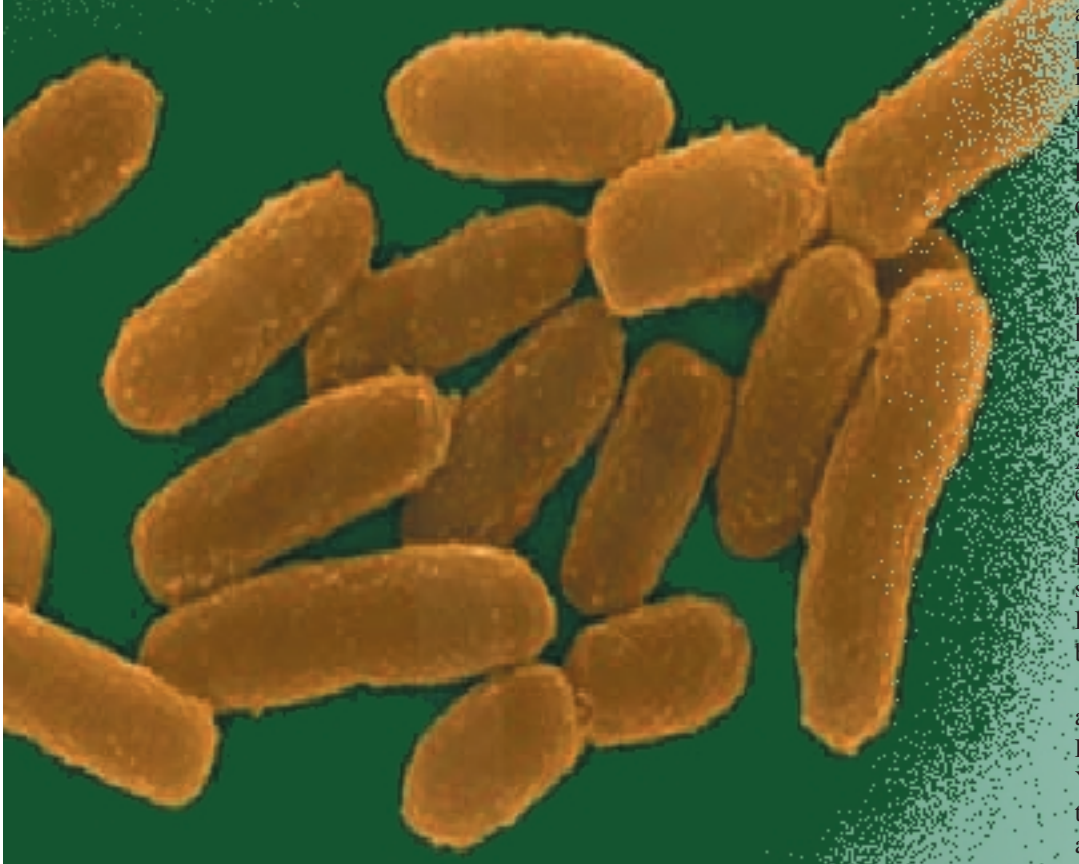
Tracking Down Virulence in Plague

How do the plague pathogen and its host interact? Scientists will apply the answer to understanding a larger set of possible agents of biological terrorism.



PLAGUE is potentially a deadly agent of bioterrorism. Unlike anthrax, which has been so much in the news lately, plague is highly infectious and can be readily passed from one person to another. The bite of a plague-infected flea or the inhalation of just a few cells of plague bacterium can kill. Like smallpox, plague can spread and kill large numbers of people very quickly. Fortunately, it can usually be treated with antibiotics.

History tells us how devastating a plague epidemic can be. In what is known as the Justinian epidemic, from 540 to 590 AD, plague spread from Lower Egypt to Alexandria to Palestine and on to the Middle East and Asia. At its peak, 10,000 deaths occurred every day in Byzantium. Eight hundred years later, in 1347, plague came to Italy from Asia or Africa, probably by ship. By 1351, fully one-third of Europe's population had died from bubonic plague.

This European epidemic is known as the Black Death or the Great Pestilence. In 1894, when Andre Yersin identified the tiny bacterium that causes plague, he named it *pestis* after the Great Pestilence. He tried to

name the genus *Pasteurella* after his mentor, Louis Pasteur. But *Yersinia*, after its discoverer, is the name that stuck.

Today, *Yersinia pestis* is one of several infectious diseases and agents of bioterrorism that researchers across the Department of Energy complex are studying as part of the Chemical and Biological National Security Program. This program comes under the purview of DOE's National Nuclear Security Administration (NNSA). At Livermore, the work on *Y. pestis* also receives support from Laboratory Directed Research and Development.

Scientists at Livermore have developed DNA signatures for *Y. pestis* that can be used to quickly detect and identify plague outbreaks. (See "Uncovering Bioterrorism," *S&TR*, May 2000, pp. 4–12.) Signatures for nine strains of the disease have been submitted to the Centers for Disease Control and Prevention in Atlanta, Georgia, where they are undergoing a rigorous validation process.

Livermore's DNA-based detection method proved its mettle in northern Arizona last June when it was used to identify a plague outbreak in prairie dogs in just four hours. Standard detection processes, which require growing the suspected bacteria in a laboratory, take 36 to 48 hours.

For a plague detector to be truly effective, it must do more than simply indicate the presence of a specific organism known to cause plague, says Pat Fitch, who leads Livermore's Chemical and Biological National Security Program. The detector also must be able to identify the specific traits found in atypical plague-causing organisms. Scientists know of several hundred strains (or isolates) of *Y. pestis*, and they do not all behave in precisely the same way. A few strains are believed to have been genetically modified or engineered to be more deadly. There have also been two clinical cases of naturally occurring antibiotic-resistant plague. Knowing the precise identity of a strain of plague—or of any infectious disease, for that matter—could help physicians treat a patient properly.

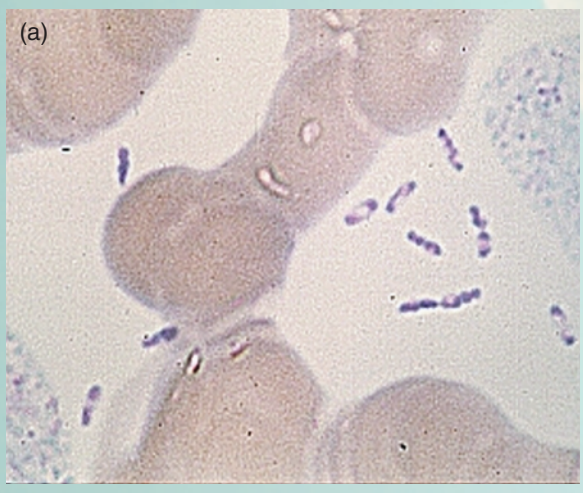
Plague research at Livermore currently is focusing on what makes *Y. pestis* so virulent and able to overcome the defenses of a host organism. Fitch is leading the Pathogen Pathway Project, using plague as a prototype for the functional genomics of a larger set of pathogenic agents that could be used in biological terrorism. (See the box on p. 6 for more information on functional genomics.)

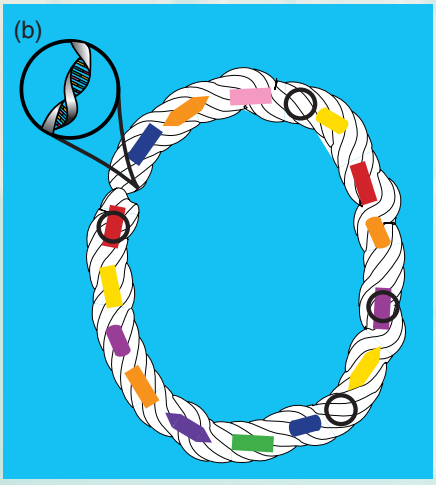
Besides building better detectors, work on the *Y. pestis* genome will also lead to a better understanding of pathogenicity and better vaccines and treatments for the disease. The ultimate goal, says Fitch, "is to produce a computer model that simulates the workings of a cell so that we can better manage exposure to pathogens."

Plague as Prototype

The highly contagious *Y. pestis* is an excellent model for studying the interactions of a pathogen and its host. In the case of *Y. pestis*, the host may be a flea, a rodent, or a human. Fleas carry plague bacteria and help transmit the disease. Once an infected flea bites a rodent or human, the bacteria begin to multiply in the new host, and their virulence shifts into high gear. *Y. pestis* circumvents the host's defenses by injecting into host cells a series of virulence factors that inhibit the response of the immune system.

Earlier research has shown that when *Y. pestis* is grown at the body temperature of a flea (26°C), its cells divide, but it does not express (turn on) many of the genes that make it virulent in rodents and humans. When the





(a) *Yersinia pestis*, which causes plague, is a pathogen likely to be used by terrorists. (b) Its DNA forms loops, unlike human DNA, which forms strands. Scientists studying it screen between two to five million of its nucleic acid bases to find unique regions (circled). Using polymerase chain reaction technology, the unique regions can be amplified thousands of times and processed to identify and characterize *Y. pestis*.

temperature increases to 37°C (human or rodent body temperature), the bacterium begins to produce the proteins essential to its virulence. This virulence mechanism can be induced in the laboratory, making plague relatively easy to study.

Examination of the *Y. pestis* genome before and after virulence has been induced shows what genes have been turned on. But that information is not enough to show precisely which genes are responsible for various aspects of virulence.

For comparative purposes, a Livermore team led by microbiologist Emilio Garcia collaborated with the Institut Pasteur in France to sequence *Y. pseudotuberculosis*, the parent organism of *Y. pestis*. Although their DNA sequences are about 95 percent identical, *Y. pestis* and *Y. pseudotuberculosis* behave differently. *Y. pseudotuberculosis* lodges in the intestine and causes flulike intestinal distress. *Y. pestis* is also closely related to the mild-mannered *Y. enterocolitica*, an intestinal bug that is itself very much like *Y. pseudotuberculosis*. *Y. enterocolitica* is currently being sequenced by the Sanger Center in Great Britain.

"Bacteria evolve very efficiently and make use of about 80 percent of their DNA," says Fitch. By comparison, humans use only about 30 percent of their DNA. Aiding speedy evolution are the many insertion sequences in a bacterial genome. Insertion sequences are bits of DNA that allow large regions of DNA to replicate themselves and move around the genome, relocating themselves somewhere else. When an insertion sequence lands within a gene, it deactivates that gene. These transfers can also occur across species, and it is not difficult for a bacterium to grab DNA from another bacterium.

Y. pestis evolved from *Y. pseudotuberculosis* within the past 15,000 years, a rapid evolution even for bacteria. "Something happened then to cause *Y. pestis* to learn how to live in a flea," says Garcia.

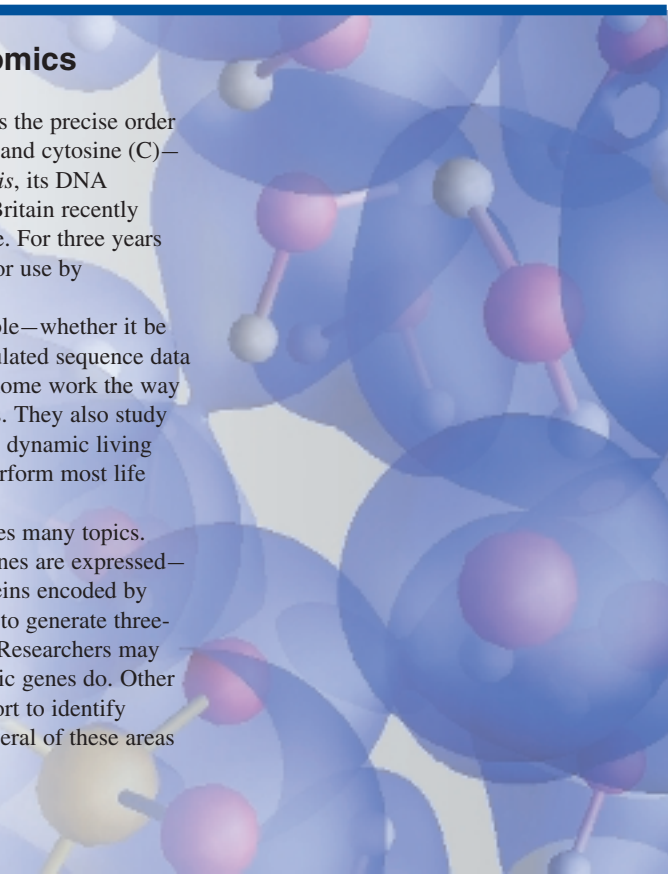
In addition to their normal chromosomal DNA, bacteria may have smaller circles of DNA known as plasmids. Plasmids replicate separately from chromosomal DNA and often

From Sequencing to Functional Genomics

DNA decoding, known as sequencing, is the process that determines the precise order of the four nucleic acid bases—adenine (A), thymine (T), guanine (G), and cytosine (C)—that comprise the DNA of all living things. In the case of *Yersinia pestis*, its DNA sequence comprises 4.66 million bases. The Sanger Institute in Great Britain recently published the complete and annotated sequence of the *Y. pestis* genome. For three years before that, a preliminary version of the sequence had been available for use by researchers throughout the world.

As the DNA genome, or parts list, for an organism becomes available—whether it be for plague, mice, or humans—researchers begin to examine the accumulated sequence data very closely. They are trying to identify what makes this particular genome work the way it does—its wiring diagram, so to speak. They search for specific genes. They also study how DNA works with proteins and the environment to create complex, dynamic living systems. Proteins are large molecules composed of amino acids that perform most life functions and make up the majority of cellular structures.

Functional genomics, as this field of research is known, encompasses many topics. Some researchers examine when, where, and under what conditions genes are expressed—that is, turned on. Others study the expression and function of the proteins encoded by certain genes. Still others use x-ray crystallography and other methods to generate three-dimensional structures of proteins, which offer clues to their function. Researchers may inactivate or knock out genes and study the results to learn what specific genes do. Other researchers compare the DNA sequence of several organisms in an effort to identify unique genes and interpret their function. Research is under way in several of these areas as Livermore examines what makes *Y. pestis* so virulent.



house genes that encode enzymes critical to the host cell or organism. For example, when a bacterium has become resistant to antibiotic drugs, it is usually because the bacterium has acquired a new plasmid.

One *Y. pestis* plasmid encodes at least two genes that allow *Y. pestis* to survive in fleas. Another plasmid is home to the gene that activates the disease's invasiveness. Researchers have found that *Salmonella* has a similar plasmid, which one bacterium probably obtained from the other.

"The interesting thing is that if you insert the three *pestis* plasmids into *Y. pseudotuberculosis* or *Y. enterocolitica*, you don't get *pestis*," says Garcia. "So something else is going on. Unfortunately, it's never simple."

Once its virulence genes have been turned on, plague infects its host using what is known as Type III secretion, an injection mechanism more colorfully called "*Yersinia's* deadly kiss." *Salmonella typhi*, enteropathogenic *Escherichia coli*, *Chlamydia psittaci*, various species of *Bordetella*, and other pathogenic bacteria appear to share this syringelike injection mechanism. This common trait may indicate another area of transferred genomic material.

Before Livermore's research on plague started, many of the genes critical for virulence had been identified but were poorly understood. The same was true for the underlying mechanisms of virulence. There was also little understanding of the gene and protein interactions that take place between the pathogenic bacteria and its host.

The Pathogen Pathway Project is using functional genomics tools to identify genes important to virulence and understand the pathways of virulence. The team's hypothesized pathway, from DNA to the host organism, is shown in the bottom figure at right.

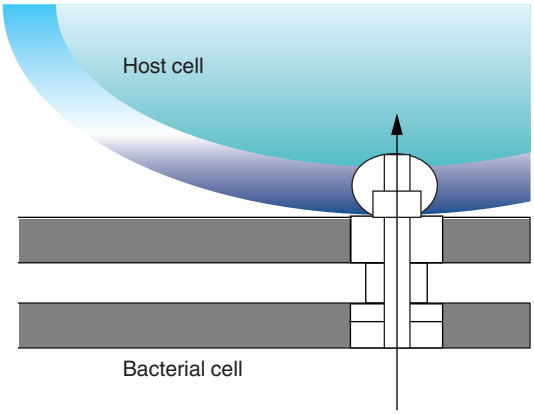
Expression and Function

An early task for Livermore bioscientists and computations experts was to develop a relational database of the DNA sequence of *Y. pestis*. In collaboration with the DOE Genome Consortium at Oak Ridge National Laboratory, these data were used to computationally predict where the 4,500 genes in *Y. pestis* are located and which genes might be associated with virulence.

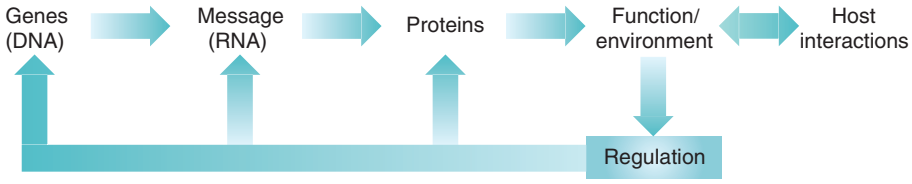
Next, Livermore bioscientist Vladimir Motin and colleagues designed chemical reagents for extracting over 300 genes from *Y. pestis* DNA, including all known virulence-associated genes on the plasmids. In an initial test, they extracted 85 genes associated with virulence and spotted them on a glass microscope slide alongside 11 control spots, making up a 96-spot microarray.

A microarray permits scientists to study the response of thousands of genes or other pieces of DNA quickly and efficiently in a process known as transcript profiling. In the process, each gene receives some kind of stimulus, causing it to turn on and produce messenger RNA (mRNA). In the case of plague, the stimuli are changes in temperature and calcium concentration. The production of mRNA leads, in turn, to the synthesis of unique proteins. The level of mRNA can be measured for each individual gene. The more active or expressed genes there are, the more mRNA will be present.

For the 96-spot microarray, the team developed a protocol to study the response of *Y. pestis* genes under conditions that mimic the infection process: at both flea and human/rodent body temperatures, 26°C and 37°C, and



The Type III secretion, a syringelike injection mechanism more colorfully called "*Yersinia's* deadly kiss," which is how plague infects a host once its virulence genes have been turned on.



A schematic diagram of information that is hypothesized to describe the pathways of virulence in a pathogen. The regulatory (feedback) loop is often nonlinear, and there can be multiple feedback paths with complex interactions.

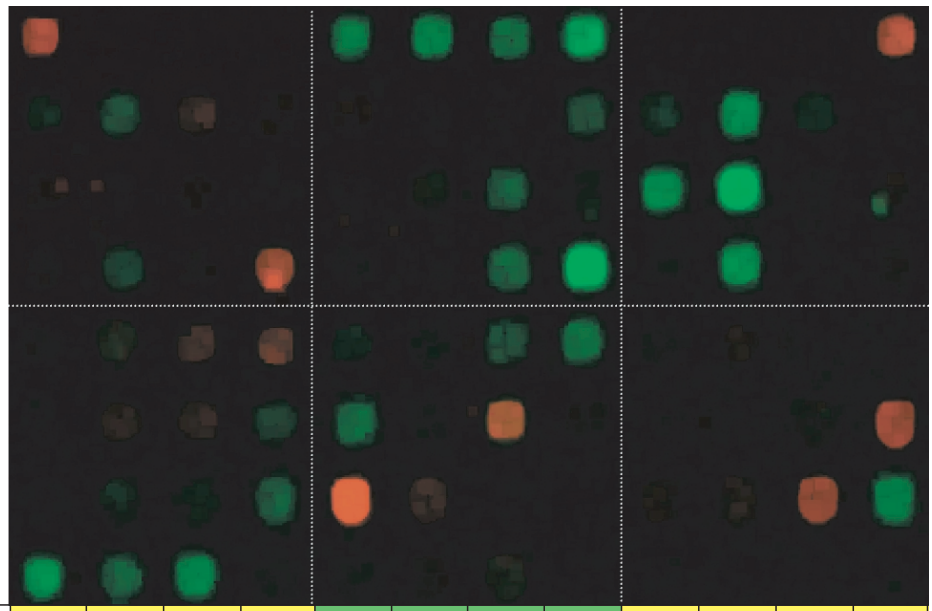
at calcium levels that correspond to those of blood (higher level) and organs (lower level), the latter location being where more virulence genes are expressed.

More recently, they developed a microarray for all 4,500 *Y. pestis* genes. All of the genes are being mapped at six time intervals as temperature rises and calcium concentration drops. The team is thus beginning to establish a timeline for how and when genes change and are expressed while the plague bacterium is infecting a human host. Some genes are expressed early, while others are late-onset genes. A detailed picture of how the bacterium behaves during the infection process will provide useful information for the development of diagnostic techniques and treatment methods.

Garcia and other researchers also completed a detailed analysis of three *Y. pestis* plasmids, which allowed them to confirm the location of several known virulence genes and to uncover four novel ones believed to contribute to virulence. Computerized comparisons with other genomic databases indicated the presence of a large number of virulence-related genes that are similar in both closely related bacteria such as *Y. pseudotuberculosis* and distantly related bacteria such as *E. coli*. The team also found numerous gene coding regions whose function they could not determine.

Using a proteomic approach of protein separation techniques and mass spectrometry (MS), Livermore researchers led by Sandra McCutchen-Maloney are analyzing complex mixtures of proteins isolated from *Y. pestis*. By comparing samples grown at the two physiological conditions mimicking the flea and the human (at 26°C and 37°C, respectively) and at low calcium concentration to induce virulence, the team is detecting differential protein expression to identify candidate proteins important for *Y. pestis* pathogenicity. Comparisons are also being made between human cells that have and have not been exposed to *Y. pestis* in order to understand the host immune response. Because it is the proteins that are actually responsible for virulence effects, the group is also working to correlate their proteomic data with genomic data obtained from microarray experiments. To learn more about the individual proteins responsible for virulence, the team is using various biochemical assays to test functional models of the candidate virulence

A 96-spot microarray for transcript profiling of 85 genes of *Yersinia pestis*; 11 of the spots are controls. Genes tagged with fluorescent dyes change color in response to various stimuli.



A	A	BRCA	XRCC	PKC	ypkA	yscC	yscO	lcrV	PKC	XRCC	BRCA	A
B	yopE	caf1A	sodA	envZ	ompR	yenI	rpoE	yopJ	yscB	lcrH	sycE	caf1R
C	tpx	rfaH	phoP	yenR	g6pd	rpoN	yopH	lcrF	lcrE	yopB	sycH	luxS
D	oxyR	psaA	glts	rpoS	lcrQ	yscW	tyeA	yopD	yadA	katY	hemF	toxR
E	rstB	nqrA	dha4	rpoH	yscL	yscU	sycN	yopM	pst	hns	cbl	flhC
F	nhaB	nadB	rpoD	yscH	lcrD	sycT	pla	catA	gcvA	copR	araC	nhaC
G	dam	yscG	lcrR	yopT	ymt	sodC	lysR	ilvY	tsaA	caf1	tuf	yscD
H	yscP	lcrG	yopK	caf1	sodB	crp	nhaR	fliA	h5	RAD	CDC2	Empt
	1	2	3	4	5	6	7	8	9	10	11	12

factors. In addition, McCutchen-Maloney's group is looking at host–pathogen interactions by using surface-enhanced laser desorption ionization (SELDI) MS to study various protein–DNA and protein–protein interactions within *Y. pestis* and between *Y. pestis* and the human host. For example, regulatory proteins that bind to genes and control differential expression are under investigation, as are the specific protein–protein interactions of suspected virulence factors. These molecular interactions are key to the genetic feedback that occurs as a pathogen infects its host, as shown in the bottom figure on p. 7.

Differences Are Key

Before Garcia's team completed its comparative sequencing of *Y. pseudotuberculosis*, microbiologists Gary Andersen, Lyndsay Radnedge, and others examined the differences between *Y. pseudotuberculosis* and *Y. pestis* using a different technique. This process, developed in Russia, is known as suppression subtractive hybridization (SSH). SSH identifies regions of DNA that are present in one species but absent in another.

SSH has the advantage of requiring only small amounts of genomic DNA. It can be used with any genome, even one that has not yet been characterized. It is especially useful for identifying the large genomic differences typically found between bacterial genomes. For example, SSH identified the genetic material that causes Kaposi's sarcoma, a skin lesion associated with HIV and AIDS. At Livermore, SSH has been useful for finding differences among anthrax strains and other potential agents of bioterrorism.

Comparison of *Y. pestis* and *Y. pseudotuberculosis* revealed seven DNA regions in *Y. pestis* that do not occur in *Y. pseudotuberculosis*. Four of them occur very closely to one another on the *Y. pestis* genome. "It is fair to assume that *pestis* acquired this region during its evolution from *Y. pseudotuberculosis,*" says Radnedge.

To learn more about the function of genes in these areas, Garcia and others are beginning "knock-out" studies. They will inactivate, or knock out, one gene at a time and test the resulting bacterium on an animal to see how the host and its genes respond. This is slow, laborious work, but it will help to determine what the function of each *Y. pestis* gene is, if any, and what gene or genes in the host are expressed as a response. This detailed examination of pathogen–host interaction for plague will be the first of its kind.

Being Prepared

Research to date on plague lays the groundwork for additional work planned at Livermore in the areas of microbiology, proteomics (the global study of proteins), bioinformatics (the integration and analysis of biological data), and biological modeling for the NNSA's Chemical and Biological National Security Program. Some of the research will elaborate on plague, some will examine a broader spectrum of human pathogens, and some will further the development and use of biodetectors, mass spectrometry, and other technologies.

In the U.S. today, plague pops out of the rodent population and into the human populace occasionally in the desert Southwest. It is a larger problem in a few other countries. But the real fear is that plague could be used as an agent of mass destruction. At least in industrialized countries, it is unlikely that plague would cause the huge number of deaths that occurred during earlier epidemics. Better sanitation, a more educated populace, and a far superior medical system would likely prevent that. But the world needs to be prepared.

—Katie Walter

Key Words: bioterrorism agents, Chemical and Biological National Security Program, plague, surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS), suppression subtractive hybridization (SSH), virulence, *Yersinia pestis*, *Yersinia pseudotuberculosis*.

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