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Review article

New biochip technology for label-free detection of pathogens and their toxins

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Abstract

μSERS is a new biochip technology that uses surface-enhanced Raman scattering (SERS) microscopy for label-free transduction. The biochip itself comprises pixels of capture biomolecules immobilized on a SERS-active metal surface. Once the biochip has been exposed to the sample and the capture biomolecules have selectively bound their ligands, a Raman microscope is used to collect SERS fingerprints from the pixels on the chip. SERS, like other whole-organism fingerprinting techniques, is very specific. Our initial studies have shown that the Gram-positive *Listeria* and Gram-negative *Legionella* bacteria, *Bacillus* spores and *Cryptosporidium* oocysts can often be identified at the subspecies/strain level on the basis of SERS fingerprints collected from single organisms. Therefore, pathogens can be individually identified by μSERS, even when organisms that cross-react with the capture biomolecules are present in a sample. Moreover, the SERS fingerprint reflects the physiological state of a bacterial cell, e.g., when pathogenic *Listeria* and *Legionella* were cultured under conditions known to affect virulence, their SERS fingerprints changed significantly. Similarly, nonviable (e.g., heat- or UV-killed) microorganisms could be differentiated from their viable counterparts by SERS fingerprinting. Finally, μSERS is also capable of the sensitive and highly specific detection of toxins. Toxins that comprised as little as 0.02% by weight of the biomolecule–toxin complex produced strong, unique fingerprints when spectra collected from the complexes were subtracted from the spectra of the uncomplexed biomolecules. For example, aflatoxins B_1 and G_1 could be detected and individually identified when biochips bearing pixels of antibody or enzyme capture biomolecules were incubated in samples containing one or both aflatoxins, and the spectra were then collected for 20 s from an area of the biomolecule pixel ~ 1 μm in diameter. In the future, we plan to investigate the use of hyperspectral imaging Raman microscopy for collecting fingerprints from all the pixels on the biochip, individually yet simultaneously, to enable the rapid detection of diverse pathogens and their toxins in a sample, using a single biochip.

Keywords: Biochip; Bacteria; Spores; Oocysts; Toxins; Surface-enhanced Raman scattering (SERS)

1. Introduction

"Whole-organism fingerprinting" (Magee, 1993) makes it possible to identify microorganisms with minimal sample preparation. Vibrational spectroscopies such as Fourier-transform infrared (FTIR) (Helm et al., 1991; Naumann et al., 1991a,b; Holt et al., 1995), Raman scattering (Naumann et al., 1995; Maquelin et al., 2000) and UV resonance Raman (Nelson et al., 1992) are among the more frequently reported whole-organism fingerprinting techniques. These techniques can be exceptionally specific; vibrational spectroscopies have sometimes been reported to be more useful than conventional approaches such as biochemotyp-

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ing, plasmid or lipopolysaccharide pattern analysis, multilocus enzyme electrophoresis or outer membrane or whole-cell protein pattern analysis in identifying strains for epidemiology applications (Seltmann et al., 1994). Raman microscopy even makes it possible to analyze individual bacterial cells (Schuster et al., 2000). However, before any of these whole-organism fingerprinting techniques can be used to analyze environmental samples, the microorganisms must be cultured in order to isolate the microorganism of interest from other sample constituents and/or produce sufficient biomass for analysis.

We are developing a new approach that eliminates the need for culturing, while enabling analysis of complex media such as environmental samples. In this approach (Grow, 1999), the pathogens of interest are selectively isolated from the sample using capture biomolecules immobilized on a biochip. The captured pathogens are then identified by Raman microscopy. To enable the rapid collection of strong, high-quality spectra from individual organisms, the biochip surface is a roughened metal film capable of inducing surface-enhanced Raman scattering (SERS), a phenomenon that can enhance the normal Raman signal by many orders of magnitude (Kneipp et al., 1997, 1998a,b, 1999; Nie and Emory, 1997; Xu et al., 1999). Because biomolecule capture adds an additional layer of specificity to whole-organism fingerprinting, analysis is exceptionally specific, making it possible to differentiate among species/strains that cross-react with a given biomolecule. A simple flow diagram of this new approach is shown in Fig. 1. This new biochip technology has been dubbed "μSERS", since it couples SERS with microscopy. This paper summarizes the studies that have been performed to date to (1) evaluate the feasibility of using μSERS to identify diverse microorganisms at the subspecies level, even if the biomolecules used in the biochip are cross-

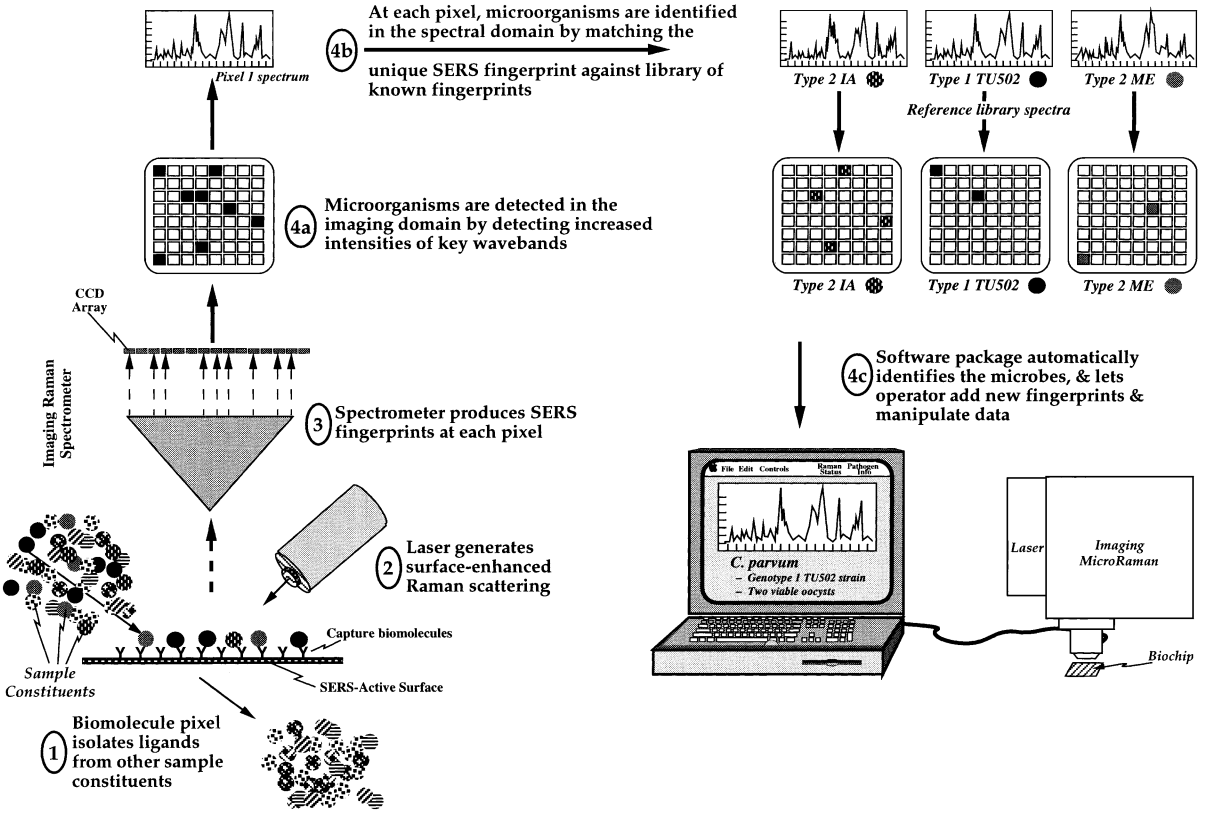

Fig. 1. Flow diagram of the μSERS process.

reactive; (2) determine the physiological state/phenotype of captured bacteria; (3) distinguish between viable and nonviable organisms; and (4) detect and identify toxins produced by pathogens as well. A few representative spectra are included as examples of the results that have been obtained.

2. Identification based on SERS fingerprints

The model systems being studied during the initial development of μSERS include the Gram-positive *Listeria* and Gram-negative *Legionella* bacteria, *Bacillus* spores, and *Cryptosporidium* oocysts. The first experiments were designed simply to confirm that the SERS fingerprints of these microorganisms would be stable and reproducible. Dilute microbial suspensions were applied to SERS surfaces, and spectra were collected for 60 s (six 10-s scans coadded) from individual organisms. Several dozen spectra each were collected from five species of *Listeria* (i.e., *Listeria monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria ivanovii*), including three strains of *L. monocytogenes* belonging to serotypes 1/2a, 3 and 4b, and two strains of *L. innocua* belonging to serotypes 6a and 6b; the spores and vegetative cells of six species of *Bacillus* (i.e., *Bacillus brevis*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus thuringiensis*), including two strains each of *B. cereus*, *B. subtilis* and *B. thuringiensis*; six species of *Legionella* (i.e., *Legionella pneumophila*, *Legionella bozemanii*, *Legionella israelensis*, *Legionella micdadei*, *Legionella maceachernii* and *Legionella dumoffii*); and oocysts from *Cryptosporidium parvum* genotypes 1 and 2 and *Cryptosporidium meleagridis*.

Spectra were collected from bacteria and spores in different aliquots from a given harvest and from different harvests. With the exception of spores from *B. cereus* ATCC11778, the fingerprints were reproducible from organism to organism, from aliquot to aliquot and from harvest to harvest, provided that the growth conditions were held constant. This was found to be true for *Listeria*, *Legionella* and *Bacillus* vegetative cells, and for *Bacillus* spores.

As expected, the fingerprints from different species belonging to a given genus were readily differentiated, even those from species as closely related as *L. monocytogenes* and *L. innocua* (Fig. 2) Also as expected, preliminary results indicated that SERS analysis, like other whole-organism fingerprinting techniques, can be used to identify bacteria and spores at the subspecies level. It was interesting to find that the fingerprints from different strains belonging to the same species were sometimes considerably more different than those of fingerprints

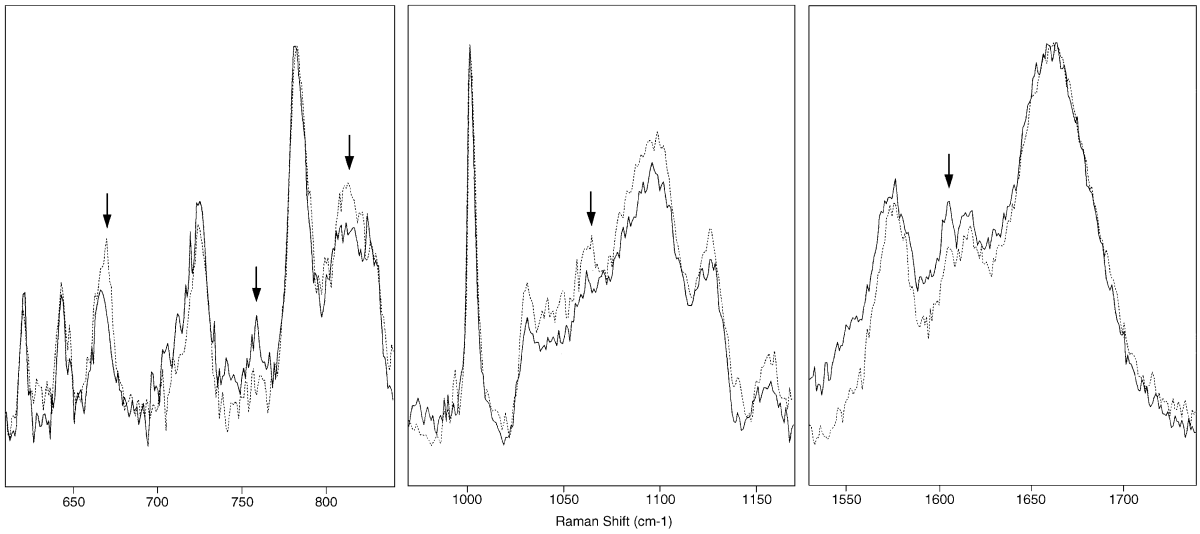

Fig. 2. Windows from the SERS spectra collected for 60 s from *L. monocytogenes* (—) and *L. innocua* (- - -) grown in BHI media at 37 °C. The spectra from the two species consistently differ from each other at the peaks indicated by arrows. Background corrected.

belonging to different species, for both bacteria and spores.

In the case of *B. cereus* ATCC11778, five peaks varied in intensity from spore to spore, even within a given sample, with the peaks being very strong in roughly two-thirds of the spores in each batch, and weaker, although still very distinctive, in the remaining third. These peaks, at ~663, 825, 1015, 1396 and 1574 cm^{-1}, were seen in all spore spectra and have tentatively been assigned to calcium dipicolinate (Shibata et al., 1986). Because all spore spectra contain the dipicolinate signature, these peaks do not appear to be particularly useful for identification at the species/subspecies levels; and, therefore, variability in their intensities are unlikely to affect the ability to differentiate among the different species/strains. The dipicolinate signature does, however, appear to be very useful for rapidly differentiating between *Bacillus* spores and other types of organisms, since it is very strong and distinctive.

Spectra were also collected from oocysts in different aliquots from a given passage and from different passages for three *C. parvum* genotype 2 strains. For two of these strains, samples ranged from several months to several years old. Since oocysts are relatively large organisms, spectra could be collected from different regions of the surface of each individual oocyst. Each strain produced two distinctive types of spectra: one in which there were a series of strong peaks in the short wavelength region, and the other in

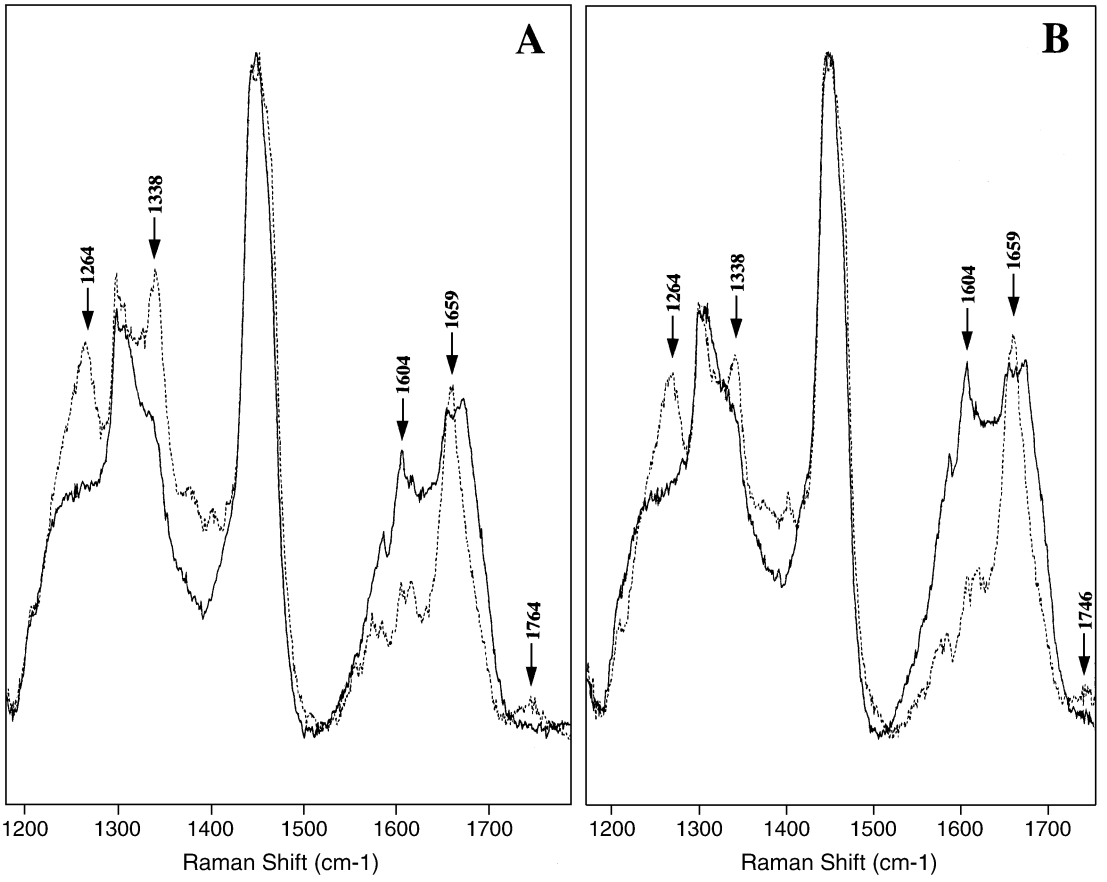

Fig. 3. A window from the SERS spectra of two *C. parvum* genotype 2 strains: (A) a calf isolate from Iowa and (B) an isolate from a foodborne outbreak in Maine, comparing the fingerprints of recently passaged (- - -) and old (—) samples. Note that the old samples give similar fingerprints, whereas the recently passaged oocysts can be readily differentiated, e.g., by the ratios of the peak heights at 1659 cm^{-1} and in the ~1250–1350 cm^{-1} range. Background corrected.

which only a few, very weak, broad peaks were seen in this region. The two different types of spectra are presumably associated with different types of structures found in these complex organisms. For both types of spectra, there was some minor variability from oocyst to oocyst within a given sample but, in general, the spectra were reproducible. However, the fingerprints of recently passaged oocysts were strikingly different from those of oocysts belonging to the same strain, but old enough to no longer be considered a threat (Fig. 3). Recently, passaged oocysts (including three *C. parvum* genotype 2 and two *C. parvum* genotype 1 strains) yielded unique fingerprints on which identification could be made. The spectra of old samples resembled each other much more closely, although there were still some subtle differences among the various strains. Recent studies indicate that oocyst fingerprints change substantially during the first few weeks after passage, with those stored at room temperature changing more rapidly than those stored at 4 °C. Although the database is very small, these findings suggest the intriguing possibility that infectious oocysts might be differentiated from noninfectious organisms, as well as identified at the subspecies level, on the basis of their fingerprints.

3. Biomolecule capture coupled with SERS transduction

Having confirmed that the microbial SERS fingerprints were stable and reproducible when growth and storage conditions were held constant and could be used to identify microorganisms at the species/subspecies levels, the next question was whether biomolecule capture would affect the SERS fingerprints. The first experiments were conducted with *Listeria* and chips prepared with either of two polyclonal antibodies, i.e., one specific for *Listeria* species and one specific for *L. monocytogenes*. In general, the fingerprints of the captured bacteria were weaker than those collected from microorganisms deposited directly onto the SERS surface (presumably because the capture molecule separates the microorganism from the metal surface by several nanometers, thereby degrading the SERS signal enhancement); and contributions from the capture biomolecules could be seen in the SERS spectra. However, the bacteria could be identified at the species and serotype levels on the basis of spectra collected for 100 s (ten 10-s scans coadded).

The spectra from the *Bacillus* spores and *Cryptosporidium* oocysts, on the other hand, overwhelmed those of the antispore and antioocyst antibodies, which did not appear to contribute to the microbial fingerprints. Spectra from the biomolecule-captured spores were somewhat weaker (usually by a factor of 2–4) than those collected from spores deposited directly onto the SERS surface. Nevertheless, subspecies identification was still possible from spectra collected for 60 s. Oocyst fingerprints did not appear to be affected by biomolecule capture, possibly because these organisms are so large (i.e., oocysts used on these studies are ~ 5–8 μm in diameter, whereas the spores are typically ovoids ~ 1 μm in diameter and 1.5 μm long.).

The initial studies utilized antibodies as the capture biomolecules, and antibodies may cross-react with strains that are nonpathogenic. However, the μSERS technology can utilize any type of biomolecule, including lectins and cell surface receptors as well as antibodies and aptamers. Accordingly, biomolecules that are associated with virulence can be used to selectively capture pathogens from the sample. For example, *Listeria* virulence has been correlated with lectin binding profiles (Facinelli et al., 1998; Cowart et al., 1990), with *L. monocytogenes* serotype 4b exhibiting a pattern of lectin binding that is distinct from other listeriae (Thuan et al., 2000). In addition, *L. monocytogenes* is known to bind to fibronectin (Gilot et al., 1999, 2000), an extracellular matrix molecule to which many bacterial pathogens adhere. μSERS biochips were prepared with Concanavalin A (ConA), wheat germ agglutinin (WGA) and fibronectin, and incubated in suspensions of four strains of *Listeria* (i.e., representative strains of *L. monocytogenes* serotypes 4b, 3 and 1/2a and *L. innocua* serotype 6a) to demonstrate selective pathogen binding. ConA biochips captured a dense, uniform coating of *L. monocytogenes* serotypes 3 and 1/2a, whereas serotype 4b cells were scattered more sparsely over the chip surface. WGA chips, on the other hand, were more efficient at capturing serotype 4b, with the number of serotype 3 and 1/2a cells captured from suspension being significantly lower. Fibronectin chips captured serotypes 4b and 1/2a, but not serotype 3 cells. None of the biochips showed affinity for *L. innocua*. As before, when SERS spectra were collected

from captured bacteria, the protein contributed to the spectrum, and the fingerprints were weak in comparison with those collected from bacteria deposited directly onto the SERS surface. Nevertheless, identification of the captured cells could be made on the basis of the SERS fingerprints.

4. Evaluating physiological state/phenotype

One of the more important advantages of the μSERS approach is that because culturing is not needed, the physiological state of the bacteria can be evaluated. Bacteria respond to environmental triggers, such as temperature, pH and nutrient concentrations, by switching to different physiological states; and one state can be far more virulent than another (e.g., Byrne and Swanson, 1998; Eichenbaum et al., 1996; James et al., 1995, 1997; Litwin and Calderwood, 1994; Pettersson et al., 1997; Mauchline et al., 1994; Kapatral et al., 1996; Samoilova et al., 1996; Maresca, 1995; Miller and Mekalanos, 1988). Moreover, virulent strains of many different pathogens can spontaneously convert to avirulent forms, sometimes at a relatively high rate (Hacker et al., 1993; Tamplin et al., 1996; Eissenberg et al., 1996; Bach et al., 1999; de Almeida et al., 1993; Vogel et al., 1996), and avirulent forms can also spontaneously revert to virulent ones (Simpson and Cleary, 1987; Wright et al., 1990; Leonard et al., 1998). The switch from one physiological state or phenotype to another involves numerous changes in the bacteria's biochemical profile, including the number and composition of outer membrane proteins, lipopolysaccharides and cellular fatty acids, and the presence or absence of flagella and/or capsules (e.g., Barker et al., 1993; James et al., 1995; Dai et al., 1992; Eichenbaum et al., 1996; McGovern and Oliver, 1995; Jøstensen and Landfald, 1996; Miller and Mekalanos, 1988; Tamplin et al., 1996; Leonard et al., 1998; Mauchline et al., 1992; Wright et al., 1990; Vogel et al., 1996).

Such major changes in cellular composition were expected to be clearly reflected in a whole-organism fingerprint, and the experiments conducted to date have confirmed that growth conditions known to trigger bacterial responses do, indeed, affect the organisms' SERS spectra. For example, growth at higher temperatures can suppress the formation of flagella in many pathogens, including *L. monocytogenes*, which grows flagella at 25 °C but rarely at 37 °C (Peel et al., 1988). The fingerprints of the five *Listeria* species grown at 25 °C differed from the same species grown at 37 °C in windows throughout the spectrum. (In contrast with the fingerprints from cells grown at 37 °C in all earlier studies, which were very reproducible, the fingerprints from cells grown at 25 °C showed fluctuations in the intensity of a peak at 740 cm^{-1}; however, this peak was not found to be useful for identification at either temperature, and so it was still possible to individually identify all five species of *Listeria* and all three serotypes of *L. monocytogenes*). Growth of *L. monocytogenes* at 4 °C can significantly increase the pathogen's virulence (Stephens et al., 1991; Czuprynski et al., 1989; Picard-Bonnaud et al., 1989; Basher et al., 1984; Durst, 1975). The fingerprints of all three *L. monocytogenes* serotypes grown at 4 °C differed significantly from those of the same serotypes grown at 25 and 37 °C. As before, all three serotypes grown at any of the three temperatures could be differentiated and individually identified.

Since changes in the physiological state are dictated by genes, closely related microorganisms might be expected to respond to the same environmental triggers in the same way, and their fingerprints, therefore, be affected in a similar fashion. Again, preliminary results confirmed this prediction. For example, many of the spectral features of the *L. monocytogenes* fingerprints that were affected by temperature were affected in the same way for all three strains. In addition, many of these spectral features were affected in the same way when *L. innocua* serotype 6a was grown under the same conditions (Fig. 4).

Since each bacterial strain can produce different fingerprints for different physiological states or phenotypes, more effort will be needed to develop the spectral reference libraries and identification algorithms for μSERS analyses than for whole-organism fingerprinting techniques that rely on cultured biomass. On the other hand, the μSERS approach will produce substantially more information about the pathogen and its physiological state. And if the responses to environmental triggers of one strain can indeed be used to predict those of other closely related strains, the level of effort needed to develop the libraries and algorithms will be minimized.

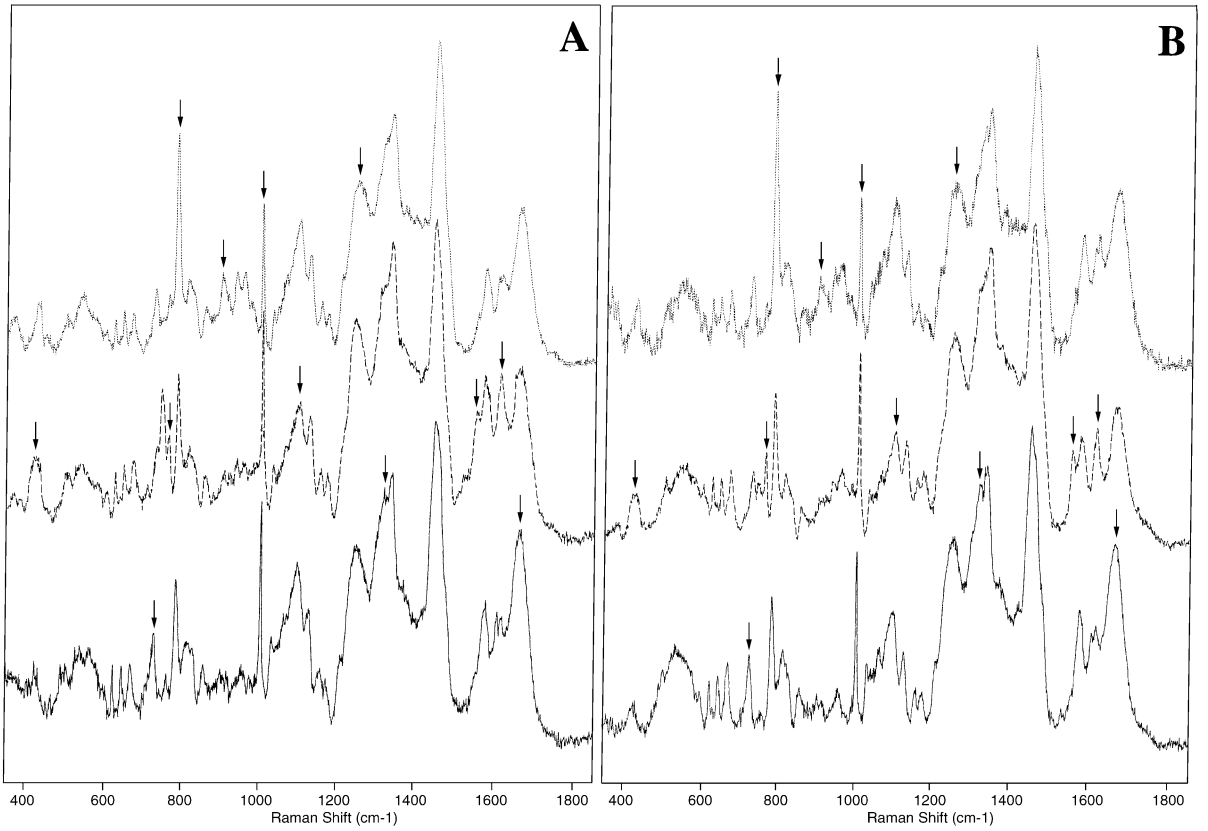

Fig. 4. SERS spectra of (A) *L. monocytogenes* serotype 4b and (B) *L. innocua* serotype 6a grown at 37 °C (—), at 25 °C (– – –) or at 4 °C (- - -). Major changes in response to temperature that are seen in the spectra of both organisms are marked with arrows. Background corrected.

Although spores, unlike bacteria, are dormant organisms that do not respond to environmental signals until germination is triggered, the conditions under which a mother cell is grown might affect the composition of the spore that it produces. Relatively little is known about the impact that mother cell growth conditions may have on spore composition; however, many researchers have studied factors that affect spore heat resistance, such as the temperature (Palop et al., 1999a; Raso et al., 1998; González et al., 1999; Williams and Robertson, 1954) and cationic environment (Palop et al., 1999b; Cazemier et al., 2001; Marquis and Bender, 1985) in which the mother cell is grown. Spores were produced using the widest range of temperatures that each *Bacillus* strain under study could tolerate, or at optimal temperature using metal cation amendments reported to affect heat resistance (Palop et al., 1999b; Cazemier et al., 2001), and their SERS fingerprints compared. The impact of either temperature or cationic environment on the spore spectra, if any, was extremely subtle. This is not surprising, since such factors apparently affect spore heat resistance by influencing the protoplast water content (Beaman et al., 1984; Beaman and Gerhardt, 1986). Therefore, any impact on the spectrum would be through alterations in the packing and layering of core macromolecules and, hence, the intermolecular interactions within the core. With dipicolinate dominating the spectrum, the impact of such interactions would be difficult to see. In any event, since the spore spectra were very reproducible when the mother cells were cultured under a range of growth conditions, it appears that it will be a relatively simple matter to develop the spectral reference libraries and identification algorithms for spores.

When germination is triggered, the event is clearly reflected in the spore fingerprint. Dipicolinate is released from spores during germination (Boschwitz et al., 1991; Kozuka et al., 1985; Alekseev et al., 1985; Scott and Ellar, 1978; Bekhtereva et al., 1975), and Shibata et al. (1986) noted that the calcium dipicolinate peaks seen in the normal Raman spectra of dormant spores were not seen in the spectra of germinated spores. Spore germination was readily apparent in the μSERS studies as well due to the loss of the dipicolinate signature, as well as to other changes found throughout the spore spectrum.

5. Differentiating between viable and nonviable organisms

When analyzing pathogens in environmental samples, the ability to discriminate between viable and nonviable organisms can be very important. It might be anticipated that conditions which are severe enough to cause the death of a microorganism will affect its spectral fingerprint. And indeed, Baek et al. (1988) confirmed that the impact of heat killing was easily discernable in the UV-excited resonance Raman spectrum of *Staphylococcus epidermidis*.

In preliminary μSERS studies, *Listeria* and *Bacillus* vegetative cells, *Bacillus* spores and *C. parvum* oocysts were heat-killed, and their SERS fingerprints compared with those of viable organisms. In addition, oocysts were treated by other methods reported to kill oocysts, such as desiccation (Robertson et al., 1992; Anderson, 1986; Deng and Cliver, 1999) and UV irradiation (Lorenzo-Lorenzo et al., 1993; Belosevic et al., 2001; Linden et al., 2001). In all cases, the spectra of the killed microorganisms differed sharply from those of untreated, viable microorganisms.

As noted earlier, five peaks in the SERS spectra of *Bacillus* spores have been tentatively assigned to dipicolinate on the basis of Shibata et al. (1986) reporting that calcium dipicolinate contributes these same peaks to the normal Raman spectra of spores. Kozuka et al. (1985) found that dipicolinate is released when spores are autoclaved. When spores were autoclaved in our experiments, the five peaks disappeared from the spore fingerprints. In addition, a puddle formed on the sur-

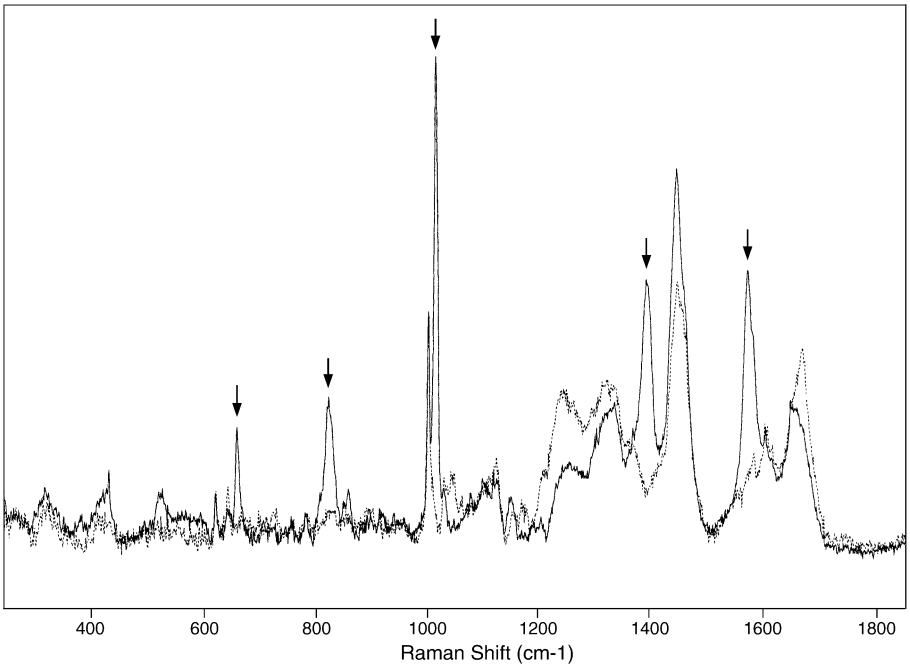

Fig. 5. SERS spectra of *B. thuringiensis* spores before (—) and after (- - -) autoclaving for 30 min at 112 °C. The five peaks in the calcium dipicolinate signature are indicated by arrows. Background corrected.

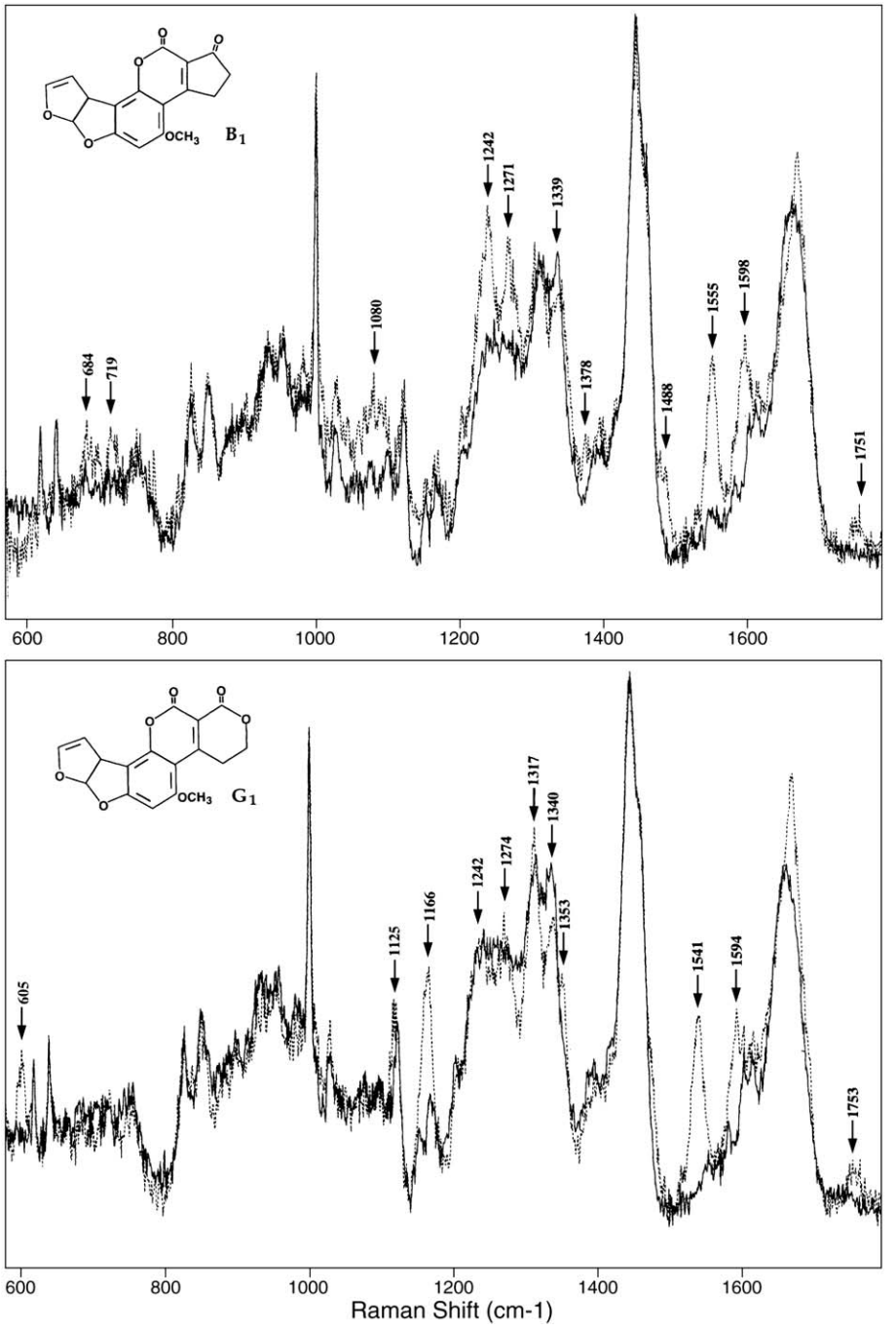

Fig. 6. SERS spectra of RNA polymerase biochips before (—) and after (- - -) incubation in solutions containing aflatoxin B_1 or aflatoxin G_1. Major features of the aflatoxin fingerprints are indicated by arrows. Note that peaks may decrease as well as increase when a ligand binds to a biomolecule. RNA polymerase has a molecular weight of ~ 440,000 Da, whereas aflatoxins B_1 and G_1 have molecular weights of 312 and 328 Da, respectively. Background corrected.

face surrounding the spores, and the SERS spectra collected from the puddles matched that of calcium dipicolinate. The disappearance of the dipicolinate signature, as well as many other changes throughout the SERS fingerprint, made it very easy to differentiate between viable and heat-killed spores (Fig. 5).

The spectral changes caused by killing the microorganisms were consistent from experiment to experiment, as long as the treatment conditions were the same. However, when oocysts were exposed to UV irradiation for different periods of time, the degree to which their spectra were affected differed, with the changes in the spectra becoming more intense as the length of exposure to the irradiation was increased. Therefore, it would appear that the degree of damage correlates to changes in the spectral fingerprint. Future studies will evaluate the impact that different methods of killing microorganisms may have on the biomolecule capture step.

6. Toxin detection and identification

The μSERS technology can be used to detect and identify the toxins that are produced by pathogens, as well as detect and identify the pathogens themselves. Unlike microorganisms, the toxins of interest are often much smaller than the capture biomolecules on the biochip. Nevertheless, toxins that comprised as little as 0.02% by weight of the biomolecule–toxin complex were shown to produce strong, distinctive fingerprints when spectra collected from the complex were compared to the spectra of the uncomplexed biomolecules.

Moreover, since each toxin has a unique Raman spectrum, each produced a unique SERS fingerprint. This enabled the identification of cross-reactive toxins. Even when biochips were incubated in mixtures, the presence of multiple toxins could be determined, since the contributions of each toxin could be seen in the SERS spectra. For example, aflatoxins B_1 and G_1 could be detected and individually identified when biochips bearing pixels of antibody or enzyme capture biomolecules were incubated in samples containing one or both aflatoxins, and the spectra were then collected for 20 s from an area of the pixel ~ 1 μm in diameter (Fig. 6). Similar results have been obtained with toxins ranging from cadaverine (102 Da) to *Staphylococcus* enterotoxin B (28,366 Da).

7. Multipixel biochip analysis

There have been many advances in Raman microscope instrumentation in recent years. Among the more exciting is the development of imaging Raman microscopes that are capable of simultaneously collecting hundreds or even hundreds of thousands of spectra from discrete areas of a surface with a spatial resolution of 250 nm–1.5 μm (Treado et al., 1992; Morris et al., 1994; Goldstein et al., 1996; Williams et al., 1996; DeVerse et al., 1998; Schaeberle et al., 1999; McClain et al., 2000). Analysis of Raman spectra in both the spatial and the spectral domains is called "hyperspectral imaging Raman".

We have already shown that by immobilizing different biomolecule pixels on a single biochip, incubating the chip in a complex sample, and then rastering the chip through the laser beam to analyze the various pixels sequentially, a single biochip and a single Raman microscope can be used to detect and identify diverse ligands. In the future, we plan to investigate the use of hyperspectral imaging Raman microscopy for collecting fingerprints from all the microorganisms on the biochip, individually yet simultaneously, to enable rapid sample analysis.

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