Uncovering Bioterrorism

DNA-based signatures are needed to quickly and accurately identify biological warfare agents and their makers.

WITH the end of the Cold War, the threat of nuclear holocaust faded but another threat emerged—attack by terrorists or even nations using biological agents such as bacteria, viruses, biological toxins, and genetically altered organisms. The former Soviet Union once had a formidable biological weapons program. Now, several countries and extremist groups are believed to possess or to be developing biological weapons that could threaten urban populations, destroy livestock, and wipe out crops.

Even terrorists with limited skills and resources could make biological weapons without much difficulty, says Tony Carrano, Lawrence Livermore's associate director for Biology and Biotechnology Research. "It's not complex, it's not expensive, and you don't need a large facility." For these reasons, biological weapons have been dubbed the poor man's atomic bomb.

Contributing to the ease of making and concealing biological weapons is

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the dual-use nature of materials to produce such weapons, because they are found in many legitimate medical research and agricultural activities as well. CIA Director George Tenet touched on this topic in Congressional testimony in February when he noted the overlap between manufacturing vaccines and producing biological weapons.

The agents used in biological weapons are difficult to detect and to identify quickly and reliably. Yet, early detection and identification are crucial for minimizing their potentially catastrophic human and economic cost. Lawrence Livermore scientists are participating in the Department of Energy's program to improve response capability to biological (as well as chemical) attacks on the civilian population.

A major part of DOE's program is developing better equipment, both fixed and portable, to detect biological agents (see *S&TR*, June 1998, pp. 4–11). However, any detection system is dependent on knowing the signatures of organisms likely to be used in biological weapons. These signatures are telltale bits of DNA unique to pathogens (disease-causing microbes). "Without proper signatures, medical authorities could lose hours or days trying to determine the cause of an outbreak, or they could be treating victims with ineffective antibiotics," says Lawrence Livermore's Bert Weinstein, deputy associate director of Biology and Biotechnology Research.

Because of the importance of biological signatures, DOE has launched a biological foundations program as a key thrust of its effort to improve response to terrorist attacks. The program involves experts at the Lawrence Livermore, Brookhaven, Los Alamos, and Sandia national laboratories, as well as colleges and universities. Researchers from the four national laboratories get together at least quarterly to share information and yearly for a formal review of their work. Weinstein reports that important progress has been made since the program began in early 1997, and new signature sets are being transferred to the Centers for Disease Control and Prevention and the DOE.

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Over the next several years, DOE scientific teams expect to produce species-level signatures for all of the most likely biological warfare pathogens. The teams also expect to have an initial set of species-level signatures for likely agricultural pathogens, because an attack on a nation's food supply could be just as disruptive as an attack on the civilian population.

Several Levels of Signatures

The teams also aim to develop strain-level signatures for the top suspected agents. Strains are a subset of a species, and their DNA may differ by about 0.1 percent within the species. A species, in turn, is a member of a larger related group (genus), and its DNA may differ by a percent or so from that of other members of the genus.

Characterizing pathogens at the strain level requires significantly more work than recognizing a species. But strain-level signatures are essential for determining the native origin of a pathogen associated with an outbreak; such information could help law enforcement identify the group or groups behind the attack.

The biological foundations work aims to provide validated signatures useful to

public health and law enforcement agencies as well as classified signatures for the national security community. In developing these signatures, biological foundation researchers are also shedding light on poorly understood aspects of biology, microbiology, and genetics, such as immunology, evolution, and virulence. Increased knowledge in these fields holds the promise of better medical treatments, including new kinds of vaccines.

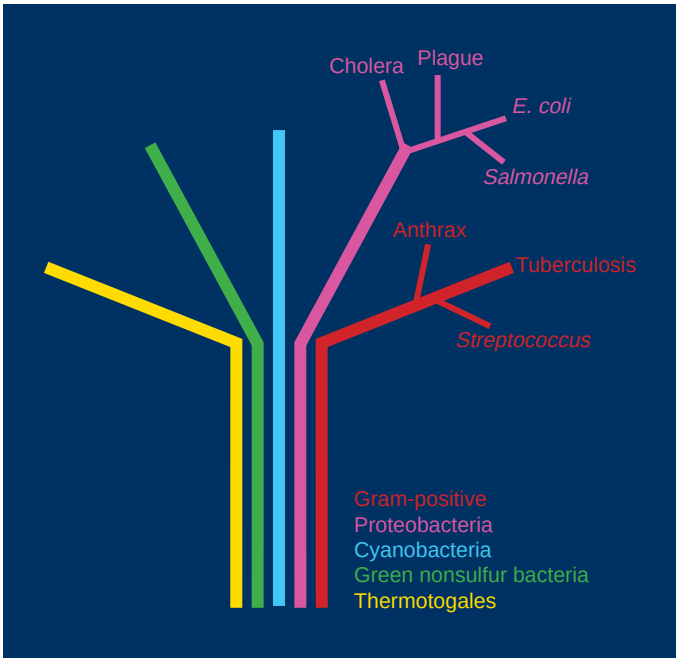
The biological foundations work is one element in DOE's Chemical and Biological Nonproliferation Program. Livermore's component of this work is managed by its Nonproliferation, Arms Control, and International Security Directorate. Other components of the overall program include detection, modeling and prediction, decontamination, and technology demonstration projects.

Livermore researchers were among the first to recognize, in the early 1990s, the tremendous potential of detectors based on DNA signatures. "We knew that a lot of work was necessary to develop the signatures the new detectors would need," says Weinstein. In particular, the researchers recognized several pitfalls. For example, if signatures are overly specific, they do not identify all strains of the pathogen and so can give a false-negative reading. On the other hand, if signatures are based on genes that are widely shared among many different bacteria, they can give a false-positive reading. As a result, signatures must be able, for example, to separate a nonpathogenic vaccine strain from an infectious one.

Several Levels of Identification

To enhance their detection development effort, researchers are exploring advanced methods that distinguish slight differences in DNA. They are using the multidisciplinary approach that characterizes Livermore research programs. In this case, DNA signature development involves a team of microbiologists, molecular biologists, biochemists, geneticists, and computer experts. In addition, the Livermore work benefits from collaborations with experts worldwide, extensive experience with DNA sequencing, and affiliation with DOE's Joint Genome Institute (see *S&TR*, April 2000, pp. 4–11).

Much of the work is focused on screening the two to five million bases that comprise a typical microbial genome to design unique DNA markers



This phylogenetic tree is a simple representation of the bacterial kingdom. All human bacterial pathogens belong to the Gram-positive (red) or Proteobacteria (magenta) divisions. The other divisions consist of nonpathogenic bacteria associated with diverse environments. Biological signatures must be able to differentiate infectious bacteria from hundreds of thousands of harmless ones. Each genus of bacteria has many species, and each species can have thousands of different strains.

DNA signature development involves a multidisciplinary team of microbiologists, molecular biologists, biochemists, geneticists, and computer experts. Here, biomedical scientists Peter Agron and Lyndsay Radnedge are performing suppressive subtractive hybridization to distinguish DNA of various species of virulent organisms.

that will identify the microbe. The markers, called primer pairs, typically contain about 30 base segments and bracket specific regions of DNA that are a few hundred bases long. The bracketed regions are replicated many thousands of times with a detector that uses polymerase chain reaction (PCR) technology. Then they are processed to unambiguously identify and characterize the organism of interest.

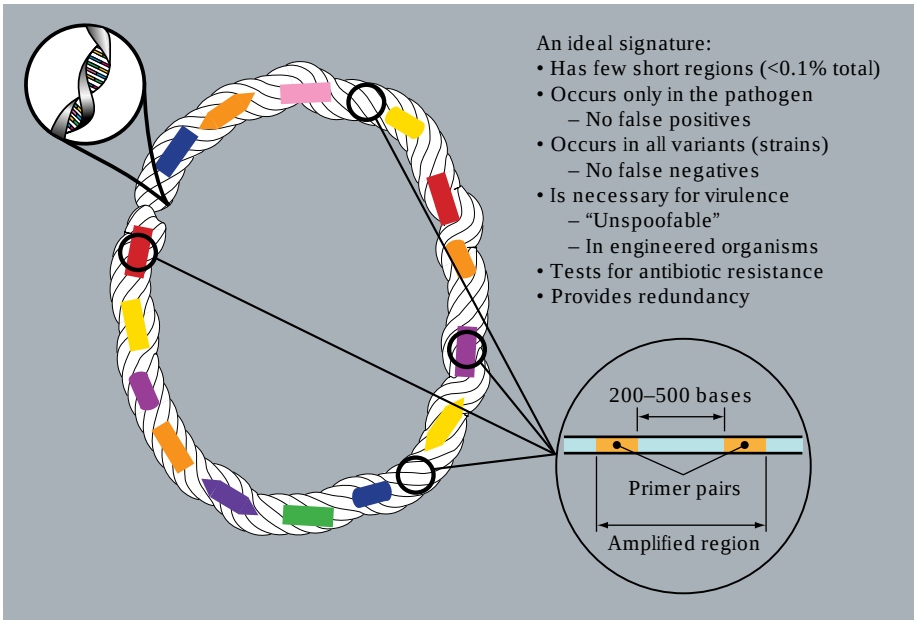
Weinstein notes that different signatures will be needed for different levels of resolution. For example, authorities trying to characterize an unknown material or respond to a suspected act of bioterrorism will begin with fairly simple signatures that flag potentially harmful pathogens within a few minutes. Typically, such a signature would encompass one or two primer pairs and be sufficient for identification at the genus level (*Yersinia* or *Bacillus*, for example) or below.

A signature in the next level of resolution is needed for unambiguously identifying a pathogen at the species level (*Yersinia pestis*, for example). This signature involves about 10 primer pairs. Currently, it takes several days to obtain conclusive data for a species-level signature. The goal is to reduce that time to less than 30 minutes.

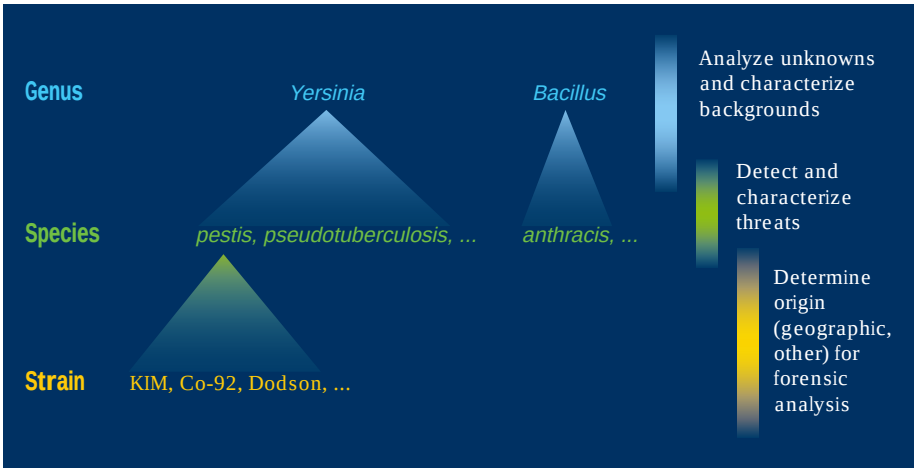
The third signature level is used in pathogen characterization, identifying any features that could affect medical response (for example, harmless vaccine materials versus highly virulent or antibiotic resistance pathogens). This signature level involves some 20 to 30 primer pairs. Together, the primer pairs offer a certainty of correct identification. Currently, providing such a high level of confidence requires several days; the goal again is to reduce the time to less than 30 minutes.

The final signature level, intended primarily for law enforcement use, will permit detailed identification of a specific strain of a pathogen (for example, *Yersinia pestis* KIM) and correlate that strain with other forensic evidence. Such data will help to identify and prosecute attackers. The present typical time lag for results is currently a few weeks, and the goal is to reduce that to a few days.

Biological foundations program scientists have worked with DOE and other agencies to assemble a list of natural pathogens most likely to be used

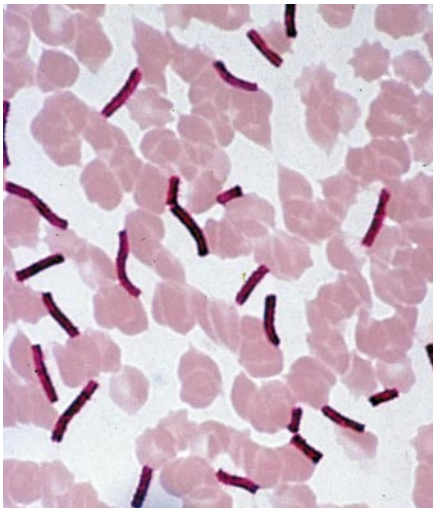


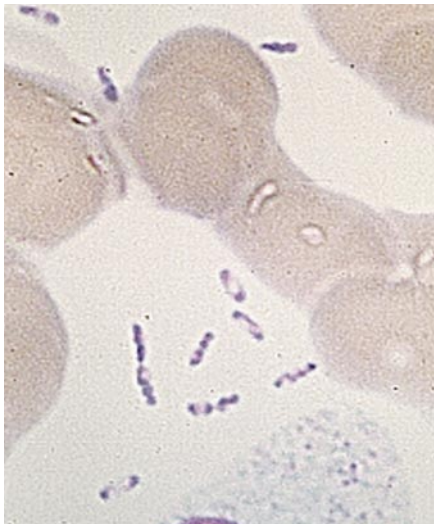
Bacterial chromosomes (DNA) form loops, unlike human chromosomes which form strands. In the loop, between two to five million bases of bacterial DNA are screened to locate unique regions (circled), which are marked with primer pairs. The marked regions are amplified thousands of times using polymerase chain reaction technology and then processed to identify and characterize an organism.



Different levels of signature complexity provide different problem resolutions. A simple signature, involving only one or two unique DNA marker regions, provides genus-level identification. Signatures for species- and strain-level identifications involve more marker regions and take longer to process, but provide more detail and accuracy. Law enforcement uses require signatures that provide strain-level identification.

in a domestic attack. The list includes bacteria, viruses, and other classes of threats, such as agricultural pathogens. Two extremely virulent pathogens head the list: *B. anthracis* and *Y. pestis*, which cause anthrax and plague in humans, respectively. *Bacillus anthracis* has few detectable differences among its strains, whereas *Y. pestis* strains can vary considerably in genetic makeup. Unraveling the significant differences between the two organisms will give national laboratory researchers experience vital for facing the challenges of the next few years, as they develop signatures for a wide spectrum of microbes.





Two extremely virulent organisms head the list of pathogens most likely to be used by terrorists: *B. anthracis* (top) and *Y. pestis* (bottom), which cause anthrax and plague in humans, respectively.

Livermore Focuses on Plague

Research has been divided and is carefully coordinated among laboratories to avoid duplication. Livermore researchers are focusing on *Y. pestis*, *Francisella tularensis* (a bacterium causing a plaguelike illness in humans), and several other microbes that threaten human and animal health. They are working in collaboration with the U.S. Army Medical Research Institute of Infectious Diseases, the Centers for Disease Control and Prevention, the California Department of Health Services, Louisiana State University, Michigan State University, and research centers in France, China, and Russia. "We want to be prepared for the most likely pathogens from throughout the world," says Weinstein.

Eleven species and many thousands of strains belong to the *Yersinia* genus. The most notorious species, *Y. pestis*, causes bubonic plague and is usually fatal unless treated quickly with antibiotics. The disease is transmitted by rodents and their fleas to humans and other animals. Although rare in the U.S., cases are still reported in the Southwest.

Livermore researcher Emilio Garcia notes that the seemingly subtle DNA differences among many *Yersinia* species mask important differences. One species causes gastroenteritis, another is often fatal, and a third is virtually harmless; yet all have very similar genetic makeup. Garcia's team is using a technique called insertion-sequence-based fingerprinting to understand these slight genetic differences. Insertion sequences are mobile sections of DNA that replicate on their own. Analyzing for their

Biological Warfare Has a Long History

The use of biological agents as weapons is not a new phenomenon, Lawrence Livermore's Tony Carrano points out. The Romans, for example, used corpses of diseased animals to poison the drinking wells of their enemies. During the horrific Black Death of the Middle Ages, the bodies of bubonic plague victims were catapulted over fortress walls of besieged cities.

During the French and Indian wars, 1754–1763, the British gave smallpox-infested blankets as gifts to the Indians because of their suspected alliance with the French. During World War II, Germany and Japan produced bacteria capable of infecting humans.

Biological attacks in the United States have been few and isolated. One occurred in 1984, when followers of Baghwan Shree Rajneesh poisoned several salad bars in Oregon with salmonella bacteria. In Europe, terrorist groups in Germany began producing botulinum toxin. In the late 1980s in Japan, the Aum Shinrikyo cult acquired anthrax bacteria and botulinum toxin and attempted to collect samples of Ebola virus.

Following the 1991 Persian Gulf War, United Nations inspectors revealed the vast scope of Iraq's biological arsenal. Iraq was found to possess more than 150 bombs and 25 missile warheads filled with botulinum toxin, anthrax, or aflatoxin. What's more, Iraq had built sophisticated laboratories to study and produce a wide range of biological agents and toxins.

presence will not only help refine signatures for *Y. pestis* but also shed light on how microorganisms evolve into strains that produce lethal toxins. This understanding, in turn, should give ammunition to researchers seeking an antidote or vaccine.

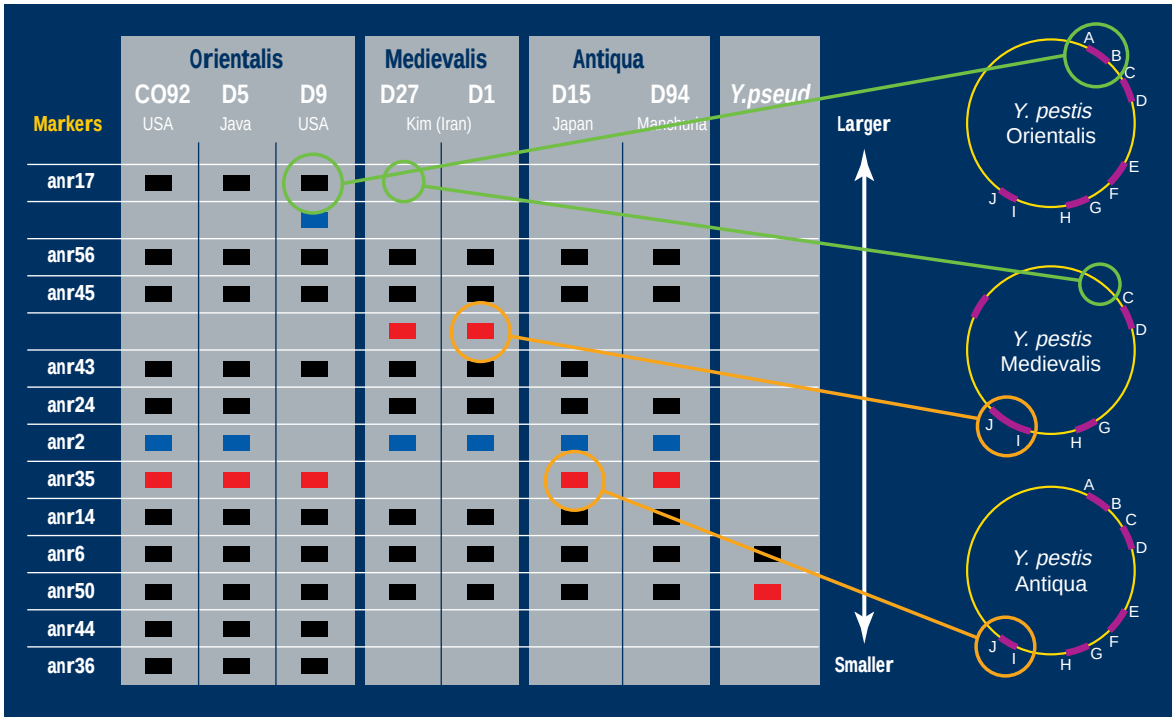
Garcia's team is collaborating with other world-renowned research centers to better understand the genetic differences among species and strains. A collaboration with France's Pasteur Institute is comparing the genetic complement of *Y. pestis* with another member of the *Yersinia* group (*pseudotuberculosis*) that causes an intestinal disease. "They are closely related, and yet they cause such different diseases," Garcia says.

Better and Faster, with More Uses

Livermore scientists are using a number of methods that allow more rapid identification and characterization of unique segments of DNA. Each method has advantages and drawbacks, with some more applicable to one organism than another. Weinstein expects that within two years, the Livermore team will have settled on a handful of techniques as the workhorses of signature generation.

In addition to the insertion sequence method, another promising technique is called suppressive subtractive hybridization. The method takes an organism and its near neighbor, hybridizes the DNA from both, and determines the fragments not in common as the basis of a signature. A team headed by Lawrence Livermore biomedical scientist Gary Andersen is working with colleagues at Moscow State University in Russia to advance the technique; one goal is to simultaneously analyze 96 strains of DNA.

Andersen's team has used suppressive subtractive hybridization to distinguish the DNA of *Y. pestis* from that of *Y. pseudotuberculosis*. The team has also used the technique to aid California's poultry industry by providing a handy way to detect *Salmonella enteritidis*. This bacterium can cause illness if eggs are eaten raw or undercooked. Subtractive hybridization results have been so



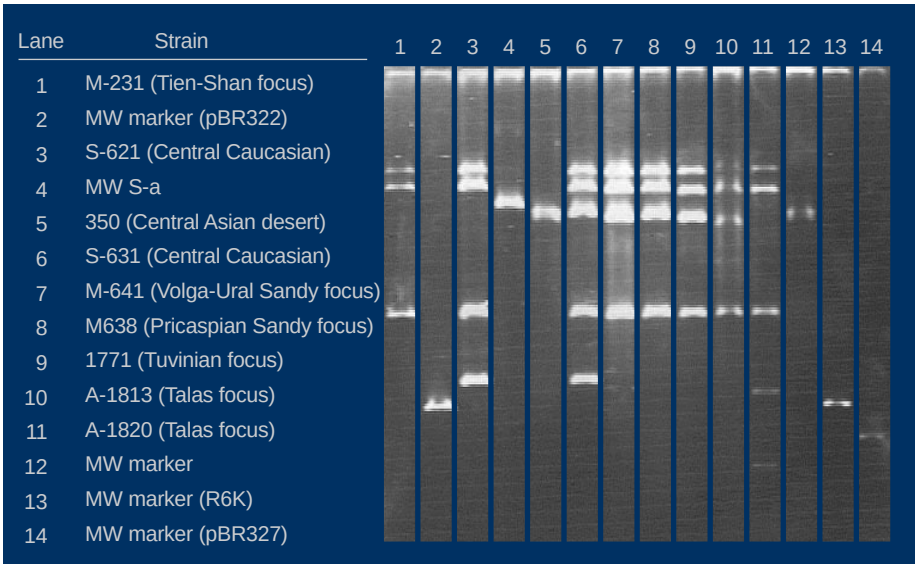
Insertion sequences are repeated sections of DNA whose location in the chromosome varies between different strains. Analyzing for their presence provides information about the type and biological function of a strain. The table at above left shows differences in the insertion sequence "fingerprints" of *Y. pestis* strains associated with the last three plague outbreaks. Red and blue rectangles indicate fragment shifts and changes from strain to strain. Some of these differences are graphically represented in the three strains of *Y. pestis* diagrammed at above right. For example, a fragment found in Orientalis is absent from Medievalis, and a fragment in Antiqua has shifted and become shorter than what it was in Medievalis.

successful that the signature can now be used to distinguish between subtypes of salmonella bacterium.

In addition to the DNA-based pathogen detection methods, researchers are developing detection capabilities using antibodies that can tag a pathogen by attaching to a molecular-level physical feature of the organism. Antibody assays are likely to play an important role in pathogen detection because they are generally fast and easy to use (commercial home-use medical tests use this form of assay).

Biological foundation researchers are working to improve these detection methods as well. For example, a collaboration with the Saratov Anti-Plague Institute in Russia is studying a bacteriophage (bacteria-killing virus) that only attacks *Y. pestis* and none of its cousins. Researchers recently discovered that the virus produces a unique protein component to attach to the bacterium cell wall at a certain site and gain entry. Garcia says that recognizing the distinct site could form the basis of a foolproof antibody signature. "If it's possible to achieve it with *Y. pestis*, we may be able to do it with other pathogens," he adds.

A Lawrence Livermore team has aided California's poultry industry with a biological signature to detect *Salmonella enteritidis*, a bacterium that can cause illness if eggs are eaten raw or undercooked. The signature can distinguish between subtypes of the bacterium and their different pathways to humans and other hosts.



Russia's Saratov Anti-Plague Institute is an important collaborator with Livermore in elucidating the subtle genetic differences among strains of *Y. pestis*. Above are some of the strains isolated by the institute.

Sensing Virulence

As more information about pathogens and their disease mechanisms becomes available and as genetic engineering tools to transplant genes become cheaper and simpler to use, the threat of genetically engineered pathogens increases. Biodetectors must be able to sense the virulence signatures of genetically engineered pathogens, or they will be blind to an entire class of threats. "Our ultimate objective is to identify several specific virulence factors that might be used in engineered biological warfare organisms so that we can detect these engineered organisms and break their virulence pathway," says Weinstein.

One key factor useful for detecting engineered organisms is an antibiotic resistance gene. When transplanted into an infectious microbe, the gene could greatly increase the effectiveness of a biological attack and complicate medical response. Some antibiotic resistance genes are widely shared among bacteria and are easily transferred with elementary molecular biology methods. In fact, a standard biotechnology research technique is introducing antibiotic resistance genes into bacteria as an indicator of successful cloning. "We need to be able to rapidly recognize such genes so that the medical response is appropriate," says Weinstein.

Another telltale indication of genetic tampering is the presence of virulence genes in a microbe that should not contain them. Virulence genes are often involved in producing toxins or molecules that cause harm or that simply evade a host's defense. "If a series of genes is made available to perform their functions at the right time, they could cause real damage," says Lawrence Livermore molecular geneticist Paula McCready. If interfering with the action of one of these genes or its proteins interrupts the virulence pathway, the disease process can be halted. Identifying and characterizing important virulence genes and determining their detailed molecular structure will greatly aid the development of vaccines, drugs, and other medical treatments.

As an example, *Y. pestis* disables the immune system in humans by injecting proteins into macrophages, one of the body's key defenders against bacterial attack. Because the protein acts as an immunosuppressant to disable the macrophage, understanding its structure not only would help scientists fashion a drug that physically blocks the protein but also would shed light on autoimmune diseases such as arthritis and asthma. A Lawrence Livermore team led by Rod Balhorn is working to determine the three-dimensional shapes of toxins such as the one produced by *Y. pestis* (see *S&TR*, April 1999, pp. 4–9).

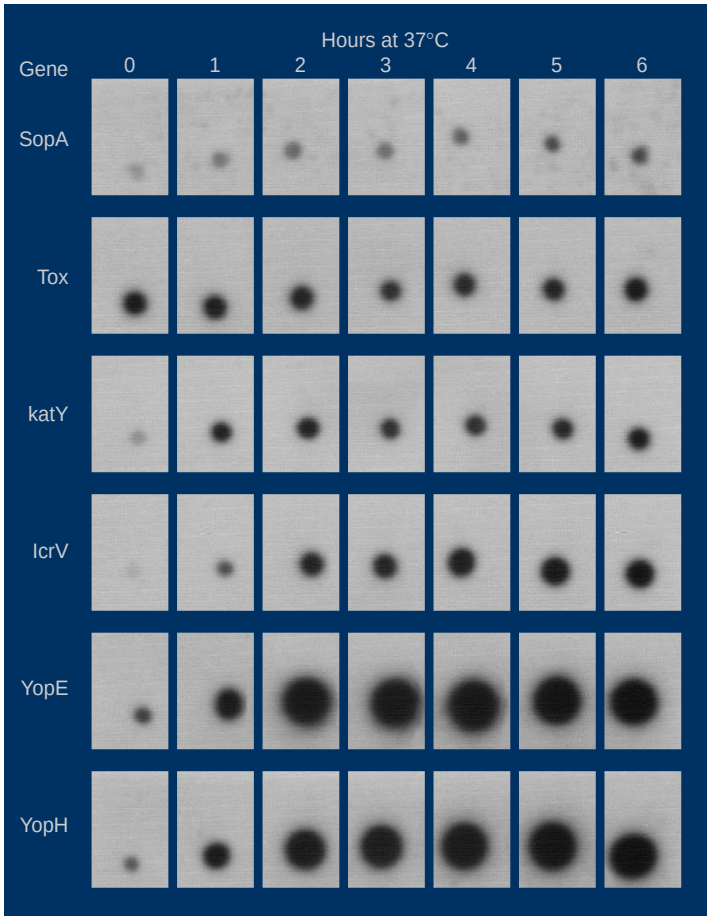
Virulence Genes in Common

Virulence genes spread naturally among pathogens and thus are also found in unrelated microbial species. Therefore, virulence genes alone are not sufficient for species-specific DNA-based detection. "We have to differentiate the virulence genes in natural organisms from engineered organisms," says Garcia.

Livermore researchers are using different methods for differentiating virulence genes from among the thousands of genes comprising the genomes of pathogens. One technique looks for genes that "switch on" (start making proteins) at the internal temperatures of mammals. For example, Livermore scientists are studying genes of *Y. pestis* that become much more active at 37°C. It seems a safe bet that many of these genes are associated with the bacterium multiplying within a warm-blooded host.

In 1998, a Lawrence Livermore team made an important contribution to understanding the genetics of *Y. pestis.* They sequenced the three plasmids (bits of DNA located outside the microorganism's circular chromosome) that contain most of the virulence genes required for full development of the bubonic plague in animals and humans. Plasmids sometimes transfer their genes to neighboring bacteria in what is called lateral evolution. (Antibiotic resistance genes are also located on plasmids.)

Garcia, who led the plasmid sequencing team, says that studying virulence genes can shed light on how new strains develop. The *Y. pestis* strain that causes bubonic plague, for example, may have evolved some 20,000 years ago. Such understanding is relevant to HIV, which may not have become infectious for humans until the 20th century.



As a way of identifying virulence genes, Livermore researchers look for bacterial genes that produce proteins at the internal temperatures of mammals (37°C). Four such genes (katY, IcrV, YopE, and YopH) in *Y. pestis* become much more active over a 6-hour period at 37°C.

Working with End Users

McCready notes that there needs to be a strong relationship between development of biological signatures and detection technologies and their end uses. Livermore researchers work with agencies that will be using signatures from Livermore and Los Alamos for both handheld detectors and field laboratories. "We want to make sure our tools get to the experts and agencies that need them," she says.

McCready is working closely with colleagues at the Bioterrorism Rapid Response and Advanced Technology Laboratory of the federal Centers for Disease Control and Prevention. Livermore is collaborating with the CDC to make diagnostic tools available to regional public health agencies and thus create a national mechanism for responding quickly to bioterrorism threats. Currently, many health agencies use detection methods that are not sufficiently sensitive, selective, or fast. For example, one culture test for detecting anthrax takes two days. Major damage and even death may have occurred in that time.

McCready emphasizes that DNA signatures will be thoroughly validated before being released, because their use might lead to evacuations of subways, airports, or sporting events, and such evacuations cannot be undertaken lightly. As part of the validation effort, Livermore scientists are characterizing natural microbial backgrounds to make sure that the signatures are accurate under actual conditions. To that end, researchers are collecting background microbial samples in air, water, and soil, as well as in human blood, urine, and saliva. McCready points out that *B. anthracis* is related to *B. thruginensis*, a naturally occurring harmless microbe that lives in dirt and can give a false positive reading to anthrax if the signature used is not adequately specific. The characterization effort is being aided by a device called the Gene Chip. Manufactured by Affymetrix Inc. and using technology developed by Livermore, the device simultaneously monitors the expression of thousands of genes.

Livermore researchers are looking ahead to a time when their efforts will have helped to equip federal and state agencies with a robust set of biological signatures crucial for America's response to any biological warfare threat. Equally important, the researchers envision a strong mechanism linking biomedical scientists with public health and law enforcement officials to develop new signatures speedily and cost-effectively to stay several steps ahead of terrorists.

—Arnie Heller

Key Words: anthrax, bacteriophage, biological signatures, biological weapons, Centers for Disease Control and Prevention (CDC), DNA, Gene Chip, plague, plasmids, virulence.

For further information, contact Bert Weinstein, (925) 422-5352 (weinstein2@llnl.gov).

About the Scientist

BERT WEINSTEIN is the deputy associate director of Livermore's Biology and Biotechnology Research Program (BBRP) Directorate. He received his B.S. in physics and mathematics from Brigham Young University and his M.S. and Ph.D. in physics from the University of Illinois at Urbana. He currently serves as leader of the biological foundations thrust area for the DOE Chemical and Biological Nonproliferation Program and as liaison for BBRP with the DOE Joint Genome Institute in Walnut Creek, California. At the Laboratory since 1974, he has held both research and leadership positions in four major programs: inertial confinement fusion, nuclear design, intelligence and national security, and the biology and biotechnology research program. He also served as a member of the Science Council for the Department of Energy's Office of Nonproliferation and National Security (now the Office of Defense Nuclear Nonproliferation).