Laser-Based Identification of Pathogenic Bacteria

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Bacteria are ubiquitous in our world. From our homes, to our work environment, to our own bodies, bacteria are the omnipresent although often unobserved companions to human life. Physicists are typically untroubled professionally by the presence of these bacteria, as their study usually falls safely outside the realm of our typical domain. In the last 10 years, however, several events have occurred that demand the attention of the general populace – including the ranks of physicists among them.

- Most famously, in late September and early-October of 2001, two separate waves of bioterrorism attacks were conducted in the United States. Spore forms of the lethal bacterium *Bacillus anthracis* were mailed to U.S. news organizations and offices in the U.S. Congress, killing five people and infecting 17 others.
- In September 2006, there was an outbreak of food-borne illness caused by *Escherichia coli (E. coli)* bacteria found in uncooked spinach in 26 U.S. states. By Oct. 6, 2006, 199 people had been infected, including three people who died and 31 who suffered a type of kidney failure called hemolytic uremic syndrome after eating spinach contaminated with *E. coli* strain O157:H7, a potentially deadly bacterium that causes bloody diarrhea and dehydration.
- In 2000, the fresh drinking water supply for the small town of Walkerton, Ontario, Canada became contaminated with this same highly dangerous strain of *E. coli* O157:H7, from farm runoff into an adjacent well that was known for years to be vulnerable to contamination. Starting May 15, 2000, many residents of the town of about 5,000 began to simultaneously experience bloody diarrhea and other symptoms of E. coli infection. As a result of this contamination and the subsequent lag in positive pathogen detection, seven people died and about 2,500 (more than 40% of the population at the time) became ill.

Most of the illness and death associated with these events could have been avoided if we possessed a 100% accurate, easy-to-use, rapid detector/identifier of bacteria. Unfortunately, this does not exist. The current methods of identifying bacteria can yield accurate results but are often too slow to direct responses or treatments and are often organism-specific, offering only a single diagnosis per test. More significantly, it is known that the microbiological expertise and cost required to perform these identifications preclude their common use as a screening mechanism to prevent human infection.[1] This lack of convenient testing has led to ever-increasing rates of food-borne outbreaks and secondary infections in hospitals (such as the current rise in nosocomial MRSA infections).[2]

Based on the current state of affairs, scientists and engineers in the non-life sciences have began to turn their attention to the problem of rapid pathogen detection by utilizing "nonmicrobiological" techniques. In particular, as a physicist, I began to wonder whether a bacterium could be identified *based on what it actually is and what it is composed of,* as compared to a current suite of microbiological tests that can identify a bacterium *based on how it reacts with or changes in*

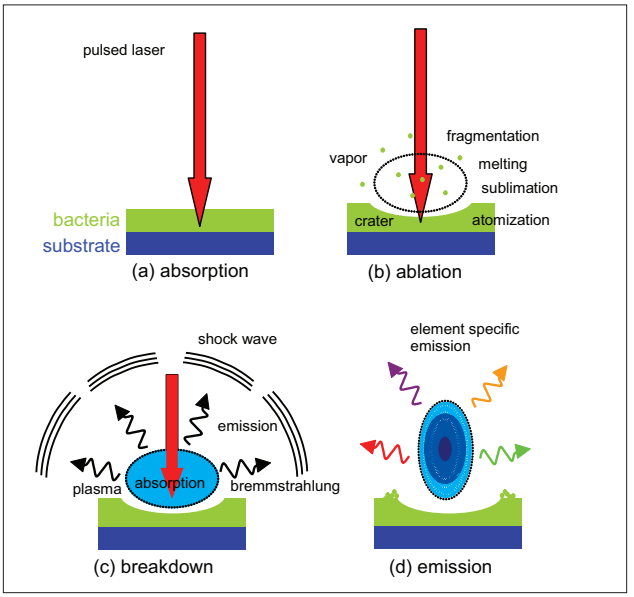

Fig. 1. The four stages of the LIBS process. (a) The process is initiated by absorption of energy by the target from a pulsed laser. Typical pulse durations are on the order of nanoseconds. (b) The absorbed energy is rapidly converted into heating, resulting in vaporization of the sample (ablation) when the temperature reaches the boiling point of the material. The removal of particulate matter from the surface leads to the formation of a vapor above the surface. (c) The laser pulse continues to illuminate the vapor plume. The vapor tends to condense into sub-micrometer droplets that lead to absorption and scattering of the laser beam, inducing strong heating, ionization, and plasma formation. (d) The dynamical evolution of the plasma plume is then characterized by a fast expansion and subsequent cooling. Approximately one microsecond after the ablation pulse, spectroscopically narrow atomic/ionic emissions may be identified in the spectrum. All elements present in the target are observed simultaneously.

response to its environment. The difference is subtle yet profound, and is most likely a result of the physicist's penchant for a "reductionist" or "simplification" approach to problem solving. Most physicists would like to reduce the number of variables that can be responsible for a unique identification by getting at the absolute heart of the issue: what is this bacterium made of?

Around 2003, physicists, engineers, and chemists began to investigate whether a pulsed-laser spectrochemical technique known as laser-induced breakdown spectroscopy (LIBS) could be used to identify a bacterium based solely on its atomic composition.[3] During LIBS, a short pulse of laser light is focused to a small spot on a bacteria-containing target, which creates a high-temperature (10,000–20,000 K) micro-plasma within the focal region of the laser. During this process, the sample illuminated by the laser is completely vaporized ("ablated"). The sample is reduced to its constituent atomic components, which are entrained in the micro-plasma plume. A careful spectroscopic analysis of the light emitted from this plasma plume yields identifiable emission lines only from those elements that were present in the target.[4,5] Figure 1 shows a schematic of four stages that occur during the LIBS process. The experimental time from the initiation of the laser pulse to the collection of the atomic emission is typically only microseconds.

Recently, LIBS has been used to identify and discriminate between a wide variety of microorganisms such as bacteria, fungal/mold spores, pollens, and proteins based on the emission intensity of trace inorganic elements found in the microorganism. My own group at Wayne State University has demonstrated that LIBS can be used to diagnose medically relevant bacteria such as the enterohemorrhagic *E. coli* strain O157:H7 mentioned earlier,[6,7] the opportunistic pathogen *Pseudomonas aeruginosa*,[8] the dental sp. *Streptococcus mutans,* and two spp. of *Staphylococci* (*aureus* and *saprophyticus*).

There is no doubt that a bacterium is a highly ordered, incredibly complex system. Atomic physicists typically deal with individual, isolated atoms. In fact, many of the quantum mechanical rules that govern atomic theory tend to break down when many atoms begin to strongly interact. In such a complicated molecular system like a bacterium, it is natural to ask how atomic spectroscopy can possibly give any information about the identity or function of the organism. The answer is fairly surprising. The majority of the atoms present in a bacterium (carbon, hydrogen, oxygen, nitrogen) are contained in carbon-based organic molecules and water. Each bacterium, however, contains trace but measurable quantities of *inorganic elements* that are crucial for metabolic or electrolytic activity (magnesium, calcium, potassium, sodium) as well as for stabilizing polar (negatively charged and repelling) bacterial membrane molecules. Also, a significant quantity of phosphorus is present in the phosphate molecules in the bacterium. It turns out that LIBS is particularly good at measuring trace quantities of these atoms. In fact, what LIBS is essentially doing is mak-

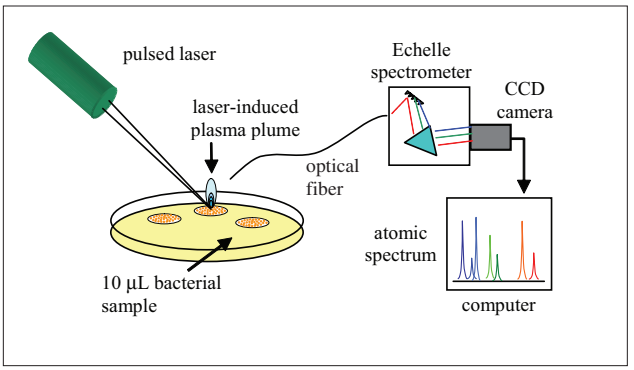

Fig. 2. A schematic of the LIBS experiment used to identify bacteria. The infrared pulsed laser produces a plasma that contains all the atoms present in the bacteria. The visible wavelength emission from this plasma is collected by an optical fiber, dispersed in an Echelle spectrometer, and recorded on a computer for analysis.

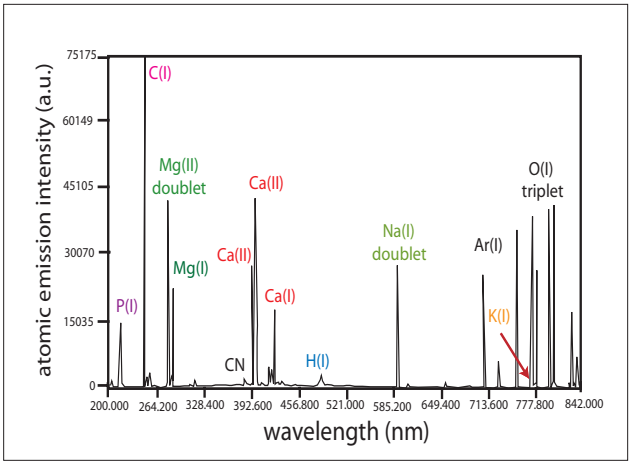

Fig. 3. A LIBS emission spectrum of *E. coli* C. The relative intensities of 31 atomic emission lines (in arbitrary units) from six elements are used as independent variables in an atomic spectral fingerprint. The identity of the observed lines is noted using (I) to specify emission from a neutral atom and (II) from an ion. The wavelength identifies the line, but is not used in the analysis. The spectrum is dominated by emission from trace inorganic elements.

ing a sensitive (accurate to about 1 ppm) measurement of the relative atomic concentrations of the trace inorganic elements in the bacterium. [9]

To make this measurement, all the molecules of the target are completely atomized and it is only the spontaneous emission from de-exciting free atoms and ions that is emitted from the plasma. This light is collected by an optical fiber placed in proximity to the plasma, and it is then dispersed in a spectrometer that produces a spectrum (a graph of light intensity versus the wavelength of the light). The whole apparatus can be fairly simple, and is shown schematically in Fig. 2. In fact, the entire apparatus can actually be made so light and compact that a man-portable prototype version has been constructed and tested by the U.S. Army. [10] A typical LIBS spectrum obtained from *E. coli* C, a well-characterized strain of *E. coli*, is shown in Fig. 3. To a first approximation, the relative peak height, or peak intensity, is related to the number of atoms of that element in the plasma and thus in the target sample.

Once spectra such as these are collected from a variety of bacterial samples, the differences in them provide a way to discriminate one sample from another. The basis of the identification technique rests in the fact that we have measured unique ratios for the quantities of the inorganic elements (as determined by the peak intensities) for all the bacteria tested to date in our lab. Figure 4 shows schematically the idea of the identification technique. We hypothesize that each bacterium possesses a unique relative abundance of these atoms. We are able to "count" or "bin" all of the different types of atoms present in the bacteria and then create a "spectral fingerprint" based on the number of atoms in that "bin." We create a library of spectral fingerprints obtained from positively identified bacteria. An unknown bacterium can then be measured, and the ratios of these inorganic elements are compared to previously measured spectra contained in the database.

Due to the fact that the bacterial spectra are highly similar (they contain the same elements, for the most part), a computerized data analysis technique known as "discriminant function analysis" (DFA) must be used. Such computational discrimination techniques, which make use of the entire observed elemental composition of the sample are often called "chemometric techniques" and are commonly used to provide highly efficient and automated identifications.[11] The DFA reduces the entire information content of the spectrum, such as that shown in Fig. 3, to a quantity known as the "discriminant function score." The scores from each spectrum are plotted against each other, and the grouping and separation caused by the differences in the spectra are immediately visible and obvious, as shown in Figs. 5 and 6. In these graphs, each symbol represents the information of an entire spectrum.

In Fig. 5(a), spectra from several different biotypes

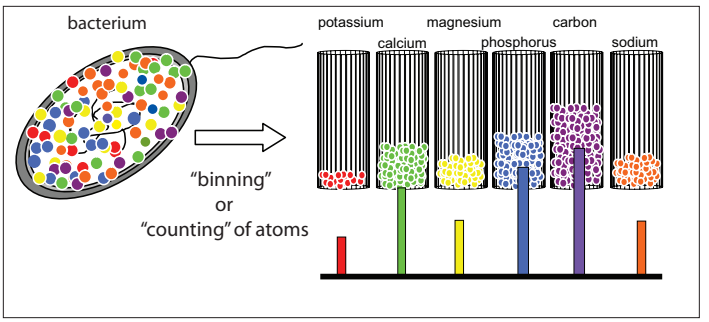

Fig. 4. A "spectral fingerprint" is created by determining the elemental composition of the bacterium and measuring the quantity of that element. Trace elements present at the ppm level in the bacterium are measured in this technique. The unique ratios of the quantities allow bacterial identification.

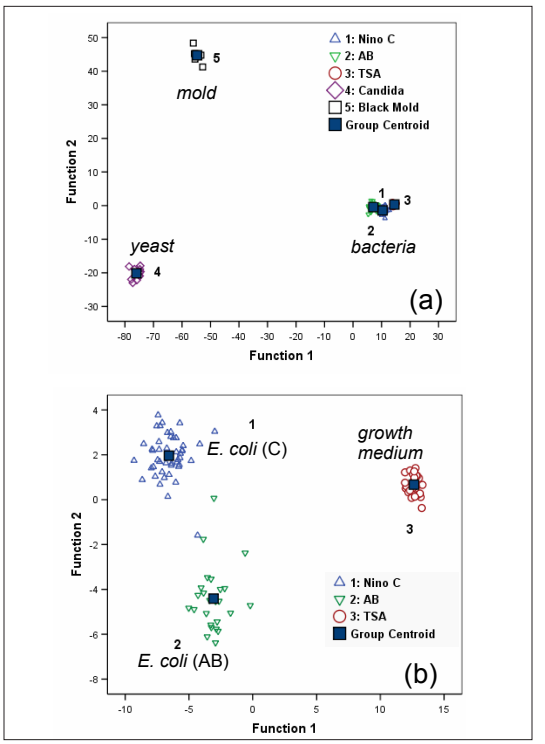

Fig. 5. Results of LIBS atomic analysis of various biological samples. In these graphs, each data point is constructed from the information contained in an entire LIBS spectrum. (a) A yeast and a mold are easily discriminated from two strains of *E. coli* bacteria and the medium on which they were grown. (b) The differences between the two strains of *E. coli* (AB and C) are apparent from their grouping. These bacteria are identified with almost 100% accuracy.

are compared, and it is immediately clear that spectra from a yeast (*Candida albicans,* shown as purple diamonds), an envi-ronmental mold (black squares), and two strains of *E. coli* bacteria are all discriminated easily. In Fig. 5(b), it is clear from the spacing between the groups (the blue triangles are spectra from *E. coli* strain C and the green triangles are spectra from *E. coli* strain AB) that there is a significant, reproducible difference between these two strains of *E. coli*.

Figure 6 demonstrates the ability of the LIBS/DFA technique to immediately differentiate between several species of bacteria. In Fig. 6(a), the discrimination between two species of *Staphylococci* (*aureus* and *saprophyticus*) and two strains of nonpathogenic *E. coli* (C and HF4714) is shown. In Fig. 6(b), we have added yet another bacterial species to the test, *Streptococcus mutans*. The spectra from these samples are all sufficiently distinct such that given a LIBS spectrum from an unknown specimen, the automated DFA would have no problem rapidly identifying and classifying the organism as belonging to the genus *Staphylococcus*, *Streptococcus*, or *Escherichia*.

Work is currently under way in our lab, and in other locations, to combine the atomic composition information obtained via LIBS with the molecular composition information obtained using molecular techniques, such as Raman spectroscopy, to develop a complete "spectral fingerprint" of the microbe. I call this program now under way at Wayne State University to create a "whole-organism spectral fingerprint" the BIOMAS project: Bacterial Identification by Optical, Molecular, and Atomic Spectroscopy.

This type of identification lies at the heart of the ultimate goal of this project: can an organism be absolutely and uniquely defined if the complete atomic and molecular composition of the organism is known with absolute accuracy? If the answer is yes, then the future of identification of microorganisms lies in the pursuit of developing technologies that allow for ever-increasing sensitivity and accuracy when making such measurements.

Perhaps the future lies with some hybrid cross between a genetic identification, immunological-based (antibody) identification, and biochemical identification (such as LIBS or Raman spectroscopy). Whichever technique or combination of techniques achieves the greatest use in the future, it is safe to say that the expertise required to advance the science will be interdisciplinary. No one discipline possesses all of the requisite knowledge to perfect these techniques, and so a research team consisting of members with expertise in microbiology, biochemistry, physics, and engineering

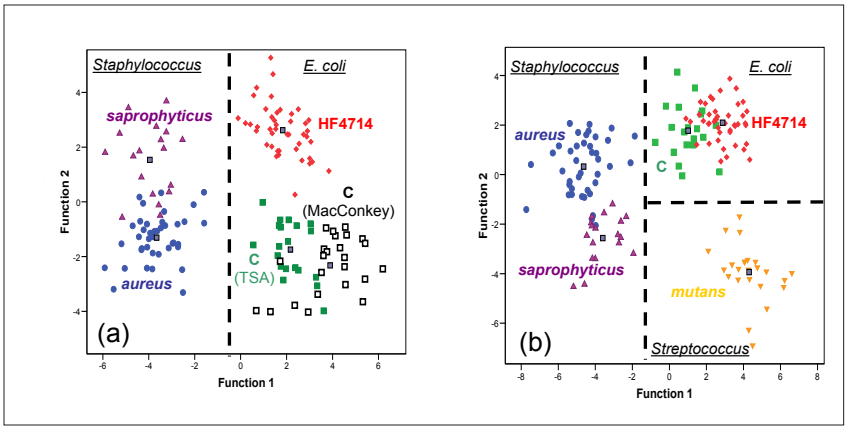

Fig. 6. Results of LIBS atomic analysis of various bacteria. (a) Two species of *Staphylococci* (*aureus* and *saprophyticus*) are easily discriminated from two strains of *E. coli* (C and HF4714). The *E. coli* C was cultured on two different nutrient media. (b) Two species of *Staphylococci*, a dental sp. *Streptococcus mutans*, and two strains of *E. coli* (C and HF4714) are easily discriminated. Each data point is a LIBS spectrum that took approximately 20 seconds to collect.

will be perfectly suited to exploit the rapid pace of advancements in technology and knowledge. A physicist with a strong interest in and knowledge of microbiology or biochemistry could be a valuable asset to such a team. A student with such varied interests may no longer need to choose between these types of disparate fields when choosing a discipline but may choose a research course of study that straddles several of these fields. The problems are big enough for all of us.

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