATCC 11568, *Pseudomonas mendocina* ATCC 25411, *Pseu-*

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* Corresponding author.

domonas pickettii ATCC 27511, *Pseudomonas vesicularis* ATCC 11426, *Pseudomonas pseudoalcaligenes* ATCC 17440, and *Flavobacterium breve* ATCC 14234 were obtained from the American Type Culture Collection. Single strains of *Flavobacterium oryzihabitans*, CDC group EO-2, CDC group M-5, CDC group IVC-2, *Chryseomonas luteola*, *Sphingobacterium multivorum*, *Oligella ureolytica*, and *Weeksella virosa* were kindly provided by the Centers for Disease Control, Atlanta, Ga. *Acinetobacter anitratus* 11a, *Alcaligenes faecalis* 16a, *Shewanella putrefaciens* 6a, *Bordetella bronchiseptica* 17a, *Flavobacterium indologenes* 12a, *Flavobacterium meningosepticum* 13a, *Flavobacterium odoratum* 20a, *Xanthomonas maltophilia* 7a, *Pseudomonas cepacia* 3a, *Pseudomonas fluorescens* 2a, *Pseudomonas paucimobilis* 9a, and *Pseudomonas stutzeri* 5a were kindly provided by G. L. Gilardi, North General Hospital, New York, N.Y. Single strains of *Acinetobacter lwoffii*, *A. faecalis*, and *B. bronchiseptica* were obtained from the College of American Pathologists proficiency surveys. Two strains of *Oligella urethralis* were kindly provided by Becton-Dickinson Microbiology Systems, Baltimore, Md. Cultures were maintained at −70°C in glycerol-Trypticase soy broth and at room temperature in nutrient agar stabs.

Conventional identification. All isolates included in the study were initially screened by the following conventional tests: Gram stain, growth and morphologic characteristics on blood agar and MacConkey agar, catalase, oxidase, triple sugar iron agar reactions, motility, indole production, gelatin liquefaction, utilization of citrate, growth at 42°C, pigment production, oxidative-fermentative carbohydrate utilization (glucose, xylose, maltose), utilization of sodium acetamide, decarboxylation of lysine, dihydrolase reaction of arginine, urease activity, susceptibility to 10 μg of colistin per ml, and hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. When necessary, additional tests included acid production from 10% lactose; phenylalanine deamination; nitrate reduction and gas production; hydrolysis of esculin, starch, DNA, and phosphatidyl choline; allantoin utilization; growth in the presence of 6.5% NaCl; and flagellum staining. These tests were considered conventional identification methods as described by others (5, 11).

Culture conditions. In the case of clinical strains, bacteria were initially isolated on Trypticase soy agar supplemented with 5% sheep blood (BBL, Cockeysville, Md.) at 35°C prior to subculture for cellular fatty acid analysis. Similarly, reference strains were subcultured from Trypticase soy agar slants or from frozen suspensions onto Trypticase soy agar supplemented with 5% sheep blood prior to analysis. Cells for fatty acid analysis, with the exception of several *Moraxella* strains, were grown on Trypticase soy broth agar plates containing 3% (wt/vol) Trypticase soy broth and 1.5% agar (Difco, Detroit, Mich.). The plates were incubated at 28°C for 22 to 26 h. The more fastidious *Moraxella* strains were grown on Trypticase soy agar supplemented with 5% sheep blood. Other slow-growing organisms were inoculated onto duplicate plates to provide a sufficient cell mass for analysis.

Fatty acid analysis. The extraction and derivatization method was done as described by Miller (22). Briefly, approximately 40 mg (wet weight) of cells was gently scraped from the surface of the agar and transferred to a screw-cap tube (13 by 100 mm) with a Teflon-lined cap. Cells were saponified by heating them at 100°C for 30 min following the addition of 1 ml of 15% NaOH in 50% aqueous methanol. The hydrolysate was cooled to ambient temperature, 2 ml of methanolic HCl was added, and the mixture was

heated at 80°C for 10 min. The methylated fatty acids were quickly cooled to ambient temperature and extracted through the addition of 1.25 ml of hexane–methyl-*tert*-butyl ether (1:1; vol/vol) with end-over-end mixing. The phases were allowed to separate, the lower aqueous layer was carefully removed, and 3 ml of dilute NaOH was added to the remaining organic layer. This was then mixed, and saturated NaCl was added to samples requiring further clarification of the phase interface. Approximately two-thirds of the organic layer containing the fatty acid methyl esters (FAMEs) was then transferred to a septum-capped sample vial for analysis.

Cellular fatty acid chromatography. FAMEs were analyzed on a Hewlett-Packard 5890A gas chromatograph (Avondale, Pa.) equipped with a flame ionization detector, automatic sampler, integrator, and computer. Separation of the FAMEs was achieved with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5% phenylmethyl silicone. The specific operating parameters of the instrument were controlled and set automatically by the computer software. The operating parameters for this study were as follows: injector temperature, 250°C; detector temperature, 300°C; and oven (column) temperature, programmed from 170 to 300°C at 5°C/min and held at 300°C for 5 min prior to recycling. The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage, and report generation. Peak naming and column performance were achieved through the use of calibration samples (Microbial ID) containing straight-chain saturated and hydroxy fatty acids. A fresh calibration mixture was used daily prior to analysis of clinical samples and was automatically rerun and evaluated after every 10th sample.

Data analysis. Cellular fatty acids were identified on the basis of equivalent chain length data. This value is a representation of a fatty acid's retention time as it relates to a series of straight-chain saturated FAMEs found in the calibration mixture. Since equivalent chain length plots of fatty acids of a homologous series, e.g., 3-hydroxy fatty acids, are linear, the computer automatically interpolates from a pre-established equivalent chain length plot the identity of each sample peak. The area of each peak was also determined and stored. Peak area values were expressed as a percentage of the total of the area of all peaks in the chromatogram exclusive of the solvent front.

Following computer analysis, a hard-copy report is generated for each sample. In addition to sample identification data, a variety of parameters concerning each chromatographic peak is printed. These include retention time, area, area/height ratio, equivalent chain length, peak name (specific fatty acid), and percentage of total fatty acids present, as well as calibration data.

Organism identification was based on computer comparison of the unknown organism's FAME profile with those of predetermined library profiles. Library organism profiles (*P*) were established on the basis of a multivariate Gaussian model of at least 12 strains of each species by using the following equation (21): $P(x \mid \text{class} = t) = [1/(2\pi)^{n/2} \mid C \mid^{1/2}] \exp [-0.5(x − m)^{\mathrm{Tr}} C^{-1} (x − m)]$, where n is the number of features in x, x is the feature vector of a sample, m is the expected value of x for class t, and C is an $n \times n$ matrix where each c_{ij} is the expected value of $(x_i − m_i)(x_j − m_j)$ for class t. The correlation of an unknown organism's profile with a library entry was expressed as a similarity index (SI) on a numeric scale of 0 to 1.0. The square of δ, the distance of an organism's profile from the established popu-

TABLE 1. Comparative identification of members of the family *Pseudomonadaceae*

Conventional identification	No. of strains tested	No. (%) of strains in agreement		SI	
		First choice with SI value of ≥0.50	First choice regardless of SI value	Mean	Range
Pseudomonas aeruginosa	61	37 (61)	59 (97)	0.57	0.04–0.94
Pseudomonas alcaligenes	1	1 (100)	1 (100)		
Pseudomonas cepacia	27	11 (41)	22 (81)	0.52	0.02–0.84
Pseudomonas diminuta	13	13 (100)	13 (100)	0.82	0.62–0.91
Pseudomonas fluorescens/Pseudomonas putida group	65	31 (48)	65 (100)	0.47	0.01–0.86
Pseudomonas mendocina	5	4 (80)	4 (80)	0.82	0.72–0.89
Pseudomonas paucimobilis	10	7 (70)	10 (100)	0.60	0.18–0.84
Pseudomonas pickettii	15	3 (20)	3 (20)	0.81	0.68–0.82
Pseudomonas pseudoalcaligenes	11	5 (45)	7 (64)	0.62	0.27–0.86
Pseudomonas stutzeri	23	21 (91)	21 (91)	0.86	0.61–0.97
Pseudomonas vesicularis	3	1 (33)	2 (67)	0.42	0.23–0.61
Shewanella putrefaciens	12	12 (100)	12 (100)	0.76	0.58–0.96
Comamonas acidovorans	17	14 (82)	17 (100)	0.69	0.25–0.93
Comamonas testosteroni	13	10 (77)	13 (100)	0.59	0.38–0.70
Flavimonas oryzihabitans	10	10 (100)	10 (100)	0.88	0.73–0.98
Xanthomonas maltophilia	47	27 (57)	45 (96)	0.55	0.01–0.93
Total	333	207 (62)	304 (91)		

lation mean, is equal to $[-0.5(x - m)^{\mathrm{Tr}} C^{-1} (x - m)]$. The SI of a particular organism's profile is then expressed as follows: $e^{-\alpha\delta}2$, where α is equal to a constant such that when δ is 3.0 the SI is 0.600. SI values of ≥0.6 were considered excellent matches, with a value of 0.5 representing approximately 3 standard deviations from the library profile mean.

For the purposes of this study, MIS identifications were compared with the conventional identification on the basis of two parameters: (i) the number of strains in agreement as the first choice with MIS SI values of ≥0.50 and (ii) the number of strains in agreement as the first choice regardless of the SI value. In instances of discrepant identifications, both the conventional and fatty acid analyses were repeated. Seven strains which could not be analyzed again because of nonviability were not included in the study.

Reproducibility of SI values. The reproducibility of the SI was determined by repeated analysis of two reference strains, *P. aeruginosa* ATCC 27853 and *X. maltophilia* 7a (Gilardi). These analyses were performed on different days under the specified growth conditions. The coefficient of variation was calculated for the SIs of each of the organisms.

Effects of temperature of incubation and media on the SI values. In order to evaluate the possible effect of temperature and media on the variability of the SI values, a library entry was established with 15 clinical strains of *P. aeruginosa* grown at 35°C for 22 to 24 h on Trypticase soy agar supplemented with 5% sheep blood by using the Hewlett-Packard Library Generation Software provided with the system. *P. aeruginosa* ATCC 27853 was then analyzed on 10 separate occasions following growth at 35°C on Trypticase soy agar with 5% sheep blood and following growth at 28°C on Trypticase soy agar. Cellular fatty acid data for each analysis was evaluated against the appropriate library entry at 28 or 35°C, with the subsequent generation of identification and SI data. The coefficient of variation was then calculated for the SIs of the 10 analyses at 28°C and the 10 analyses at 35°C.

RESULTS

The identifications of members of the family *Pseudomonadaceae* by the MIS compared with conventional biochemical identifications are given in Table 1. If the first choice and an SI value of ≥0.5 were used as the criteria for agreement with the conventional identification system, there was only agreement for 62% of the isolates tested. Species in which 80% agreement or greater occurred included *P. diminuta*, *P. mendocina*, *P. stutzeri*, *Shewanella putrefaciens*, *C. acidovorans*, and *F. oryzihabitans*. With the exception of *P. aeruginosa*, there were no significant morphologic or phenotypic differences between strains of a species which were identified with SI values of ≥0.5 versus those identified with SI values of <0.5. Five of the *P. aeruginosa* isolates included in this study were atypical mucoid isolates recovered from patients with cystic fibrosis. Two were identified as *P. fluorescens* or *Pseudomonas putida* (Table 2), and the remaining three had SI values of 0.04, 0.153, and 0.157. Table 2 lists the disparities between the conventional biochemical identification system and the MIS first choice identification, regardless of SI value, for the identification of the *Pseudomonadaceae*. Five of 27 *P. cepacia* isolates were identified as *Pseudomonas gladioli*, a closely related plant pathogen (29). Three of these isolates had SI values of >0.5, and for three isolates, *P. cepacia* was listed as a close second choice. As shown in Table 2, the key phenotypic characteristics which were used to differentiate these two species were acid production from maltose and 10% lactose, esculin hydrolysis, lysine decarboxylation, and a positive oxidase reaction. The close genetic relationship on the basis of both rRNA and DNA homology (34) between *P. mendocina*, *P. pseudoalcaligenes*, *P. stutzeri*, and *P. alcaligenes* is reflected in the discrepancies shown in Table 2 between the conventional identification system and MIS. Similarly, incorrect identification of *P. pickettii* and *X. maltophilia* frequently resulted in these species being listed as second choices, with the first choice being the genetically closely related plant pathogens *Pseudomonas solanacearum* and *Xanthomonas campestris*, respectively.

Identifications of non-*Pseudomonadaceae* clinical isolates are given in Table 3. Among the 199 strains examined, only 107 (54%) of the MIS first choice identification with SI values of ≥0.5 were in agreement with the conventional identification, while 174 (87%) were in agreement if the first choice regardless of SI value was used as the criterion. Identifications of all strains of *Agrobacterium radiobacter*, *B. bronchiseptica*, *Flavobacterium indologenes*, *Flavobacterium meningosepticum*, *Flavobacterium odoratum*, and *Methylobacterium extorquens* were in agreement with the conventional identifications if the latter criterion was used. However, poor agreement was found for *F. meningosepticum*, *F. odoratum*, and *M. extorquens* when an SI value of ≥0.5 was used as the criterion for a correct identification. Discrepancies between the MIS and the conventional system, as well as the key phenotypic characteristics used for differentiation, are given in Table 4. Of the 42 strains of *Acinetobacter* evaluated, 7 were incorrectly identified with low SI values (≤0.25). Because of its heterogeneity and taxonomic complexity, the two identification systems were compared on the basis of genus-level rather than species-level identification for this group (2). Although *Branhamella catarrhalis* and *Streptococcus uberis* are readily differentiated from *Acinetobacter* isolates on the basis of conventional tests, all seven of the incorrectly identified *Acinetobacter* isolates contained 2-hydroxydodecanoic acid (2-OH-12:0), a key differentiating fatty acid of the genus according to Moss et al. (28). Of the 82 isolates of *Alcaligenes* tested, 11 resulted in discrepancies between
the MIS and the conventional system. One of the isolates of *Alcaligenes xylosoxidans* subsp. *denitrificans* was identified as *A. faecalis*, while three of the seven discrepant *A. faecalis* isolates were identified as *A. xylosoxidans* subsp. *denitrificans*, with *A. faecalis* being listed as a close second choice on the basis of the SI value. The only useful conventional test for differentiation of these two species is nitrate reduction with gas production, which was positive for the one discrepant strain of *A. xylosoxidans* subsp. *denitrificans* and negative for the three strains of *A. faecalis*. For all but one discrepant *A. faecalis* isolate, this species was listed as the second choice with an SI value very close to that of the incorrect first choice. This suggests an inability of the MIS to accurately separate this group of *A. faecalis* strains from *A. xylosoxidans* subsp. *denitrificans*, *Yersinia pseudotuberculosis*, *Kingella kingae*, *Enterobacter agglomerans*, and *B. bronchiseptica* on the basis of fatty acid profiles in the existing library. Similarly, on the basis of the closeness of the SI value, some strains of *A. xylosoxidans* subsp. *xylosoxidans* cannot be differentiated from *B. bronchiseptica* on the basis of existing MIS fatty acid library entries and software. Only 12 isolates of *Moraxella* were evaluated, with the MIS correctly identifying only 2 of the isolates of *Moraxella osloensis* with acceptable SI values and none of the 3 *Moraxella phenylpyruvica* isolates tested.

Forty-one isolates representing 12 nonfermentative gram-negative species not contained in the MIS cellular fatty acid library were also evaluated. As shown in Table 5, with the exception of CDC group VB-3 and *Chryseomonas luteola*, none of the isolates tested were identified or were named with very low SI values. CDC group VB-3 is phenotypically and genetically closely related to the "*Pseudomonas stutzeri* group," which includes *P. alcaligenes*, and this is reflected in the cellular fatty acid profile similarities in the MIS library entries (12). Similarly, the phenotypic and chemotaxonomic relatedness of *C. luteola* and *Flavimonas oryzihabitans*,

TABLE 2. Discrepancies between the conventional identification and the MIS

Conventional identification (no. of strains)	MIS identification (SI)		Key phenotypic characteristics used for differentiation
	First choice	Second choice	
Pseudomonas aeruginosa (2)	*P. putida* (0.01) *P. fluorescens* (0.02)		Denitrification, growth at 42°C, gelatin hydrolysis, pigment production
Pseudomonas cepacia (5)	*P. gladioli* (0.78) *P. gladioli* (0.37) *P. gladioli* (0.84) *P. gladioli* (0.87) *P. gladioli* (0.12)	*P. cepacia* (0.47) *P. cepacia* (0.35) *P. cepacia* (0.82)	Acid from maltose and 10% lactose, oxidase positive, lysine decarboxylation, esculin hydrolysis
Pseudomonas mendocina (1)	*P. stutzeri* (0.71)	*P. mendocina* (0.70)	Acid from glucose and xylose but not maltose, arginine dihydrolase activity, no starch hydrolysis
Pseudomonas pickettii (12)	*P. solanacearum* (0.25) *P. solanacearum* (0.34) *P. solanacearum* (0.79) *P. solanacearum* (0.45–0.74) (9 isolates)	*P. pickettii* (0.16) *P. pickettii* (0.19) *P. pickettii* (0.66)	Growth at 42°C, acid from xylose
Pseudomonas pseudoalcaligenes (4)	*P. mendocina* (0.77) *P. alcaligenes* (0.46) *P. stutzeri* (0.20) *P. mendocina* (0.80)	*P. pseudoalcaligenes* (0.69) *P. pseudoalcaligenes* (0.38)	No acid from glucose or xylose, no pigment, no nitrate reduction without gas, acid from fructose
Pseudomonas stutzeri (2)	*P. mendocina* (0.80) *P. mendocina* (0.66)	*P. stutzeri* (0.71)	Utilization of maltose, no arginine dihydrolase activity, starch hydrolysis
Pseudomonas vesicularis (1)	*Streptococcus faecium* (0.22)	*P. vesicularis* (0.16)	Gram-negative, oxidase positive, nonfermenter
Xanthomonas maltophilia (2)	*X. campestris* (0.22) *X. campestris* (0.02)	*X. maltophilia* (0.21) *X. maltophilia* (0.01)	Decarboxylation of lysine

TABLE 3. Comparative identification of *Acinetobacter, Alcaligenes, Agrobacterium, Bordetella,*
Flavobacterium, Methylobacterium, and *Moraxella* species

Conventional identification	No. of strains tested	No. (%) of strains in agreement		SI	
		First choice with SI value of ≥0.50	First choice regardless of SI value	Mean	Range
Acinetobacter sp.	42	15 (36)	35 (83)	0.46	0.04–0.97
Agrobacterium radiobacter	4	4 (100)	4 (100)	0.77	0.70–0.87
Alcaligenes xylosoxidans subsp. *denitrificans*	3	2 (67)	2 (67)	0.71	0.54–0.89
Alcaligenes xylosoxidans subsp. *xylosoxidans*	51	41 (80)	48 (94)	0.67	0.12–0.90
Alcaligenes faecalis or *Alcaligenes odorans*	28	10 (36)	21 (75)	0.47	0.12–0.80
Bordetella bronchiseptica	4	4 (100)	4 (100)	0.66	0.59–0.73
Flavobacterium indologenes	20	17 (85)	20 (100)	0.65	0.37–0.81
Flavobacterium meningosepticum	14	3 (21)	14 (100)	0.35	0.10–0.62
Flavobacterium odoratum	8	5 (63)	8 (100)	0.58	0.24–0.84
Methylobacterium extorquens	13	4 (31)	13 (100)	0.42	0.06–0.88
Moraxella osloensis	9	2 (22)	5 (56)	0.45	0.28–0.67
Moraxella phenylpyruvica	3	0 (0)	0 (0)		
Total	199	107 (54)	174 (87)		

formerly biovars of CDC group Ve (8, 13), were reflected in the MIS identification of all five strains of *C. luteola* tested as *F. oryzihabitans.* Dees et al. (9) have demonstrated 17:0 and 19:0 carbon cyclopropane acids to be a differentiating characteristic between these organisms. In this study, 3 of 5 *C. luteola* isolates contained a cyclopropane fatty acid, while none were present in any of the 10 isolates of *F. oryzihabitans* tested.

The reproducibility of the SI values of the same isolate tested on repeated occasions in the MIS is given in Table 6. *P. aeruginosa* ATCC 27853 and *X. maltophilia* 7a were used as control organisms during the course of this study, being run daily on the MIS after growth under the specified conditions. There was a significant variation in the SI values when these control strains were tested on 100 separate occasions. In an effort to determine the potential effects of temperature of incubation and medium composition on the reproducibility of the SI values, *P. aeruginosa* ATCC 27853 was grown at 35°C for 22 to 26 h on Trypticase soy agar with 5% sheep blood on 10 separate occasions and identified by using a cellular fatty acid library entry which was generated by using 15 different strains of *P. aeruginosa* grown under these same conditions. At the same time, *P. aeruginosa* ATCC 27853 was grown under the recommended conditions at 28°C and evaluated against the MIS library. There was a correlation between these 10 samples and the 100 samples run previously under the conditions recommended for MIS with regard to the mean SI value and the coefficient of variation. Of particular note was the significant improvement in SI values obtained for cells grown at 35°C on Trypticase soy agar with 5% sheep blood agar by using a library entry generated at this temperature with the Library Generation Software.

DISCUSSION

While the results of identification by the conventional system and MIS were in good agreement if the MIS first choice regardless of SI value was used (90%), the use of the recommended SI value of ≥0.5 as the criterion for agreement resulted in only a 59% correlation. This variation was further emphasized in the evaluation of SI reproducibility by using control strains of *P. aeruginosa* and *X. maltophilia,* in

which the coefficients of variation were 26 and 43%, respectively. Bacterial fatty acid composition has been shown to vary with culture medium, incubation temperature, and physiological age of the cell population; however, if growth conditions are standardized, several investigators have demonstrated that the same bacterial strain grown and analyzed repeatedly will yield highly reproducible cellular fatty acid profiles (10, 29). It is unlikely in this evaluation that the variation in SI values, and presumably, cellular fatty acid profiles, that was seen was due to either the effects of medium or changes in the incubation temperature since all isolates were cultured for analysis under conditions identical to those used to establish the library entries used for identification. However, there is potential for variation as a result of differences in the physiological age of the cell population at the time of analysis. A number of investigators have demonstrated variation, both quantitative and qualitative, in the fatty acid composition of bacteria with culture age (6, 14, 19). The recommended culture conditions for the MIS, 28°C on Trypticase soy agar for 22 to 26 h, does offer the potential for variation in physiological age differences as a consequence of the relatively low incubation temperature and the resulting increase in generation time. This possibility is supported by the comparative reproducibility of the SI value of *P. aeruginosa* ATCC 27853 grown at 28 and 35°C and identified with library entries generated at those respective temperatures. This suggests that a marked improvement in the SI values could be achieved if the standardized growth temperature for the development of library profiles and organism analysis was changed from 28 to 35°C, which would allow all organisms to attain the same physiological age, i.e., stationary growth phase.

The accuracy of any identification system is in large part determined by the validity of the data base on which unknowns are identified in terms of inclusion of sufficient numbers of authentic and correctly identified strains. Inclusion of incorrectly identified or poorly characterized isolates in an identification data base will result in incorrect or no identification of these species or an inability to differentiate between closely related species. Overall, the MIS data base did perform well if the first choice regardless of the SI value was used as the criterion for definitive identification. However, several species contained in the MIS data base resulted in high discrepancy rates when identifications were com-

TABLE 4. Discrepancies between the conventional identification and the Microbial Identification System

Conventional identification (no. of strains)	MIS identification (SI)		Key phenotypic characteristics used for differentiation
	First choice	Second choice	
Acinetobacter spp. (7)	B. catarrhalis (0.25) B. catarrhalis (0.19) B. catarrhalis (0.17) B. catarrhalis (0.11) B. catarrhalis (0.09) S. uberis (0.22) S. uberis (0.04)	A. lwoffii (0.16)	Gram-negative coccobacillus, oxidase negative, no hydrolysis of DNA
Alcaligenes xylosoxidans subsp. denitrificans (1)	A. faecalis (0.45)		Nitrate reduction with gas production
Alcaligenes xylosoxidans subsp. xylosoxidans (3)	B. bronchiseptica (0.09) B. bronchiseptica (0.66) B. bronchiseptica (0.45)	A. xylosoxidans subsp. xylosoxidans (0.06) A. xylosoxidans subsp. xylosoxidans (0.55) A. xylosoxidans subsp. xylosoxidans (0.40)	Acid from xylose, urease negative
Alcaligenes faecalis (7)	Yersinia pseudotuberculosis (0.33) Alcaligenes denitrificans (0.80) A. denitrificans (0.64) A. denitrificans (0.69) K. kingae (0.91) E. agglomerans (0.38) B. bronchiseptica (0.80)	A. faecalis (0.28) A. faecalis (0.68) A. faecalis (0.48) A. faecalis (0.68) A. faecalis (0.41) A. faecalis (0.36)	No acid from glucose, urease negative, no gas production from nitrate
Moraxella osloensis (4)	B. catarrhalis (0.20) B. catarrhalis (0.03) B. catarrhalis (0.04) Acinetobacter calcoaceticus (0.03)		Oxidase-positive, gram-negative bacillus, DNA hydrolysis negative, susceptible to polymyxin
Moraxella phenylpyruvica (3)	No match (3 isolates)		Oxidase-positive, gram-negative bacillus, nonsaccharolytic, phenylalanine deamination, urea hydrolysis

TABLE 5. Identification of gram-negative nonfermentative bacilli not in the MIS cellular fatty acid profile library

Conventional identification (no. of strains tested)	MIS identification (SI)
Ochrobactrum anthropi (17)	*Bradyrhizobium japonicum* (0.01–0.32) (seven strains)
	Enterococcus faecium (0.01–0.02) (four strains)
	Brucella abortus (0.01–0.05) (three strains)
	Methylobacterium extorquens (0.001)
	Agrobacterium tumefaciens (0.002)
	No match
CDC group EO-2 (2)	*Branhamella catarrhalis* (0.026)
	Erwinia stewartii (0.001)
CDC group VB-3 (3)	*Pseudomonas stutzeri* (0.695)
	Pseudomonas stutzeri (0.850)
	Pseudomonas alcaligenes (0.785)
CDC group M-5 (1)	*Neisseria mucosa* (0.096)
CDC group IVC-2 (1)	*Pseudomonas solanacearum* (0.107)
Chryseomonas luteola (5)	*Flavimonas oryzihabitans* (0.15–0.83) (five strains)
Flavobacterium breve (1)	No match
Sphingobacterium multivorum (1)	No match
Oligella urethralis (3)	*Campylobacter jejuni* (0.001–0.047) (three strains)
Oligella ureolytica (1)	*Campylobacter jejuni* (0.092)
Weeksella virosa (5)	No match (four strains)
	Flavobacterium meningosepticum (0.001)
Weeksella zoohelcum (1)	*Flavobacterium indologenes* (0.005)

pared with those obtained by the conventional test system. For both species of the genus *Moraxella*, there was a poor correlation between the conventional identification system and MIS, although only 12 strains were included in this study. Comparison of the MIS fatty acid library profiles of *M. osloensis* and *M. phenylpyruvica* with the fatty acid composition reported by Moss et al. (28) demonstrated significant quantitative and qualitative differences not attributable to differences in culture conditions alone, suggesting that either too few or poorly characterized strains were used to develop the MIS library entry.

In addition to SI variation caused by potential differences in physiological cell age and the possible inclusion of poorly characterized organisms in the library profile data base, the data from this study suggest a third possibility for discrepancies between the MIS and the conventional identification system. In a number of instances, the SI values for the first and second identification choices were almost identical. This could be seen with some strains of *P. cepacia* and *P. gladioli*; *X. maltophilia* and *X. campestris*; *A. xylosoxidans* subsp. *denitrificans* and *A. faecalis*; *P. mendocina*, *P. pseudoalcaligenes*, and *P. stutzeri*; *P. pickettii* and *P. solanacearum*; *A. xylosoxidans* subsp. *xylosoxidans* and *B. bronchiseptica*; and *C. luteola* and *F. oryzihabitans*. These groups are closely related genetically, and the closeness in SI values for some isolates indicates that they are highly related chemically in terms of cellular fatty acid composition (4, 23, 34, 36). Because of this apparent relatedness in fatty

acid composition, organisms falling into these fatty acid groups require phenotypic differentiation to the species level on the basis of conventional tests.

The existing MIS library includes a wide variety of plant and environmental bacteria which have not been associated with humans. Because of their close taxonomic relationship, many of these species cannot be consistently separated on the basis of cellular fatty acid composition by using the current MIS methods and require the use of selected phenotypic characteristics for differentiation. The development of a library by using the culture conditions used in clinical bacteriology laboratories with large numbers of well-characterized clinical isolates would potentially alleviate this problem and improve identification.

In summary, the MIS proved to be a relatively rapid and accurate method for the identification of gram-negative nonfermentative bacteria when it was compared with conventional identification methods. Identification discrepancies, when they occurred, were attributable to SI variation, incorrect or poorly defined library profiles, or an inability to differentiate precisely genetically and chemically closely related species. In large part, these represent standardization and data base problems and are not a reflection of the value of cellular fatty acid analysis as a tool for bacterial identification, which has been conclusively demonstrated (3, 24, 25, 29, 33, 38). The development of a cellular fatty acid library by using the culture conditions routinely used in clinical microbiology laboratories would potentially reduce

TABLE 6. Reproducibility and effect of temperature on SI values of the MIS

Organism	Growth conditions	Library entry tested	No. of isolates tested	SI		
				Mean	Coefficient of variation (%)	Range
Pseudomonas aeruginosa ATCC 27853	22–26 h, 28°C	MIS	100	0.72	26	0.30–0.97
Xanthomonas maltophilia 7a	22–26 h, 28°C	MIS	100	0.36	43	0.08–0.74
Pseudomonas aeruginosa ATCC 27853	22–26 h, 28°C	MIS	10	0.71	23	0.41–0.91
Pseudomonas aeruginosa ATCC 27853	22–26 h, 35°C	JHH[a]	10	0.88	4	0.83–0.92

[a] JHH, entry created at Johns Hopkins Hospital.

the time required for identification as well as improve the overall accuracy of the system. The MIS continues to be improved, and additional library updates are expected to address the limitations discussed here.

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