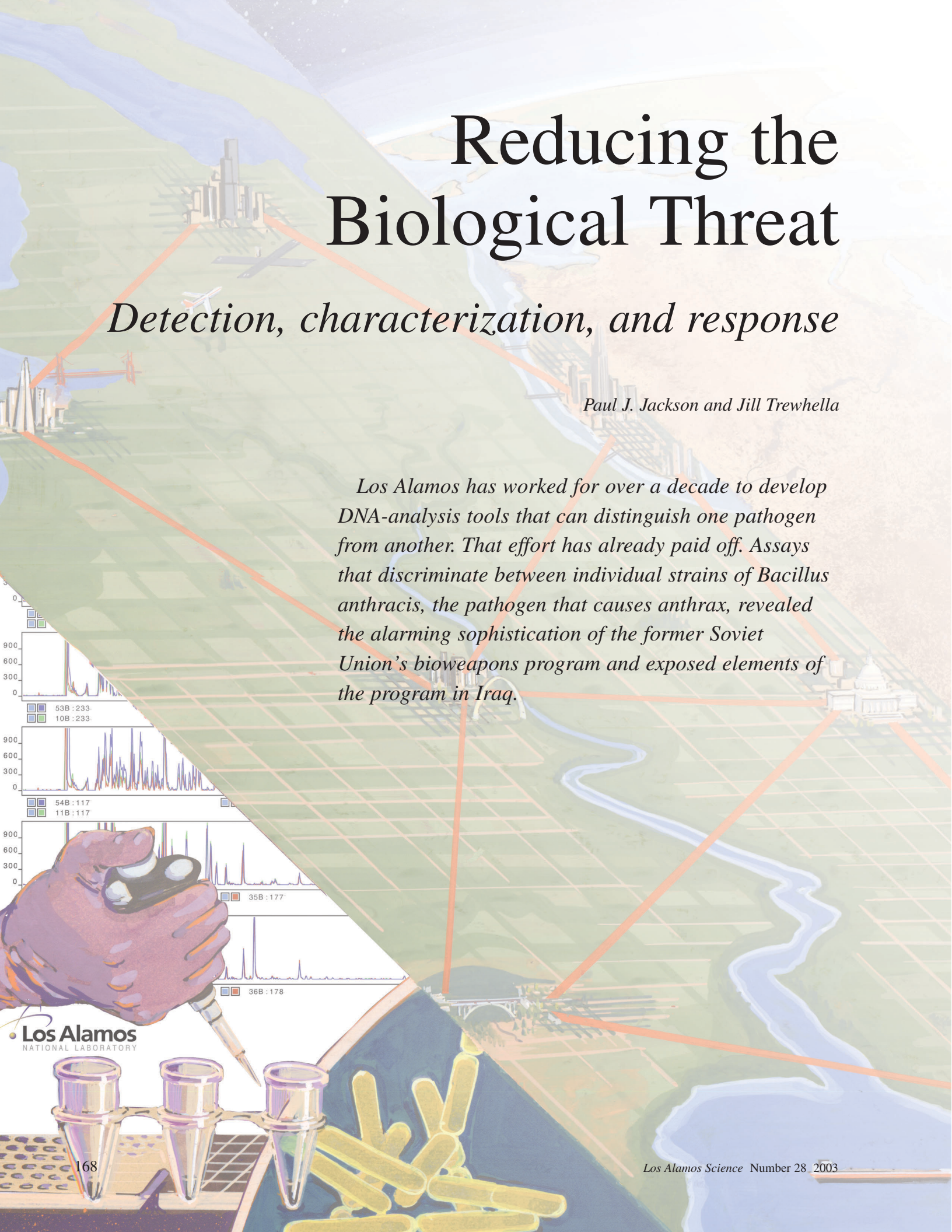
Reducing the Biological Threat

Detection, characterization, and response

Paul J. Jackson and Jill Trewhella

Los Alamos has worked for over a decade to develop DNA-analysis tools that can distinguish one pathogen from another. That effort has already paid off. Assays that discriminate between individual strains of Bacillus anthracis, the pathogen that causes anthrax, revealed the alarming sophistication of the former Soviet Union's bioweapons program and exposed elements of the program in Iraq.



From our beginnings, disease-causing microbes have taken their toll on human populations, sometimes in devastating numbers. In the fourteenth century, the Black Plague killed 25 million people, or one-third of the European population, and the 1918 Spanish Flu pandemic resulted in 30 to 40 million deaths worldwide. As man evolved and looked to defending or expanding his territory, it did not escape his attention that the agents that cause disease could be used effectively as weapons. As early as 1346, during the siege of Kaffa, the Tartar army hurled corpses of plague victims over the walls of the city, an action that led to the eventual surrender of Kaffa's inhabitants. In the spring of 1763, during the French and Indian War, blankets laden with the remnants of smallpox sores from infected British troops were collected and given to the Indians allied with the French while they attended a conference at Fort Pitt. Thus, a devastating smallpox outbreak was unleashed in the previously unexposed Native American population.

In the nineteenth century, Louis Pasteur, Robert Koch, and others established the relationship between microbial pathogens and disease. From that point in history, efforts were made to isolate and propagate cultures of the different pathogens both for research into ways to understand them and protect human health and for use of those pathogens as agents of war. During World War I, German saboteurs in France infected horses and mules with *Bacillus anthracis* and *Burkholderia mallei*, the microbes that cause anthrax and glanders, respectively. Beginning in the 1930s and continuing through World War II, the infamous Imperial Japanese Army Unit 731 experimented with biological warfare in Manchuria. Those experiments, some of which were gruesome, resulted in the deaths of thousands of Chinese nationals. It is now well established that the former Soviet Union supported one of the largest and most sophisticated biological weapons efforts, whose legacy in terms of control and accountability of materials and expertise is a concern today. In the 1990s, the United Nations Special Commission, or UNSCOM, inspectors in Iraq eventually forced Saddam Hussein to acknowledge an active and diverse biological warfare program that included having agents loaded in munitions ready for delivery.

In September 1984, the prospect of non-state-sponsored terrorism gained attention as the Rashneeshee cult contaminated salad bars in ten restaurants in The Dalles, Oregon, by pouring vials of liquid *Salmonella typhimurium* culture over the foods. This contamination caused an estimated 751 cases of salmonella poisoning and is believed to be an attempt at influencing the outcome of the November elections. Most vivid in our memories, however, is the impact of five deaths and the infections resulting from the letters laced with *B. anthracis* spores and mailed to people in the media and Congress soon after the September 11 terrorist attack on the World Trade Center. Although quick medical responses contained the number of deaths, the closing down of the Senate Hart Building, the disruption and loss of confidence in the safety of the U.S. mail, the cost of cleanup, and treatment of tens of thousands of potentially exposed people raised our awareness of the potential impact of individual or state-sponsored bioterrorism. Los Alamos scientists became actively involved in the nation's response to the anthrax mail attacks because of expertise we started to develop more than twelve years ago.

The Challenge of Biothreat Reduction

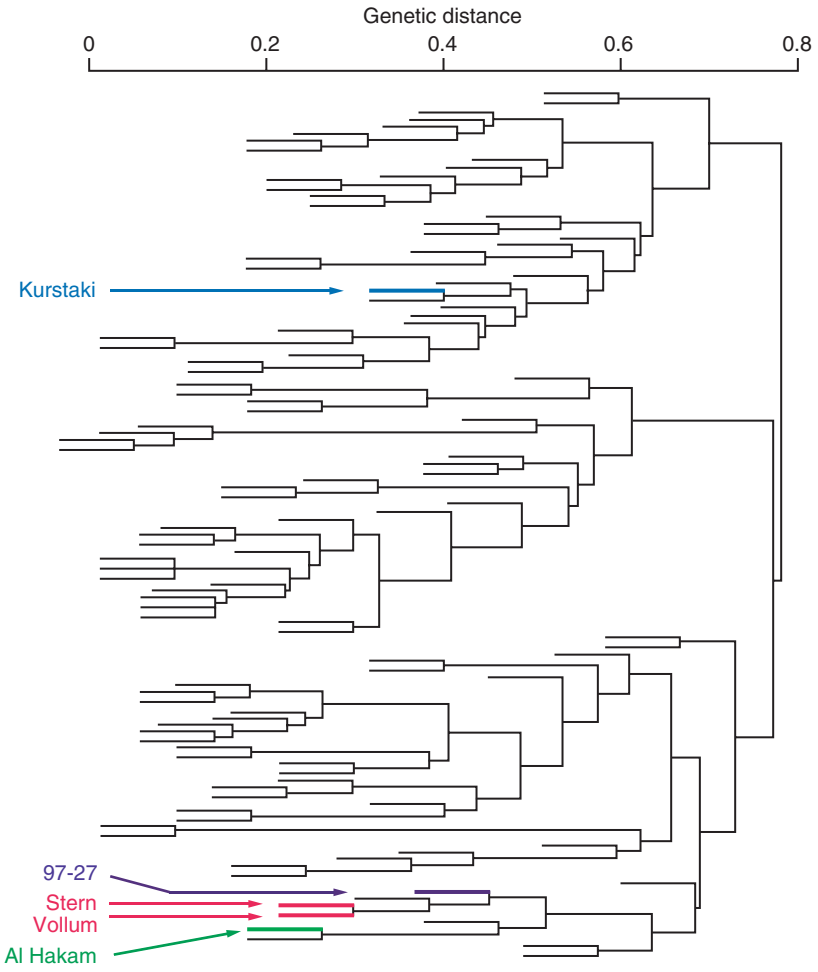
As early as 1991, scientists at Los Alamos began developing DNA-based methods for detecting and characterizing those biological agents that can be used as weapons. Our motivation was twofold. In addition to possibly identifying the presence of a threat agent and how we might deal with it, we needed to know whether the pathogen had been deliberately released, and if so, where it might have come from. Attributing an outbreak to its source could provide information to help mitigate the spread of disease in the population. In the realm of threat reduction, "attribution" can also deter individuals or nation states from using biological weapons for fear of being caught and having to bear the consequences.

Los Alamos was a logical place for federal agencies to come and ask for these capabilities. Almost from its inception, the Laboratory has had a significant bioscience program. At that time, our efforts were directed toward understanding the health effects of radiation. By the 1980s, this long-standing effort was intensely focused on molecular and cell biology and on exploring the hypothesis that individual susceptibility to radiation was programmed into our DNA. In particular, Charles DeLisi of the Department of Energy was among those arguing that the basis for this genetic susceptibility could be discovered if there were a complete human genome reference sequence. The ambitious idea to sequence the human genome later evolved into the multinational Human Genome Project, which led to unprecedented capabilities for genomic analysis. But this capability could be applied equally to microbial genomes, and as a result, Los Alamos entered the genomic era with two complementary missions: advancing the Human Genome Project and developing methods to detect and identify pathogens in environmental and laboratory samples.

Pathogen detection is challenging, however, because there are few genetic differences that distinguish a pathogen from a closely related nonpathogenic organism. It is not enough to detect the genes that make the organism threatening—the so-called pathogenicity or virulence genes—because these are often found in nonpathogenic microbes and

Figure 1. Genetic Variability and Phylogenetic Trees

A phylogenetic tree depicts the evolutionary relationship between different species. This is a partial tree, showing some of the pathogenic subgroup I of the bacillus family. Each branching point results from one or more mutations that generate a new, genetically distinct organism. Species that are near each other on the tree are closely related. "Closeness" is quantified by genetic distance, which can be estimated by summing the horizontal lengths of the branches that go from one species to another. (Vertical lengths carry no information.) The red branches are the *B. anthracis* region of the tree. (The blue, purple, and green branches are species discussed later in the text.) All *B. anthracis* species and strains are closely related and show limited genetic variability. On this tree, they fall within the two branches labeled Stern and Vollum. *B. thuriengiensis*, however, exhibits high genetic variability and is difficult to identify. Many bacteria on this tree have been called strains of *B. thuriengiensis* because of phenotypic (nongenetic) properties. Interpreting the results of a test without a thorough understanding of the tree is difficult even with DNA-based detection assays.



sometimes in microbes that are not even closely related to the target pathogen. Furthermore, given the aforementioned genetic mutability of microbes, there may be numerous "strains" of the same organism, some of which may be pathogenic and others not.

One must therefore know something about the pathogen's genetic diversity as such knowledge affects the detection results. Unfortunately, we still do not completely understand the diversity of all the threat agents. We know, for example, that a species such as *Burk. pseudomallei* (the causative agent of melioidosis, an infectious disease similar to glanders) is very variable. If *Burk. psuedomallei* must be detected and characterized, reagents that detect one strain are not likely to detect the others. We also know that *B. anthracis* exhibits very limited genetic variability, so reagents that detect one strain will virtually always detect the others. But there is less than 0.3 percent DNA sequence difference between *B. anthracis* and one of its closest nonpathogenic relatives. This common near neighbor is often found in environmental and sometimes even medical samples. If only the near neighbor is in the sample but the detection reagents cannot distinguish between it and *B. anthracis*, the sample will give a false positive.

The strategy to detect and identify pathogens must therefore incorporate several steps. One must first identify species-specific markers that are present in all strains of a pathogen but are not present in its close relatives. One then needs to develop a comprehensive phylogenetic tree (refer to Figure 1) that maps out the relationships among the pathogen strains and closely related species. Then one can begin to build a capacity for attributing a microbe to a particular source by overlaying information about the global distribution of species and strains on this tree and including isolates from known biological weapons activities.

Before addressing the science and technology challenges inherent in DNA-based detection and identification, we will note that antibody-based assays provide an alternative means of detection. Because they are easy to implement, such assays are commonly used in the field by first responders. Typically, a solution containing the

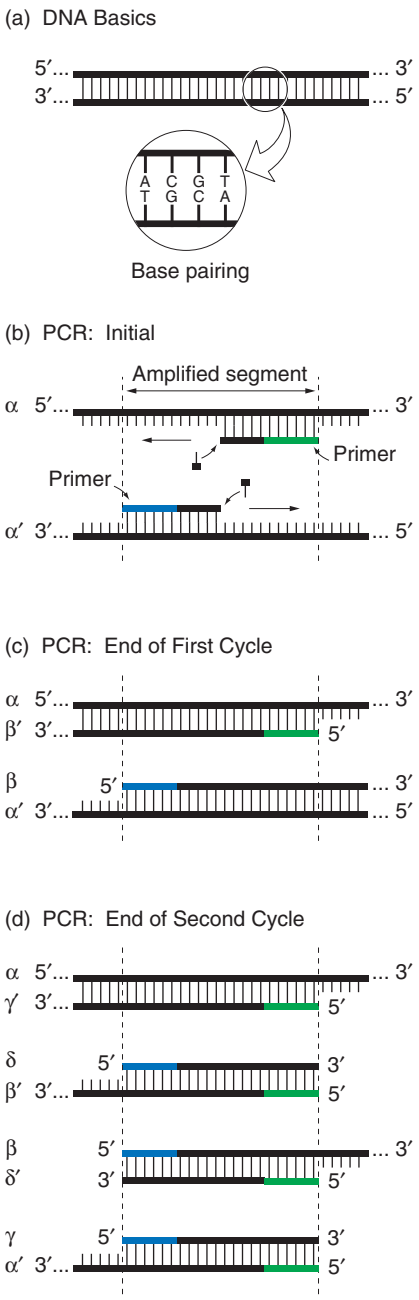


Figure 2. DNA Basics and PCR
(a) Double-stranded DNA is a sequence of nucleic acid bases (represented by the letters A, C, G, and T) attached to a sugar-based backbone. The bases always pair up: A to T and C to G. Pairing means that each strand can act as a template to replicate the other strand. Because of its molecular structure, the backbone runs in a particular direction, designated as 5′ → 3′, and the two strands run in opposite directions. (b) PCR is a technique for producing large quantities of specific DNA segments. Double-stranded DNA is placed in a vial, together with short pieces of single-stranded DNA (primers), an ample supply of DNA bases, and enzymes known as polymerases. The PCR cycle starts when the vial is heated to allow the DNA to split into its constituent strands (labeled α and α′). The temperature is lowered. Because of base pairing, the primers bind to specific sites that flank the segment to be amplified. The primers provide a starting point for the polymerase (not shown), which moves along each strand in the 5′→3′ direction, affixing the proper base to the growing DNA. (c) Both strands get replicated and form two double strands. (d) A second PCR cycle starts when the vial is reheated. The DNA splits into four single strands (α, α′, β, and β′), which are all templates for replication. At the end of the second cycle, there are four double strands, and two of the eight single strands (δ, and δ′) are the exact DNA segment of interest. After three cycles, there are 16 strands and eight copies of the desired segment. Thereafter, the desired segment amplifies exponentially and after about 30 cycles completely dominates the product.

sample is placed on an antibody-laden matrix (paper, plastic membrane, and others). In principle, the antibodies will lock only onto proteins that are associated with a specific pathogen, and that binding will trigger some detectable event, such as a change in the color of the matrix. But to date, antibody-based assays are neither specific enough nor sufficiently reliable to form the basis of a detection strategy. (This lack of reliability caused the government last year to recommend that HAZMAT teams stop using antibody tickets for *B. anthracis* testing.) Los Alamos is developing a new assay that has many of the desired properties of antibody detection but with improved reliability and specificity (see the article "Fluorobodies" on page 178).

Science and Technology Challenges

Our choice of DNA-based methods for pathogen detection stems from the relative stability of DNA in the environment. The DNA molecule contains coded information that can potentially be linked to a specific pathogen, and we can extract that information from samples even when the DNA is badly degraded, that is, broken into pieces and partly destroyed.

An initial concern was whether our methods would require more DNA than was available in the samples. Fortuitously, at the time we were focusing on this problem, a newly developed method, the polymerase chain reaction (PCR), was showing great promise for amplifying specific DNA sequences from samples containing millions of other microbial species (see Figure 2). Our first breakthrough was demonstrating that PCR-based methods could indeed perform this feat and amplify selected portions of pathogenic DNA sequences from very complex mixtures.

In principle, genomic sequencing can be used to uniquely identify every pathogen. But currently, high-resolution sequencing of even a small genome is expensive and time-consuming. Furthermore, so many microbes share so many different DNA sequences that most of the information is not very useful for species identification. For an unknown pathogen, finding the unique stretches of DNA is very difficult. A fast and efficient method of characterizing DNA sequences from a large number of different strains and species is to cut the total DNA from each microbe into fragments and look at the pattern of fragment lengths that is generated for each isolate.

We use restriction enzymes to cut DNA. These proteins cut the DNA double helix in two whenever they happen upon a specific, short sequence of typi-

cally 4 to 6 bases. On average, enzymes that recognize a sequence of 6 bases cut every 4100 bases, whereas those that recognize 4 bases cut every 250 bases. If one starts with a single DNA molecule of several million bases, then digestion with a single restriction enzyme will generate several thousand DNA fragments whose lengths are defined by the locations of the enzyme recognition sites within the original DNA molecule. Because of differences in their respective genomes, each microbial species or strain will have a different distribution of fragment lengths.

We create a "fingerprint" of a microbe or pathogen's genome by using an electric field to push the fragments through a slab of jellylike substance called a sizing gel. Because smaller fragments move through the gel faster than longer ones, the fragments separate over time. The emerging pattern corresponds to the original distribution of fragment lengths. This pattern is called a fingerprint and can be used to identify the microbe if a matching or similar fingerprint exists in an archive. Numerous methods for pathogen identification and strain discrimination depend upon this kind of DNA fingerprinting.

At Los Alamos, we have had considerable success developing and applying the fingerprinting approach known as amplified fragment length polymorphism (AFLP) (Jackson et al. 1999). AFLP is a way of culling from the large pool of DNA fragments a very small subset of fragments that have optimal lengths for sizing (see Figure 3). The technique uses a battery of reagents that are not specific to a single microbial species and can therefore identify pathogens even when one does not know what might be present in a sample. (The sample, however, must contain only a single species; otherwise, the result is an unidentifiable mishmash of fragments.) Furthermore, entirely new microbes (not previously observed)

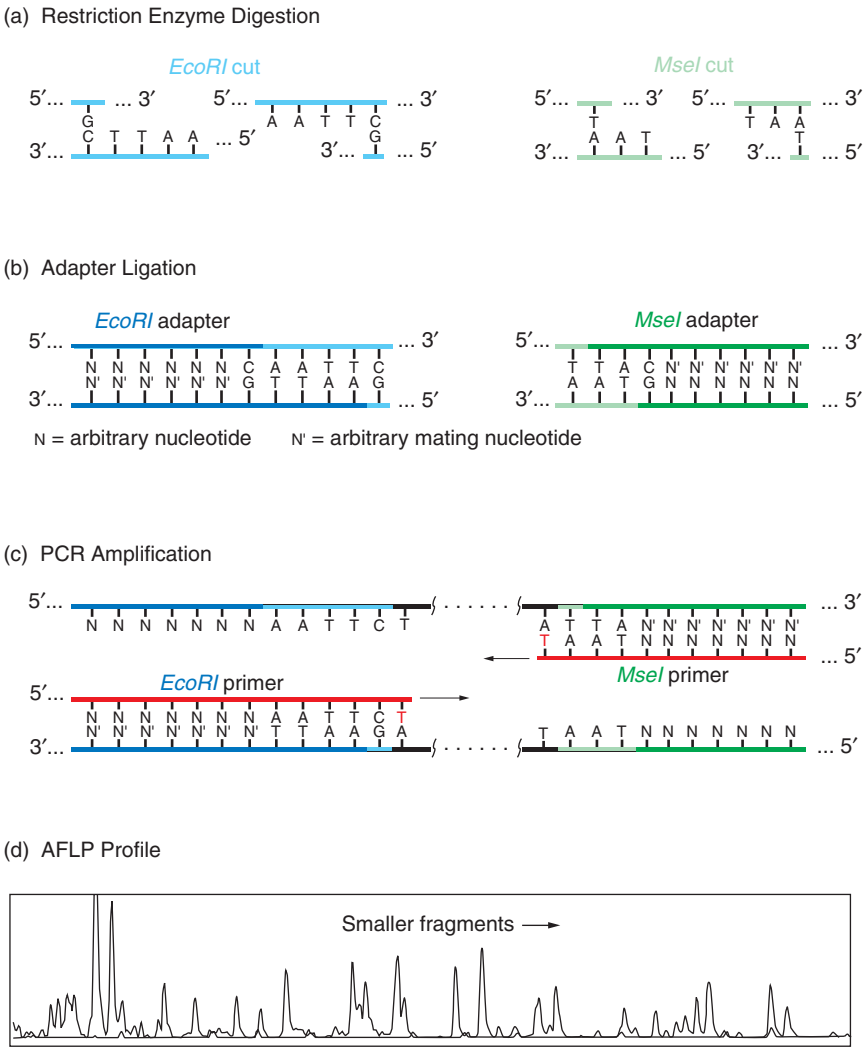


Figure 3. Identifying Pathogens by AFLP

(a) AFLP is a DNA fingerprinting technique that uses two restriction enzymes (for example, *EcoRI* and *MseI*) to cut a pathogen's genome into thousands of fragments. Each enzyme recognizes a short sequence of paired bases and cuts the double-stranded DNA at that site. The 5′ end of each cut strand is left with a few unpaired bases. (b) A set of double-stranded "adapters," which mate to the unpaired bases, are added and then "glued" (ligated) to each fragment. (c) A special set of PCR primers are added: one matching the *EcoRI* and adapter site and the other, the *MseI* and adapter site. The primers also have one or several extra bases (shown in red). Only the subset of fragments that have an *EcoRI* end, an *MseI* end, and the correct extra base (or bases) will get amplified. Enzymes, adapters, and the number of extra bases are chosen in such a way that PCR produces 100 to 200 fragments. (d) The fragments are sized on a capillary electrophoresis system (similar in function to the sizing gel discussed in the text). Smaller fragments travel through the capillary faster, so a detector focused on one spot of the capillary detects progressively larger fragments. A trace of the detector signal vs time consists of a series of peaks, and each peak corresponds to a different fragment size. The height of the peak is related to how much of a particular DNA fragment is present. To identify the pathogen, we compare this genetic "profile" with others from a database.

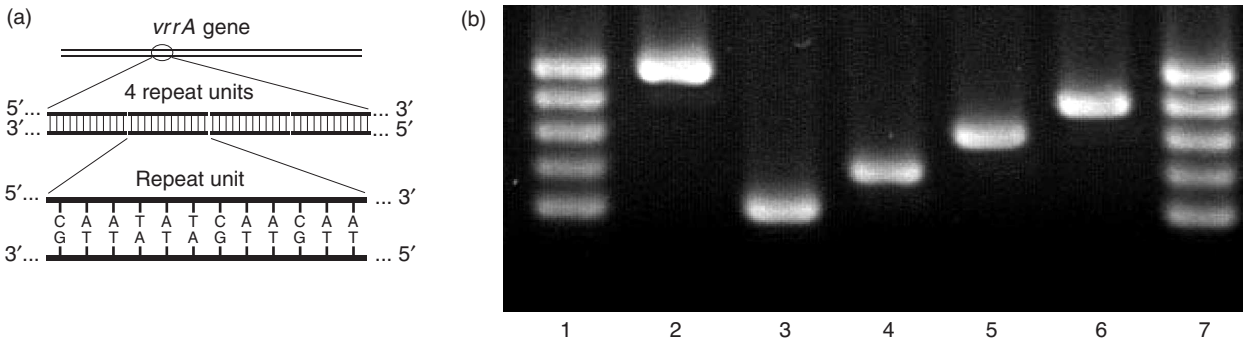


Figure 4. VNTR Strain Analysis

(a) The *vrrA* gene in *B. anthracis* contains a 12-base-long "unit" of DNA that will repeat, back to back, from 2 to 6 times. Different *B. anthracis* strains are associated with the number of repeats. (b) We use PCR to amplify the repeat region of the gene. The resulting DNA fragment will have one of five different lengths. We typically run fragments on a sizing gel to determine their size. Columns 2 through 6 in this gel show five different strains. Each band in the vertical direction corresponds to a fragment with an additional repeat unit. Columns 1 and 7 contain DNA fragments that were created to calibrate the size.

can be located on a phylogenetic tree and placed into a genetic context. For example, if we analyze an unknown microbe and 90 percent of its fragments match the AFLP fragments generated by *B. anthracis*, then this unknown is very closely related to *B. anthracis*. (In fact, the tree shown in Figure 1 was constructed with information obtained from AFLP profiles.) AFLP can also be used for strain discrimination, and was the first method to identify strain-variable DNA target sequences in *B. anthracis* (Keim et al. 1997).

A detailed analysis of AFLP profiles from different *B. anthracis* isolates showed that, although most DNA fragments in the profile were identical across all isolates, a minor set of fragments showed variations. These fragments contained what we call a variable number tandem repeat (VNTR) (Jackson et al. 1997). The VNTRs are relatively fast mutating loci in the genome that contain a variable number of short sequence repeats. For example, the VNTR found within the *vrrA* gene of *B. anthracis* can have five different lengths, depending on how many times a 12-nucleotide repeat is present in a particular strain (see Figure 4). These different lengths allow us to place all known *B. anthracis* isolates into one of five groups. (A group in this context contains those isolates that have the same number of repeats in the *vrr*A gene and can include different strains that are distinguished by variations in other parts of the genome.)

Methods focusing on VNTRs, such as multiple-locus VNTR analysis (MLVA), have been further developed and very successfully applied to recent real-world samples. Typically, pathogens have numerous locations in their genomes that harbor repeating units of DNA, and each pathogen has a unique set of loci. Together, the VNTRs from this set constitute a unique microbial signature that can be used for precise strain identification. For example, analysis of *B. anthracis* AFLP profiles from many different isolates identified eight VNTRs (Keim et al. 2000). These eight genetic markers divided all known *B. anthracis* isolates into 89 different groups.[1] Similar VNTRs are being identified for *Yersinia pestis* (Klevytska et al. 2001) other pathogens (Farlow et al. 2001).

Providing one knows what pathogen to look for, MLVA has some advantages over AFLP analysis. Because we design PCR primers to amplify only the VNTR-containing regions of a targeted pathogen, purified DNA from a single microbial species is not required for the analysis. However, in the absence of information about the target species present in the sample, MLVA analysis has limited value.

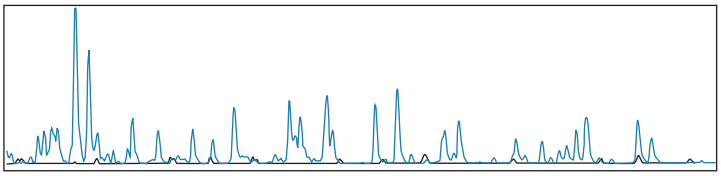
Recent advances in sequencing technology and methods for automation have presented us with another approach to MLVA that uses single nucleotide polymorphisms (SNPs) in genetic sequences. These are loci in the genome that show variations in sequence between strains involving only a single nucleotide substitution. In *B. anthracis*, such changes are rare and provide far less resolution than MLVA analysis. However, in more highly variable species, SNPs may provide as much or more resolution to allow distinguishing among strains. Where there is significant genetic overlap between closely related species, SNPs may provide the only means of differentiating between such species.

Largely on the basis of extensive AFLP and MLVA archives and analy-

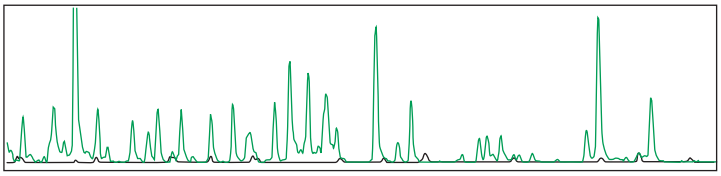
[1] A survey of the recently completed *B. anthracis* whole genomic sequence identified 28 more VNTR loci (Paul Keim, personal communication), increasing the number of different possible categories significantly. However, to date, all *B. anthracis* isolates fall into one of more than 100 categories.

(a)

(b) *B. thuringiensis kurstaki*



(c) Putative *B. thuringiensis* from Al Hakam



(d) *B. anthracis*—Vollum

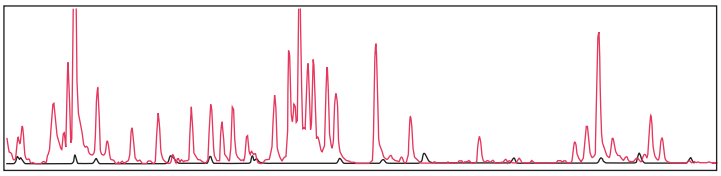


Figure 5. The Iraqi Sample
(a) The al Hakam facility in Iraq, shown in this photo, was the centerpiece of the Iraqi biological weapons effort. The Iraqis claimed that bacteria in samples obtained at al Hakam by weapons inspectors were *B. thuringiensis kurstaki*, a common biopesticide. (b) This capillary electrophoresis trace shows the AFLP profile of *B. thuringiensis kurstaki*, whereas (c) shows the AFLP profile of the isolate obtained from al Hakam. The profiles are dissimilar. (d) Our analysis revealed that the isolate was more closely related to *B. anthracis* –Vollum. (See the labeled branches in Figure 1.) In fact, the Iraqis had had in their possession a nonpathogenic surrogate for *B. anthracis*.

ses, *B. anthracis* has become one of the best characterized pathogens in terms of its genetic diversity and its relationship to its close relatives in the bacillus family (Jackson et al. 1997, Keim et al. 2000). The results of this mapping demonstrate that the genomic sequences of the closest *B. anthracis* relatives differ from the true pathogen by less than 1 percent, making identification of *B. anthracis*–specific DNA signatures quite difficult. To date, only a very few truly species-specific sequences have been identified. But recent work shows great promise in using computational methods to identify DNA signatures that can be used to position an unknown pathogen on a phylogenetic tree. (See the article "Analyzing Pathogen DNA Sequences" on page 182.)

Work to develop a detailed understanding of the phylogeny of different pathogens considered candidates for use as weapons continues today and is critical to the successful implementation of DNA-based methods for reliable detection and characterization of the range of pathogens we are concerned about. Detection systems that do not detect all strains of a pathogen may miss the presence of the pathogen when it is really there. Conversely, the use of DNA sequences or other targets that are not threat-agent specific can result in false positives, causing disruption and expense for those who rely on them.

Applying Our Methods to Bioterrorism

The first real-world application of our methods for pathogen detection and strain discrimination was the analysis of tissue samples from the victims of an anthrax outbreak in the former Soviet Union, near Sverdlosk, in 1979. Soviet authorities attributed the outbreak to contaminated meat. We analyzed tissue samples at Los Alamos in the 1990s (Jackson et al. 1998). VNTR profiles showed that the victims were infected by multiple *B. anthracis* strains. Because all natural outbreaks tested until then resulted from only a single *B. anthracis* strain, this finding strongly suggested that there had been intentional mixing of strains. Thus, our finding validated other indicators that the outbreak was due to an accidental release of *B. anthracis* spores from a Soviet biological weapons production facility. This scenario was eventually proved and acknowledged around the time of the fall of the Soviet empire.

We learned much about the sophistication of the former Soviet Union's bioweapons program from the analysis of these samples. For example, infection with multiple strains would complicate initial sample analysis. As a result, selection of an effective therapy would become problematic, opening up the possibility for the spore population to become resistant to multiple drugs or vaccines.

In the mid and late 1990s, we conducted an AFLP analysis on samples collected by UNSCOM inspectors in Iraq. In one case, a putative *Bacillus thuringiensis* isolate was collected at the Iraqi al Hakam facility. The Iraqis claimed the isolate was a subspecies of *B. thuringiensis* known as *kurstaki*, a bacterium widely used as a biopesti-

cide. Standard analysis methods could not dispute this claim. But AFLP analysis demonstrated that the sample was neither *B. thuringiensis kurstaki* nor any other closely related *B. thuringiensis* isolate. Comparison of its AFLP profile with our continuously growing collection of bacilli AFLP profiles shows that the sample was a nonpathogenic close relative of *B. anthracis*, with the same growth and spore production properties (see Figure 5). The Iraqis had an excellent surrogate for *B. anthracis* that had proved hard to identify by standard assays. Al Hakam was eventually acknowledged as the centerpiece of the Iraqi biological weapons production effort.

Another intriguing sample came to us even more recently (1998), again with a *B. thuringiensis* label. This time it was cultured from the infected wounds of a French soldier in Bosnia. Initial antibody-based analyses by other laboratories suggested that this was *B. thuringiensis*. Such results led to public concerns that *B. thuringiensis*–based biopesticides might be dangerous to humans in spite of 40 years of apparently safe use. As a result, European and North American regulatory agencies have been re-evaluating the use of this microbe. However, AFLP analysis of this sample shows that, like the al Hakam isolate, it is not closely related to the *B. thuringiensis* isolates that are used as biopesticides. Instead, its AFLP fingerprint suggests a very close relationship to *B. anthracis* and not to the insecticidal bacilli. (The purple arrow in Figure 1 indicates the identified species.) The story illustrates the problems that can arise when an assay with poor specificity yields a false positive (or when microbes with the same species name are erroneously assumed by regulatory agencies to have the same pathogenic properties).

New and Improved Pathogens

Until recently, the prevailing view has held that naturally occurring pathogens are sufficient to generate an arsenal of biological weapons and that there is no need to further manipulate these microbes to enhance their effectiveness. However, rapid developments of molecular tools to make specific changes to a microbe's genome, the expansion of our knowledge concerning the biochemical bases for virulence and pathogenesis, and identification of genes and pathways that affect these factors provide opportunities to deliberately enhance pathogenicity or perhaps to introduce the genes that can make a normally innocuous microbe pathogenic. These developments, coupled with publication by scientists from the former Soviet Union's biological weapons program of methods that outline the results of genetically engineering *B. anthracis* to confer resistance to the current anthrax vaccine (Pomerantsev et al. 1997), strongly suggest that genetically engineered biological agents for weaponization are already a reality.

Resistance to a variety of antibiotics can result from inserting specific genes into a microbe or from selecting naturally occurring resistant isolates from laboratory-grown cultures. Methods that detect these changes are essential to ensure the best medical response to an outbreak. Los Alamos assays can detect eight different single nucleotide changes within *B. anthracis* responsible for conferring ciprofloxacin resistance (unpublished results). We have also developed rapid PCR-based assays that detect many of the DNA molecules used to genetically manipulate this pathogen. As the scientific community continues to unravel the mechanisms underlying pathogenicity and virulence, such knowledge can be used to defeat these microbes—or to enhance them. We must continue to develop assays that will detect such manipulations.

The Future

The first step in responding to a naturally occurring disease outbreak or to an intentional release of a biological threat agent is detecting the presence of the microbe and determining those genetic characteristics that will provide information about its pathogenic properties and, perhaps, its source. However, the nature of biological agents requires their release before they can be detected. Therefore, we must pursue two important strategies, one being long term and the other, short term. Our short-term strategy should be to continue the development of rapid, sensitive, and accurate detectors and to develop strategies for their effective deployment. It should also include developing an effective means of intelligence to better track activities associated with the intentional production of biological threat agents. Our long-term strategy will be to better understand the pathogenic microbes responsible for these diseases so that we can effectively treat or prevent the diseases they cause. Our ultimate goal is to remove each of the threat agents from the threat list. The best approach to this end is to develop methods of effectively treating those who are exposed to the agent so that there is no health impact from an exposure. Such treatment strategies require a thorough understanding of the mechanisms underlying pathogenicity and virulence and the genes that encode these mechanisms.

The challenges to achieving these goals are huge and worthy of a major initiative. In the 1960s we set out for the moon, in the 1970s we started the "war on cancer," and in the 1980s we took on the fight against AIDS. At the beginning of the twenty-first century, we have been challenged to limit the spread of infectious diseases and prepare to defeat an adversary that might use biological weapons against our citizens, our crops, or livestock. As did the

preceding challenges, this new challenge will help drive advances in understanding and technology that will have broad benefits. While we are striving to more rapidly detect and characterize these pathogens and to understand the mechanisms underlying virulence and pathogenicity, our work will drive advances in biology, medicine, instrumentation, information technology, communications systems, and public health protection that will benefit us all. ■

Further Reading

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Jill Trewhella received her bachelor's degree in physics and applied mathematics in 1975 and master's in physics in 1978 from the University of New South Wales. She received her Ph.D. in chemistry from the University of Sydney in 1981. Following a postdoctoral research position in molecular biophysics and biochemistry at Yale University, Jill came to Los Alamos as a staff member in then Life Science Division. She had a joint appointment in physics to build a program in structural biology using neutron scattering. Jill has published a body of work focused on the structural biology of intracellular messengers and signal transduction in biomolecular networks. For her contributions, Jill was named Laboratory fellow in 1995 and a fellow of the American Association for the Advancement of Science in 1999. She is currently the Bioscience Division Leader.

Paul Jackson graduated from the University of Washington in 1974 with a bachelor's degree in cellular biology and received a Ph.D. in molecular biology from the University of Utah in 1981. Paul came to Los Alamos National Laboratory as a Director-funded postdoctoral fellow in 1981 and became a technical staff member in the Life Sciences Division in 1983. He was appointed a Laboratory fellow in 2001. He has worked on research related to biological threat reduction for many years and is recognized for his contributions to understanding variations among different strains of selected threat agents and for development of DNA-based detection and characterization of *Bacillus anthracis* and other threat agents.