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Dear Dr. Migliore:

Enclosed please find three reprints of the manuscript entitled "Rapid Identification of Common Human Pathogens by High-Resolution Proton Magnetic Resonance Spectroscopy" published in May, 1995 in the Journal of Clinical Microbiology.

The authors of this manuscript wish to thank The Moran Foundation, Department of Pathology, Baylor College of Medicine, for the financial support for our project.

Sincerely,

Charles E. Stager, Ph.D.

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Rapid Identification of Common Human Pathogens by High-Resolution Proton Magnetic Resonance Spectroscopy

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Routine procedures for recovery of bacteria from clinical specimens involve culturing the latter on various nonselective and selective agar media. The bacteria are then identified by means of biochemical and immunological test procedures. Reduction of the time required to identify the bacteria is highly desirable for rapid clinical diagnosis. Towards this end the potential of proton nuclear magnetic resonance (NMR) spectroscopy for providing a "fingerprint" within the proton spectrum of five bacterial genera, reflecting their characteristic cell wall constituents, has been investigated. Establishing a database of high-resolution proton NMR spectra of a large number of bacterial species is a prerequisite for attaining this objective. A database has been established for five common human pathogens: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*. On the basis of the presence of characteristic resonances in their spectra, a simple algorithm has been developed to differentiate and identify these microorganisms. The NMR spectra of *E. coli* and *S. aureus* showed no dependency on the type of growth medium, growth density, or incubation time.

A rapid and accurate method for identifying microorganisms that cause serious infections would help physicians verify their presumptive clinical diagnosis and lead to prompt initiation of a tailored antibiotic regimen. This is conventionally carried out by culturing the bacterium in vitro and subjecting it to the usual identification protocols. The pathogenicity of bacteria and their susceptibility to antimicrobial agents may be ascribed in part to the molecular composition of the cell wall, cytoplasm, periplasm, and membranes that separate these compartments (4). Such components would include lipopolysaccharides (9, 11), membranes and cytoplasmic proteins (1, 2, 5, 7), peptidoglycans (6, 8), teichoic acids (3), and several different types of phospholipids (10).

Nuclear magnetic resonance (NMR) spectroscopy is an unusually powerful technique for studying these individual components, especially when they are isotopically enriched. Because of their magnetic moment, the protons (hydrogen nuclei) in organic molecules and biological systems, when subjected to an intense magnetic field, have the possibility of orienting themselves with the field (lower energy state) or against the field (higher energy state). The nuclei are predominantly in the lower energy state. However, when this system is subjected to electromagnetic energy in the form of radio frequency irradiation, the protons orient themselves in the higher energy state. As the microenvironments of the various protons often differ, varying amounts of radio frequency energy (resonance frequency) must be applied to bring all the protons to the higher energy state. The results are presented on a plot of the resonance frequency (in parts per million [a dimensionless unit]) versus signal intensity. The majority of the protons found in organic and biological molecules resonate within a narrow spectral window: 0 to 10 ppm. The lower-energy side of the spectrum (larger parts-per-million value) contains signals for protons that require lesser amounts of energy for resonance as compared with the ones on the higher-energy side of the spectrum. The reference signal at 0 ppm originates from a chemical compound called trimethylsilyl-propionate and is due to the nine protons in this molecule which all appear at the same parts-per-million value as a narrow intense singlet signal. The sample under investigation is placed in a narrow (5-mm [outer diameter]) cylindrical tube which is inserted into the magnetic field and irradiated with radio frequency energy spanning the entire 10-ppm window. A deuterated species—in this particular case deuterium oxide—is added to provide the spectrometer a field-frequency lock to prevent any drift or changes in the magnetic field during measurement. The intensity of a particular resonance signal is proportional to the number of protons with that specific resonance frequency. Therefore, concentrated samples yield more intense signals. The line width, or sharpness, of an NMR signal is dependent upon its relaxation characteristics, i.e., how soon the excited nucleus relaxes to the lower energy state. In general, the more time the nucleus in the higher energy state requires to relax, the sharper is its signal. Further, the more rapidly a nucleus (and therefore the whole molecule itself) moves in solution, the longer it takes to relax. Polysaccharide chains extending out from the bacterial coat often experience rapid segmental motion in solution, and hence their proton signals are sharp relative to the other protons embedded deeper in the outer membrane, thus contributing more toward signal intensity.

In the present study, we have used high-field (9.4 T) proton NMR (^{1}H NMR) to distinguish bacterial cell types which differ in their molecular composition. Because of differences in the composition of their cell wall and cytoplasmic constituents, individual bacterial species exhibit spectra with specific char-

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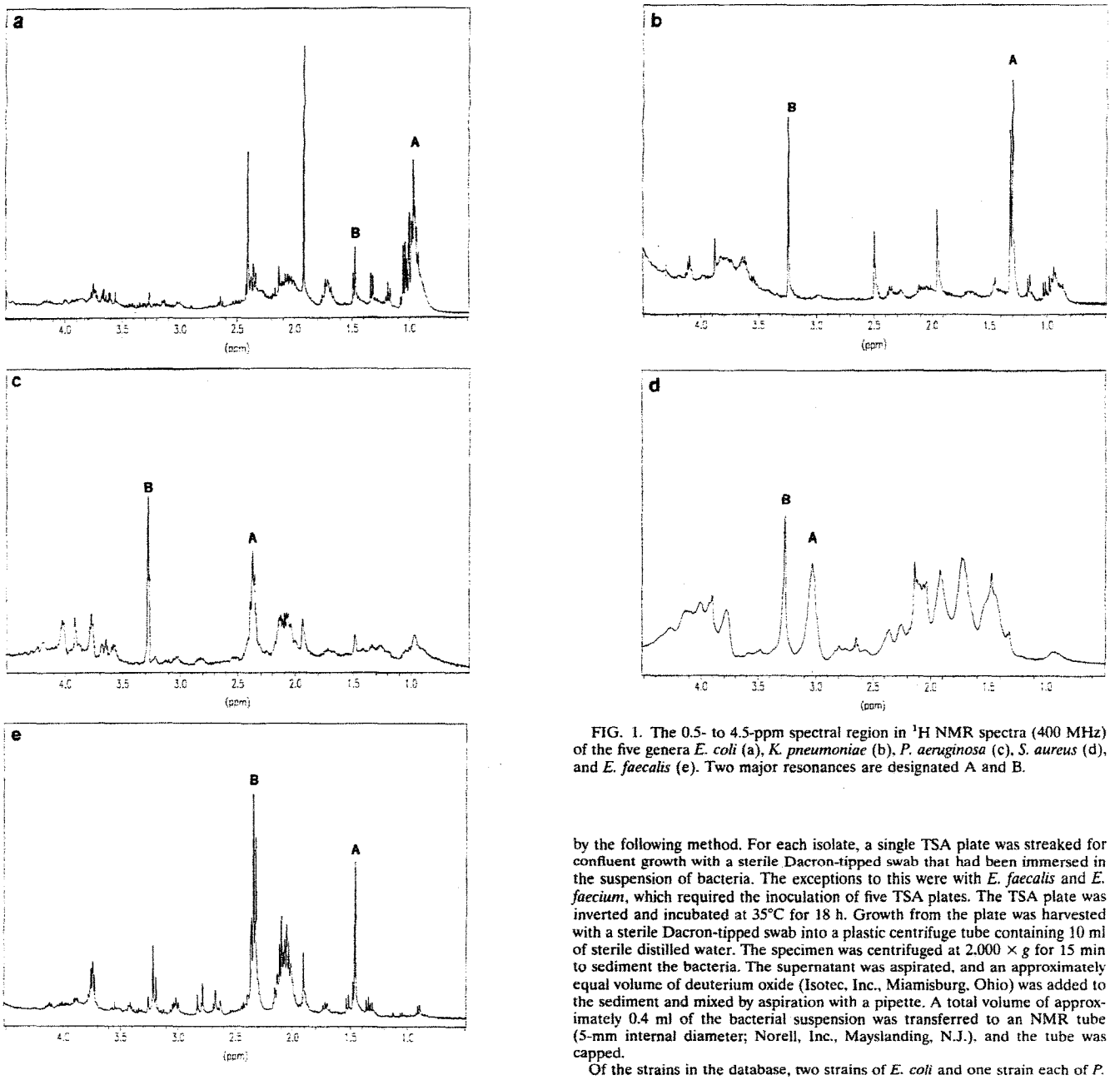

FIG. 1. The 0.5- to 4.5-ppm spectral region in ^{1}H NMR spectra (400 MHz) of the five genera *E. coli* (a), *K. pneumoniae* (b), *P. aeruginosa* (c), *S. aureus* (d), and *E. faecalis* (e). Two major resonances are designated A and B.

acteristic resonances. Accordingly, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* can be identified by their ^{1}H NMR spectra in 20 min. A limited database of the ^{1}H NMR spectra of 10 strains of each of the microorganisms was established.

MATERIALS AND METHODS

Ten strains each of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *E. faecalis* and a single strain each of *Shigella sonnei*, *Shigella flexneri*, and *Enterococcus faecium* were obtained from clinical specimens at Ben Taub General Hospital in Houston, Tex. A single colony was subcultured to a Trypticase soy sheep blood agar (TSA) plate (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and incubated at 35°C for 18 h. Several colonies were suspended in 0.9% sodium chloride and adjusted to a turbidity equivalent to a 0.5 McFarland standard. The microorganisms were then prepared for NMR spectroscopy by the following method. For each isolate, a single TSA plate was streaked for confluent growth with a sterile Dacron-tipped swab that had been immersed in the suspension of bacteria. The exceptions to this were with *E. faecalis* and *E. faecium*, which required the inoculation of five TSA plates. The TSA plate was inverted and incubated at 35°C for 18 h. Growth from the plate was harvested with a sterile Dacron-tipped swab into a plastic centrifuge tube containing 10 ml of sterile distilled water. The specimen was centrifuged at 2,000 × *g* for 15 min to sediment the bacteria. The supernatant was aspirated, and an approximately equal volume of deuterium oxide (Isotec, Inc., Miamisburg, Ohio) was added to the sediment and mixed by aspiration with a pipette. A total volume of approximately 0.4 ml of the bacterial suspension was transferred to an NMR tube (5-mm internal diameter; Norell, Inc., Mayslanding, N.J.), and the tube was capped.

Of the strains in the database, two strains of *E. coli* and one strain each of *P. aeruginosa* and *S. aureus* were inoculated to a TSA plate as described above but, after incubation, were harvested directly into a plastic tube containing approximately 0.7 ml of deuterium oxide. A total volume of approximately 0.4 ml of the bacterial suspension was transferred to an NMR tube.

One each of the above-mentioned strains of *E. coli* and *S. aureus* was inoculated to TSA plates and brucella sheep blood agar plates (brucella: Becton Dickinson Microbiology Systems). The plates were streaked for confluent growth as described above as well as with a loop to obtain isolated colonies in the third quadrant of growth. For third-quadrant growth it was necessary to streak five plates per isolate. Growth was harvested and processed as described above at both 24- and 48-h intervals.

NMR spectroscopy. Spectra (8K datum points) were acquired at 35°C with a Bruker AM-400 wide-bore NMR spectrometer (Bruker, Billerica, Mass.) at a field strength of 9.4 T (400 MHz for ^{1}H). The residual water signal was suppressed by presaturation for 2 s prior to the data acquisition. A total of 256 scans were collected per microorganism. The time interval between loading the sample in the spectrometer and the printout of the spectrum was about 20 min. The spectra were referenced with an internal standard, using sodium tetradeuterotrimethylsilyl-propionate (δ = 0 ppm). In addition to the Bruker DISNMR program, off-line data processing was carried out on a Sun 3/60 workstation, using the NMRi software developed by New Methods Research (Syracuse,

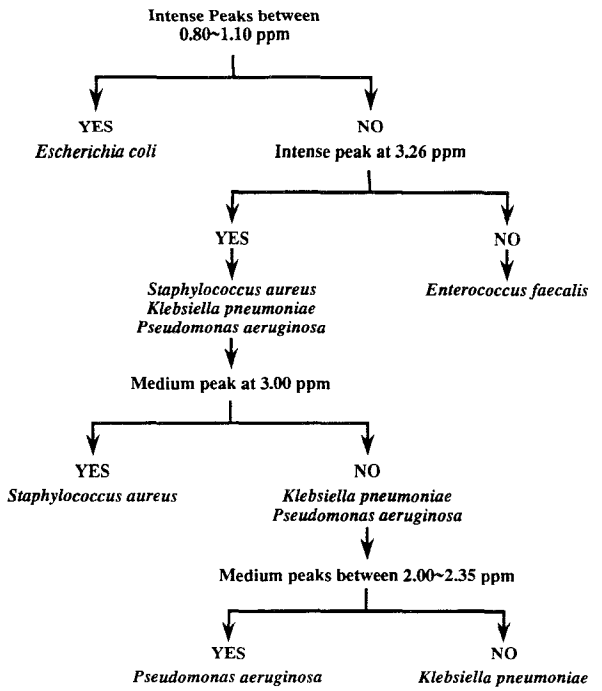

FIG. 2. Algorithm for identification of common human pathogens, compiled for the ^{1}H NMR spectral database.

N.Y.), as well as the WIN-NMR (Bruker) software used with a DOS-based personal computer.

RESULTS

NMR spectra for 10 strains each of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*, which had been processed by the method previously described, were acquired. The spectral regions 0.5 to 4.5 ppm were compared for each of the five genera studied. Typical spectra obtained under conditions of water presaturation are shown in Fig. 1. These spectra each contain one or more specific characteristic resonances or groups of resonances. On the basis of representative spectra, an algorithm (Fig. 2) was developed to distinguish these microorganisms from one another. The presence or absence of significant peaks in their ^{1}H spectra was used as a decision criterion. The signal intensities in the spectra were classified relative to that of the most intense peak (excluding that of residual water) as high (70 to 100%), medium (60 to 30%), or low (30 to 10%). The first decision step in the algorithm (Fig. 2) is the presence or absence of high-intensity resonances in the region 0.8 to 1.1 ppm to distinguish *E. coli* from the other microorganisms. The presence of several medium-intensity resonances near to each other with a broad high-intensity envelope at 1.00 ppm appears to be characteristic of *E. coli*. The positions of these signals are characteristic for the methyl groups in valine, leucine, and isoleucine as well as for the terminal methyl groups in long-chain fatty acids. The second decision-making criterion in the algorithm is the absence of a high-intensity singlet peak at 3.26 ppm, which distinguishes *E. faecalis* from the other organisms which exhibit this resonance in their NMR spectra. This signal can be ascribed to *O*-methyl as well as *N*-methyl groups. As a confirmation, the ^{13}C NMR spectrum of *S. aureus* in deuterium oxide exhibited several signals in the region 56.0 ± 2 ppm which are characteristic for *O*-methyl carbons. The presence of a medium-intensity peak in the spectrum of the *S. aureus* strains at 3.00 ppm distinguishes it from *K. pneumoniae* and *P. aeruginosa*. The presence of medium-intensity peaks within the region 2.00 to 2.35 ppm was characteristic for *P. aeruginosa*, whereas *K. pneumoniae* did not exhibit this resonance. Twenty-one spectra (for three strains each of *E. coli* and *P. aeruginosa* and five strains each of *K. pneumoniae*, *S. aureus*, and *E. faecalis*) were blind coded by one author and were then given to three other authors for assignment with the algorithm. All organisms were correctly identified by this process.

The NMR spectra of two additional strains of *E. coli* are shown in Fig. 3 to demonstrate that each strain had essentially the same characteristic resonances, with variation only in intensity of signals in certain cases. This was essentially true of all strains tested.

To demonstrate the reproducibility of NMR resonances for 10 strains each of the five bacterial genera, two major resonances for each genus were arbitrarily selected and designated A and B (Fig. 1). There was good reproducibility of parts-per-million values for these selected resonances for 10 strains each of the five bacterial genera (Table 1).

One strain each of *S. sonnei* and *S. flexneri* was differentiated from 10 strains of *E. coli* by NMR. The spectra of *S. sonnei* and *S. flexneri* had a signal of medium intensity at 2.44 ppm which

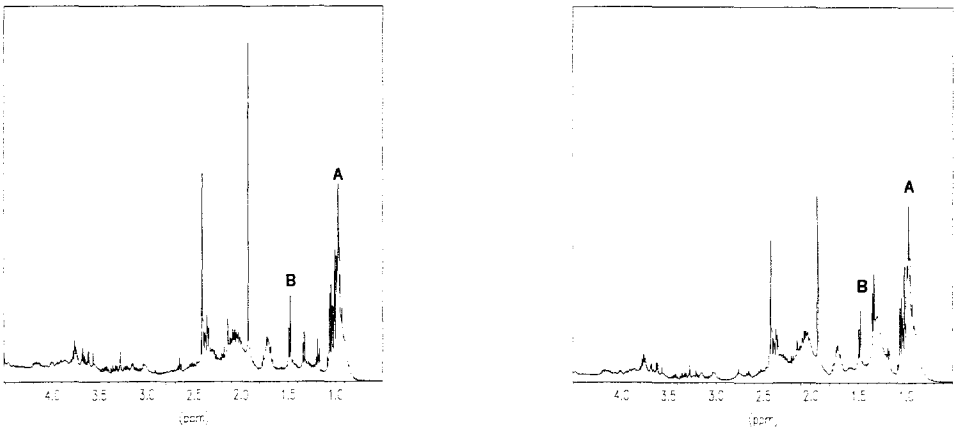

FIG. 3. Comparison of the 0.5- to 4.5-ppm spectral region in ^{1}H NMR spectra (400 MHz) of two strains of *E. coli*. Two major resonances are designated A and B.

TABLE 1. Reproducibility of select NMR resonances for five bacterial genera

Strain no.	Resonance (ppm) at indicated location for:									
	E. coli		*S. aureus*		*K. pneumoniae*		*P. aeruginosa*		*E. faecalis*	
	A	B	A	B	A	B	A	B	A	B
1	0.96	1.48	3.01	3.27	1.32	3.26	2.36	3.27	1.48	2.36
2	0.96	1.48	3.04	3.27	1.32	3.26	2.35	3.27	1.48	2.34
3	0.96	1.47	3.03	3.27	1.32	3.26	2.36	3.27	1.47	2.36
4	0.97	1.48	3.03	3.27	1.32	3.26	2.38	3.27	1.47	2.36
5	0.97	1.48	3.03	3.27	1.32	3.26	2.36	3.27	1.47	2.36
6	0.96	1.47	3.03	3.27	1.32	3.26	2.36	3.27	1.48	2.36
7	0.96	1.47	3.03	3.27	1.32	3.26	2.36	3.27	1.48	2.34
8	0.97	1.48	3.03	3.27	1.32	3.26	2.36	3.27	1.47	2.35
9	0.96	1.47	3.03	3.27	1.32	3.26	2.36	3.27	1.47	2.36
10	0.96	1.47	3.03	3.27	1.32	3.26	2.34	3.27	1.48	2.36

was absent in the spectra of *E. coli*. The spectrum of *S. flexneri* had a signal of medium intensity at 3.80 ppm which was absent in the spectrum of *S. sonnei*, thereby allowing species differentiation. Similarly, *E. faecium* (one strain) was differentiated from 10 strains of *E. faecalis*, the spectrum of the former bacterium having a signal of medium intensity at 3.67 ppm which was absent in the 10 spectra of *E. faecalis*.

The effects of type of growth medium, growth density, and incubation time on two major resonances (arbitrarily designated A and B) for one of the strains of *E. coli* and *S. aureus* in the database are demonstrated in Table 2. These major NMR resonances of *E. coli* and *S. aureus* were minimally affected by variation of any of the tested parameters.

Of the strains in the database, two strains of *E. coli* and one strain each of *P. aeruginosa* and *S. aureus* were inoculated for confluent growth onto TSA plates and, after an 18-h incubation, were harvested directly into deuterium oxide for testing by NMR. This variation in sample preparation omitted the suspension of cells into water and subsequent centrifugation and did not affect the NMR spectral characterization with the algorithm.

TABLE 2. The effect of growth density, incubation time, and growth medium on select NMR resonances of *E. coli* and *S. aureus*

Organism and growth conditions	ppm at location[a]:	
	A	B
E. coli		
24 h		
Confluent, TSA plates	0.94	1.45
3rd quadrant, TSA plates	0.94	1.45
Confluent, brucella agar plates	0.93	1.43
3rd quadrant, brucella agar plates	0.94	1.45
48 h		
Confluent, TSA plates	0.94	1.46
3rd quadrant, TSA plates	0.95	1.43
Confluent, brucella agar plates	0.94	1.46
3rd quadrant, brucella agar plates	0.94	1.45
S. aureus		
24 h		
Confluent, TSA plates	3.00	3.23
3rd quadrant, TSA plates	2.99	3.24
Confluent, brucella agar plates	3.00	3.23
3rd quadrant, brucella agar plates	3.00	3.24
48 h		
Confluent, TSA plates	3.03	3.25
3rd quadrant, TSA plates	3.01	3.25
Confluent, brucella agar plates	3.02	3.25
3rd quadrant, brucella agar plates	3.03	3.25

[a] Locations A and B are the same as locations A and B in Fig. 1a and d.

DISCUSSION

Bacteria contain a number of different macromolecular and multimolecular ensembles that could potentially contribute to their respective ^1H spectra (4). The phospholipids, which serve as the basic framework for the cell's cytoplasmic membrane (gram-positive and gram-negative organisms) and outer membrane (gram-negative organisms), have perhaps the highest molar concentration of chemically similar moieties (fatty acyl, CH_2, and CH_3 groups), but most bacterial cell membranes contain high proportions of membrane proteins that significantly restrict lipid motions, thereby reducing their contribution to the isotropic ^1H spectrum. Many of the cell proteins are typically large molecules embedded in or completely spanning a membrane bilayer; their restricted motions and relatively low molar concentrations make them difficult to study by ^1H NMR.

Most gram-positive bacteria have a relatively thick (20- to 80-nm) continuous cell wall, which is largely composed of peptidoglycan. When thick cell walls are found, other biopolymers such as teichoic acids, polysaccharides, and peptidoglycolipids are covalently coupled to the peptidoglycan, forming a rather rigid lattice of moieties with restricted motions and low spectral intensities. By contrast, in gram-negative bacteria the peptidoglycan layer is thin (5 to 10 nm), perhaps allowing significant motional freedom and detectable spectral intensity. A lipoprotein layer is present in the gram-negative cell wall, thereby stabilizing the outer membrane and cross-linking it to diaminopimelic acids on the peptidoglycan layer. Embedded in the outer leaflet of the outer membrane is lipopolysaccharide. Its lipid component (lipid A) anchors the molecule in the membrane, while the polysaccharide component is exposed to the outer surface of the bacterium, probably experiences considerable molecular motion, and probably contributes significant intensity to the ^1H spectrum (10, 11).

The present report does not address the assignment of particular molecular species in bacterial cells to salient spectral peaks. These assignments are the focus of continuing studies. This preliminary report demonstrates that NMR spectroscopy can rapidly identify bacteria that commonly cause infections in humans. Table 1 demonstrates that amongst strains of the same genus and species, major resonances are consistently present and exhibit an extremely narrow range of variability. There was great consistency in the location of medium- and high-intensity resonances in all the strains of each species studied. This is demonstrated by comparison of the spectra in Fig. 3 and 1a, representing the spectra of three strains of *E. coli*. It is significant that NMR could differentiate *E. coli*, *S. sonnei*, and *S. flexneri*, as well as *E. faecalis* and *E. faecium* (data not shown). However, the number of strains tested was small. Whether NMR could accurately differentiate amongst strains of a given species has not been established. Varying the incubation conditions had a minimal effect on the NMR spectra of *E. coli* and *S. aureus* (Table 1) and did not preclude unambiguous differentiation with the algorithm.

Though sample preparation is simple for NMR, a large amount of growth is required. This would generally require subculture from the primary plate of clinical specimens. Direct suspension of primary-plate growth into deuterium oxide for

NMR might be possible in the case of urine cultures and blood cultures, for which heavy growth of a pure culture is often obtained. However, commercial systems readily identify most organisms isolated from these specimens. In preliminary studies, we have identified *E. coli* and *P. aeruginosa* directly from blood culture vials by NMR spectroscopy. The erythrocytes were lysed with Triton X-100, and the bacteria were harvested for NMR spectroscopy by centrifugation (unpublished data).

This report is the first description of the use of high-resolution NMR spectroscopy for differentiating several genera and species of bacteria. As the spectral database is expanded it may be possible to achieve identification of organisms that present problems when commercial systems are used. While high-resolution NMR equipment is expensive, many hospitals associated with academic institutions have access to these facilities.

ACKNOWLEDGMENTS

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