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Identification and discrimination of *Pseudomonas aeruginosa* bacteria grown in blood and bile by laser-induced breakdown spectroscopy

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Abstract

Pseudomonas aeruginosa bacteria colonies have been analyzed by laser-induced breakdown spectroscopy using nanosecond laser pulses. LIBS spectra were obtained after transferring the bacteria from a nutrient-rich culture medium to a nutrient-free agar plate for laser ablation. To study the dependence of the LIBS spectrum on growth and environmental conditions, colonies were cultured on three different nutrient media: a trypticase soy agar (TSA) plate, a blood agar plate, and a medium chosen deliberately to induce bacteria membrane changes, a MacConkey agar plate containing bile salts. Nineteen atomic and ionic emission lines in the LIBS spectrum, which was dominated by inorganic elements such as calcium, magnesium and sodium, were used to identify and classify the bacteria. A discriminant function analysis was used to discriminate between the *P. aeruginosa* bacteria and two strains of *E. coli*: a non-pathogenic environmental strain and the pathogenic strain enterohemorrhagic *E. coli* 0157:H7 (EHEC). Nearly identical spectra were obtained from *P. aeruginosa* grown on the TSA plate and the blood agar plate, while the bacteria grown on the MacConkey plate exhibited easily distinguishable differences from the other two. All *P. aeruginosa* samples, independent of initial growth conditions, were readily discriminated from the two *E. coli* strains.

Keywords: Laser-induced breakdown spectroscopy (LIBS); Bacteria identification; Biological; Discriminant function analysis (DFA); *P. aeruginosa*; *E. coli*

1. Introduction

Laser-induced breakdown spectroscopy (LIBS) is a time-resolved spectroscopic analysis technique based on optical emission following pulsed-laser ablation of a sample. The technique, which has been utilized in a wide variety of applications, has numerous advantages over other competing spectrochemical analysis techniques [1,2]. A short list of these advantages includes: minimal or no sample preparation is required; very rapid measurements are possible (potentially in under 1 s); a high spatial resolution on the target can be obtained (under 10 μm is possible, although under 100 μm is more common); and the analysis detects all elements without bias, including those present in molecules (which are atomized during the process). Two advantages specific to the application of LIBS in microbiologically important systems are that measurements can be preformed remotely allowing the safe analysis of hazardous, highly-contagious, or pathogenic targets and that the analysis can be computerized, removing the requirement of the expertise of a trained microbiologist for the identification of bacteria or bio-agents.

In the past three years the use of LIBS as a practical detection and identification technology for biological samples, particularly bacteria, has been investigated by several research groups. These early works were concerned primarily with investigating the ability to discriminate particular microorganisms (usually bacteria used as simulants of pathogenic bio-agents) from "background" biological organisms, often of a different bio-type (pollen, molds, etc.). For example, in preliminary experiments performed in 2003, Morel et al. investigated the detection of six strains of bacteria and two pollens [3]. They placed particular emphasis on *Bacillus globigii* which acts as a non-pathogenic

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surrogate for *Bacillus anthracis* (anthrax). In the same year, Samuels et al. used a broadband spectrometer to study the discrimination potential of the technique applied to bacterial spores, molds, pollens, and proteins [4]. As had been previously done, all bio-samples were studied in the solid phase, in this case deposited as an aqueous suspension onto silver membrane filters to create thin films of deposited bio-matter. In a similar way, Hybl et al. investigated the technique's potential for detecting and discriminating aerosolized bacterial spores from more common background fungal/mold spores and pollens [5]. The underlying motivation for a great number of these early studies was the development of a practical, real-time early-warning technology to protect against incidents of bio-terrorism [6–9].

Our research group is focused more on the development of LIBS as a useful diagnostic technology in the fields of clinical medicine and public health and has recently performed LIBS on live *Escherichia coli* colonies with a very simple, yet realistic, sampling methodology [10,11]. *E. coli* was an ideal candidate for these initial studies due to its complete genetic characterization, non-pathogenicity, and ease of preparation [12]. We spectroscopically analyzed *E. coli* cultured on a variety of nutrient media for only 24 h (as well as a sample of environmental mold spores and the *Candida albicans* yeast) on a nutrient-free bacto-agar plate. A discriminant function analysis based on the optical intensity of 19 inorganic element emission lines was applied to identify and discriminate between four strains of *E. coli*, including the pathogenic enterohemorrhagic *E. coli* (EHEC, or *E. coli* 0157:H7). The other three strains consisted of two K-12 strains (AB and HF4714) and an environmental strain (Nino C) utilized in water quality assays. Different strains of *E. coli* can cause an impressive variety of diseases, including dysentery, hemolytic uremic syndrome (kidney failure), bladder infection, septicemia, pneumonia, and meningitis. Therefore the ability to quickly and accurately identity particular strains of a given bacteria is a very important diagnostic tool to prevent or contain outbreaks caused by a particular strain [13]. Our analysis confirmed the ability of LIBS to rapidly and easily discriminate between strains of *E. coli*.

Recent results have shown the utility of performing LIBS with femtosecond, rather than nanosecond, laser pulses, specifically to analyze *E. coli* [14–16]. Although some advantages due to the use of the shorter femtosecond laser pulses were noted, these studies avoided the issue of realistic sample preparation, instead analyzing samples which had been harvested from culture, washed, aspirated onto filters, washed again, and then dried to insure high signal-to-noise. Prior to our work, only one instance of performing LIBS on an actual *E. coli* bacterial colony had been previously reported [17].

One practical issue that is thought to limit the usefulness of LIBS as a clinical or field-portable diagnostic technology is the possible variability in LIBS spectra obtained from microorganisms that have lived and reproduced under a wide variety of conditions. Specifically, it is not known whether LIBS-based clinical methodologies developed to identify and discriminate a specific bacteria strain in a laboratory setting will effectively and accurately identify the same bacteria obtained from clinical and environmental sources (e.g. a patient's bloodstream, brain, throat, gastrointestinal tract, uncooked meat, or an environmental water source). It is therefore imperative to study the effect that the bacteria growth conditions, environment, and stage of life have on the LIBS spectrum and how this impacts the ability of the technique to accurately and reliably identity the specimen. This issue was addressed by our group in an earlier work by preparing two strains of *E. coli* in two different growth media [11]. In the present work, we extend this investigation by comparing the LIBS spectra from another Gram-negative bacterium, *Pseudomonas aeruginosa*, cultured on three different nutrient media. To more closely simulate clinical environments, one of these media contained blood, one contained bile salts, and one contained neither.

P. aeruginosa is an opportunistic pathogen capable of colonizing the skin and intestinal tract of humans and animals. *P. aeruginosa* is best known for its ability to infect burns and to cause complications in patients with cystic fibrosis (CF). CF patients typically develop persistent *P. aeruginosa* lung infections that lead to lung failure, followed by transplants or early death [18]. *P. aeruginosa* also causes endocarditis (infection of the heart valves) and is responsible for a host of other infections in immuno-compromised patients. In patients with CF, the *P. aeruginosa* bacteria adapt to the patient's defense system by forming biofilms of microcolonies. These biofilms are the main reason for persistent infections in CF patients. It is also true, however that antibiotic-resistant mechanisms are important, and a high-frequency of multiply antibiotic-resistant stains is found to infect CF patients. The identification of these strains, as well as the ability to identify the particular stain while living in a mucoid biofilm or microcolony, is therefore a highly important problem in clinical medicine.

In this paper, we report on our analysis of *P. aeruginaosa* bacteria with LIBS. We have discriminated *P. aeruginosa* from another common bacteria, specifically infectious and non-infections *E. coli*, and we have investigated the effect that growth in blood, bile salts, and TS agar had on the *P. aeruginosa* LIBS spectrum. As well, we present a detailed description of the statistical analysis technique known as discriminant function analysis which was utilized to differentiate the spectra obtained from the different strains and species of bacteria.

2. Experimental

2.1. LIBS instrumentation

The experimental setup used to perform LIBS on the bacteria samples is shown schematically in Fig. 1. 10 ns laser pulses from an Nd:YAG laser (Spectra Physics, LAB-150-10) operating at its fundamental wavelength of 1064 nm were used to ablate the bacteria. A spatial mode cleaner consisting of a 3× telescopic beam expander was used to expand the beam from its nominal beam diameter of 9 mm to 27 mm prior to the final focusing lens. An iris with a 9 mm opening immediately following this beam expander sampled the inner one-third of the beam diameter to obtain a more Gaussian transverse mode distribution. The LAB-150 specifies a >70% near-field (1 m) Gaussian fit to the actual spatial mode energy distribution, with

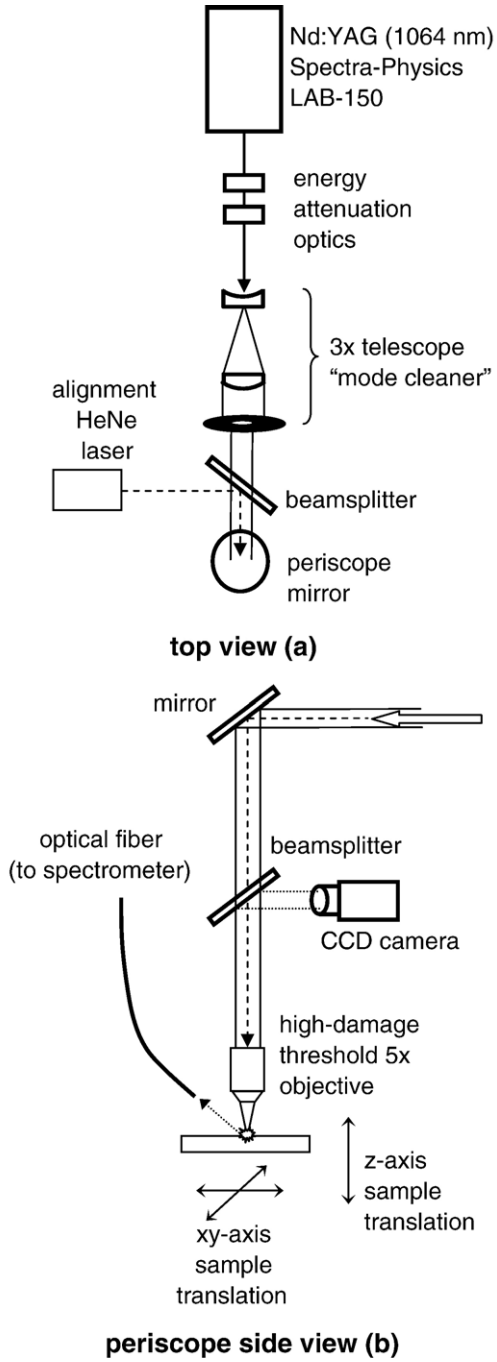

Fig. 1. The experimental setup used to perform LIBS on the *P. aeruginosa* and *E. coli* samples. (a) An overhead view of the apparatus. The path of the infra-red ablation laser beam is indicated by a solid line, while the alignment HeNe laser beam is indicated by a dashed line. (b) A side view of the periscope assembly used to focus both co-linear lasers onto the agar surface. A CCD camera was used to image the surface. The optical fiber made an angle of 30° with the vertical laser beams.

this specification increasing to >95% in the far field (>6 m). The mode cleaner was located 1.3 m after the laser and transmitted approximately 15% of the energy incident upon it (100 mJ/pulse was obtained out of the laser, with 15 mJ in the pulse after the spatial filter).

To visualize the focused infra-red laser beam location on the bacteria, a continuous wave (cw) alignment helium–neon laser at 632.8 nm was overlaid with the infra-red laser beam with a 50:50 633 nm beamsplitter. The coating-specific reflectivity did not reflect a significant fraction of the incident infra-red radiation. The collinear visible and infra-red laser beams were then reflected vertically via a periscope assembly, as shown in Fig. 1(b). After reflection from the periscope's vertical turning mirror, the two laser beams passed through a broadband visible wavelength beamsplitter. This beamsplitter allowed a CCD camera (Everfocus EX100) to image the magnified target. The magnified view was displayed on a TV monitor for visual placement of the sample target in the focal region.

Both laser beams were focused by a high-damage threshold 5× infinite-conjugate microscope objective (LMH-5X-1064, OFR). This objective had an aperture of 10 mm, an effective focal length of 40 mm, a numerical aperture of 0.13 and a working distance of 35 mm. This large working distance allowed easy sample manipulation and plasma light collection while the fast focusing provided by the objective's short confocal region allowed extremely precise placement of the beam waist within the bacterial target but not in the substrate material below. The sample itself was placed on an X–Y–Z manual translation stage with 0.5 in. of travel in every coordinate. Visual examination of single-shot ablation craters on a variety of substrates yielded an average diameter of 100 μm in the objective's focal plane with typical pulse energies at the ablation surface of 8 mJ/pulse.

Optical emission from the LIBS microplasma was collected by a 1-m steel encased multimode optical fiber (core diameter = 600 μm, N.A. = 0.22) placed a distance of 23 mm from the ablation spot with no other light collection optics. The fiber was angled at 30° relative to sample normal and was aligned by illuminating the far exit end with a second helium–neon laser and overlapping the resulting cone of light from the fiber entrance with the alignment laser spot on the sample. Due to their close proximity to the ablation plasma and the high percentage of liquid in the target materials, the end of the fiber and the exit aperture of the microscope objective were prone to being coated with a thin film of splattered bacteria and substrate after acquisition of approximately 15–20 spectra from a sample. Cleaning of both fiber and objective were necessary to maintain constant pulse energy and to insure no optical emission (particularly in the UV) was lost due to absorption.

This fiber was coupled to an Echelle spectrometer equipped with a 1024 × 1024 (24 μm^2 pixel area) intensified CCD-array (LLA Instruments, Inc., ESA3000) which provided complete spectra coverage from 200 to 840 nm with a resolution of 0.005 nm in the UV. The Echelle spectrometer was controlled by a PC running manufacturer-provided software. The PC controlled not only the gating (shuttering) of the ICCD, but also controlled operation of the pulsed-laser via an on-board fast pulse generator to eliminate jitter in the time between laser pulse and plasma observation.

LIBS spectra were acquired at a delay time of 1 μs after the ablation pulse, with an ICCD intensifier gate width of 20 μs duration. This delay time was chosen to maximize emission from the strongest, most obvious emission lines while minimizing the contribution from the broadband background emission. Spectra from 10 laser pulses were accumulated on the

CCD chip prior to readout. The sample was then translated 250 μm and another set of 10 laser pulses was averaged. 10 accumulations were averaged in this way, resulting in a spectrum comprised of 100 laser pulses that took approximately 40 s to obtain. Typically 20–30 such measurements could be made from one colony 24 h after initial streaking (approximately 1 million bacteria in the colony).

2.2. Bacteria sample preparation

The *P. aeruginosa* and *E. coli* samples were prepared in the following way. All *E. coli* colonies were cultured on a trypticase soy agar (TSA) nutrient medium for 24 h. TSA is a rich bacteriological growth medium containing pancreatic digest of casein, soybean meal, NaCl, dextrose, and dipotassium phosphate. The *P. aeruginosa* colonies were cultured on TSA as well as a blood agar (BAP) plate, which is a TSA plus blood medium, and a MacConkey plus lactose (MAC) agar plate with bile salts. The BAP substrate was chosen to simulate a sample obtained from a *P. aeruginosa* blood infection, while the TSA substrate more effectively simulated a sample obtained environmentally or from sputum. The use of the MacConkey plate plus bile salts was chosen specifically to investigate possible alteration of the integrity of the membrane by the substrate. It is generally accepted that the hydrophobic bile salts can cause a disruption of the membrane integrity after solubilization of the membrane lipids [19]. These salts can possibly affect the virulence of certain organisms, particularly the strains of enterohemorrhagic *E. coli*, which produce a Shiga toxin in humans, yet colonize and live in the gastrointestinal tract of cattle without causing disease [20]. It is possible that the presence of these bile salts produces a chemical alteration in the bacteria membrane or protoplasm that could then be detected during the LIBS analysis.

We have previously shown that the LIBS technique is particularly sensitive to the concentration of trace inorganic elements which occur in the outer membrane, particularly the divalent cations Mg^{2+} and Ca^{2+} which function to link adjacent core oligosaccharides [21]. It has been shown previously that the concentrations of these elements (along with Fe, Na, K and P) provide a robust and effective means for discriminating bacteria species, particularly between Gram-positive and Gram-negative bacteria, but also between multiple Gram-negative species [22]. Thus the calcium and magnesium optical signal intensities were expected to provide a very sensitive probe of the composition of the bacteria membrane. This composition was expected to change with exposure to the bile salts present in the MacConkey plate but not with exposure to blood.

After incubation at 37 °C for 24 h, the bacteria were transferred to the surface of a 0.7% nutrient-free agar plate with a very thin smear. The choice of agar as an ablation substrate afforded a very flat, large area substrate with a high-breakdown threshold due to its near-transparency. The agar also kept the bacteria hydrated for many hours, improving the ease with which the laser was focused into the bacteria smear. The specific advantage of utilizing this highly watery agar is that it did not contribute directly or indirectly to the LIBS spectra of the bacteria. The optical emission from this agar was an order of magnitude smaller than from the bacteria and lacked many of the elements present in the bacteria. Ablation on blank agar substrates was immediately identifiable as lacking bacteria and provided a convenient method for determining when the bacteria target had been "missed" by the ablation laser. It is important to note that this straight-forward yet reliable sample preparation method can also be used for testing liquid samples (such as water, sputum, or blood samples) or mucoid samples, such as a biofilm of *P. aeruginosa*. The agar provides a solid platform ideally suited for supporting any of these forms of samples without contributing significantly to the optical signal.

3. Results and discussion

3.1. P. aeruginosa spectrum

A representative LIBS spectrum of *P. aeruginosa* is shown in Fig. 2. This spectrum is composed of measurements taken at 10 different locations, each location sampled with 10 laser pulses. The *P. aeruginosa* spectra were not obviously different from the spectra obtained from *E. coli* bacteria in our previous works. The dominant emission lines were atomic and ionic lines from inorganic elements and have been labeled on the graph. The elemental composition of bacterial cells was first measured by Gunsalus and Stanier in 1960 in a measurement which reflected both the basic composition of bacterial protoplasm and that of accessory materials, such as stored polysaccharides and lipids [23]. These measurements were made in ash after incineration of dried samples and thus exhibited high variability, introducing a large uncertainty into the inorganic content of the bacteria (which comprises only around 5% of the dried weight in the form of salts). The inorganic composition of the bacterial cell was found to contain sodium, potassium, calcium, magnesium, phosphorus, sulfur, chlorine and iron, with trace amounts of manganese, copper and aluminum. Our analysis was based on a measurement of the relative concentrations of the first five of these inorganic elements, along with carbon, which accounts for approximately 50% of the dry weight. Iron has been observed in femtosecond LIBS spectra of *E. coli*, but was not observed in the nanosecond LIBS performed for this study [16].

The atomic emission spectrum for both *E. coli* and *P. aeruginosa* was dominated by calcium, magnesium, and sodium which, as mentioned earlier, occur in the outer membrane of the bacteria. However, the intensity of 19 emission lines from the six elements mentioned above formed the basis of our spectroscopic identification. Table 1 identifies the specific lines used in this work, the wavelength of the emission line, and representative values of the normalized intensities of these lines. The intensities of all observed lines are summed in each spectrum, and this is called the total spectral power. Individual intensities are then divided by this total spectral power to arrive at the fraction of total spectral power represented by that emission line. Typical values are shown in column three of Table 1. Column four of Table 1 contains the Wilks' lambda for each emission line, which is a parameter resulting from the analysis which is described in Section 3.2.

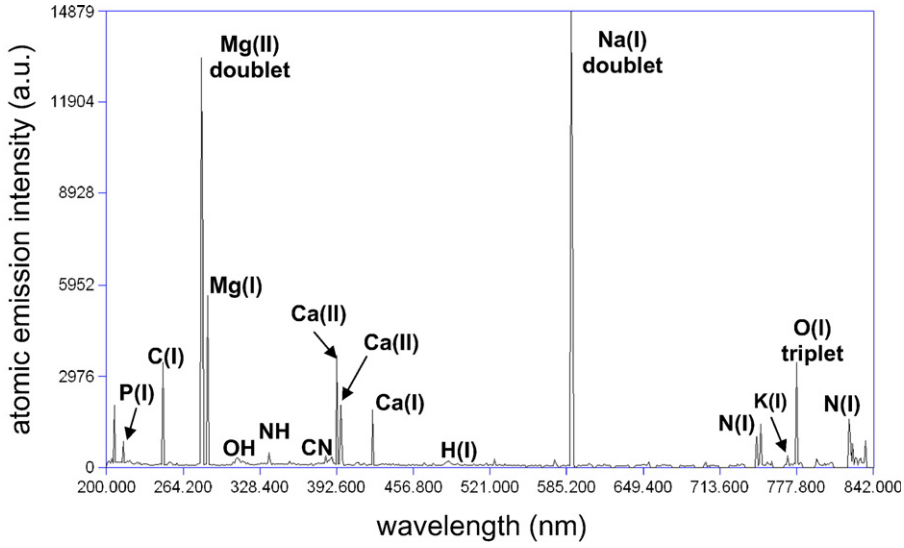

Fig. 2. A LIBS spectrum of *P. aeruginosa* with the relevant atomic emissions identified. The spectrum is dominated by emission from inorganic elements.

3.2. Discriminant function analysis

Discriminant function analysis (DFA) is a data analysis technique which is similar to analysis of variance, but which is used to predict and quantify group memberships between two or more distinct groups. It is therefore described more accurately as a multi-variate analysis of variance. In our study, for example, all the spectra acquired from *P. aeruginosa* grown on a particular substrate should ideally be identical, and thus constitute a group. Other bacteria species or strains would constitute different groups. DFA uses a set of independent variables from each spectrum (each spectrum is treated as a single data point) to predict the group membership of that particular spectrum in three basic steps. First, a set of orthogonal discriminant functions is constructed from the data sets from all the groups. In this step, a canonical correlation analysis produces a set of canonical discriminant functions which are essentially the eigenvectors of the data expressed in a basis that maximizes the difference between groups. For a discrimination between N groups, $N-1$ discriminant functions are constructed.

Second, a test for significance in the discriminant functions is performed. This is done by performing a check on the differences in the means of the groups. The null hypothesis (which is disproved if a real, significant difference exists between groups) assumes that all data is sampled from a single normal distribution with mean μ and variance σ^2. If the groups are significantly different, they will exhibit means that differ by an amount significantly greater than the variance. In the last step, if significant differences between groups are observed, the group membership of each data spectrum is predicted based on the calculated canonical discriminant function scores for each data spectrum.

In the analysis presented in this work, the LIBS spectra obtained from ablation of the bacteria were analyzed by measuring the intensity (integrated area under the line profile) of 19 emission lines from 6 different elements. The intensity of each line was divided by the sum of all 19 line intensities (the total spectral power) to normalize the spectra for shot-to-shot fluctuations and sample density variations. These relative line intensities constituted 19 independent variables (sometimes referred to as predictor variables) that completely described each spectrum and were written as a 1×19 vector. Discrimination can also be accomplished by summing the normalized intensities of all lines that belong to a particular element and using the sums as predictor variables, as done by Baudelet et al. [22].

All the independent variable vectors from all groups were then analyzed simultaneously by a commercial DFA program (SPSS Inc., SPSS v14.0) to construct the canonical discriminant functions which were in turn used to calculate for each data spectrum a discriminant function score for that particular

Table 1
Identification, relative strength, and importance to discriminant functions of nineteen atomic and ionic emission lines used in the spectral fingerprinting of *P. aeruginosa*

Wavelength (nm)	Line identification	Fraction of total spectral power	Wilks' lambda
213.618	P I	0.023	.619
214.914	P I	0.013	.492
247.856	C I	0.086	.521
253.560	P I	0.005	.771
279.553	Mg II	0.201	.040
280.271	Mg II	0.109	.061
285.213	Mg I	0.097	.037
373.690	Ca II	0.002	.909
383.231	Mg I	0.008	.782
383.829	Mg I	0.006	.588
393.366	Ca II	0.081	.034
396.847	Ca II	0.035	.060
422.673	Ca II	0.031	.062
430.253	Ca I	0.001	.803
518.361	Mg I	0.003	.773
585.745	Ca I	0.001	.920
588.995	Na I	0.182	.020
589.593	Na I	0.103	.022
769.896	K I	0.012	.931

function. The first canonical discriminant function (denoted DF_1) is constructed such that it accounts for more of the variance between groups than the second canonical discriminant function (DF_2), which accounts for more of the variance than the third canonical discriminant function (DF_3), etc. When used to discriminate similar spectra, such as those obtained from bacteria, typically almost all of the variance (upward of 90%) was described by only the first two canonical discriminant functions. Accordingly, all of the data plots presented in this work show only the scores from the first two discriminant functions.

A useful parameter generated during DFA is called the Wilks' lambda, and it varies from 0 to 1. Typical Wilks' lambda values for the 19 emission lines obtained from a discrimination between bacteria and nutrient media described in Section 3.3 are shown in Table 1. A small Wilks' lambda score indicates a greater relation to the between-group variance than a large value, i.e. an independent variable with a small Wilks' lambda is more important to the discrimination than a variable with a large Wilks' lambda. From Table 1 it can be seen that the line with the largest intensity (Mg II at 279.553 nm in this particular case) is not the most important to discrimination. The line with the lowest Wilks' lambda is the Na I line at 588.995 nm, indicating that the relative concentration of sodium is a very important quantity in this particular discrimination test. Indeed, the sodium lines are typically the most important when discriminating bacteria from nutrient media, as the sodium concentration tends to be much higher in the nutrient media.

To complete an identification based on a discriminant function analysis, samples with known identification must first be obtained to construct the required canonical discriminant functions. The DFA cannot identify unknown members of a group without first having known members to compare them to. Therefore the LIBS technique coupled with a DFA is not suited for random identification of completely unexpected or unknown pathogens. It is particularly useful, however, for identifying particular strains of a pathogenic agent, or diagnosing which species of a particular microorganism is responsible for a given infection.

3.3. Comparison of *P. aeruginosa* prepared on 3 substrates

A plot of the three discriminant function scores from a DFA of the LIBS atomic emission spectra of *P. aeruginosa* bacteria grown on three nutrient substrates, as well as the scores of three nutrient substrates themselves, is shown in Fig. 3. The clusters of data, along with a group centroid, labeled as 1, 2, and 3 are spectra from *P. aeruginosa*, all streaked from the same sample making them genetically identical, but grown on TSA, BAP, and MAC, respectively. The clusters of data indicated by 4, 5, and 6 are spectra obtained by ablating the respective nutrient media.

In this analysis, 98.9% of the variance between all the groups was described by the first two canonical discriminant functions and 94.7% of all the original grouped cases were correctly classified. It is clear that the LIBS spectra obtained from the *P. aeruginosa* samples were distinctly different from the spectra from the nutrient media. All three nutrient substrates were

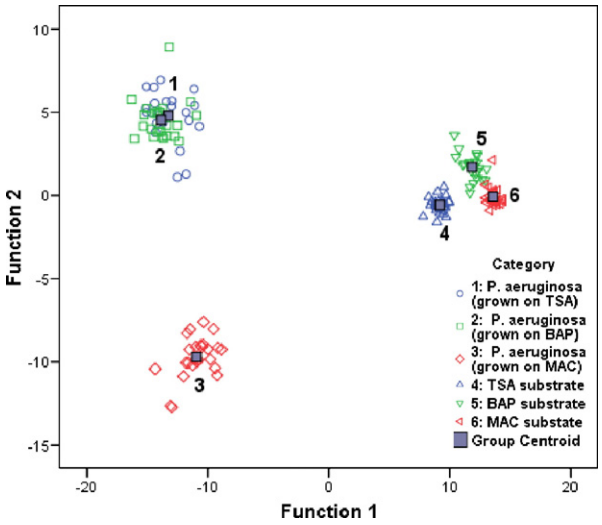

Fig. 3. (Color online) A discriminant function analysis plot showing the first two discriminant function scores of LIBS spectra obtained from a single strain of *P. aeruginosa* cultured on three different nutrient media (groups 1, 2, and 3) and spectra obtained from the media (groups 4, 5, and 6).

tightly grouped and very similar to each other. In Fig. 3, discriminant function one (DF_1) provided the discrimination between the nutrient media, which all possessed large positive DF_1 scores, and the bacteria samples, which all possessed large negative DF_1 scores. An analysis of the coefficients which comprised DF_1 indicated it was the relative sodium concentration that provided the primary discrimination between the nutrient media and the bacteria. Typically, most nutrient media have considerably higher sodium concentrations than the bacteria. The bacteria all possessed very similar DF_1 scores, as their relative concentrations of sodium were all similar (smaller). This grouping proves that the bacteria were easily distinguished from the media on which they were cultured. These bacteria were not simply "taking up" elements from the nutrient medium, making them look elementally identical to that medium.

Discriminant function two (DF_2) provided the discrimination between bacteria samples. The *P. aeruginosa* grown on TSA and BAP were spectroscopically very similar, as evidenced by the highly overlapping clusters of data groups 1 and 2, while samples obtained from the colony grown on MAC (cluster 3) were significantly different. Despite this clear similarity, in this analysis 95.7% of the samples from bacteria cultured on TSA were correctly classified, 95.8% of the samples from bacteria cultured on BAP were correctly classified, and 100.0% of the MAC-cultured bacteria were correctly classified. The *P. aeruginosa* grown on both the TSA and BAP possessed large positive DF_2 scores, while the MAC-cultured samples had a large negative DF_2 score. An analysis of the coefficients which comprised DF_2 indicated it was the relative calcium and magnesium concentrations that provided the primary discrimination between these two clusters of bacteria. As described above, this was the anticipated result, as the MacConkey plate with bile salts was expected to alter membrane composition, the suspected primary reservoir of

bacterial calcium and magnesium. It appears as if the presence of the bile salts in the MacConkey medium during culturing acted to increase the relative calcium content and decrease the relative magnesium content of the *P. aeruginosa* cultured on that medium. The bacteria cultured on the blood agar, however, exhibited no significant differences from the bacteria cultured on the TSA plate. This result is indicative that bacteria obtained clinically from a patient's blood, sputum, or mucous membranes should exhibit no chemical or elemental differences, allowing a rapid identification via the LIBS technique. Experiments are underway in our laboratory to further examine the extent to which membrane composition can be intentionally altered via nutrient medium culturing. We are also investigating the extent to which bile salts in a non-MacConkey medium can alter the bacteria membrane composition.

3.4. Comparison of *P. aeruginosa* and *E. coli*

Two strains of *E. coli* were added to the DFA to compare the spectral signatures from the three samples of *P. aeruginosa* to the spectra from this entirely different species of bacteria. For simplicity, and due to their high spectral similarity, only one of the nutrient medium groups (TSA) was included in this analysis. The results are shown in Fig. 4. In this analysis, 96.0% of all the original grouped cases were correctly classified. DF_1 provided a clear discrimination between the *P. aeruginosa* and the *E. coli*, as well as the TSA nutrient medium. A line has been added to the plot of Fig. 4 indicating that all spectra to the right of the line (not including the well-isolated TSA spectra) were associated with *P. aeruginosa* and all spectra to the left of the line were associated with *E. coli*.

In this analysis, it was not a simple function of relative sodium content that allowed discrimination between bacteria

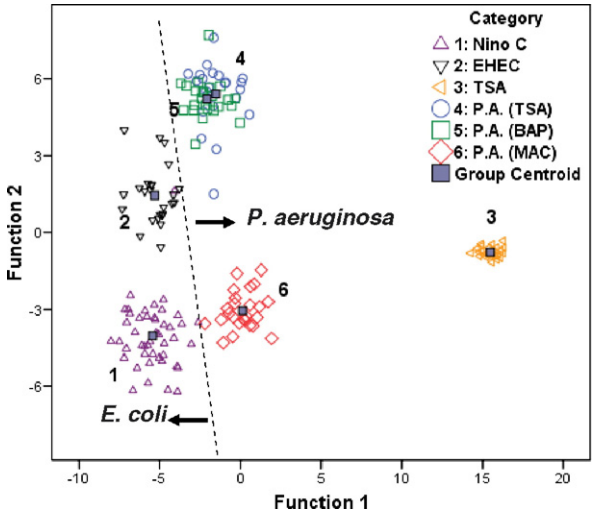

Fig. 4. (Color online) A discriminant function analysis plot showing the first two discriminant function scores of LIBS spectra obtained from a single strain of *P. aeruginosa* cultured on three different nutrient media (groups 4, 5, and 6), two strains of *E. coli* (groups 1 and 2) and one nutrient medium (group 3). A dashed line indicates that based on these two scores, all spectra from *P. aeruginosa* were easily discriminated from all spectra from either strain of *E. coli*, despite differing growth environments.

species via DF_1. In fact, only 73.5% of the variance between groups was described by DF_1 with 18.6% of the variance described by DF_2, 5.6% by DF_3, 1.8% by DF_4, and only 0.5% by DF_5. DF_2 primarily provided the discrimination between the two *E. coli* strains as well as between the *P. aeruginosa* cultured with and without bile salts. On the basis of all 5 discriminant function scores, 97.8% or the *E. coli* strain Nino C samples and 100.0% of the EHEC samples were correctly classified. Within the *P. aeruginosa* bacteria samples, 82.6% of the TSA-cultured samples (group 4), 91.7% of the BAP-cultured samples (group 5), and 100.0% of the MAC-cultured samples (group 6) were correctly classified in this analysis. Thus these two species of bacteria, two strains of *E. coli* and one strain of *P. aeruginosa*, prepared in a variety of ways and in a number of conditions were thus easily discriminated on the basis of their LIBS spectra alone. As well, we have shown that while strain discrimination is still possible, samples of *P. aeruginosa* grown in different environments (particularly blood and sputum) are easily recognizable as identical bacteria. This discrimination provides powerful evidence to the potential usefulness of the LIBS technique as applied to bacteriological systems. We have also shown that membrane composition can indeed be altered by growth on a particular medium, in this case a bile salts-containing medium.

4. Conclusions

One of the most important questions surrounding the practical application of LIBS as a diagnostic tool for the identification and discrimination of bacterial species and strains is the variation in emission spectra that could potentially be observed from samples prepared in different ways. A reliable technology must be capable of identifying the desired bacteria regardless of its growth history or the stage of its life-cycle. In this work, we have examined the effect that culturing *P. aeruginosa* on three different nutrient media had on the LIBS spectra and the subsequent ability to classify or identify these bacteria based on those spectra.

Highly similar spectra were observed from *P. aeruginosa* grown on two nutrient media (TSA and BAP) and significant differences were observed in samples obtained from bacteria grown on MacConkey agar plates. This difference was interpreted as a real and not-unexpected elemental alteration of the membrane of bacteria cultured on that medium and did not represent an inherent limitation of the LIBS technology. The alteration of fundamental bacteria chemistry was attributed to the presence of the bile salts in the MacConkey medium which is known from biochemistry to disrupt membrane integrity. Because standard serological (antibody-based) diagnostics are also membrane, not genetically, based, these competing microbiological techniques might also misidentify such bacteria if their outer membrane or surface has been altered significantly.

The addition of *E. coli* spectra to the discrimination analysis showed that two distinct strains of *E. coli* were still immediately differentiable from all three samples of *P. aeruginosa*. Discrimination between the strains of *E. coli* was also still possible in

this analysis and the differences between *P. aeruginosa* samples cultured with and without bile salts were still observed.

Much work remains to be done to investigate the impact that the growth conditions have on the LIBS-based discrimination of bacteria. Careful studies of the variation in LIBS spectra based on the amount of time spent on the growth medium need to be performed. We are particularly interested in the ability of the LIBS technique to identify the *P. aeruginosa* bacteria in the biofilm it forms as a protective response to its environment. The bacteria in the biofilm can present as three phenotypes: mucoid, non-mucoid and small colony variant [18]. Whether the presence or type of biofilm alters the LIBS spectrum is a significant but important question as the bacteria in different phenotypes do not respond to antibiotics in the same way. In this case an alteration of the LIBS spectrum based on the biofilm phenotype may be desirable, so that a particular antibiotic regimen may be constructed based on the identified biofilm. This type of in vivo identification is not currently possible.

LIBS has a long way to go before being accepted as a clinical diagnostic technology, but these early works seem to indicate the possibility of its application to certain microbiological problems and the necessity of further work.

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