The Effect of Bacterial Environmental and Metabolic Stresses on a Laser-Induced Breakdown Spectroscopy (LIBS) Based Identification of *Escherichia coli* and *Streptococcus viridans*

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In this paper we investigate the effect that adverse environmental and metabolic stresses have on the laser-induced breakdown spectroscopy (LIBS) identification of bacterial specimens. Single-pulse LIBS spectra were acquired from a non-pathogenic strain of *Escherichia coli* cultured in two different nutrient media: a trypticase soy agar and a MacConkey agar with a 0.01% concentration of deoxycholate. A chemometric discriminant function analysis showed that the LIBS spectra acquired from bacteria grown in these two media were indistinguishable and easily discriminated from spectra acquired from two other non-pathogenic *E. coli* strains. LIBS spectra were obtained from specimens of a non-pathogenic *E. coli* strain and an avirulent derivative of the pathogen *Streptococcus viridans* in three different metabolic situations: live bacteria reproducing in the log-phase, bacteria inactivated on an abiotic surface by exposure to bactericidal ultraviolet irradiation, and bacteria killed via autoclaving. All bacteria were correctly identified regardless of their metabolic state. This successful identification suggests the possibility of testing specimens that have been rendered safe for handling prior to LIBS identification. This would greatly enhance personnel safety and lower the cost of a LIBS-based diagnostic test. LIBS spectra were obtained from pathogenic and non-pathogenic bacteria that were deprived of nutrition for a period of time ranging from one day to nine days by deposition on an abiotic surface at room temperature. All specimens were successfully classified by species regardless of the duration of nutrient deprivation.

Index Headings: **Laser-induced breakdown spectroscopy; LIBS; Bacteria identification; Biophysics; Chemometrics; *E. coli*; *S. viridans*.**

INTRODUCTION

Laser-induced breakdown spectroscopy (LIBS) has recently been used for the spectrochemical analysis of biological samples such as woods, trees, plants, microorganisms, and plant and animal tissues.[1–8] The spectrochemical technique has numerous applications in the biomedical sciences, of which the identification of pathogenic bacteria is an important one.[9–17] A rapid diagnostic that could confirm the presence of bacteria and diagnose the species or strain, discriminate contaminated from non-contaminated specimens (e.g., of water, food, or working surfaces), or detect incidents of bioterrorism with little or no sample preparation would be an extremely useful technology in many sectors of society.

Such a diagnostic tool could be a particularly important advance for medical clinicians, who are currently called upon to diagnose pathogenic infections using a variety of techniques. In the coming years, the expectations of clinical and public health microbiology laboratories will increase as they will be challenged by the need to detect and quickly identify newly emerging pathogens due to mutation of existing organisms or perhaps introduced by bioterrorism. Clinical microbiologists will find themselves not only detecting pathogens but also screening for mutations indicative of drug resistance and of diseases associated with growth in biofilms.[18,19] To do this, clinical health care workers are developing an ever-more sophisticated arsenal of diagnostic tests, such as a rapid-polymerase chain reaction (PCR) test for genetic identification and matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF).[20,21] The speed of a LIBS-based diagnostic test could well exceed the speed of these previously mentioned tests and certainly exceeds traditional culturing techniques. The use of advanced computerized chemometric algorithms for real-time identification based on the LIBS signatures also implies that diagnoses can be made without any microbiological expertise required to interpret the results. Due to the known complexities of reliably identifying an unknown target present on the surface of a variety of previously uncharacterized matrices, a real-world LIBS-based pathogen identification device will probably initially be most useful for identifying pathogens in a controlled laboratory environment. In such an environment (e.g., hospital pathology laboratory, quality assurance/purity laboratory, etc.), unknown pathogens could be mounted on well-characterized, purpose-designed testing substrates. As classification algorithms improve and ablation processes become more controlled, the technique will be extended to identification on uncharacterized or unknown surfaces.

Recent results have shown progress toward realizing the potential of a rapid LIBS point-of-contact diagnostic. The diagnostic has exhibited excellent sensitivity and specificity, as evidenced by a 100% accuracy in a blind identification trial of four different methicillin-resistant *Staphylococcus aureus* (MRSA) strains and a non-pathogenic *E. coli* strain and has exhibited a low limit of identification (LOI) evidenced by achieving a 100% accuracy in discriminating only 2500 bacterial cells of *Mycobacterium smegmatis* from a genetically modified mutant of the same strain.[22,23] Work remains to be done, however, to investigate the loss of specificity and the increase in the LOI that may arise due to naturally occurring biodiversity in live (as opposed to freeze-dried or "lyophilized") bacterial cells and biochemical variations that may arise due to environmental influences during growth, and to develop protocols for sample preparation that will minimize risks to health-care professionals. To this end, in this paper we report on the effect on bacterial identification of: (1) intentionally changing the nutrition medium environment during the growth of closely related *E. coli* strains; (2) killing or inactivating the bacteria by bactericidal ultraviolet (UV) irradiation and

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autoclaving prior to LIBS testing; and (3) depriving the bacterial cells of nutrition sources or carbon sources for extended periods of time (nutrient deprivation or "starvation") prior to LIBS testing, representing changes that may occur when bacterial cells are tested on an abiotic or inert surface.

LASER-INDUCED BREAKDOWN SPECTROSCOPY INSTRUMENTATION

Nanosecond laser pulses at 1064 nm from a Nd:YAG laser were used to ablate bacteria mounted on a nutrient-free agar substrate in an argon environment at atmospheric pressure.[24] Argon has been shown to increase classification accuracy by providing more and stronger elemental plasma emission lines, particularly of phosphorus and carbon, although all lines demonstrate an increase of signal-to-noise ratio compared to ablation in air. We have also shown that ablation in a helium environment provides a nearly equivalent enhancement.[24] The laser energy was approximately 8 mJ/pulse and the laser was focused by a 5× high-damage-threshold microscope objective to a diameter of approximately 100 μm. The experimental setup used to perform LIBS on the bacteria specimens has been described in detail elsewhere.[11,12] Five laser pulses were fired per sampling location and the specimen was then translated 250 μm to provide a fresh surface. Five such locations were averaged per spectrum, although a sufficient signal-to-noise ratio was usually achieved after only two locations. LIBS spectra were acquired at a delay time of 2 μs after the ablation pulse with an integration gate width of 20 μs. The optical spectrum was dispersed and analyzed with an Échelle spectrometer, equipped with a gated intensified charge-coupled device (CCD) detector.

All the bacteria analyzed in this manuscript were mounted on a 1.4% nutrient-free bacto-agar substrate as a semi-liquid droplet. Bacterial specimens were collected from colonies cultured on a trypticase–soy agar nutrient medium (unless otherwise noted) during log phase growth. Specimens were suspended in deionized water and centrifuged at 5000 rpm for 3 minutes to form a semi-liquid pellet containing roughly 10^9 bacteria/mL. The supernatant fluid was drawn off after centrifugation and discarded.

A 10 μL micro-pipette was used to withdraw the bacteria-containing liquid and deposit the suspension on the bacto-agar. After approximately thirty minutes, the liquid was absorbed by the agar, leaving a transparent thin film or "bed" of bacteria. This process is shown in Fig. 1. High-resolution optical microscopy was performed on these specimens before and after LIBS testing and a representative micrograph is shown in Fig. 2. Figure 2a shows a 5× magnification of the bacterial bed, showing the magnification scale and approximate size of the laser spot for reference. Figure 2b shows a 100× magnification with the same scale and laser spot size for reference. Individual *E. coli* bacteria are visible within the laser beam diameter. A conservative estimate of the number of bacterial cells ablated per sampling location based on our initial knowledge of the titer of the semi-liquid pellet, the volume of liquid deposited, the area of the thin film, and the area of the focused laser spot size is approximately 1500.[23]

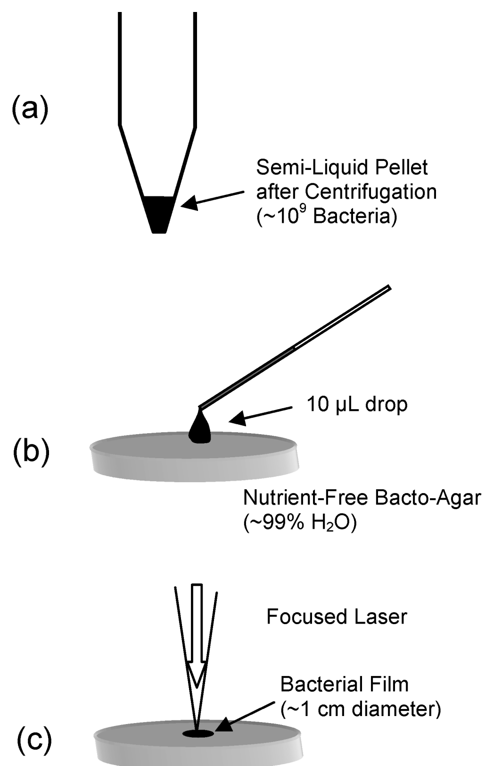


FIG. 1. The bacterial mounting procedure used in this study. (**a**) $\sim 10^9$ bacteria were harvested from cultures on solid media and suspended as a semi-liquid pellet by centrifugation. (**b**) 10 μL drops were deposited on a nutrient-free bacto-agar substrate. (**c**) After 30 minutes, the transparent "bed" of bacteria was ablated by a pulsed laser.

RESULTS AND DISCUSSION

The LIBS spectra acquired from these bacteria were similar to those reported by us and others for other bacteria,[8,9,25] being dominated by emission from trace inorganic metals and salts, specifically calcium and magnesium, as well as phosphorus, carbon, and sodium. Numerous argon lines from the inert gas environment were also observed. The absolute emission intensity (integrated area under the curve) for thirteen lines in these five elements was recorded for each spectrum and divided by the sum of all thirteen intensities (the total spectral power) to normalize the data. These thirteen normalized intensities were used as independent variables in a computerized discriminant function analysis (DFA) (SPSS v18.0 SPSS, Inc.). The DFA classified bacterial LIBS signatures based on spectral similarities and differences, calculated the accuracy of classification utilizing a "leave one out" test, and quantified the significance of specific elemental emission lines via the DFA structure matrix.[26]

NUTRITION MEDIUM ENVIRONMENT: EFFECT ON *E. COLI* STRAIN DISCRMINATION

Laser-induced breakdown spectroscopy spectra from three similar strains of non-pathogenic *E. coli* (C, HF4714, and ATCC 25922) were acquired as described earlier. In addition to these three specimens, one specimen of *E. coli* C was cultured on a bile-salts-containing nutrient medium (a MacConkey agar with a 0.01% concentration of deoxycholate). Trypticase–soy agar is a basic nutrition medium used for culturing most types of bacteria. It is also used as an initial growth medium for different purposes such as developing pure cultures. Conversely, MacConkey agar is a selective medium that inhibits the growth of Gram-positive

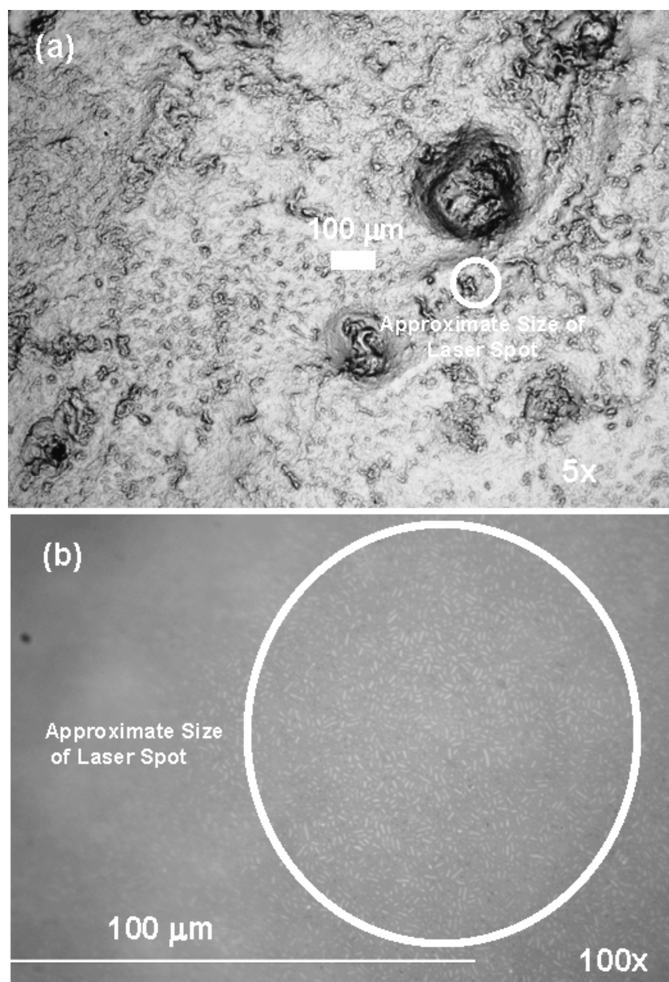


FIG. 2. Optical micrographs of the bacterial bed mounted on an agar substrate. (**a**) A 5× magnification of the bacterial bed showing the magnification scale and an approximate size of the laser spot for reference. Some non-uniformities in the bacterial bed can be seen. (**b**) A 100× magnification with the magnification scale and laser spot size for reference. Individual *E. coli* bacteria are visible within the laser beam diameter.

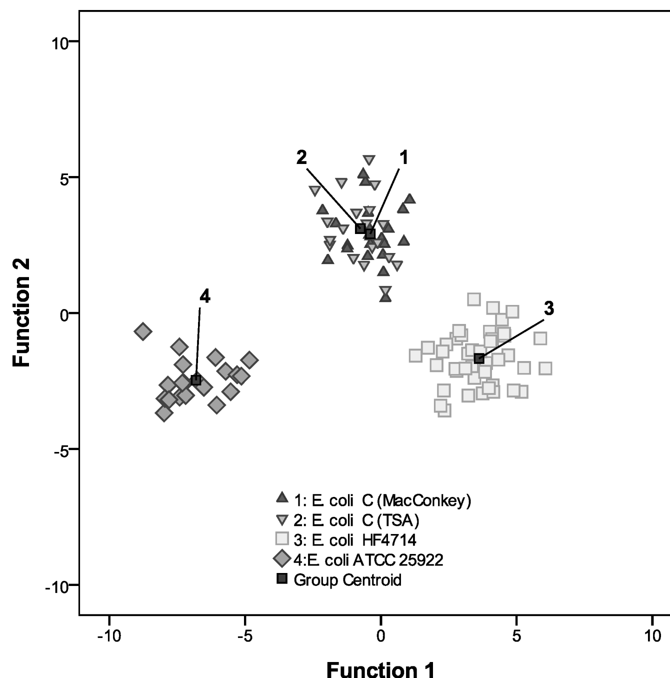


FIG. 3. A DFA plot of the LIBS spectra from four *E. coli* strains. The C strain was cultured on two different nutrition media, including a bile-salts-containing MacConkey agar and a trypticase soy agar medium. These two specimens of *E. coli* C cultured on different media were indistinguishable from each other and were discriminated with 100% accuracy from *E. coli* strain HF4714 and *E. coli* strain ATCC 25922, both of which were cultured on the trypticase soy agar medium.

bacteria due to the presence of bile salts and crystal violet. Figure 3 is a discriminant function analysis (DFA) plot showing the first two discriminant function scores of a DFA performed on spectra acquired for the four specimens cultured with these media. Regardless of nutrition medium, the *E. coli* specimens were effectively discriminated from each other on the basis of their DF_1 and DF_2 scores. The specimens of *E. coli* C cultured on the two different media (TSA and MacConkey) were still closely grouped despite possible membrane alteration due to the detergent action of the deoxycholate on the lipid bilayer outer membrane of the *E. coli*.[27] In this DFA, 100% of the bacteria were correctly classified by strain, regardless of nutritional environmental conditions. This analysis therefore suggests that clinical specimens obtained from infected persons could be identified on the basis of their LIBS spectra independent of the chemical environment present in the host. While this data is promising, extensive blind trials on clinical specimens isolated from patients positively diagnosed via other methods are required to confirm this conclusion.

LIBS-BASED IDENTIFICATION OF LIVE AND DEAD BACTERIA: EFFECT ON CLASSIFICATION ACCURACY AND LIMIT OF IDENTIFICATION

To study whether the LIBS spectral pattern is dependent upon the bacterial phase of growth, bacteria were prepared in three ways prior to LIBS testing. Live bacteria were harvested while reproducing in log-phase and then tested as described earlier. Autoclaved bacteria were autoclaved in a standard microbiological autoclave to kill the bacteria prior to mounting on the agar substrate. This is the standard method for rendering a biological specimen completely safe prior to disposal. Specifically, a 1.5 mL tube of *E. coli* C was subjected to high-pressure steam at 121 °C for 45 minutes. To ensure the total death of bacteria, a small number of these bacteria were picked up on a sterile loop for streaking on a standard TSA plate after autoclaving. No evidence of any bacterial growth was observed after incubation of this TSA plate at 37 °C for 24 hours, indicating death of all the bacterial cells in the autoclaved samples. Autoclaving the bacteria after mounting the cells on the nutrient-free bacto-agar substrate would have resulted in melting of the agar, so this test was not conducted.

Ultraviolet-irradiated bacteria were mounted on the nutrient-free agar as usual, but after absorption of the fluid by the agar, the specimen was irradiated for 20 minutes by 248 nm radiation from a bactericidal lamp. Such lamps are commonly used in microbiology to disinfect surfaces after cleaning. Exposure to this UV light does not technically "kill" the

bacteria, but it does destroy their ability to divide, rendering them harmless to the personnel working with them.

In all cases, multiple pads of bacteria were placed on a single agar substrate. In the autoclave test, one pad was used for LIBS testing and one pad was not ablated as a control. In the case of the UV-exposed specimens, four pads were placed on the substrate. Two were exposed to UV light and two were not. One UV-exposed and one unexposed pad were tested with LIBS, leaving two non-LIBS-tested pads as a control. All control pads were tested for activity via the standard microbiological method of restreaking and counting the number of colony forming units (CFU) to confirm the inactivation of greater than 99% of the bacteria in the specimen.

Figure 4a shows the DFA of the LIBS spectra acquired from three specimens of *E. coli* C ("live", "autoclaved," and "UV"), one specimen of *E. coli* strain ATCC 25922, and one specimen of *Mycobacterium smegmatis*, a genetically modified strain of an organism commonly used as a surrogate for *M. tuberculosis* utilized by us in previous studies (denoted as strain "TA"). The results of the DFA show that all three *E. coli* C specimens possessed nearly identical LIBS spectra and were identified 100% of the time as *E. coli* C regardless of whether they were live, dead, or inactivated. All three specimens were indistinguishable and showed excellent discrimination from the closely related *E. coli* ATCC 25922. The two *E. coli* strains possessed similar LIBS spectra, as evidenced by the similarity in their discriminant function one scores in Fig. 4a, compared to the *Mycobacterium* specimens. Despite their similarity, the two *E. coli* strains were well separated and classifiable with 100% accuracy.

This result is highly suggestive that LIBS testing can provide accurate results on specimens rendered innocuous via commonly available non-chemical anti-microbial procedures. Moreover, not only can this analysis be performed with no significant decrease in accuracy, but no decrease in the intensity of the LIBS signal was observed. Figure 4b shows the total spectral power (in arbitrary units) for the "live" and UV-inactivated specimens. Also shown is the average of all the data points for each specimen category and the 1σ standard deviation (a measure of total LIBS signal fluctuation). The total spectral powers for both the "live" and "UV" specimens were the same within error and the sample sets exhibited the same scatter. While spectral variations of absolute intensities on the order of 16% to 21% are slightly larger than the $<$15% that is expected in reproducible LIBS data, it is not unusual for our LIBS experiments on bacteria where specimen mounting uniformity plays a role.

The *E. coli* specimens tested in Fig. 4 were Gram-negative and non-pathogenic. To illustrate the universality of this result, this test was repeated with specimens of Gram-positive bacteria, specifically an avirulent derivative of the pathogen *Streptococcus viridans*. Figure 5a shows the DFA of the three specimens of *S. viridans* treated with the anti-bacterial methods described above, in addition to one specimen of *E. coli* ATCC 25922 and one specimen of *M. smegmatis*. From the DFA plot, it can be easily seen that all three specimens of *S. viridans* possessed nearly identical LIBS spectra. This result implies that a bacterial sample can be accurately identified whether it is pathogenic or non-pathogenic and regardless of whether it is alive or killed. This suggests the possibility of reducing the biosafety hazard level of the LIBS-based test to biosafety level

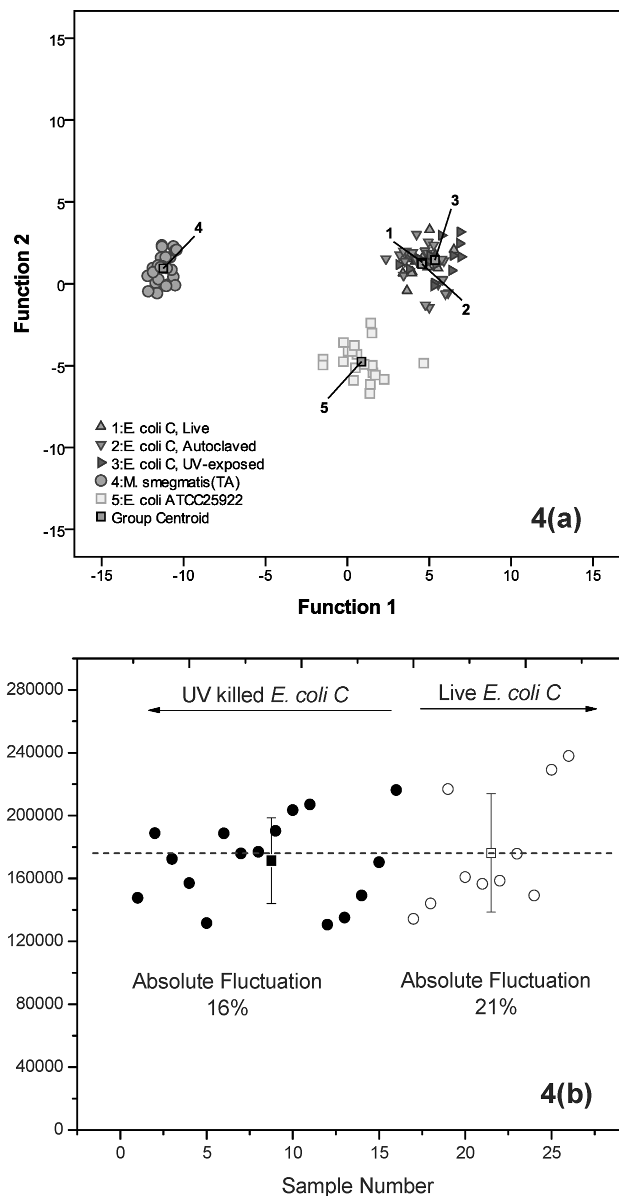
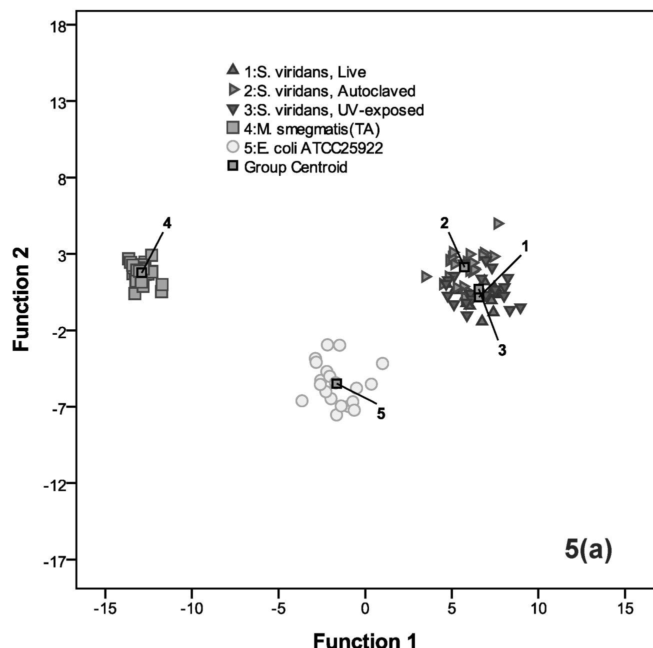


FIG. 4. (**a**) A DFA of three specimens of *E. coli* strain C, one specimen of *E. coli* strain ATCC 25922, and one specimen of *M. smegmatis*. The three *E. coli* specimens were prepared in different manners prior to LIBS testing. Group 1 ("live") was tested while alive. Group 2 ("autoclaved") was killed by autoclaving prior to testing. Group 3 ("UV") was inactivated via exposure to 248 nm UV irradiation prior to testing. The three *E. coli* C specimens possessed indistinguishable spectra regardless of their treatment, as revealed in the DFA. All the *E. coli* C specimens were discriminated with 100% accuracy from the *E. coli* strain ATCC25922 and the *M. smegmatis*. (**b**) The total spectral power (in arbitrary units) of the individual spectra from the live and UV-irradiated specimens. Also shown are the average of all the data points for each specimen category (the square symbol) and the 1σ standard deviation of the measurements (a measure of total LIBS signal fluctuation). The total spectral powers of the two specimens were the same within error and the sample sets exhibited similar scatter.

one (BSL-1) by killing or inactivating the bacteria prior to LIBS testing. This will ultimately save time and expense in a clinical diagnostic test. The total spectral powers for the spectra from "live" and UV-inactivated specimens of *S. viridans* were calculated and compared. In Fig. 5b, the average of the total spectral power for the two types of specimens ("live" and



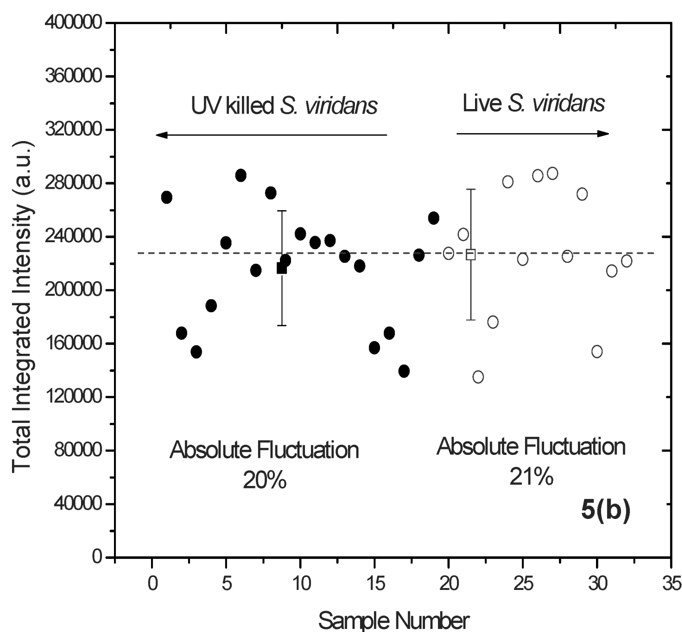


FIG. 5. (**a**) A DFA of three specimens of *S. viridans*, one specimen of *E. coli* strain ATCC 25922, and one specimen of *M. smegmatis*. The three *S. viridans* specimens were prepared in different manners prior to LIBS testing. Group 1 ("live") was tested while alive. Group 2 ("autoclaved") was killed by autoclaving prior to testing. Group 3 ("UV") was inactivated via exposure to 248 nm UV irradiation prior to testing. The three *S. viridans* specimens possessed indistinguishable spectra regardless of their treatment, as revealed in the DFA. All the *S. viridans* specimens were discriminated with 100% accuracy from the *E. coli* strain ATCC25922 and the *M. smegmatis*. (**b**) The total spectral power (in arbitrary units) of the individual spectra from the live and UV-irradiated specimens. Also shown is the average of all the data points for each specimen category (the square symbol) and the 1σ standard deviation of the measurements (a measure of total LIBS signal fluctuation). The total spectral powers of the two specimens were the same within error and the sample sets exhibited similar scatter.

"UV") is the same, as is their scatter about the mean value, indicating again that the LOI of this test was not affected by this treatment of the bacteria. All tests were conducted with no loss of useful signal.

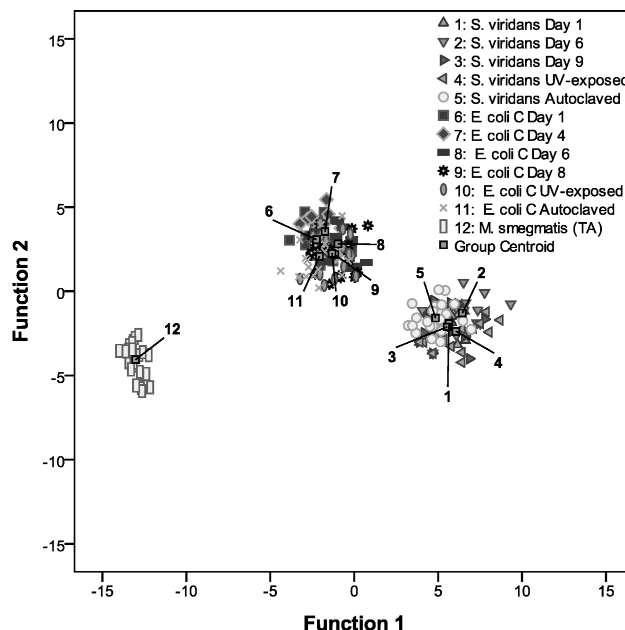


FIG. 6. A DFA plot of the spectra from many different bacterial specimens: starved *S. viridans* (three different starvation durations), autoclaved *S. viridans*, UV-irradiated *S. viridans*, starved *E. coli* C (four different starvation durations), autoclaved *E. coli* C, UV-irradiated *E. coli* C, and *M. smegmatis*. No significant differences were observed between the LIBS spectra of a given species acquired from bacterial specimens that had been deprived of a metabolic source, autoclaved, or exposed to bactericidal UV light. For all three species of bacteria, 100% of the spectra were correctly classified and discriminated from the other species.

LIBS-BASED IDENTIFICATION OF PATHOGENIC AND NON-PATHOGENIC BACTERIA UNDER NUTRIENT DEPRIVATION CONDITIONS: EFFECT ON CLASSIFICATION ACCURACY AND LIMIT OF IDENTIFICATION

To test the effect of depriving the bacteria of nutrition ("starvation") on the bacterial LIBS spectrum, specimens of the non-pathogenic *E. coli* C and the pathogenic *S. viridans* were prepared in the standard manner detailed above, mounted on nutrient-free agar substrates, and placed in a 21 °C isolated environment. LIBS spectra were then acquired one day, six days, and nine days after mounting the *S. viridans* specimens. LIBS spectra were acquired one, four, six, and eight days after mounting the *E. coli* C specimens. The bacteria did not die during this starvation trial, but having no external nutrients to consume, they would have initially consumed internal reserves of nutrients and then entered a dormant, non-reproducing state. In the dormant state, the bacterial cells are metabolically active but cannot be cultured by known laboratory techniques.[28] All LIBS spectra were analyzed with a DFA. The results are shown in Fig. 6. 100% of the starved *E. coli* C specimens were classified as *E. coli* C regardless of the time of starvation and 100% of the starved *S. viridans* specimens were correctly classified as *S. viridans*. Therefore, in our experiment we found that the bacteria retained distinguishable LIBS spectra even after they had entered a metabolically dormant, non-culturable state. In addition, the total spectral power of the bacterial spectra for both *E. coli* C and *S. viridans* did not significantly decrease from the first day to the last day of starvation. Therefore, we conclude that the LOI is independent of the time

TABLE I. The LIBS classification accuracies of the three tests described in this article.

Specimens	# of spectra tested	Accuracy
Nutrition medium test		
E. coli C	20	100%
E. coli HF4714	44	100%
E. coli ATCC 25922	20	100%
M. smegmatis (TA)	25	100%
S. viridans	15	100%
Live/UV-irradiation/autoclave test		
E. coli C UV-exposed	16	100%
E. coli C Autoclaved	24	100%
S. viridans UV-exposed	20	100%
S. viridans Autoclaved	23	100%
Starvation test		
E. coli C Day 1	15	100%
E. coli C Day 4	15	100%
E. coli C Day 6	16	100%
E. coli C Day 8	18	100%
S. viridans Day 1	14	100%
S. viridans Day 6	15	100%
S. viridans Day 9	14	100%

the bacterial specimens have spent in a nutrient-free environment.

For completeness, and in an attempt to "confuse" the DFA, the autoclaved and UV-irradiated specimens from the previous study were included in the analysis shown in Fig. 6. In this analysis, no *E. coli* C spectra were identified as anything but *E. coli* C (100%) and no *S. viridans* spectra were identified as anything but *S. viridans* (100%). All the *M. smegmatis* LIBS spectra were correctly classified. The results of this test and the previous two tests are summarized in Table I, which shows the identification accuracies of all three tests described in this paper (nutrition medium test, autoclaved/UV/live test, and starvation test). Based on Table I, one can see that the LIBS spectra of both *E. coli* C and *S. viridans*, chosen to give a representative cross-section of both Gram-negative and Gram-positive bacteria as well as pathogenic and non-pathogenic bacteria, are not altered by a wide variety of biologically diverse conditions that the bacteria may be exposed to prior to LIBS testing.

CONCLUSIONS

The specificity and sensitivity of a LIBS-based *E. coli* strain identification was not changed by culturing the bacteria on different media prior to LIBS testing. The LIBS-based DFA was also not dependent upon the metabolic activity of the bacteria, whether live, autoclaved, or inactivated by UV exposure. This was demonstrated for a representative species of both Gram-negative and Gram-positive bacteria. In addition, the signal-to-noise ratio of the bacterial LIBS spectra was not reduced, indicating that the LOI of this test was not increased in any statistically significant amount by treatment with these common bactericidal techniques. *E. coli* bacteria that were deprived of nutrition for up to 8 days prior to LIBS testing were still identifiable and were discriminated from the pathogenic Gram-positive *S. viridans*, which were deprived of nutrition for up to 9 days with 100% accuracy. In all three of these tests, all LIBS spectra were correctly classified. The signal-to-noise ratio of the bacterial spectra was not decreased in any significant way due to the nutrition deprivation conditions, indicating that this also would not increase the LOI of the LIBS test.

We believe that these results should be fairly universal for many types of bacteria, despite our testing of only two species (one Gram-negative and one Gram-positive). Fundamentally, these processes (particularly UV irradiation, autoclaving, and starvation) do not change the elemental composition of the bacteria on which the classification is based. The most significant change induced by most of our stressors involved the hydration or water content of the cell. However, we do not use lines of hydrogen or oxygen in our analysis; therefore, the test is relatively insensitive to hydration. More likely, sample hydration would affect the strength of the plasma emission and the temperature of the LIBS plasma, which could result in varying emission ratios observed from samples with an otherwise identical composition. This was the motivation for the experiments described herein. Our data lead us to conclude that such hydration-induced plasma-formation differences, if present, did not alter the LIBS plasma enough to significantly disrupt accurate classification.

Lastly it is worth noting that the stressor most likely to truly alter bacterial elemental composition was the composition of the medium in which the bacteria reproduced. That the nutrient medium did not significantly alter the LIBS spectrum is not surprising, as many bacteria can actually only survive within a narrow window of environmental conditions, including temperature, pH, osmotic pressure, and ionic concentration. All nutrient rich media are optimized for bacterial growth; therefore, the range of environmental conditions is not as great as supposed. As well, it is likely that the bacteria cannot survive if their elemental composition is significantly altered beyond a narrow range, particularly given the important role that the divalent cations of Ca and Mg play in regulating cell function and membrane porosity.[27] Undoubtedly media could be obtained or created that would significantly alter bacterial elemental concentrations while still encouraging growth. However, because we are attempting to develop a biomedical diagnostic, we are primarily interested in testing bacteria in conditions that they are likely to experience in vivo, and not in arbitrary or unrealistic chemical environments. This paper has shown that for a variety of bacterial stressors likely to be encountered environmentally or administered intentionally, the LIBS-based diagnostic retains its selectivity and sensitivity.

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