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Raman Spectroscopic Method for Identification of Clinically Relevant Microorganisms Growing on Solid Culture Medium

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Routine clinical microbiological identification of pathogenic microorganisms is largely based on nutritional and biochemical tests. In the case of severely ill patients, the unavoidable time delay associated with such identification procedures can be fatal. We present a novel identification method based on confocal Raman microspectroscopy. With this approach it is possible to obtain Raman spectra directly from microbial microcolonies on the solid culture medium, which have developed after only 6 h of culturing for the most commonly encountered organisms. Due to the limited thickness of microcolonies, some of the underlying culture medium is sampled together with the bacteria. Spectra measured at different depths in a microcolony contain different amounts of the medium signal. A mathematical routine, involving vector algebra, is described for the nonsubjective correction of spectra for variable signal contributions of the medium. To illustrate the possibilities of our approach for the identification of microorganisms, Raman spectra were collected from 6-h microcolonies of five bacterial strains on solid culture medium. The classification results show that confocal Raman microspectroscopy has great potential as a powerful new tool in clinical diagnostic microbiology.

Routine microbiological identification of clinical samples is largely based on nutritional and biochemical characteristics of microorganisms. Following receipt of patient material, microbes are usually first cultured for 16−24 h on solid culture medium. A biomass of 10^6−10^8 cells is then used for biochemical assays, and after or in parallel with the identification assays, an antibiotic susceptibility profile of the microorganism is generated. Consequently, it is usually not until 2−3 days later that the clinician is presented with the full results of this labor-intensive procedure. In critical life-threatening infections, such a delay can be fatal for the patient. Therefore, common clinical practice is to start broad-spectrum empiric antimicrobial therapy based on experience with similar cases before the test results from the microbiology laboratory are known. Early identification of a causative microorganism will enable the clinician to modify and target the initial therapy to the microorganism that causes the specific infection. This practice of streamlining and drug targeting is important to optimize the efficiency of antimicrobial therapy, to reduce the development of drug resistance and other undesired side effects on the microflora. Furthermore, possible toxic effects of broad-spectrum empiric therapy, which sometimes includes a combination of two different antimicrobial agents, can be reduced.

Molecular biological techniques are now being evaluated and used as methods for the identification of microorganisms and the detection of specific antibiotic resistance genes.[1−5] Although these techniques are potentially rapid, they are relatively expensive and require highly skilled personnel. Problems with false positive reactions due to DNA contamination and false negative reactions due to inhibitors introduced while preparing or collecting a sample are complicating factors in DNA amplification-based molecular diagnostics.[6−9] At present, molecular diagnostics are usually second lines of investigation and are seldom the sole basis for microbial identification. Therefore, there is a need for new techniques that can rapidly identify pathogenic microorganisms and provide information on drug susceptibility.

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(1) Tang, Y. W.; Procop, G. W.; Persing, D. H. *Clin. Chem.* **1997**, *43*, 2021−2038.
(2) Nikkari, S.; Relman, D. A. *Curr. Opin. Rheumatol.* **1999**, *11*, 11−16.
(3) Tang, Y. W.; Ellis, N. M.; Hopkins, M. K.; Smith, D. H.; Dodge, D. E.; Persing, D. H. *J. Clin. Microbiol.* **1998**, *36*, 3674−3679.
(4) Tenover, F. C.; Huang, M. B.; Rasheed, J. K.; Persing, D. H. *J. Clin. Microbiol.* **1994**, *32*, 2729−2737.
(5) Clark, N. C.; Olsvik, O.; Swenson, J. M.; Spiegel, C. A.; Tenover, F. C. *Antimicrob. Agents Chemother.* **1999**, *43*, 157−160.
(6) Vaneechoutte, M.; Van Eldere, J. *J. Med. Microbiol.* **1997**, *46*, 188−194.
(7) Noordhoek, G. T.; van Embden, J. D.; Kolk, A. H. *J. Clin. Microbiol.* **1996**, *34*, 2522−2525.
(8) Ieven, M.; Goossens, H. *Clin. Microbiol. Rev.* **1997**, *10*, 242−256.
(9) Fredricks, D. N.; Relman, D. A. *J. Clin. Microbiol.* **1998**, *36*, 2810−2816.

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An alternative approach in microbial characterization is the use of spectroscopic methods. Pyrolysis mass spectrometry has been evaluated as a method for bacterial characterization;[10−14] however, thus far the high instrument costs have hindered the widespread use of this method.[15] The use of Fourier transform infrared (FT-IR) spectroscopy for microbial identification and characterization has been gaining acceptance since Naumann and co-workers published their pioneering work in this field.[16−18] Manfait et al.[19−21] have used FT-IR spectroscopy to identify drug resistance in bacteria, indicating the high information content of this technique.

The application of Raman spectroscopy in microbiology has also been explored previously. Studies have been reported in which FT-Raman[22,23] or ultraviolet (UV) resonance Raman[24−26] spectroscopy was used to study suspensions and dried films of microorganisms as well as hydrated microbial smears taken from a solid culture medium.[27] Using UV resonance Raman spectroscopy to study bacterial suspensions and bacterial cell constituents, Nelson and Sperry were able to identify microorganisms on the basis of their Raman spectra.[28] They also reported measurements with only very small numbers of cells (1−50).[29,30] However, the application of UV resonance Raman spectroscopy requires the cells to be suspended in liquid medium in order to avoid damage due to heating[31] and photochemical effects as a result of the strong absorption of UV radiation by nucleic acids and proteins. For the clinical application of Raman spectroscopy targeted at rapid identification of pathogenic microorganisms, this is not a practical solution.

(10) Goodacre, R. *Microbiol. Eur.* **1994**, *2*, 16−22.
(11) Goodacre, R.; Hiom, S. J.; Cheeseman, S. L.; Murdoch, D.; Weightman, A. J.; Wade, W. G. *Curr. Microbiol.* **1996**, *32*, 77−84.
(12) Timmins, E. M.; Howell, S. A.; Alsberg, B. K.; Noble, W. C.; Goodacre, R. *J. Clin. Microbiol.* **1998**, *36*, 367−374.
(13) Barshick, S. A.; Wolf, D. A.; Vass, A. A. *Anal. Chem.* **1999**, *71*, 633−641.
(14) Demirev, P. A.; Ho, Y. P.; Ryzhov, V.; Fenselau, C. *Anal. Chem.* **1999**, *71*, 2732−2738.
(15) Busse, H. J.; Denner, E. B.; Lubitz, W. *J. Biotechnol.* **1996**, *47*, 3−38.
(16) Naumann, D.; Helm, D.; Labischinski, H. *Nature* **1991**, *351*, 81−82.
(17) Helm, D.; Labischinski, H.; Schallehn, G.; Naumann, D. *J. Gen. Microbiol.* **1991**, *137*, 69−79.
(18) Helm, D.; Labischinski, H.; Naumann, D. *J. Microbiol. Methods* **1991**, *14*, 127−142.
(19) Zeroual, W.; Manfait, M.; Choisy, C. *Pathol. Biol.* **1995**, *43* (4), 300−305.
(20) Sockalingum, G. D.; Bouhedja, W.; Pina, P.; Allouch, P.; Mandray, C.; Labia, R.; Millot, J. M.; Manfait, M. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 240−246.
(21) Bouhedja, W.; Sockalingum, G. D.; Pina, P.; Allouch, P.; Bloy, C.; Labia, R.; Millot, J. M.; Manfait, M. *FEBS Lett.* **1997**, *412*, 39−42.
(22) Williams, A. C.; Edwards, H. G. M. *J. Raman Spectrosc.* **1994**, *25*, 673−677.
(23) Edwards, H. G. M.; Russell, N. C.; Weinstein, R.; Wynn-Williams, D. D. *J. Raman Spectrosc.* **1995**, *26*, 911−916.
(24) Chadha, S.; Manoharan, R.; Moënne-Loccoz, P.; Nelson, W. H.; Peticolas, W. L.; Sperry, J. F. *Appl. Spectrosc.* **1993**, *47*, 38−43.
(25) Manoharan, R.; Ghiamati, E.; Dalterio, R. A.; Britton, K. A.; Nelson, W. H.; Sperry, J. F. *J. Microbiol. Methods* **1990**, *11*, 1−15.
(26) Ghiamati, E.; Manoharan, R.; Nelson, W. H.; Sperry, J. F. *Appl. Spectrosc.* **1992**, *46* (2), 357−364.
(27) Löchte, T. Ph.D. Thesis, Universität Essen, 1997.
(28) Nelson, W. H.; Sperry, J. F. In *Modern techniques for rapid microbiological analysis*; Nelson, W. H., Ed.; VCH Publishers: New York, 1991; pp 97−143.
(29) Dalterio, R. A.; Baek, M.; Nelson, W. H.; Britt, D.; Sperry, J. F.; Purcell, F. J. *Appl. Spectrosc.* **1987**, *41* (2), 241−244.
(30) Chadha, S.; Nelson, W. H.; Sperry, J. F. *Rev. Sci. Instrum.* **1993**, *64*, 3088−3093.
(31) Nelson, W. H.; Manoharan, R.; Sperry, J. F. *Appl. Spectrosc. Rev.* **1992**, *27* (1), 67−124.

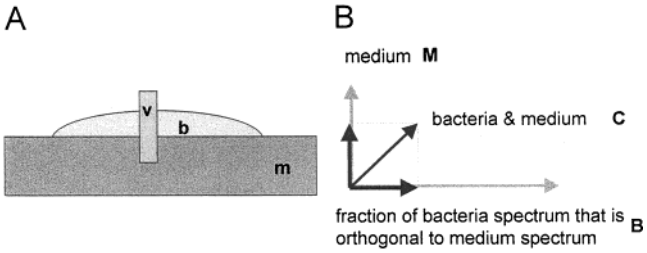

Figure 1. (A) Diagrammatic representation of the measurement volume (v) during sampling of a bacterial microcolony (b) on a solid culture medium (m). (B) Vector diagram of the background subtraction routine depicting the orthogonal vectors of the medium (**M**) and bacteria (**B**) and the vector of the combined bacteria and medium signal (**C**). See text for details.

For our studies, we have chosen to use near-infrared (NIR) multichannel confocal Raman microspectroscopy. The use of NIR laser light minimizes the excitation of sample autofluorescence, which tends to mask the much weaker Raman signal when using visible light excitation. Moreover, the use of a confocal signal detection scheme enables Raman spectroscopic measurements of very small sample volumes (even down to about 1 μm^3).[32,33] Bacteria are therefore required to be cultured only until microcolonies are formed; microcolonies here are defined as colonies that develop in 6 h of growth after plating and have average colony diameters of 10−110 μm (depending on the type of microorganism). Raman spectra can be directly acquired from the microcolonies on solid culture media. When performing measurements on microorganisms still growing on the solid culture medium, there are minimal sample preparation steps prior to spectral acquisition. Since spectra with good signal-to-noise ratios can be obtained of microcolonies within a few minutes of signal collection time, this Raman spectroscopic approach offers the potential for rapid identification of microorganisms. However, a major obstacle of this approach is the presence of signal contributions from the underlying culture medium in the bacteria Raman spectrum due to the limited thickness of the microcolonies (Figure 1A). Because the medium signal contribution is neither negligible nor constant, it will interfere with strain identification. We have developed a new approach to deal with this problem. The method does not aim to subtract the exact amount of signal that is contributed by the culture medium. Instead, we subtract all signal contained in the combined microorganism and culture medium spectrum that is indistinguishable from the culture medium spectrum.

In this paper, the methodological aspects of obtaining and analyzing Raman spectra of microorganisms directly on solid culture medium are discussed. In so doing, confocal Raman microspectroscopy can be developed for the rapid, routine identification and characterization of microorganisms.

MATERIALS AND METHODS

Sample Preparations. The various bacterial strains used in the studies were derived either from the American Type Culture Collection (ATCC) or from the collection of the Department of Medical Microbiology and Infectious Diseases of the University

(32) Puppels, G. J. In *Fluorescent and luminescent probes for biological activity*, 2nd ed.; Mason, W. T., Ed.; Academic Press: London, 1999; pp 377−406.
(33) Puppels, G. J.; de Mul, F. F. M.; Otto, C.; Greve, J.; Robert-Nicoud, M.; Arndt-Jovin, D. J.; Jovin, T. M. *Nature* **1990**, *347*, 301−303.

Hospital Rotterdam (UHR- and BM-labeled strains). Strains were stored at −80 °C in a brain−heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) containing 10% glycerol until use. Following an overnight passage (37 °C) on Mueller−Hinton (MH) medium (Merck, Darmstadt, Germany), the strains were recultured on MH medium for 6 h at 37 °C prior to Raman measurement of microcolonies.

For studies involving bacterial smears on CaF_2 substrate, a second overnight (16 h) culturing step was performed on MH medium. From these overnight colonies, a biomass from several well-isolated colonies was picked up using an inoculating loop and smeared onto a CaF_2 substrate. The samples were allowed to dry in air prior to the Raman measurements.

Ribonucleic acid (RNA) from baker's yeast (Sigma, St. Louis, MO) was dissolved in water to a concentration of 80 mg/mL prior to measurement.

Raman Measurements. Raman spectra were acquired using a Renishaw System 1000 Raman microspectrometer (Renishaw plc, Gloucestershire, UK). The accompanying Leica DM-LM microscope was fitted with an 80× near-infrared objective (MIR Plan 80×/0.75, Olympus). The spatial resolution of the setup was determined to be approximately 1.5 μm in the lateral direction and 7−8 μm along the optical axis. This depth resolution is, to a larger degree, dictated by the entrance slit width of the spectrometer. The spectrometer was equipped with a 300 lines/mm grating. Raman signal was collected in the spectral interval from 250 to 2150 cm^{-1}, with a spectral resolution of 8 cm^{-1}. Raman measurements were performed using 830-nm excitation from a titanium−sapphire laser (model 3900, Spectra Physics, Mountain View, CA) pumped by an argon ion laser (series 2000, Spectra Physics), delivering 100 mW of laser power on the sample.

The constant background signal contribution originating from optical elements in the laser light delivery pathway was subtracted from all spectra. The reference spectrum of a tungsten band lamp of known temperature was used to correct for the wavelength-dependent signal detection efficiency of the Raman setup.[34,35]

Correction for Background Medium Signal Contribution. A nonsubjective method was developed to subtract Raman signal contributions of the culture medium from spectra obtained from bacterial microcolonies growing on the culture medium. This procedure involves the use of vector algebra. In mathematical terms, a Raman spectrum of bacteria on culture medium consisting of *n* data points can be thought of as a vector in an *n*-dimensional space. Similarly, the spectrum of the culture medium alone can be thought of as another vector in this *n*-dimensional space. The combined bacteria and medium vector can now be decomposed into a vector parallel to and a vector orthogonal to the medium vector (**M**) (Figure 1B). (Throughout the text, uppercase boldface type is used to denote vectors). The projection of the "combined signal vector" (**C**) of bacterial and medium signal on **M** ($proj_{\mathbf{M}}$) gives the amount of signal in **C** that cannot be distinguished from **M**. Subsequent subtraction of this projection from **C** results in the desired non-medium-related bacteria spectrum (**B**) (i.e., the vector component of **C** orthogonal to **M**, see eq 1). When eq 1 is elaborated in terms of the dot product of **C** and **M**, we obtain eq 2.

$$\mathbf{B} = \mathbf{C} - \mathrm{proj}_{\mathbf{M}}\mathbf{C} \tag{1}$$

$$\mathbf{B} = \mathbf{C} - \frac{\mathbf{C}\cdot\mathbf{M}}{||\mathbf{M}||^2}\mathbf{M} \tag{2}$$

A similar approach for spectral subtraction was described earlier by Berger et al.[36] for the subtraction of a pure component spectrum from the spectrum of a mixture, containing that component, as a first step in a linear multivariate calibration algorithm.

In the case of measurements on fully hydrated microcolonies, a second variable that can interfere with microorganism identification is the water concentration in the measuring volume. Since fluctuations in ambient temperature and humidity levels can influence the water content of the culture medium, and hence the water signal contribution in the Raman spectra, it is necessary to eliminate this variable as well. The vector correction routine we have developed therefore involves a "double-correction" approach. This procedure starts with independently correcting both the combination spectrum (i.e., the spectrum of bacteria and medium) and the medium spectrum for water signal contributions by subtracting their respective projections on the water vector (eqs 3 and 4, $proj_{\mathbf{W}}$ is the vector projection on the water vector, **W**). The two resulting spectra are then used in the next vector correction step to actually correct the combination spectrum for the medium signal (eq 5).

$$\mathbf{C}' = \mathbf{C} - \mathrm{proj}_{\mathbf{W}}\mathbf{C} \tag{3}$$

$$\mathbf{M}' = \mathbf{M} - \mathrm{proj}_{\mathbf{W}}\mathbf{M} \tag{4}$$

$$\mathbf{B}' = \mathbf{C}' - \mathrm{proj}_{\mathbf{M}'}\mathbf{C}' \tag{5}$$

In the work presented here, this method was applied to first derivative spectra instead of the actual measured spectra. In most cases, Raman spectra of biological molecules, cells, or tissues contain a broad, relatively featureless background signal, usually ascribed to fluorescence. Its intensity and shape may vary somewhat (and sometimes quite considerably) from measurement to measurement and from strain to strain. When the method described above is applied to spectra that differ only in their Raman-to-fluorescence background signal ratio, the resulting "corrected" spectra would differ. Therefore, the fluorescence background signal contribution needs to be eliminated before application of the vector correction method. This is achieved by making use of first derivative spectra.

It is important to note that the vector correction procedure is not the same as subtraction of the exact amount of signal contributed by the medium. When a pure bacteria spectrum (i.e., without medium signal contribution) is decomposed into vectors parallel and perpendicular to the medium vector, the parallel

(34) Wolthuis, R.; Bakker Schut, T. C.; Caspers, P. J.; Buschman, H. P. J.; Römer, T. J.; Bruining, H. A.; Puppels, G. J. In *Fluorescent and luminescent probes for biological activity*, 2nd ed.; Mason, W. T., Ed.; Academic Press: London, 1999; pp 431−455.

(35) Puppels, G. J.; Colier, W.; Olminkhof, J. H. F.; Otto, C.; de Mul, F. F. M.; Greve, J. *J. Raman Spectrosc.* **1991**, *22*, 217−225.

(36) Berger, A. J.; Koo, T. W.; Itzkan, I.; Feld, M. S. *Anal. Chem.* **1998**, *70*, 623−627.

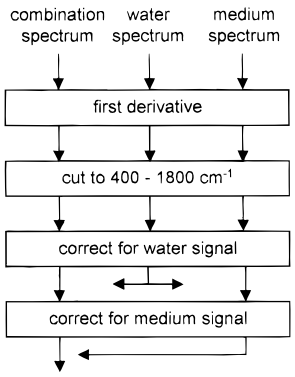

Figure 2. Schematic representation of the protocol used for correcting data. First derivative spectra are cut to the spectral region of 400−1800 cm^{-1}. Cut spectra are then corrected for water and culture medium signal contributions using the vector correction routine described in the Materials and Method section.

component would normally not be (exactly) zero. This implies that the result of the vector correction method described is not the exact "pure" bacteria spectrum but the component of the bacteria spectrum that is orthogonal to the medium vector. Therefore, this subtraction method may affect the possibilities of a precise biochemical interpretation of the spectra. However, since the components of the microorganism spectrum that are parallel to the medium and/or water vector cannot be distinguished from the actual signal contributions of water and medium (whose intensity varies from measurement to measurement), they cannot be considered as useful information when it comes to microorganism identification.

A schematic of the whole vector correction procedure is given in Figure 2. The code for the calculations in this scheme was developed under the Matlab software package (The Mathworks Inc., Natick, MA).

Validation of the Medium Subtraction Method. The application of the vector correction method to nonsubjectively subtract background signal contributions is expected to result in reproducible spectra regardless of the amount of medium and water signal initially present in the raw data. This method was tested in several ways.

First, a simulation was performed, in which the measurement of spectra at different depths within a microcolony on solid culture medium (and therefore with varying medium signal contributions) was mimicked. The resulting spectra were subsequently subjected to vector correction in order to remove water and culture medium signal contributions. Second, actual measurements at different depths in a microcolony growing on a solid culture medium served as an illustration of the practical situation. Finally, the effect of separately correcting for water signal contributions in addition to medium signal correction with the double-correction procedure was investigated.

(1) Simulation. To simulate measurements at different depths in a bacterial colony, a pure bacteria spectrum (i.e., without culture medium signal contributions) and a spectrum of the culture medium were added in different ratios. Raman spectra of dried bacterial smears on CaF_2 substrate served as the pure bacteria spectra, since in the spectral region of interest CaF_2 has no Raman features. Raman spectra were collected of a dried bacterial smear (*Escherichia coli* ATCC 25922) on CaF_2, of Mueller−Hinton culture medium, and of water. Raman spectra of the bacterial smear and of the culture medium were obtained at random locations in the sample, comprising 60 min total signal collection time. The same signal collection time was used to obtain a spectrum of water. The Raman spectrum of the culture medium used for subtraction in the vector correction routine was also obtained with a high signal-to-noise level. The spectra were scaled to standard normal variance (SNV; i.e., zero mean and unit variance).[37] The bacterium and medium spectra were then added in the ratios of 1:1, 1:5, and 1:10. First-derivative spectra were calculated for the combination spectra, the second MH spectrum, and the water spectrum, followed by cutting all the spectra to the region of interest, 400−1800 cm^{-1} (schematic representation in Figure 2). These combination spectra were corrected for the water and medium signal contributions by an independently measured culture medium spectrum, as described above. Having a second independent spectrum parallels the scenario of correcting acquired microcolony spectra with a reference culture medium spectrum.

(2) Depth Measurements. To illustrate the validity of the vector correction method on spectra of bacterial samples growing on culture media, Raman spectra were acquired directly from a 6-h microcolony (*E. coli* ATCC 25922) with the laser focused at three depths within the microcolony. Spectra were acquired from the top (2 μm below surface), middle (4 μm below surface), and bottom (10 μm below the surface) of the microcolony, each in 5 min signal collection time. Water and medium Raman spectra were obtained in 60 min signal collection time as described above. A Raman spectrum of RNA was obtained in 30 min signal integration time. Data treatment was performed as described in Figure 2.

(3) "Double Correction". The effect of correcting for both water and medium signals was illustrated by culturing *E. coli* ATCC 25922 on three media that differed only in their water content. Separately, 2.25, 3.8, and 5.6 g portions of dehydrated MH medium (Difco Laboratories, Detroit, MI) were dissolved in 100 mL of distilled water to prepare the solid culture media. Raman spectra of water and the three media were collected as described above. Spectra were treated as outlined in Figure 2, and for illustration purposes, the water correction step was initially omitted.

Identification/Classification of Bacterial Strains. Spectra were obtained of 6-h microcolonies of five bacterial strains (*Staphylococcus aureus* ATCC 29213, *S. aureus* UHR 28624, *Staphylococcus epidermidis* UHR 29489, *E. coli* ATCC 25922, and *Enterococcus faecium* BM 4147). These measurements were performed in triplicate on separate days. Measurements were carried out on five microcolonies per bacterial strain, with five spectra obtained from various positions within each microcolony. For each measurement, Raman signal was collected for 30 s. Raman spectra of the Mueller−Hinton culture medium were obtained at random locations in the medium, comprising 60 min signal collection time. A water spectrum was also obtained in 60 min total signal collection time.

Vector corrections for water and medium signal contributions were performed as described above. Per strain, the five spectra collected from each microcolony were averaged. The complete

(37) Barnes, R. J.; Dhanoa, M. S.; Lister, S. J. *Appl. Spectrosc.* **1989**, *43*, 772−777.

spectral range (400−1800 cm^{-1}) of the microcolony spectra was then used in the subsequent multivariate analysis. Data from all 3 days were combined and analyzed as one data set.

For multivariate analyses, the amount of data was first reduced using principal component analysis (PCA)[38] performed using the Matlab PLS toolbox (Eigenvector Research Inc., Manson, WA). A total of $n - 1$ PCA scores were calculated (n being the number of spectra in the analysis), typically accounting for 99−100% of the variation in the data set. These PCA scores were used in a cluster analysis (SPSS, Chicago, IL). Ward's clustering algorithm method and squared Euclidean distance measure were used in generating the dendrogram of the hierarchical cluster analysis.

Linear discriminant analysis (LDA) was performed on principal component scores using SPSS. Two-thirds of the data was used as a training set and one-third as a test set. Selection of principal components that were included in the LDA model was based upon Wilk's Lambda[39] method and 95% F-test inclusion criterion,[39] thus maximizing group separation. The strength of the model based on the training set was evaluated using the leave-one-out method.[39]

RESULTS AND DISCUSSION

The Raman spectrum obtained directly from a bacterial microcolony on solid culture medium contains signal contributions from both bacteria and the culture medium. The relative signal contribution of the culture medium will vary, as it critically depends on the exact depth at which the laser light is focused as well as on the thickness of the microcolony. Since the medium signal contribution is not negligible and is variable, it would interfere with multivariate analysis intended for strain identification.

Correction for Signal Contributions of the Culture Medium. In Figure 3A, a spectrum of a dried smear of bacteria and a spectrum of medium from a culture plate are shown. The spectrum of the medium does not contain any clear marker bands that could be used to determine the exact intensity of the medium signal contribution. Therefore, it is not possible to accurately and nonsubjectively subtract the medium signal contribution on the basis of the intensity of such a band. For this reason we have developed the vector correction method described in the materials and methods section, which was evaluated in three experiments.

As explained in the Materials and Methods section, the result of the vector correction method is a spectrum that, when viewed as a vector, is perpendicular to the medium spectrum (i.e., not only medium signal is subtracted but also the component of the bacteria spectrum that is parallel to the medium spectrum). However, for the purpose of readability we will, from here on, refer to the action of the vector correction method as "subtraction of medium signal".

Simulated Experiment. The two spectra of Figure 3A were used to simulate an experiment in which measurements are carried out at different depths within a microcolony on solid culture medium. To achieve this, the bacteria spectrum and a medium spectrum were co-added in various ratios (Figure 3B) (see Materials and Methods section for details). Spectral features belonging to the bacteria become less obvious when the fraction of the medium is increased in the combination spectrum. For example, there was an increasing sloping background contribution due to the greater water content of the medium. In the spectra with higher proportions of medium added in, the water contribution was also more noticeable by a marked broadening of the 1550−1700 cm^{-1} region. This spectral region overlaps with Raman features predominantly arising from C═O amide backbone groups within proteins. In addition, various other spectral changes are observed around 1300 and 1000 cm^{-1} and between 800 and 900 cm^{-1}.

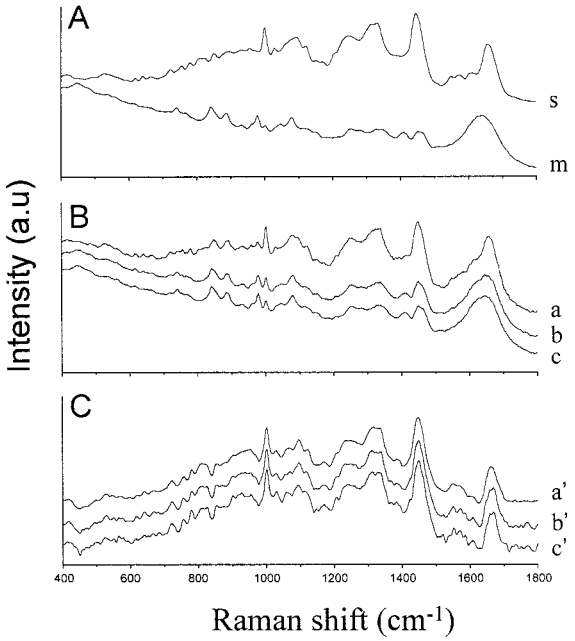

Figure 3. Validation of the vector correction routine. Simulation of measurements at different focusing depths within a bacterial microcolony. (A) Raman spectrum of *E. coli* ATCC 25922 bacterial smear on CaF_2 substrate (s) and Raman spectrum of Mueller−Hinton medium (m) to be used for the simulation. (B) Spectra obtained by adding the bacteria spectrum and a medium spectrum in the ratios (a) 1:1, (b) 1:5, and (c) 1:10. (C) Resulting bacteria spectra (a′, b′, c′) after vector correction for water and medium signal contributions. (au = arbitrary units.)

Using a second medium spectrum, the vector correction method was used to subtract out the corresponding signal contributions. This routine resulted in bacteria spectra with high similarity, illustrating that, with the correction scheme, the background signal could be reproducibly subtracted (Figure 3C). There were no residual features of the medium following vector correction.

Measurement on Microcolony Growing on Solid Culture Medium. Having demonstrated that the vector correction procedure is able to nonsubjectively subtract medium signal contributions to yield similar bacteria spectra, Raman spectra were acquired at different depths within an actual bacterial microcolony (*E. coli* ATCC 25922). We observed that, at the various focusing depths, there are clear differences in the untreated spectra (Figure 4A). Following subtraction of water and medium signal contributions, the spectra were very similar (Figure 4B). However, it was noticed that there were still some spectral differences between spectra taken at different depths within the microcolony, as became clear when the difference was taken between the spectra acquired from the top and from the bottom of the microcolony (Figure 4C). Also

(38) Jolliffe, I. T. *Principal component analysis*; Springer Verlag: New York, 1986.
(39) Norusis, M. J. *SPSS advanced statistics 6.1*; SPSS Corp., Inc.: Chigaco, IL, 1994; pp 1−42.

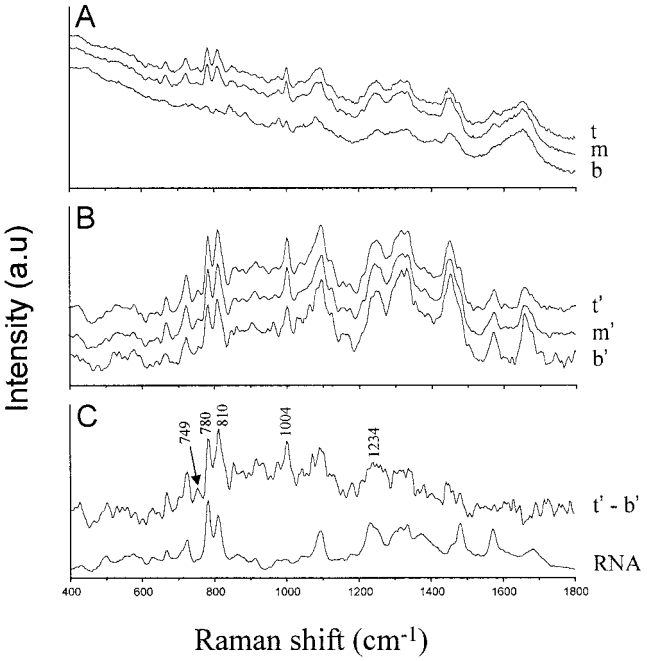

Figure 4. Raman spectra of *E. coli* ATCC 25922 measured at various focusing depths: t (top) = 2 μm, m (middle) = 4 μm, and b (bottom) = 10 μm, below the surface of the microcolony. (A) Spectra obtained at the various depths before vector correction. (B) Spectra (t′, m′, b′) following vector correction for water and the underlying Mueller−Hinton culture medium signals. (C) Difference spectrum between the vector-corrected spectra acquired from the top and the bottom of the microcolony (t′−b′) and the Raman spectrum of a solution of ribonucleic acid (RNA) after a water spectrum has been subtracted. (au = arbitrary units.)

depicted in Figure 4C is a spectrum of a solution of RNA from which a water spectrum has been subtracted. Comparison of the difference spectrum with this RNA spectrum reveals many similarities, suggesting that different RNA levels account for the differences observed. However, not all of the features in the difference spectrum can be accounted for by RNA bands alone. Among others, the difference spectrum also shows peaks of thymine (749 cm^{-1}) and phenylalanine (1004 cm^{-1}) (Table 1), indicating the presence of more biological differences. Hence, these spectral differences observed are not artifacts of the correction routine. They arise from the naturally occurring biochemical heterogeneity of the bacterial colonies. One explanation could be that, even in microcolonies with a thickness of 6−8 μm, the various layers contain bacteria in different growth stages (older versus younger cells), and these differences are reflected in changes in the Raman spectrum. Manoharan et al. reported increased RNA levels in bacteria entering the logarithmic phase.[40] We speculate that the cells in the higher layers of the microcolony are more actively dividing than cells in the deeper layers. Further investigations into microcolony heterogeneity are currently underway.

Correction for Varying Water Contribution. In our subtraction method, it is necessary to use a double-correction approach involving water and the culture medium. The validity of this approach was demonstrated in a study in which spectra were measured from bacteria grown on various culture media that differed only in water content. Figure 5A shows bacteria spectra after vector correction for the medium alone. Although the spectra look remarkably similar, difference spectra revealed residual variation in the spectra especially in the region around 1650 cm^{-1}. This broad band is characteristic of the Raman spectrum of water. When the extra correction step for the water signal was performed, very similar spectra were obtained, although the intensity of one peak at 780 cm^{-1} was higher in the medium richer in nutrients (i.e., lower water content) (Figure 5B). This peak could be attributed to uracil (RNA) (Table 1), but one would expect to observe other RNA peaks in the difference spectrum as well. Further research should give additional information and is currently being performed.

Limitations in the comparison of organisms cultured on different media therefore do not originate from the correction method presented here but rather from intrinsic physiological cell differences. Minimization of these influences can be achieved by thorough standardization in the preparation of culture media and in the culture conditions. Commercially available, ready-to-use media have the advantage that major manufacturers use standard protocols and quality controls in their medium preparations. To what extent minor fluctuations from batch to batch can influence the identification process is not yet clear and needs further investigation. The presence of heterogeneity within microcolonies suggests that the Raman signal from several locations in the colony needs to be averaged in order to optimize reproducibility.

Identification of Bacteria. Now that complicating factors such as the variability in the water and medium signal contributions are eliminated, the Raman spectra can be treated by multivariate analysis for (nonsubjective) classification of bacterial strains. Representative spectra acquired from five bacterial strains are shown in Figure 6A. Closer inspection of the spectra reveals that there are, indeed, spectral differences characteristic of the various strains. For example, the *Staphylococcus* strains are characterized by markedly high intensities at 780 cm^{-1}. To the naked eye, the two *S. aureus* stains resemble one another very closely. The spectra of *E. coli* have a characteristically intense band at around

Table 1. Tentative Wavenumber Assignments of Some of the Raman Features in the Spectra Presented[a]

wavenumber (cm^{-1})	tentative assignment
533	δ(COC) glycosidic ring
668	ν(CS)
725	$\rho(CH_2)$
749	thymine (T) ring
780	uracil (U) ring
810	C−O−P−O−C in A-RNA backbone
856	ν(CC)
	ν(COC) 1,4-glycosidic link
1004	ν(CC) aromatic ring (Phe)
1095	ν(CC) skeletal
	ν(COC) 1,4-glycosidic link
1220−1290	amide III
1334	δ(CH)
1452	$\delta(CH_2)$
1573	δ(NH) and ν(CN), amide II
1630−1680	amide I

[a] δ, Deformation; ν, stretching; ρ, rocking; Phe, phenylalanine (refs 22 and 23).

(40) Manoharan, R.; Ghiamati, E.; Chadha, S.; Nelson, W. H.; Sperry, J. F. *Appl. Spectrosc.* **1993**, *47* (12), 2145−2150.

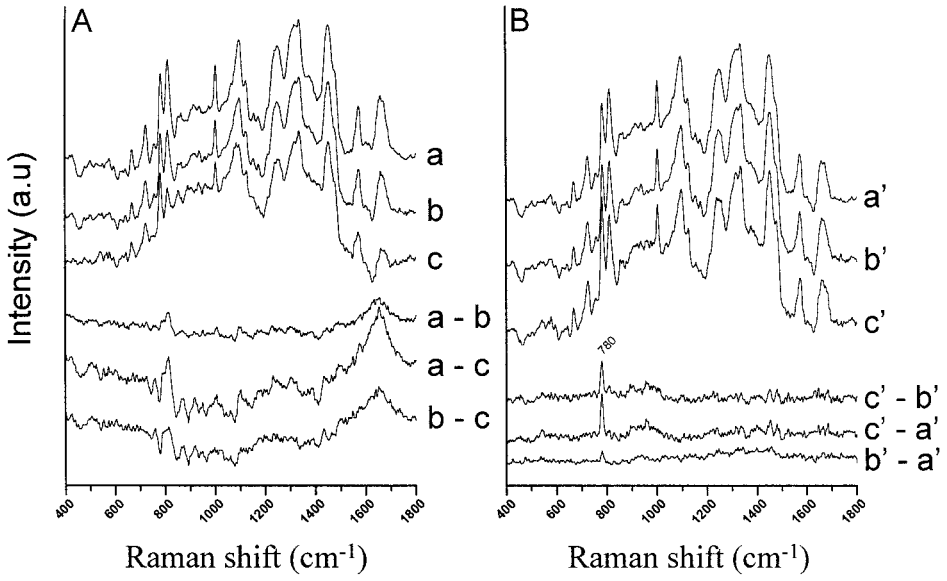

Figure 5. Raman spectra of *E. coli* ATCC 25922 cultured on MH media with different water content. (A) Spectra after vector correction for *only* the corresponding medium (in order of decreasing water content, a, b, c) and their difference spectra. (B) Bacteria spectra (in order of decreasing water content, a′, b′, c′) after vector correction for *both* the water and corresponding medium signals and their difference spectra. (au = arbitrary units.)

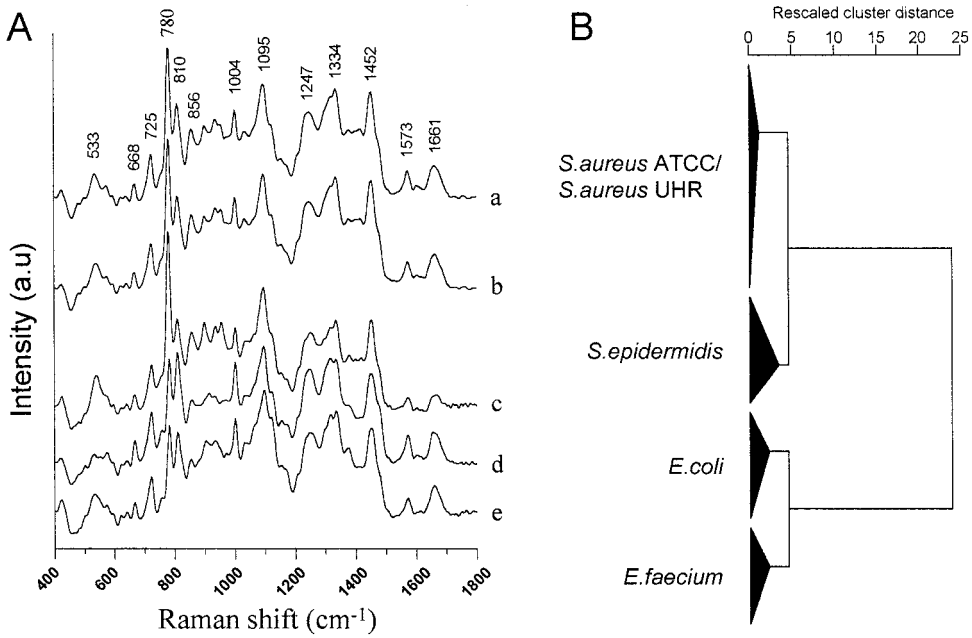

Figure 6. (A) Representative Raman spectra of five bacterial strains obtained from 6-h microcolonies growing on MH medium: (a) *Staphylococcus aureus* ATCC 29213, (b) *Staphylococcus aureus* UHR 28624, (c) *Staphylococcus epidermidis* UHR 29489, (d) *Escherichia coli* ATCC 25922, and (e) *Enterococcus faecium* BM 4147. (B) Dendrogram of hierarchical cluster analysis (squared Euclidean distance measure and Ward's cluster algorithm) performed on the principal component scores of the vector-corrected Raman spectra. Repeated measurements of the same strain on three separate days were included in the analysis. Bacteria spectra were collected in 2.5 min signal integration time. (au = arbitrary units.)

1004 cm^{-1} arising from the ring breathing vibration found in the amino acid phenylalanine. The biological relevance of the increased intensity of this peak in *E. coli* Raman spectra is presently unknown. Raman spectra arising from *S. epidermidis* and *E. faecium* have slightly poorer signal-to-noise levels when compared to those of the other strains. This is because the 6-h microcolonies of these strains are very thin. The colony thickness was approximately 1−3 μm for *S. epidermidis* and *E. faecium*, as opposed to between 6 and 8 μm for the strains of *S. aureus* and *E. coli*. These differences lie in the intrinsic biological growth difference of the strains and are reflected in the resulting Raman spectra.

Hierarchical cluster analysis of the microcolony spectra of the 5 strains over 3 days resulted in the dendrogram shown in Figure 6B. We observed that there are two major clustering branches, consisting of the *Staphylococcus* strains in one group and *E. faecium* and *E. coli* in the other. Within this latter group, a clear division is observed between the *E. faecium* and *E. coli* strains. In the *Staphylococcus* branch, subclusters are formed of *S.*

epidermidis and *S. aureus*. Therefore, it is possible to distinguish these bacterial genera and the *Staphylococcus* species on the basis of their Raman spectra.

Within the clusters containing spectra of one strain, spectra measured on the same day tended to cluster together, indicating the presence of some day-to-day variation. This variation, however, did not interfere with identification down to the species level of the *Staphylococcus* strains, suggesting that spectral differences between the various species are greater than any subtle day-to-day variation in the spectra of the strain.

When data from all 3 days were analyzed together, the two *S. aureus* strains could not be clearly separated with the unsupervised classification approach. PCA followed by the supervised linear discriminant analysis on only the *S. aureus* spectra resulted in a 100% correct classification of the training set and 83% of the test set. When hierarchical cluster analysis was performed on the PCA scores of each day separately, 100% separation of the two *S. aureus* strains was obtained. This observation suggests that when data from the 3 days were combined, the complete discrimination of the two *S. aureus* strains was hindered by day-to-day variations in the spectra. The effect of small deviations in wavenumber calibration of the spectra as well as fluctuations in the biochemical composition of the cells are currently being investigated as possible sources of this day-to-day variation. The issue of repeatable wavenumber calibration of multichannel Raman instruments and instrument-to-instrument calibration transfer is an area of active research. A thorough analysis of the problems involved as well as potential solutions was recently published by Mann and Vickers.[41]

Other studies using FT-IR spectroscopy combined with multivariate analysis have also reported the successful separation of different bacterial species, such as *Eubacterium* spp.,[42] *Lactobacillus* spp.,[43] *Streptococcus* spp., and *Enterococcus* spp.[44] Hence, although the various Raman spectra of microorganisms look similar upon first inspection, as with FT-IR spectra, there is a high information content in these spectra which can be used in multivariate analysis for the discrimination of microorganisms.

Overall, then, the classification results presented indicate that it is possible to identify bacteria from their Raman spectra acquired directly from microcolonies growing on solid culture medium.

CONCLUSION

We have presented a novel approach of the use of Raman microspectroscopy for the identification and characterization of clinically relevant microorganisms. An approach for measuring Raman spectra of 6-h microcolonies directly on the solid culture medium could, in principle, enable identification results to be obtained within the same day of receipt of patient material. In contrast, conventional microbiological approaches require significantly more time to arrive at the same result. With respect to other spectroscopic methods mentioned in the introduction, Raman spectroscopy also has the advantage of minimizing culturing time, sample handling, and the use of chemicals and disposables. Using online data processing routines, the Raman spectra can be analyzed and clustered rapidly. However, key features to the successful application of this technique are the careful correction for variable parameters, such as water signal contributions of the culture medium and the amount of culture medium signal collected with the Raman signal of bacteria. Averaging of signal over several positions in the microcolony is fundamental as well, to compensate for colony heterogeneity. The methods must be rigorously standardized, such that reproducible spectra of good signal-to-noise levels are obtained. Furthermore, the use of a standard growth medium with carefully controlled composition is essential for accurate classification of bacteria. The different signal contributions of different media can, in principle, be compensated for through the application of double-vector correction schemes. However, it is likely that differences in medium composition also have an influence on the overall biochemical composition of the bacteria. This is, therefore, a point of concern for all spectroscopic methods.

To our knowledge, this is the first report on spectroscopic measurements of bacteria directly on solid culture media. Good signal-to-noise levels could be obtained after an incubation period of 6 h, followed by 2.5 min of Raman signal collection. From the limited data set presented here, it appears that bacteria may be distinguished at the genus and species level on the basis of unsupervised analysis of their Raman spectra. Supervised methods, such as wavenumber region selection methods[34] and minimization of day-to-day variation in instrumental and/or biological parameters, should enable discrimination down to the strain level. Therefore, we conclude that, when thoroughly standardized and optimized, confocal Raman microspectroscopy has potential as a powerful new tool in diagnostic microbiology.

(41) Mann, C. K.; Vickers, T. J. *Appl. Spectrosc.* **1999**, *53*, 856−861.
(42) Alsberg, B. J.; Wade, W. G.; Goodacre, R. *Appl. Spectrosc.* **1998**, *52*, 823−832.
(43) Curk, M. C.; Peladan, F.; Hubert, J. C. *FEMS Microbiol. Lett.* **1994**, *123*, 241−248.
(44) Goodacre, R.; Timmins, E. M.; Rooney, P. J.; Rowland, J. J.; Kell, D. B. *FEMS Microbiol. Lett.* **1996**, *140*, 233−239.

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