

Decoding the Origin of a Bioagent

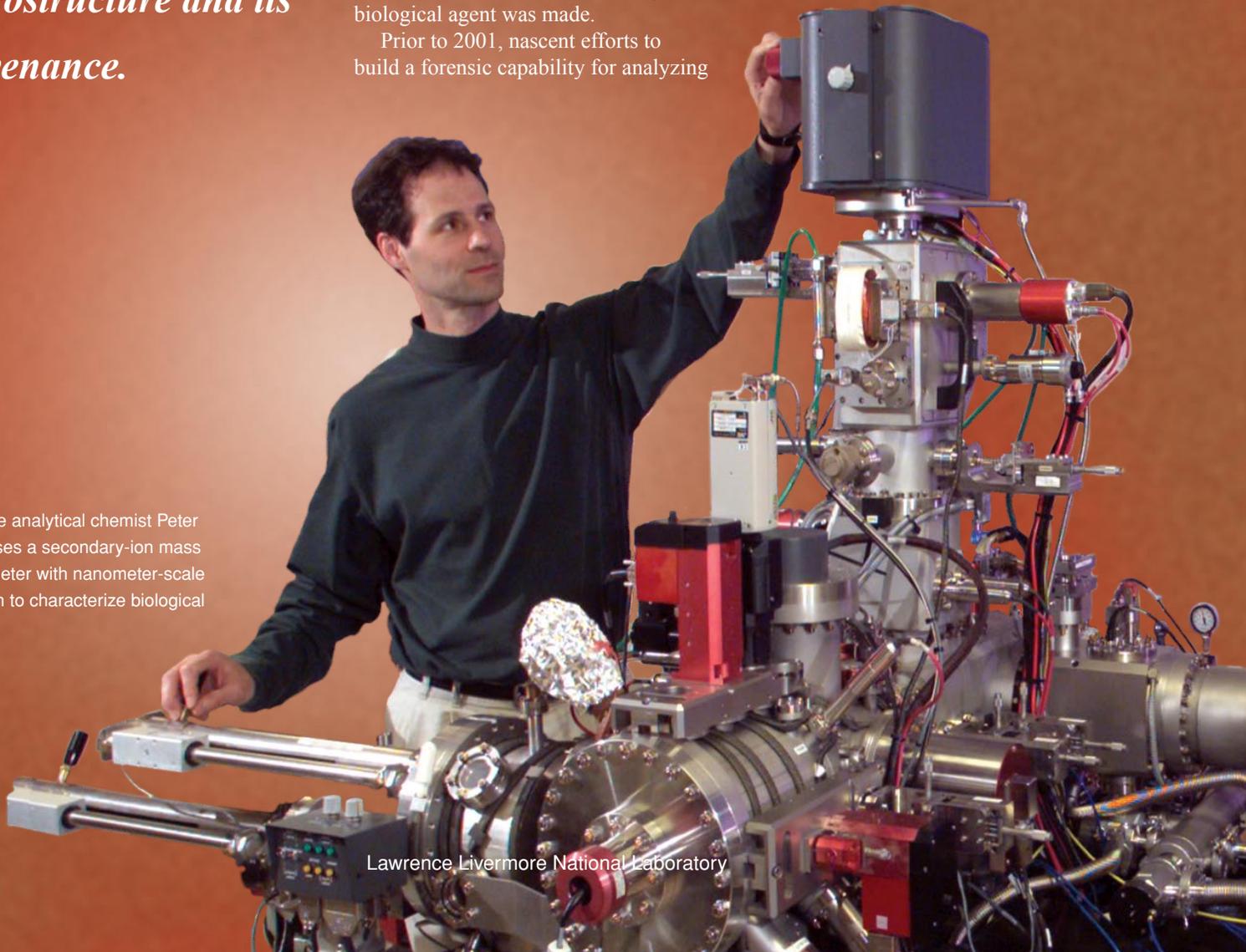
Researchers are discovering relationships between a pathogen's microstructure and its provenance.

AMONG the possible threats to life, few are as frightening as a weapon we can't see directed at us from an enemy we can't identify. Nanometer- to micrometer-size microorganisms can make powerful covert weapons, and the capability to detect and trace their origin has become a national security priority. Microbial forensics is a new area of research, the goal of which is to provide scientific tools to uncover how, when, where, and by whom a biological agent was made.

Prior to 2001, nascent efforts to build a forensic capability for analyzing

microorganisms were already under way within the National Nuclear Security Administration's Chemical and Biological National Security Program. In 2001, the attacks in the U.S. in which *Bacillus anthracis* spores were included in mailed letters revealed the need for a more extensive national microbial forensics research and development program. Experts used genetic typing to determine

Livermore analytical chemist Peter Weber uses a secondary-ion mass spectrometer with nanometer-scale resolution to characterize biological samples.



that the letters contained the *B. anthracis* Ames strain, but they had few other methods available for tracing the spores further to their source.

Bioterrorist Wake-up Call

The 2001 letter attacks galvanized the national security community to assess the status of the nation's microbial forensic capability and develop protocols to better handle such attacks. One of the first actions after the Department of Homeland Security (DHS) was formed was to establish the National Bioforensics Analysis Center (NBFAC). Working with the NBFAC, the Federal Bureau of Investigation (FBI) formed a Scientific Working Group for Forensic Analysis of Microbial Evidence (SWGfAME), bringing together dozens of experts around the country. Lawrence Livermore has an important role in the working group, providing new technologies and scientific expertise.

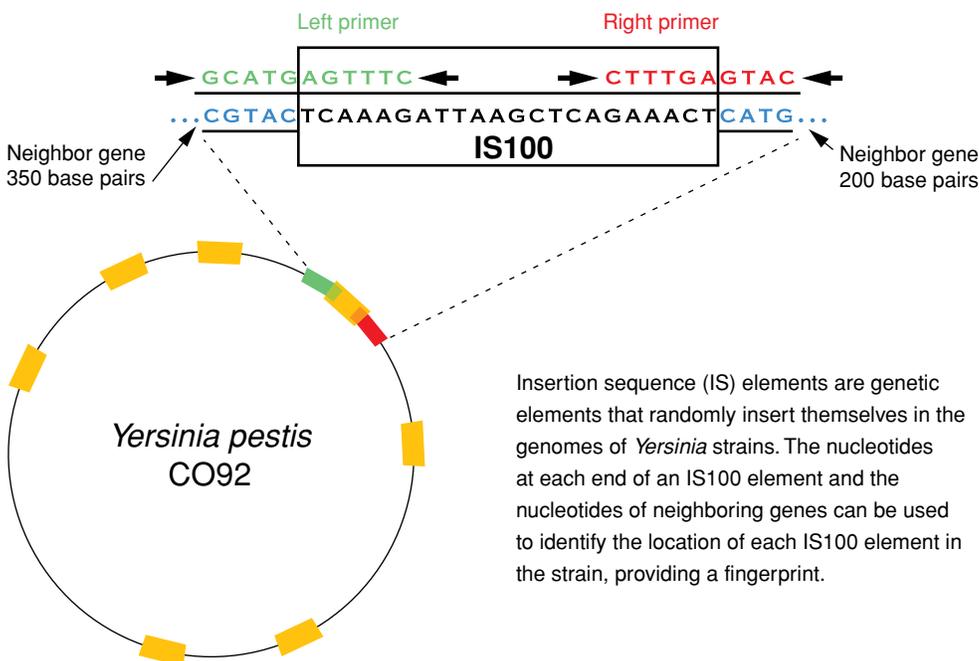
Chemical physicist Steve Velsko, one of several Livermore scientists serving on the SWGFAME, leads a team that is applying the Laboratory's expertise in

biology, chemistry, and materials science to solve microbial forensics problems. The researchers use techniques such as atomic force microscopy and secondary-ion mass spectrometry to determine whether structural features of microorganisms can be correlated with the methods used for growing and preparing the microbes. They are also developing new genetic typing techniques that could be used to quickly determine if a bacterial strain used in a bioterrorist incident is related to one found in a suspect laboratory.

"The difficulty," says Velsko, "is that much of the fundamental science needed to interpret both DNA and non-DNA evidence remains to be elucidated. Biological organisms such as *B. anthracis* can be found in many locations, which makes unambiguous tracing of pathogens to a single source very difficult. Sometimes it is even difficult to recognize that an attack has occurred." For example, about 300,000 hospitalizations and 5,000 deaths are attributable each year to food-borne illnesses in the U.S., and it is not always obvious whether these are a result of natural or intentional causes.

The Centers for Disease Control and Prevention maintains a database of select biological agent holdings at U.S. laboratories. Biological threat agents fall into three categories: viruses, bacteria, and toxins. The agents of most concern include those that may have been involved in foreign state-sponsored bioweapon programs: *B. anthracis*, *Yersinia pestis* (plague), *Franciscella tularensis* (tularemia), *Burkholderia* species (glanders and melioidosis), *Brucella* species (brucellosis), and *Variola* (smallpox).

"In 2001, the Ames anthrax strain was believed to be held by seven or more laboratories," says Velsko. "If the spores used in the attacks originated from one of those labs, just a short amount of time may have passed between the time the spores left the lab and when material grown from them was released. If so, the DNA of the strain in the letters may not be very different from the strains in these labs." When pathogens are transferred from nature to a laboratory, or between laboratories with different growing conditions, mutations (polymorphisms) can occur that shift the majority genotype away from that of the original strain. Improved genetic typing methods are needed to quickly identify these changes among closely related samples of bacteria.



High Cost of Lengthy Genomes

Typically, bacterial genomes contain 3 to 5 million DNA base pairs. Viruses are smaller, containing fewer than 200,000 nucleotides of RNA or DNA. Comparing the entire genomic sequence of two or more organisms is the most accurate identification method available. However, the cost of this method makes it impractical in a forensic investigation involving a potentially large number of pathogen samples, except in the case of small RNA viruses. For example, the estimated cost is about \$140,000 to determine with high confidence the sequence of one *B. anthracis* genome containing approximately 5.5 million base pairs. Sequencing 7 to 10 samples could

cost well over a million dollars. Therefore, researchers generally use sequencing to identify just the highly variable regions of bacterial pathogenic genomes.

Recently, researchers have developed less costly and faster approaches to genetically differentiate related strains of microorganisms. For example, advancements in partial genomic sequencing have led to a genetic typing system for *B. anthracis*. These systems look for polymorphisms in segments of the genome known to be particularly susceptible to mutation. However, one drawback to virtually every genetic typing system is that only fragments of a genome are examined, and these fragments may or may not capture all the significant changes in the genome. Thus, a challenge for researchers is to choose the most effective genetic typing system for detecting a particular polymorphism.

One important tool incorporated into a number of genetic technologies is the polymerase chain reaction (PCR). PCR is a technique for replicating DNA fragments in a test tube. The technique is used to copy a small number of DNA molecules up to millions of times, thereby amplifying a specific portion of the genetic material to an amount that can be detected. (See *S&TR*, May 2000, pp. 4–12.) Researchers determine which small DNA fragment to amplify by selecting primers—DNA strands usually between 18 and 25 base pairs long—that are complementary to the beginning and end of the DNA fragment of interest. They subject the DNA fragment to a process that separates the two DNA strands. The primers then adhere to complementary sequences on the single DNA strands, where a DNA polymerase catalyzes DNA replication and extends the targeted genetic sequence.

Locating Mutation Positions

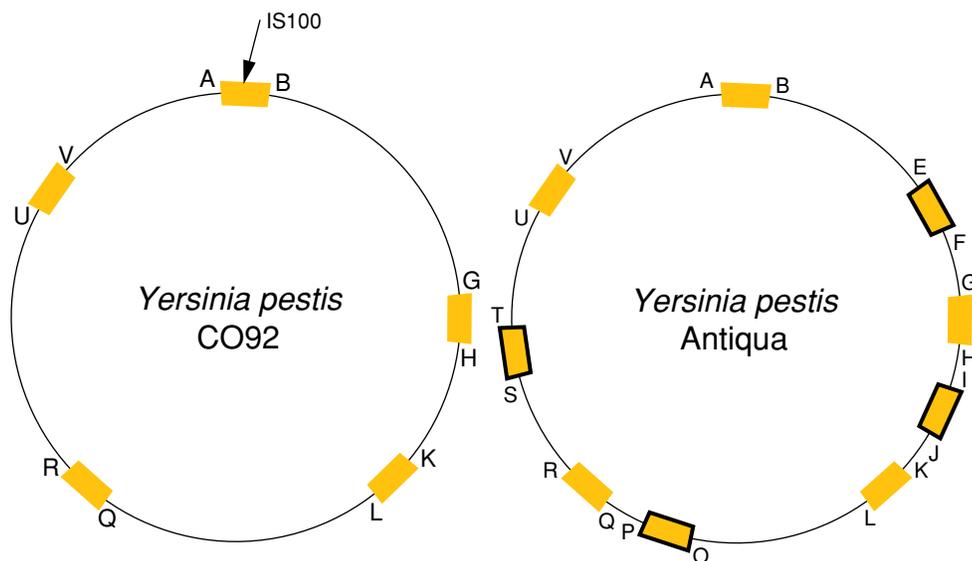
Through funding from DHS, Livermore microbiologist Emilio Garcia leads a team that is developing a novel PCR-based genetic typing system. The new

system uses a class of genetic elements called insertion sequence (IS) elements to distinguish among different strains of the same bacterial species. IS elements are repetitive units of DNA that have randomly inserted themselves at multiple locations throughout a bacterial genome. Different families of IS elements can vary in size and composition. IS elements have been observed in a variety of pathogenic bacteria, including *F. tularensis* and *Y. pestis*, and may play a role in providing bacteria the capability to quickly adapt to changing environments.

Researchers have used several molecular methods to confirm the presence of three IS elements unique to *Yersinia*—IS100, IS285, and IS1541. These insertion elements have been sequenced, but no efficient process has been available to locate their positions within a strain's genome. Garcia's new typing method allows researchers for the first time to effectively group and identify strains based on the positions of known IS elements in the *Y. pestis* genome.

Last year, the team compared the location of IS100 elements in a large collection of strains from the three *Y. pestis* groups, called biovars. Each of the biovars—Orientalis, Medievalis, and Antiqua—evolved in different geographic regions of the world. Garcia's team used the nucleotides at the ends of an IS100 element, along with primers the team designed, to hook the ends on both sides to neighboring genes. The team then identified the location within the genome of each IS100 element in the strains. The *Y. pestis* strain CO92, whose genome has been completely sequenced, was used as the reference genome.

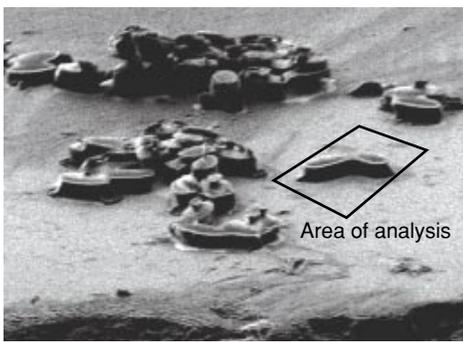
The team used the IS100 sequences and the DNA sequences surrounding those in the CO92 genome to design the primers for the experiments. They included 44 different IS100 insertions in 113 *Yersinia* DNA samples. Results showed that it was possible to distinguish among *Y. pestis* strains within each biovar and from distinct geographic regions. The team has also applied the typing system



A schematic shows how the locations for IS100 elements in the *Yersinia pestis* CO92 strain, whose genome has been completely sequenced, can be used as a reference to determine if an unknown *Yersinia* strain (here *Yersinia pestis* Antiqua) is related. The letters refer to specific locations on the genomes. The black-framed elements denote locations unique to Antiqua.

to locate IS285 in *Yersinia* strains. “IS element fingerprinting can provide the right amount of discriminatory power because IS elements evolve more slowly and are more stable than random variations,” says Garcia.

Paul Jackson, forensics group leader in the Laboratory’s Biosciences Directorate, also serves on the SWGFAME. Jackson is applying genetic typing methods to biological toxins. “The active ingredients



5 micrometers

Using a focused ion beam instrument, researchers can select and extract areas of a spore for further analysis with a nanometer-scale secondary-ion mass spectrometer (NanoSIMS).

NanoSIMS images of the same type of spore grown in two nutrients, G agar (top row) and NB agar (bottom row), show different ion concentrations of the elements phosphorus, fluorine, and chlorine, which may provide a signature. Brighter colors indicate areas of higher ion concentration.

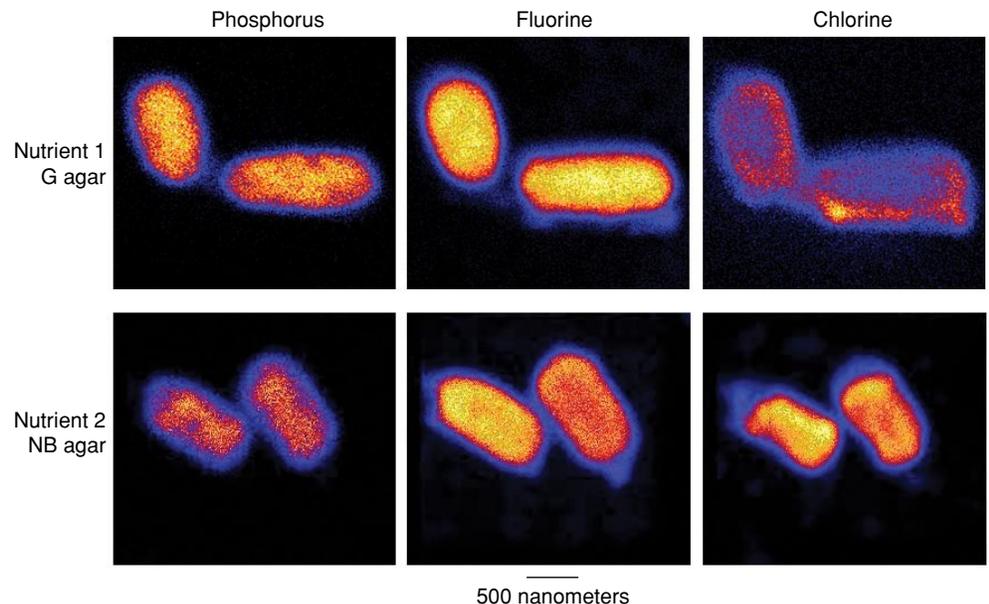
in biological toxins are proteins, not DNA,” says Jackson, “but they are derived from cellular organisms and always contain traces of their original biological source DNA. This residual DNA can be used as a fingerprint for the original source of the toxin.” Jackson is studying the DNA of the *Ricinus communis* plant, whose seeds are the source for ricin, one of the most deadly toxins. The FBI is currently investigating a number of cases involving ricin, which is believed to be the most common bioterrorism agent used worldwide. “Investigators need methods for determining whether two ricin samples were made from the same batch of seeds, and whether the seeds came from a particular geographic region,” says Jackson.

To characterize the DNA of the *R. communis* plant, Jackson uses a method called amplified-fragment-length polymorphism typing, in which the DNA is first cut into characteristic short fragments using one or more restriction enzymes. Certain fragments are then amplified using a PCR method. The lengths of the amplified fragments are determined using a DNA sequencer, similar to the way in

which DNA fragment sizes are determined in genomic sequencing. Jackson’s team is analyzing DNA in more than 200 types of *R. communis* plants from many geographic locations to determine the genetic diversity among the world’s sources. “This information will help classify the possible sources of natural ricin and select the appropriate genetic types for more detailed analyses such as sequencing,” says Jackson.

Structure and Formulation Connect

In addition to genetically based approaches to microbial forensics, the Livermore team is exploring technologies that could potentially provide other types of information on the origin and source of biological agents. “Remnants of growth media, stabilizers, or additives as well as incidental biocontaminants, such as environmental pollens that adhere to the spores, may provide clues about processes used to enhance the lethality and delivery of a biological agent,” explains Velsko. One goal of the national program is to develop a reference database of chemical and physical signatures that growth and processing methods leave on a biological agent.



With funding from DHS, FBI, and the Laboratory Directed Research and Development (LDRD) Program, analytical chemist Peter Weber uses Livermore's nanometer-scale secondary-ion mass spectrometer (NanoSIMS) to conduct studies on biological agents. In this technique, a sample is bombarded with an ion beam; atoms on the surface of the sample are ejected and then analyzed in the mass spectrometer.

Livermore's NanoSIMS provides a spatial resolution of 50 nanometers, 20 times better than that of conventional SIMS. Its sensitivity is 50 times better. Weber's team is hoping to gain insight into possible links between the chemical composition of microorganisms and how, when, and where they were produced. These studies might also help resolve some of the uncertainty that exists with regard to conclusions drawn from research using surrogates of lethal pathogens. Weber says, "Biological weapon agents can be produced using a variety of methods. In addition to the microorganism, there may be a matrix of agents intended to enhance delivery. Plus samples collected from different environments may contain contaminants from external material."

The team has also used a focused-ion-beam instrument to extract slices from targeted areas of a sample before analyzing the composition with NanoSIMS. Bacterial spores are composed of approximately 50 percent carbon, 15 percent nitrogen, 3 percent calcium, 1 percent phosphorus, and trace amounts of about 20 other elements. Chemically related elements can substitute for one another. For example, strontium and barium are related to calcium and can substitute for it. Substitutions may be signatures of certain growth or processing methods.

The high resolution achievable with NanoSIMS allows Weber's team to analyze not only individual spores but also the elemental distribution inside the spores.

These analyses can reveal microstructural production signatures. Previously, only major elemental distributions could be studied. The NanoSIMS team, however, can detect the distribution of less abundant elements, such as chlorine and fluorine, to study how spore production methods may change their distribution.

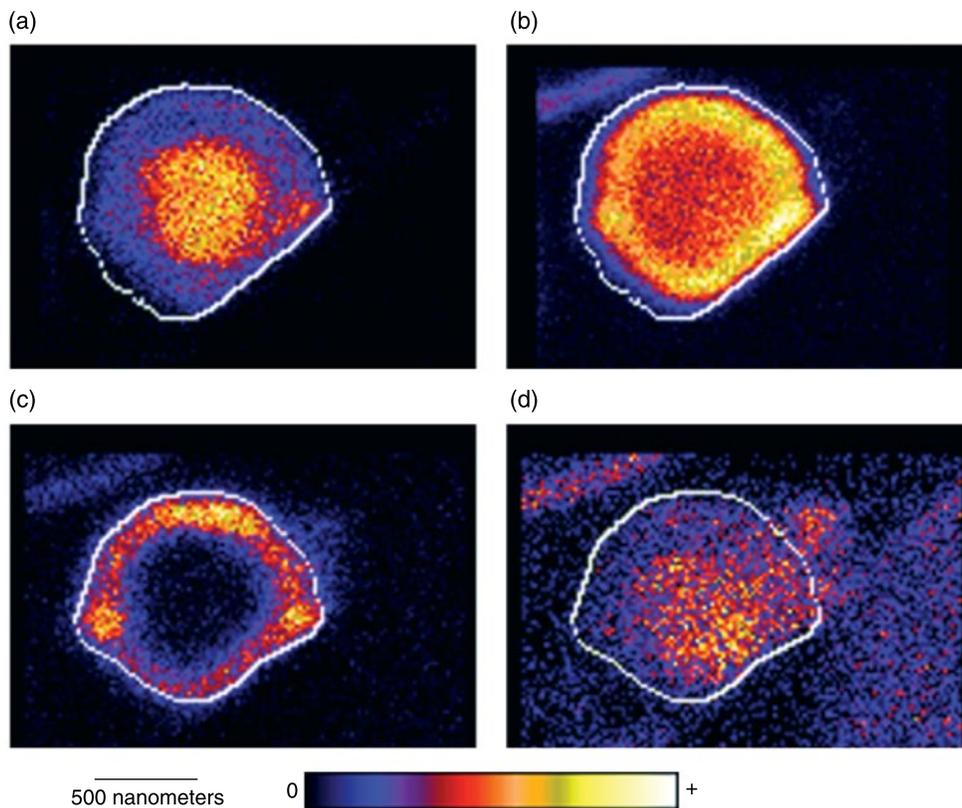
Weber's team is studying *B. thuringiensis* and the Sterne vaccine strain of *B. anthracis* to develop a matrix of elemental and isotopic signatures for spores that have undergone various growth and processing methods. Terrance Leighton from Children's Hospital Oakland Research Institute collaborates with the Livermore team and provides them with samples. In addition,

NanoSIMS is contributing to a study to determine whether diffusion rates of different elements in and out of spores could establish the date an agent was manufactured.

Unique Surface Architectures

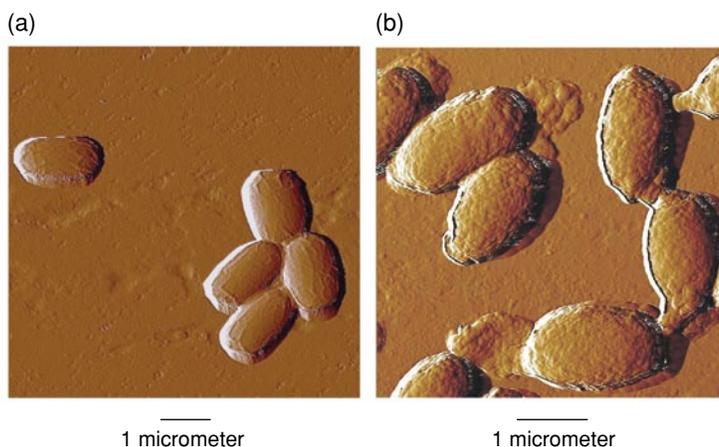
The Livermore team is also using atomic force microscopy (AFM) for its forensic studies. One advantage of AFM is that it can be used to image biological samples in fluids under native conditions as well as air-dried samples. A second advantage is that it can achieve resolutions of about 1 nanometer, allowing researchers to analyze high-resolution pathogen structures.

The AFM instrument has an ultrasharp tip that scans across a sample's surface.



NanoSIMS images of a *Bacillus thuringiensis* spore that was sectioned using a focused ion beam show ion concentrations of abundant elements such as (a) phosphorus and (b) sulfur as well as concentrations of trace elements such as (c) chlorine and (d) fluorine. Brighter colors indicate areas of higher ion concentration.

Atomic force microscopy images of air-dried (a) *Bacillus anthracis* and (b) *Clostridium novyi-NT* spores show different morphological features even at relatively low resolution.



A high-resolution image of the sample's topography is constructed as the molecules on the surface of the sample interact with those on the microscope's tip. Livermore researchers have used AFM to study crystal growth in biomineralization and corrosion resistance in metals, to measure mechanical properties of soft materials, and to investigate the surface architecture and assembly of viruses and bacterial spores. (See *S&TR*, December 2001, pp. 12–19; May 2002, pp. 11–15; May 2004, pp. 4–11.)

Although entire genomes have been sequenced for several *Bacillus* species,

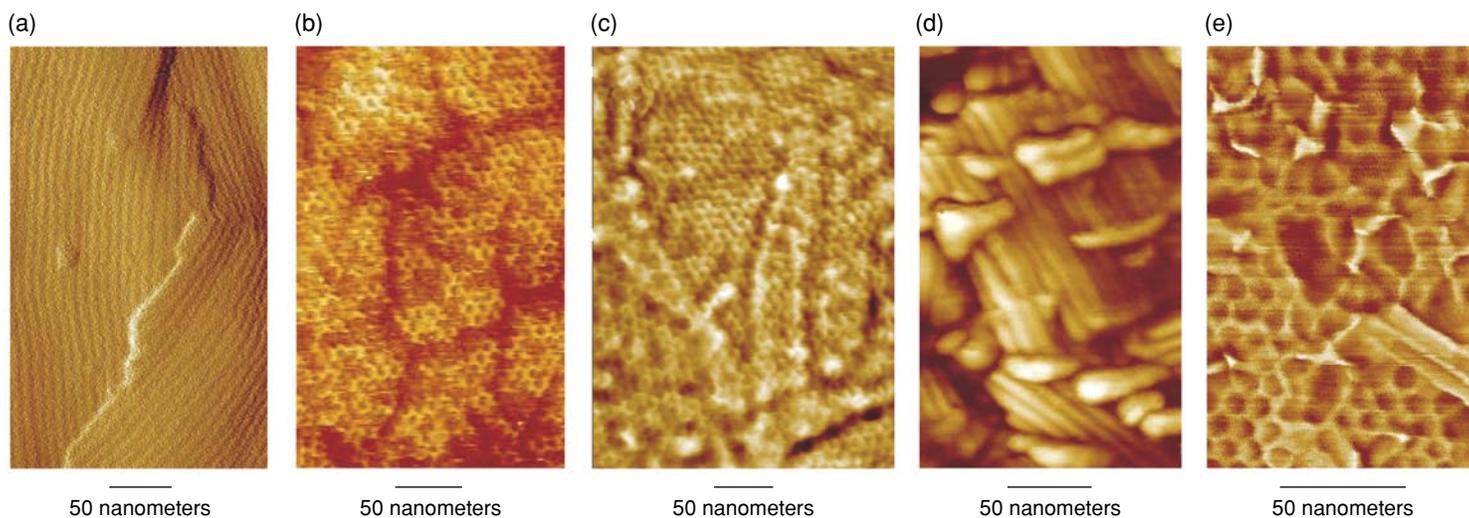
their spore-coat structures are not very well understood. Through funding from the LDRD Program and the FBI, Livermore chemist Alex Malkin and postdoctoral researcher Marco Plomp are investigating the structure–function relationships of bacterial spores.

The team provided the first high-resolution AFM images of the native spore-coat structures of four *Bacillus* species and a *Clostridium novyi-NT* isolate. “We found that strikingly different spore-coat structures are a consequence of species-specific nucleation and crystallization mechanisms,”

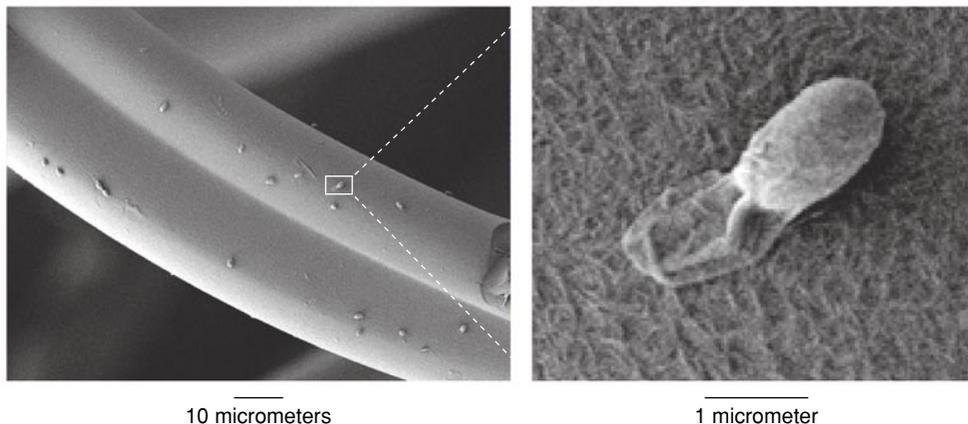
says Malkin. “These mechanisms, which regulate the assembly of the outer spore coat, are similar to those for growth of inorganic and macromolecular single crystals. We can thus apply the concepts that have been developed to study the growth of these crystals to examine the assembly of spore coats.”

The team found that the outer spore coats resembled honeycomb or rod-shaped structures, depending on the nutrient used to grow the cells. “The results established for the first time that outer coat structural patterns can be a formulation signature,” says Malkin.

Leighton has produced and characterized a library of spores from the *B. anthracis* Sterne vaccine strain using a range of media formulations. The Livermore team applies AFM to examine the high-resolution structure of air-dried and hydrated *B. anthracis* spore samples. For comparison, they use killed (irradiated or chlorine dioxide-treated) Ames strain spores. “We now have the capability to conduct AFM characterization of pathogens authorized at the necessary biosecurity level to study vaccine strains of anthrax and smallpox,” says Malkin.



Atomic force microscopy provides the first high-resolution images of native spore-coat structure. The outer spore coat of (a) *Bacillus atrophaeus* consists of crystalline layers of rod-shaped structures while the outer coats of (b) *B. thuringiensis* and (c) *Clostridium novyi* consist of honeycomb structures. (d) *B. cereus* is covered with small domains of rodlets and has (e) a honeycomb structure beneath.



10 micrometers

1 micrometer

A flexible hydrophobic outer membrane, or exosporium (spore overcoat), of *Bacillus anthracis* strengthens its adhesion to fibers.

The team is also developing an experimental approach called AFM-based immunolabeling. A spore's coat is composed of about 30 proteins and, under different conditions, the coat composition changes. The team uses antibodies labeled with gold particles to characterize the influence of growth and processing conditions on the proteomic structure of *B. anthracis* spore surfaces. They have also developed a technique to dissect a spore coat layer by layer. The technique has allowed researchers for the first time to define the complete architecture of the spore coat.

Collecting the Evidence

The FBI and other investigative agencies also require methods for collecting and extracting biological threat agents and their DNA from crime scenes and other locations.

Investigators often search for microbial evidence among fibrous materials, such as cloth, carpet, building ventilation filters, and paper, because these materials harbor agents well and it is difficult for the criminal—or a routine cleaning person—to remove these agents. However, it is also difficult for investigators to efficiently collect and extract spores and DNA from fibrous materials, and samples may include only trace amounts of an agent.

Livermore chemical engineer David Camp and his team are developing improved methods to separate colloidal

B. anthracis spores and DNA from fibrous materials and their contaminants. In an effort led by chemical engineer Elizabeth Wheeler, a technique was developed and demonstrated that is 10 times more sensitive than commercial DNA cleanup kits at recovering trace amounts of *B. anthracis* DNA from challenging dirty suspensions. The NBFAC hopes to incorporate this technique into its automated sample processing system.

Capability on Its Way

Since the SWGFAME was formed, the Laboratory has developed several new complementary technologies to increase our nation's microbial forensics capability. "Forensics data are most valuable when different techniques provide the same results," notes Malkin. NanoSIMS, AFM, gene typing, and other technologies are being combined to construct an attribution profile for microorganisms turned into bioweapons. "Strain identification must be quick enough to allow for an adequate medical and public health response, yet it must also endure the rigorous standards for evidentiary material that may be presented in a courtroom," says Velsko. It's a tall order. Still, Velsko is optimistic that the research avenues being investigated will soon provide the necessary capability.

—Gabriele Rennie

Key Words: atomic force microscopy (AFM), *Bacillus anthracis*, biological agent, *Clostridium novyi-NT*, genetic typing, insertion sequence (IS) elements, microbial forensics, nanometer-scale secondary-ion mass spectrometry (NanoSIMS), National Bioforensics Analysis Center (NBFAC), polymerase chain reaction (PCR), polymorphism, Scientific Working Group for Forensic Analysis of Microbial Evidence (SWGFAME), *Yersinia pestis*.

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