

Identification of *Enterococcus*, *Streptococcus*, and *Staphylococcus* by Multivariate Analysis of Proton Magnetic Resonance Spectroscopic Data from Plate Cultures

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A new fingerprinting technique with the potential for rapid identification of bacteria was developed by combining proton magnetic resonance spectroscopy (¹H MRS) with multivariate statistical analysis. This resulted in an objective identification strategy for common clinical isolates belonging to the bacterial species *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and the *Streptococcus milleri* group. Duplicate cultures of 104 different isolates were examined one or more times using ¹H MRS. A total of 312 cultures were examined. An optimized classifier was developed using a bootstrapping process and a seven-group linear discriminant analysis to provide objective classification of the spectra. Identification of isolates was based on consistent high-probability classification of spectra from duplicate cultures and achieved 92% agreement with conventional methods of identification. Fewer than 1% of isolates were identified incorrectly. Identification of the remaining 7% of isolates was defined as indeterminate.

In both clinical and industrial laboratories, methods for identification of microorganisms have historically been based on multiple phenotypic characters, including morphological features and a range of biochemical reactions. These tests are often time-consuming and/or relatively expensive in their application, and some are imprecise. Recently, alternative methods have been investigated in an attempt to develop a single, rapid method for characterization and identification of microorganisms. These have included Fourier transform infrared spectroscopy (11, 14), pyrolysis mass spectrometry (12), electrospray ionization mass spectrometry (7), UV resonance Raman spectroscopy (15), and protein electrophoresis (16). While reports of these techniques suggest the possibility of rapid and reliable identification of some groups of microorganisms, most have been tested with small data sets. With the exception of Fourier transform infrared spectroscopy, they are destructive techniques which analyze cellular decomposition products. All have the limitation that they do not directly yield information about the biochemistry of the intact viable organism.

In contrast, magnetic resonance spectroscopy (MRS) of viable cells can provide information on a large range of metabolites. Biological applications of MRS most commonly exploit the noninvasive nature of the technique to study aspects of cellular biochemistry in living systems (6). However, not all applications of MRS require or include identification of the

metabolites contributing to the MR spectrum. Pattern recognition techniques, which detect gross spectral characteristics associated with a priori-defined classes (such as pathological conditions), have been successfully applied to MRS of both tissues and body fluids. Accurate and reliable classifiers based on multivariate analyses of ¹H MR spectroscopic data have been developed and validated for objective diagnosis of thyroid (21), ovarian (22), prostate (9), breast (13), and brain (20) tumors. In some pathologies, MRS is able to detect malignancy before morphological manifestations are visible by light microscopy (17).

A one-dimensional ¹H MR spectrum of a bacterial cell suspension provides an overview of hydrogen-containing compounds that are tumbling rapidly on the MR timescale. Consequently, the ¹H MR spectrum will be more representative of the physiology of the cell (metabolite pools) than of its structure (comprising immobile components such as the cell wall). While many different bacterial groups may express and utilize essentially identical metabolic pathways, it might reasonably be expected that differing levels of enzyme expression and activity in different groups would give rise to distinctly different levels of particular metabolites when dissimilar groups are grown in similar environments. We therefore proposed that significantly different metabolite pool sizes would be detected as differences between the ¹H MR spectra of the different bacterial groups. This was suggested in a previous study comparing selected bacterial ¹H MR spectra (5); however, the small number of isolates examined and the qualitative identification methods described in that study did not permit automation or quantitative comparison of the species groups.

We show here that it is possible, using simple linear discriminant analysis (LDA) on 312 cultures of 104 different isolates, to

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make reliable automated identifications of bacteria on the basis of their ^1H MR spectra.

MATERIALS AND METHODS

Storage and culture of bacteria. Isolates were obtained from the collection of the Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research, Sydney, Australia and the American Type Culture Collection, or were recent clinical isolates from the clinical identification laboratory of the Centre for Infectious Diseases and Microbiology Laboratory Services. Stored isolates were suspended in 10% glycerol in nutrient broth at -70°C . Horse blood agar (HBA) was prepared by addition of sterile horse blood to autoclaved blood agar base (Oxoid, Basingstoke, United Kingdom or Amyl Media, Sydney, Australia). Isolates retrieved from storage were subcultured onto 5% horse blood agar and incubated in 5% CO_2 for 18 to 24 h at 37°C . New isolates and isolates subcultured on HBA after storage were streaked onto duplicate HBA plates, incubated at 37°C for 18 to 24 h, and then stored at ambient temperature (20 to 30°C) for 3 to 9 h before being subjected to spectroscopy.

To test for short-term method variability, we examined duplicate cultures of all isolates. To test for long-term culture and method variability, we recultured a number of isolates up to six times over an 8-month period. Included in the analysis were spectra of three isolates of *Enterococcus gallinarum* and three isolates of *E. casseliflavus*, which are closely related to *E. faecalis* (10) (Table 1). The number of distinct isolates examined from each species group and the number of times the isolate was recultured and reexamined can be determined from Table 1.

Conventional identification of bacteria. *Staphylococcus aureus* was identified on the basis of positive coagulase (using rabbit or human plasma) and DNase tests. *Staphylococcus epidermidis* was identified using the API ID32 staph test (BioMérieux, Marcy l'Etoile, France). *Streptococcus* and *Enterococcus* species were identified by conventional methods, i.e., optochin sensitivity (*Streptococcus pneumoniae*), salt tolerance and bile-esculin positivity (*Enterococcus* spp.), latex agglutination (*Streptococcus agalactiae*), and by the API ID32 strep test (BioMérieux). All tests were carried out as specified by the manufacturers. In general, isolates were identified only once, upon receipt in the microbiology laboratory and prior to storage. Some isolates retrieved from storage were reidentified by conventional tests.

^1H MRS. Bacterial colonies (2 to 200 mg [wet weight]) were gently removed from the HBA plate with a plastic inoculating loop and suspended by vortexing in 0.3 ml of phosphate-buffered saline (pH 7.2, room temperature) made up in D_2O ($\text{PBS-D}_2\text{O}$). For most cultures, $>80\%$ of cells were scraped off the plate. In cases of heavy growth, $<10\%$ of cells were harvested, usually from the first quadrant. The suspension was immediately transferred to a 5-mm-diameter susceptibility-matched MR sample tube (Shigemi). ^1H MRS measurements were performed at 37°C on a Bruker Avance 360 MHz MR spectrometer using a $^1\text{H}/^{13}\text{C}$ 5-mm probe head. One-dimensional (1D) spectra were acquired with acquisition parameters as follows: frequency, 360.13 MHz; pulse angle, 90° (6 to 7 μs), repetition time, 1s; 8k data points, 256 or 512 transients; spectral width, 3600 Hz; total acquisition time, 10 or 20 min. The field was locked to D_2O . Water suppression was effected by a selective excitation field gradient method (double-pulsed field gradient spin echo [DPFGSE]) (3). The spectra of cells suspended in $\text{PBS-D}_2\text{O}$ were stable for at least 2 h at 37°C .

Signal assignment. 2D homo- and heteronuclear correlation spectra were acquired for at least two isolates per species to assign 1D MR resonances to specific compounds. $\{^1\text{H}, ^1\text{H}\}$ gradient correlation spectroscopy (COSY) experiments were performed in magnitude mode. The acquisition parameters were as follows: sweep width in t_2 , 3,600 Hz; t_2 time domain, 2K; 256 increments of 32 or 48 acquisitions each; repetition time, 1 s. Sine bell window functions were applied in the t_1 dimension, and Gaussian-Lorentzian window functions were applied in the t_2 dimension. Zero filling was used to expand the data matrix to 1K in the t_1 dimension. Total correlation spectroscopy (TOCSY) spectra with mixing times of 40 and 150 ms were acquired with 256 increments of 2K data points and 32 acquisitions (1). $\{^1\text{H}, ^{13}\text{C}\}$ one-bond shift correlation spectra were obtained in the ^1H detection mode using a gradient heteronuclear single quantum coherence (HSQC) pulse sequence (23). The ^1H MR spectral width was 3,600 Hz, and the ^{13}C MR spectral width was 15,000 Hz. ^{13}C MR decoupling during acquisition was achieved by using globally optimized alternating phase rectangular pulses (GARP) (18). The evolution time (t_1) was incremented to obtain 400 FIDs, each of 40 to 64 acquisitions and consisting of 2K data points. The repetition time was 1 s. A sine bell window function was applied in the t_2 dimension, and a Gaussian-Lorentzian function was applied in the t_1 dimension. Zero filling to 1K was used in the t_1 dimension prior to Fourier transformation. $\{^1\text{H}, ^{13}\text{C}\}$ gradient hetero-

nuclear multiple-bond correlation (HMBC) spectra were acquired without proton decoupling using the same parameters as for the HSQC experiments, except for a ^{13}C MR spectral width of 20 kHz (23). One-bond and long-range correlation experiments were usually optimized for $^1\text{J}_{\text{C,H}}$ of 140 Hz and $^n\text{J}_{\text{C,H}}$ of 7 Hz, respectively. 1D ^1H MR spectra were acquired before and after the 2D experiments to verify absence of metabolic changes.

Data processing. Spectra were processed using Bruker XWINNMR spectrometer software. Zero filling was performed to extend the free induction decay data set to 16K. An exponential window function was applied before Fourier transformation, yielding a line broadening of 1 Hz. Chemical shift calibration was performed by setting the center of the spectrum to 4.64 ppm (the nominal position of the water resonance with respect to tetramethylsilane in $\text{PBS-D}_2\text{O}$ at 37°C). Spectra were manually phase corrected to achieve a linear and flat baseline. Sixteen contiguous fixed integration regions were subjectively chosen on the basis of major peaks present in the representative spectra (see Fig. 1). The individual integrals were normalized to the total intensity of the 16 integrals.

LDA. The table of integrals was imported from Microsoft Excel into STATISTICA (StatSoft Pacific P/L) for LDA. Each of the first 15 of 16 chosen integral regions (see Results) formed one independent variable in the seven-group LDA (standard method, tolerance 0.01, a priori classification probability proportional to group size). The 16th region (arbitrary choice) was omitted from the LDA because, in a normalized data set, one region is redundant for discriminant analysis. Information from the omitted region is "embedded" in the remaining regions. Classification functions and classification probabilities were calculated with STATISTICA.

Classification of spectra and identification of isolates. In this paper we use the following definitions. The term "classification" refers to assignment of an individual spectrum from a bacterial culture to a species group. "Identification" refers to assignment of an isolate to a species group (on the basis of classification of two independent spectra derived from duplicate cultures of the isolate). "Correct classification" refers to assignment of a spectrum to the same species group as conventional classification with a percent classification probability of $\geq 85\%$. The chosen percentage is arbitrary but is considered a reasonably high probability for confident assignment. "Misclassification" refers to assignment of a spectrum to a species group different from conventional classification with a percent classification probability of $\geq 85\%$. "Indeterminate classification" refers to assignment of a spectrum to any species group with percent classification probability of $<85\%$. "Correct identification" refers to assignment of both spectra of duplicate cultures according to conventional identification and with an average percent classification probability of $\geq 85\%$. "Misidentification" refers to assignment of both spectra of duplicate cultures to the same species group but different from conventional identification and with an average percent classification probability of $\geq 85\%$. "Indeterminate identification" refers to assignment of spectra of duplicate cultures to different groups or the same group with an average classification probability of $<85\%$.

An optimized seven-group classifier was developed based on the bootstrap method (2) modified and renamed the robust bootstrap method by Somorjai et al. (19). Starting with all 312 spectra, we randomly selected half the spectra from each species group and used this training set to train the seven-group classifier (LDA). The resulting classifier was then used to validate the remaining spectra (the test set). This process was repeated B times (with replacement), and every time the optimized LDA coefficients were saved. The weighted average of these B sets of LDA coefficients produces the final classifier ($B = 1,000$). The weight for the m th set is $W_m = K_m C_m^{1/2}$ ($m = 1, \dots, B$), where $0 \leq C_m \leq 1$ is the crispness (defined as the fraction of test samples assigned to a class with a percent probability of $\geq 75\%$) and $0 \leq K_m \leq 1$ is Cohen's chance-corrected measure of agreement (4), with $K_m = 1$ signifying the perfect classification of a test set. The weights W_m were obtained not for the bootstrap training sets but for the less optimistic test sets. The optimized classifier was then used to classify all 312 spectra. Classifier outcome is reported as a normalized percent class probability.

The Robust Bootstrap classification software was written in-house using STATISTICA, Microsoft EXCEL, and Microsoft VISUAL BASIC FOR APPLICATIONS and run on a Pentium-based personal computer. The VISUAL BASIC FOR APPLICATIONS code is available from the authors.

RESULTS

^1H MR spectra. Representative spectra of each of the seven species groups and the 16 integration regions chosen for analysis are shown in Fig. 1. Spectra of ATCC type strains are

TABLE 1. Classification and identification results with optimized classifier

Species group	Lab. no.	Classification probability ^a						Error group ^b	ID result ^c
		Initial culture	Repeat cultures:						
			1	2	3	4	5		
<i>E. faecalis</i> (18 isolates, 60 cultures)	ATCC 29212	100, 100	100, 100	100, 100	100, 100	100, 100	100, 100	100, 99	c
	083-1246	96, 98							c
	175-1753	100, 100							c
	184-0712	100, 100	100, 100						c
	184-0721	100, 100	62, 97	100, 100					c
	200-1831	100, 98	77, 87						i
	200-2616	100, 100	99, 100						c
	206-0685	100, 100	98, 94						c
	270-2132	100, 100							c
	273-2358	100, 100							c
	282-0250	100, 100							c
	282-0407	100, 100							c
	(<i>gallinarum</i>)	182-2747	98, 96	98, 100					c
	(<i>gallinarum</i>)	14/04/56	100, 100						c
	(<i>gallinarum</i>)	14/04/53	100, 100						c
(<i>casseliflavus</i>)	14/04/58	87, 76						i	
(<i>casseliflavus</i>)	14/04/52	100, 100						c	
(<i>casseliflavus</i>)	207-2246	100, 100						c	
<i>S. aureus</i> (18 isolates, 56 cultures)	ATCC 25923	100, 100	100, 100	100, 100	100, 100	100, 100	100, 100	100, 100	c
	008-1690	100, 100							c
	040-2754	100, 100							c
	099-1094	89, 91							c
	124-2873	100, 100	94, 100						c
	127-2131	100, 100	58, 82						i
	127-2297	100, 100	100, 100						c
	242-2881	100, 100							c
	261-1095	100, 100							c
	271-0835	99, 100							c
	281-2429	100, 100							c
	29213	100, 100							c
	319-2410	100, 100							c
	320-2161	100, 100							c
	320-2356	72, 100	100, 100	100, 100					c
323-0934	100, 100							c	
323-1573	100, 100							c	
338-1348	100, 100							c	
<i>S. epidemidis</i> (14 isolates, 40 cultures)	ATCC 12228	100, 100	100, 100	100, 98	100, 100	50, 84			i
	003-1283	100, 100	75, 100						c
	141-1667	100, 100							c
	162-2710	100, 100							c
	170-1085	100, 99							c
	174-2177	100, 89							c
	177-1320	61, 99							i
	270-0170	100, 100							c
	281-0122	100, 100							c
	289-1072	95, 99							c
	319-1923	100, 100							c
	323-1622	100, 100							c
	326-2592	100, 100							c
	327-2569	100, 100							c
	<i>S. agalactiae</i> (15 isolates, 36 cultures)	048-1676	100, 100						
159-2821		100, 100							c
165-1046		98, 97							i
176-0797		100, 100	100, 100	100, 100					c
183-2646		100, 100							c
208-2835		100, 100							c
242-1786		100, 100							c
260-1829		100, 100							c
269-0712		100, 100							c
269-1137		100, 100							c
269-1160		100, 100							c
270-1106		100, 100							c
285-2806		100, 100							c
290-1094		100, 100							c
291-1523		100, 100							c
<i>S. milleri</i> (group 11 isolates, 30 cultures)	073-0596	100, 100							c
	097-1166	100, 100							c

Continued on following page

TABLE 1—Continued

Species group	Lab. no.	Classification probability ^a						Error group ^b	ID result ^c	
		Initial culture	Repeat cultures:							
			1	2	3	4	5			6
	141-0714	100, 100	99, 100							c
	141-1834	99, 100								c
	150-1172	100, 100								c
	164-0507	98, 89								c
	185-1175	100, 100	100, 99							c
	291-0591	98, 96								c
	291-1767	42 , 94	100, 99							i
	349-2486	42 , 47	92, 84							i
	408-0803	100, 100								c
<i>S. pneumoniae</i> (15 isolates, 42 cultures)	ATCC 6305	99, 100	100, 100	<u>97</u> , 100	100, 99	100, 100	100, 99	100, 100	<i>S. pyogenes</i>	i
	221-2745	78, 74								c
	221-2755	100, 100								c
	230-2817	100, 99								c
	234-1207	91, 100								c
	235-2193	94, 100								c
	241-1187	100, 92								c
	259-1456	100, 100								c
	272-0604	100, 98								c
	278-1723	64 , 73								i
	278-1727	97, 100								c
	324-1010	100, 100								c
	404-0191	100, 98								c
	467143	100, 100								c
	480837	72 , 90								c
<i>S. pyogenes</i> (13 isolates, 48 cultures)	ATCC 19615	96, 99	99, 100	100, 100	100, 100	100, 100	100, 100	100, 100		c
	162-1915	99, 59							<i>S. pneumoniae</i>	i
	213-0136	100, 70	100, 100							c
	221-1798	99, 100	99, 99							c
	221-2985	99, 95	<u>95</u> , <u>95</u>						<i>E. faecalis</i> (both)	m
	223-2690	99, 100	100, 100							c
	235-3096	94, 87								c
	236-1570	98, 100								c
	260-2388	88, 100	93, 96							c
	312-2457	99, 99								c
	12/03/06	98, 99								c
	326-0413	95, 100								c
	3-61-70	89, 90								c

^a Numbers show percent classification probabilities for each spectrum of duplicate cultures. Classification probabilities less than 85% are shown in bold typeface. Misclassifications are underlined.

^b The error group is the species to which a spectrum was incorrectly assigned.

^c Isolate identification result. c, correct; i, indeterminate; m, misidentification.

shown where available; otherwise spectra of isolates close to the group centroid (based on integral intensities) of all spectra are shown. The most significant contributing metabolites identified for each integration region and used for the statistical analyses are listed in Table 2. Since it is not possible to show the range of spectral patterns found in the 30 to 60 spectra examined from each species group, we show in Fig. 2 the range of normalized integral intensities (mean \pm standard deviation SD) measured for each species group.

Classification of spectra and identification of isolates. The results of the classification of 312 spectra and identification of 104 isolates from the seven species groups based on the optimized classifier are shown in Table 1. A summary of results in terms of classification and identification performance is shown in Table 3. Less than 2% of spectra were misclassified, and less than 1% of isolates were misidentified. Nineteen spectra had a classification of indeterminate.

Reproducibility of spectra. Independent analysis of spectra from concurrent, duplicate cultures and of isolates retrieved

repeatedly from storage over a 1 to 8-month period confirmed that the classification method is robust and is not affected by short- or long-term procedural variability due to factors such as minor changes in culture conditions, number of organisms, or storage of isolates (Table 1).

DISCUSSION

¹H MRS and selection of independent variables for multivariate analysis. Visible differences between typical spectra of some species are readily observed, as seen in Fig. 1. However, differences between spectra of species such as *S. pyogenes* and *S. pneumoniae* are not obvious by visual inspection, and the only possibility of reliably distinguishing between such similar groups lies in a multivariate analysis of the data. The initial step in such an analysis is the extraction from the spectra, which are composed of many thousands of data points, of a manageable set of independent variables in which any significant group differences are manifest. While sophisticated meth-

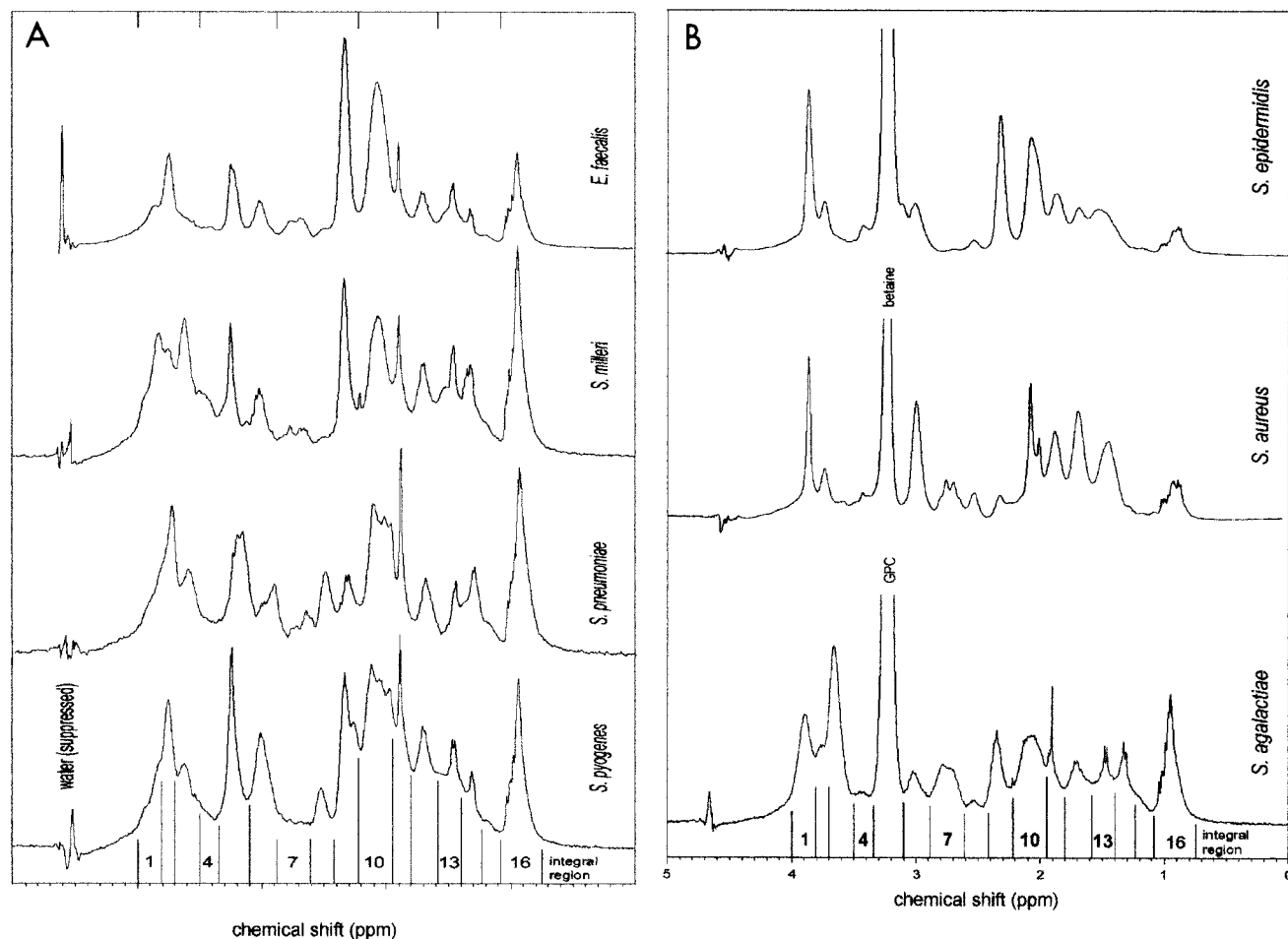


FIG. 1. (A) Representative ¹H MR spectra of *E. faecalis*, *S. milleri*, *S. pneumoniae*, and *S. pyogenes* isolates. Refer to Table 2 for the identity of the major metabolites contributing to the spectra in each integration region. (B) Representative ¹H MR spectra of *S. epidermidis*, *S. aureus*, and *S. agalactiae* isolates. The intense betaine peaks in the spectra of *S. aureus* and *S. epidermidis* and the glycerol phosphocholine (GPC) peak of *S. agalactiae* have been truncated to show details of the less intense peaks. The relative intensities of the betaine and glycerol phosphocholine peaks can be seen in Fig. 2. Refer to Table 2 for the identity of the major metabolites contributing to the spectra in each integration region.

ods have been described for the selection of optimally discriminating spectral regions (21), we chose a simple division of all spectra into 16 contiguous regions visually selected on the basis of peaks present in the spectra illustrated in Fig. 1. The advantage of this procedure is that the resultant independent variables may be assigned a specific biochemical significance (i.e., an independent variable may be associated with a particular metabolite or group of metabolites) if the metabolites contributing to the signal in each integration region can be identified. Although we have identified in Table 2 some of the major metabolites contributing to the spectra in Fig. 1, the bacterial identification method applied here does not depend on identification or quantitation of the metabolites contributing to the MR signal. It is, however, important to note that the measured cellular characteristics on which the classification is based are substantially different from those detected during routine identification and are also different from those measured by other whole-organism fingerprinting techniques. It was not our intention in this study to identify metabolites which distinguish the species groups or to construct dendro-

grams of group relationships. These will be addressed in a separate report.

Classification and identification strategy. Classification based on LDA requires that a set of functions derived by LDA of a training set of data be used to classify a test set of data, which is preferably independent of the training set (cross-validation). The function of the training set is to describe, in terms of the n independent variables derived from the MR spectra, the region of n -dimensional data space occupied by each of the a priori defined groups. If the defined groups in the training set are well separated in data space, the LDA will produce classification functions which assign every member of the training set to its a priori defined group. The region of data space associated with a particular group will increase with phenotype variation between the members of a particular species group and also with procedural (environmental, biochemical, and methodological) variations associated with repeated culture and classification of spectra of a specific member of a group. A training set comprising only a small number of randomly selected members of a particular group is therefore

TABLE 2. Integral regions and most significant contributing metabolites

Region	Range (ppm)	Metabolites with resonances in region ^a
1	4.00–3.81	AA, betaine, GPC, GPE, EA
2	3.81–3.70	AA, glycerol, G3P
3	3.70–3.50	AA, GPC, glycine, choline, inositol
4	3.50–3.34	Taurine, GPE, tryptophan
5	3.34–3.10	Histidine, tyrosine, taurine, phenylalanine, betaine, GPC, choline, inositol, PA, EA
6	3.10–2.88	Lysine, histidine, tyrosine, asparagine, PA
7	2.88–2.61	Aspartate, asparagine, methionine
8	2.61–2.42	Succinate
9	2.42–2.22	Valine, glutamine, glutamate, succinate
10	2.22–1.95	Isoleucine, glutamine, glutamate, methionine, PA, N-acetyl compounds
11	1.95–1.80	Acetate, lysine, isoleucine
12	1.80–1.58	Leucine, lysine
13	1.58–1.40	Lysine, alanine
14	1.40–1.23	Lactate, isoleucine, threonine
15	1.23–1.08	None identified
16	1.08–0.75	Valine, leucine, isoleucine

^a Abbreviations: AA, amino acid (nonspecific); PA, polyamine; GPC, glycerol phosphocholine; GPE, glycerol phosphoethanolamine; EA, ethanolamine; G3P, glycerol-3-phosphate.

unlikely to accurately represent the data space (phenotype range) occupied by all members of that species group. If the training set contains only a single measurement of each isolate member, it may also not account for procedural variability. Consequently, it is to be expected that some misclassifications will occur when a classification function based on a training subset of a group is used to classify group members which are not members of the training set.

For classifier robustness and reliability, it is desirable that the number of spectra per species group in the training set be 5 to 10 times larger than the number of independent variables (19). Such large data sets are rare in the published literature and usually difficult to acquire, especially if the derived classifier is to be validated against a test set independent of the training set. The Robust BootStrap method attenuates this problem by allowing cross-validated classifier development with all of the available data (19).

In an attempt to reduce the number of independent variables, we applied the forward stepwise method of seven-group LDA and limited the number of independent variables. There was a progressive decrease in overall classification accuracy as the number of independent variables was decreased. In contrast, pairwise LDA between any of the species groups required only two to four independent variables for 100% discrimination between any pair of species groups. We are presently developing software to classify multiple groups based on a set of classifiers derived from pairwise LDA.

The ease of preparation and examination of duplicate or even triplicate cultures of a particular clinical isolate, as used in this study, has the advantage that a consensus identification of the isolate based on multiple independent analyses is obtained. This feature of our isolate identification strategy has not been applied in other microbial whole-organism fingerprinting studies (5, 8), in which, at best, only instrument duplicates were acquired. We have demonstrated that in a few cases the duplicates may be classified as different species. Consequently, identification based on analysis of a single subculture of an

isolate cannot be assigned the same confidence level as an identification based on classification of independent duplicate cultures. When using conventional methods, which report an identification probability based on analysis of a single culture of an isolate, it is common practice to reexamine isolates for which the identification probability is <85%. Analysis is repeated until a single test returns an identification probability of >85%. By this method, it is possible that the average identification probability of all tests on an isolate will be <85% at the conclusion of testing. Our method of testing duplicate cultures and requiring that correct identification be based on an average probability of >85% imposes a more rigorous and reliable identification constraint than would be the case with single cultures. However, in Table 3 it can be seen that the accuracy of identification based on classification of spectra from single cultures would, in fact, have been similar to that based on duplicate cultures.

Phenotypic variability within species groups was addressed by examination of at least 11 isolates from each species group. The general success of the classification method used indicates that between the species groups there are significant and consistent spectral differences, which are larger than the typical range of variation within species due to procedure or phenotype.

Classification and identification results. The very small number of misclassifications of spectra could not be attributed to any specific steps of the method. Potential problems with reproducibility due to short- and long-term procedural variability (use of different batches of culture medium, storage of isolates, etc.) were excluded by undertaking (i) separate analysis of spectra from duplicate cultures of all isolates and (ii) repeated culture of 25 isolates, at times up to 8 months after original culture and spectroscopy. The single instance of misidentification (*S. pyogenes* Lab. No. 221-2985) may have been the result of contamination.

We did not examine a sufficient number of isolates in the *S. milleri* group to attempt an MRS-based assignment of the isolates to one of the three species within the group (*S. anginosus*, *S. constellatus*, and *S. intermedius*). However, our results demonstrate that on the basis of the nonroutine metabolites measured, the group is physiologically homogeneous relative to the diversity of the seven species groups examined. Although not surprising, this result is consistent with group similarities defined by other biochemical tests. Similarly, our data confirm that the *E. casseliflavus* and *E. gallinarum* isolates

TABLE 3. Summary of classification and identification results

Classification or identification type	Count	% of total
Classification type		
Correct	288	92.3
Indeterminate	19	6.1
Misclassification	5	1.6
Total	312	100.0
Identification type		
Correct	144	92.3
Indeterminate	11	7.1
Misidentification	1	0.6
Total	156	100.0

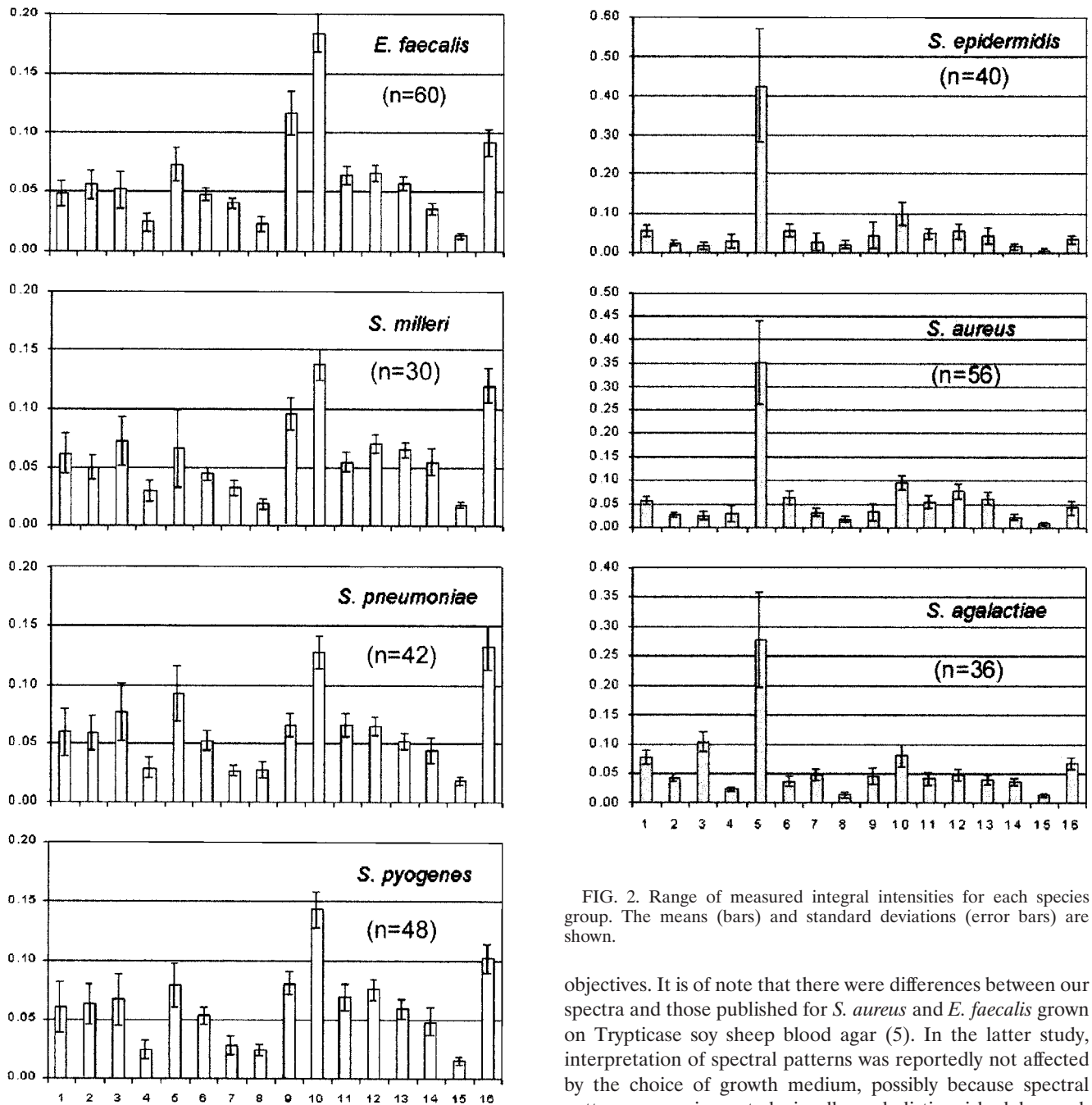


FIG. 2. Range of measured integral intensities for each species group. The means (bars) and standard deviations (error bars) are shown.

examined are physiologically more similar to *E. faecalis* than to the *Streptococcus* and *Staphylococcus* species tested.

Choice of growth medium. In selecting the most appropriate medium for use in a clinical diagnostic or reference laboratory, we reasoned that choice of a universal growth substrate and ease of sample preparation were of prime importance. Since HBA is a common medium in use in diagnostic microbiology laboratories in Australia and since bacterial cells could be easily harvested directly from HBA plates without the need for washing, we chose this growth medium as best satisfying our

objectives. It is of note that there were differences between our spectra and those published for *S. aureus* and *E. faecalis* grown on Trypticase soy sheep blood agar (5). In the latter study, interpretation of spectral patterns was reportedly not affected by the choice of growth medium, possibly because spectral patterns were inspected visually and distinguished by peak positions rather than peak intensities. We found previously that growth on or in different media (HBA versus brain heart infusion broth) affected the relative peak intensities (due to changes in metabolite pool sizes) much more significantly than it affected peak positions, which may be slightly affected by factors such as intracellular pH (R. Bourne, unpublished data). These differences suggest that the analysis is dependent on the constraint that all cultures must be grown on the same medium.

Clinical application. There are several characteristics of the method used in this study which point to the robust nature of the identification. First, the growth conditions for the samples are not strictly controlled. For example, the precise constitu-

tion of the growth medium may vary from batch to batch (base media from two different manufacturers and multiple batches of horse blood were used). The size of the inoculum may vary from plate to plate. Growth of bacteria on an agar plate is inherently inhomogeneous, due to crowding and slow diffusion of oxygen and other nutrients through colonies and agar. Our early experiments with triplicate cultures of all isolates demonstrated a lack of variation in spectra from cells grown on single batches of medium. Due to large variations between species in the amount of growth obtained overnight on HBA plates (the growth of *S. milleri* was usually very poor), the wet weight of cells resuspended varied from 2 to 200 mg. Since the MR signal is directly proportional to the sample concentration, there is no need to standardize the sample density. Poor bacterial growth required only an extended number of transients to achieve an adequate signal-to-noise ratio.

The phase correction and integration steps of spectrum processing, as implemented, required some subjective operator input. These deficiencies in the method will introduce some extra variance into the data. They may be overcome by procedures not presently available in our laboratory (use of magnitude spectra and automated integration [22]). Other whole-organism fingerprinting techniques are reported to require strict control of growth media and repeated standardization with control cultures (11, 12).

The nondestructive nature of the method enables retention of viable organisms postanalysis for subsequent checking of contamination or methodological errors.

The use of more sophisticated pattern recognition methods than those used in our study (19) may further improve discrimination and allow separate classification within the species groups, albeit at the possible expense of easily interpreted biochemical information. For an application dedicated to identification rather than characterization, this would be an acceptable compromise.

We have demonstrated that, in principle, MRS may be combined with automated pattern recognition techniques to identify bacteria to the species level. We have recently achieved identification results of similar accuracy for six gram-negative species and for two *Cryptococcus neoformans* varieties (unpublished results). The extreme ease of sample preparation, biochemically informative results, rapid automated identification, and the robust nature of the method are attractive for clinical and industrial applications. In practice, MR-based identification may be of most value for bacterial species which are relatively slow growing or difficult to identify by conventional methods.

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