Armed with a vast collection of strains and a refined DNA fingerprinting system, a research team in Arizona may help solve who's behind the anthrax attacks—and nail other bioterrorists in the future

Taking Anthrax's Genetic Fingerprints

ANTHRAX

This special news package looks behind the scenes at one of the labs helping with the anthrax investigation; a second story looks at what doctors and researchers have learned from the new inhalational anthrax patients.

▶ DISEASE DETECTIVES

AN OLD ENEMY REVISITED

FLAGSTAFF, ARIZONA, AND BATON ROUGE, LOUISIANA—Paul Keim loves talking about his work. But ask him whether he's been enlisted by the Federal Bureau of Investigation (FBI) to help identify the source of the anthrax spores that have so far killed five people in the United States, and there's a good chance he'll recite a line he knows by heart: "I can't deny or confirm that, but we would be pleased to work with any federal officials if they so request."

But it's hardly a secret that Keim, a geneticist at Northern Arizona University (NAU) in Flagstaff, is one of the key scientists in the current anthrax inquiry—and one of the very few who are not employed by a government lab. His role: to produce a genetic fingerprint of the anthrax spores used in every one of the mail attacks. Colleagues say his lab will soon receive two new samples, if it doesn't have them already: one from a letter to Senator Patrick Leahy (D–VT), discovered on 16 November, and one from the 94-year-old Connecticut woman who mysteriously died of anthrax last week. Keim's work should provide the most detailed description of the anthrax spores possible, and, when compared to the fingerprint of bacilli stored in labs around the country, it may help investigators home in on the perpetrator.

The assaults have turned life in Keim's lab on its head. Graduate students, logging long hours and working weekend shifts, find themselves in the thick of a suspense novel come to life—and having to keep quiet about it. Keim, walking around with two pagers, says he's getting calls from headhunters and has become a local hero in this small university town. The lab was inundated with media calls last month—which Keim says he has answered haphazardly—and TV vans lined up in front of the building. Meanwhile, security has been tightened: Armed police officers guarded the lab around the clock in the first days after the discovery of the first anthrax letter, while new iron doors and locks were hastily added.

But for Keim, a tall and cheerful Idaho native who joined NAU 13 years ago, the attacks were also a scientific challenge that came at just the right time. Keim's primary interest is how genomes evolve, and he has studied genetic variation in a wide variety of species, from microbes to endangered birds. But 5 years ago, with funding from the Department of Energy, he embarked on a program to develop fingerprinting techniques for a series of potential bioweapons.

Investigators. Paul Keim (left) is developing fingerprinting systems for several potential bioweapons.

CREDIT: GARY FOX

Bacillus anthracis—which heads almost every list of biothreats—was one of the first organisms he took on. In fact, his identification system for anthrax—using short pieces of repetitive DNA sequences that vary from one strain to the other—has worked so well that Keim was already shifting his focus to other potential bioweapons, such as *Brucella*, *Burkholderia*, and *Francisella tularensis*.

Other researchers in the field say Keim's strain-typing system for anthrax is the most advanced yet—and the way to go for several other organisms as well. "We, the anthrax community, have been very excited by what they have been able to achieve," says Peter Turnbull, a researcher formerly at the Centre for Applied Microbiology and Research in Porton Down, United Kingdom.

To develop a fingerprinting system for anthrax—or any other organism—state-of-the-art molecular tools are not enough. Another prerequisite is a large collection of strains that researchers can search for distinctive genetic differences. That's why Keim is quick to credit another lab for his current role as a disease detective: that of veterinary epidemiologist Martin Hugh-Jones at Louisiana State University, Baton Rouge. In the mid-1990s Hugh-Jones started building a huge anthrax collection that now comprises more than 1200 isolates.

Hugh-Jones, a transplanted Brit nearing retirement, chairs a World Health Organization (WHO) working group on anthrax and moderates animal disease reports for ProMED, a popular electronic mailing list about emerging infectious diseases. He's fascinated by the ecology and epidemiology of anthrax as a disease of domesticated and wild animals: where it arose, how it survives in the soil, and how human travel and trade have helped spread it around the globe.

Hugh-Jones was a member of a team of U.S. scientists who traveled to Russia in 1992 to investigate a 1979 outbreak of inhalational anthrax in the city of Sverdlovsk (now called Yekaterinburg). Two years later, one of the Russian scientists involved in the epidemic came to the U.S. with tissue samples from 42 of the patients. Hugh-Jones arranged for 11 of them to be tested for genetic traces of *B. anthracis* by Paul Jackson's group at Los Alamos National Laboratory in New Mexico. Keim, visiting Jackson's lab as part of a sabbatical, was specializing in strain typing and did part of the work.

The study, published in 1998, showed that the Sverdlovsk patients had been infected

Can Lab Sleuths Clinch the Case?

It would make a terrific episode of *Law and Order*: The police have finally nabbed a lonesome lab technician from Trenton, New Jersey, and charged him with sending anthrax-laced letters to members of the media and the Senate. But the evidence is circumstantial, and the prosecution's case hinges on the similarity between the DNA of anthrax spores found in his apartment and those found in the letters. Would the evidence hold up in court? At the moment, few researchers would bet on it, because microbial genetic analysis is not as standardized as human DNA analysis is. "It's very scary; none of us is fully prepared for that," says Paul Keim of Northern Arizona University in Flagstaff. "I'd hate to be across the witness stand from somebody like Barry Scheck," a high-profile lawyer with expert knowledge about human DNA fingerprinting.

Over the past decade, human DNA from blood stains, semen, or hair has become an accepted forensic tool, and debates about its reliability have subsided as the technology has advanced. But the rare cases when nonhuman DNA went to court—like one in which seed pods were traced back to a single palo verde tree in Arizona (*Science*, 14 May 1993, p. 894)—have been highly controversial. Similarly, "I would expect a fairly extensive hearing" about the admissibility of evidence linking a disease outbreak to a suspected bioterrorist, says David Kaye, a lawyer and DNA evidence expert at Arizona State University in Tempe. Even if the judge allowed such evidence, he says, lawyers would certainly try to sow doubts in the jury's mind by having other scientists highlight its weaknesses.

Whether DNA evidence would be allowed, says Kaye, would depend on how well accepted the underlying science was—in this case, the use of repetitive DNA sequences to identify pathogens. DNA evidence would be less likely to be accepted, he says, if it involved a technique that was still being developed—such as the anthrax typing system used by Keim—or if there was debate about the likelihood that two DNA samples would match.

At the moment, such debates seem highly likely if anthrax or other agents of bioterrorism are involved, says Keim. Human DNA is shuffled during sexual reproduction, giving each individual a set of genes that's unique in the world. But microbes reproduce asexually, and every new generation is an almost exact copy of its ancestor. With anthrax, especially, the genetic differences among strains—even if they're found in far-flung parts of the world—are extremely small, so an apparent match could easily be a coincidence. On the other hand, adds Keim, if the perpetrators used a very rare strain, DNA evidence might clinch the case.

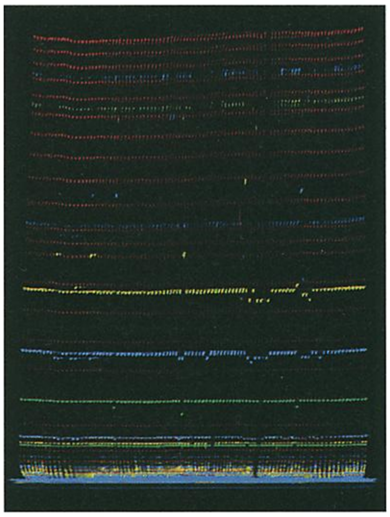


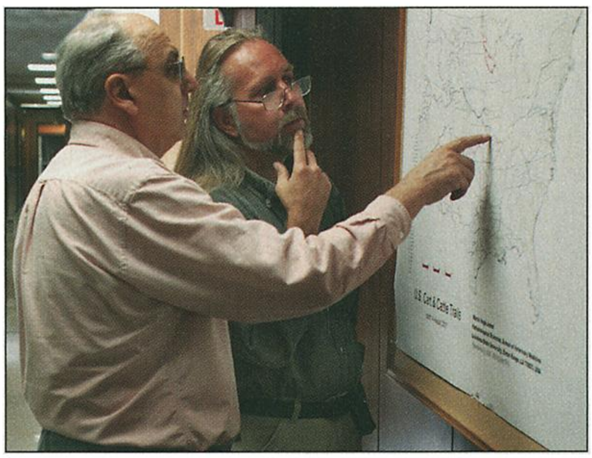
Exhibit A? Gel images like these could provide evidence about a microbe's origins.

To study the validity and credibility of microbial DNA tests, Keim plans to assemble a panel—he's hoping for support from the National Academies of Science or the National Science Foundation—to study the issue, much like past committees that hammered out consensus about human DNA testing. "We need to get some of these things figured out as a scientific community," he says. "If we're fighting like cats and dogs among ourselves, how are we going to convince a judge?" **–M.E.**

with four different strains of *B. anthracis*. This further bolstered the earlier group's conclusion, published in *Science* in 1994 (18 November 1994, p. 1202), that the outbreak had been caused by an accidental release of anthrax spores from a nearby military research facility—and not by contaminated meat, as Russian authorities had long insisted.

Keim and Hugh-Jones have worked closely together ever since. Collaborating with a well-known veteran in the field helped Keim, a relative newcomer, "break into the smoke-filled backrooms of anthrax," he says. It also gave him access to Hugh-Jones's collection. Through "a combination of persuasion, financial help, and blackmail," Hugh-Jones says he constantly pressures researchers and diagnosticians from around the world to send him anthrax collections and samples they have gathered. Over the years, 60 samples arrived from Turkey, 55 from Italy, 225 from China, and so on. The entire collection—believed to be the biggest and most diverse in the world—still fits in a freezer the size of a household refrigerator. (Keim also keeps a copy of every one of Hugh-Jones's isolates, so that the collection is now stored in both Baton Rouge and Flagstaff.)

Over the past 5 years, Keim's group has been refining a technique that Hugh-Jones needs for his epidemiologic studies—and the FBI needs for its criminal investigation: a way to tell different anthrax strains apart. (Researchers refer to an isolate as something they find in the field; a strain is thought to be a set of microbes that are genetically close, although what that means is not well defined.)



Mapping anthrax. Martin Hugh-Jones (left) and Kimothy Smith, a researcher in Keim's lab, study how cattle trails have helped spread the disease.

CREDITS: (TOP TO BOTTOM) JASON FARLOW AND DAN SOLOMON; HARRY M. COWGILL

Anthrax is probably the most genetically homogenous species known, says Keim: Until recently, every isolate ever found seemed to have exactly the same DNA as every other isolate. It was as if 1000 potential murderers all shared the same fingerprints, height, race, and eye color.

Historically, researchers have given only three isolates a specific name: Ames, isolated from a cow in Iowa; Vollum, a strain originally isolated in Britain; and Sterne, an avirulent strain widely used as a vaccine. But apart from the obvious fact that the Sterne strain causes no disease, researchers had no way of telling these three apart—nor any other member of their anthrax collections—some of which presumably are duplicates.

Researchers suspect this uniformity arises from anthrax's unique life cycle. After killing an animal, the bacteria sporulate and ooze with blood from the carcass into the soil, where they can remain dormant for years, decades, or perhaps centuries. Only when another animal comes into contact with the spores—it's unclear exactly what conditions favor this process—does the cycle start anew.

A Second Anthrax Genome Project

The bioterrorist assaults in the United States have spawned a scientific novelty: the first genome project ever triggered by a crime. Last month, the National Science Foundation (NSF) announced that it would give The Institute for Genomic Research (TIGR) in Rockville, Maryland, almost $200,000 to sequence the entire genome of the *Bacillus anthracis* strain used in the attack on American Media, a publishing company in Boca Raton, Florida.

TIGR had already sequenced more than 95% of the genome of the so-called Ames strain of *B. anthracis*, a common variety used in research labs. (The remaining hard-to-sequence part is expected to be in the bag within a few months.) The bioterrorists also used Ames—but their version may have subtle differences that set it apart from other Ames cultures. According to NSF, having a second anthrax genome will give researchers a better understanding of anthrax's diversity; and the project may just help officials investigating the case, says Maryanna Henkart, NSF's division director for molecular and cellular biosciences.

The genome project should reveal whether the perpetrators genetically altered the anthrax, for instance, to make it more virulent, says Arthur Friedlander of the U.S. Army Medical Research Institute of Infectious Diseases in Fort Detrick, Maryland. So far, he says, there's nothing to suggest that the microbes have been tinkered with—but that doesn't mean it didn't happen.

But the project is less likely to be of help in tracing the bioterrorists' identities, says Paul Keim of Northern Arizona University in Flagstaff. To do that, researchers cannot just compare the new strain with that already sequenced by TIGR; they'd have to sequence yet another strain—say, spores found in a suspect's lab or car—and compare the two new genomes. But the error rate of current sequencing technology—at least 1 in every 100,000 bases—would swamp the rare single–base pair differences between two closely related strains, says Keim.

Still, most researchers applaud the project, if only because it will for the first time give them the full sequence of the two plasmids—circular minichromosomes that harbor anthrax's virulence genes—of the Ames strain. The first TIGR project had skipped those because plasmids from different strains had already been sequenced by other groups. **–M.E.**

So, although anthrax may have caused vicious outbreaks for more than 10,000 years, its evolution has moved in slow motion.

In 1996, Kenneth Wilson's team at the Department of Veterans Affairs Medical Center in Durham, North Carolina, detected a single spot where anthrax strains varied genetically from each other. In that region, a short stretch of DNA was repeated two times in some bacteria and up to six times in others. Finally, researchers had a way to classify anthrax strains into five different groups, based on the number of repeats in this area.

Keim and his colleagues spent the next 3 years painstakingly searching for more of these so-called variable-number tandem repeats (VNTRs), or markers. By 1998, they had discovered seven new ones; using all of them and the VNTR discovered by Wilson, the team fingerprinted 426 isolates from Hugh-Jones's worldwide collection. The results, published last year, revealed that many of the strains were identical: The 426 isolates had only 89 unique genomes.

Then the genomic revolution came to anthrax. In 1999, Timothy Read of The Institute for Genomic Research in Rockville, Maryland, posted the bulk of the sequence of the *B. anthracis* genome online. Keim's colleague Jim Schupp vividly recalls searching the data for the first time: "I was enthralled," he says. "I could not sleep, I sat here until 4 in the morning staring at my screen." What Schupp saw—represented graphically as a series of boxes and lines—were hundreds of potential new VNTRs that could help discern infinitely more differences among anthrax strains.

Some of these newfound VNTRs have changed slowly over time, making them suitable for comparing strains that are far apart genetically; others have evolved rapidly, making them perfect for studying groups of closely related strains that were indistinguishable in previous analyses. Keim's group is now working with an arsenal of 50 markers and adding new ones all the time. "If we had known the stakes would be this high 6 months ago, we might have had 100 or 150 markers ready to go," says Keim.

Searching for spores. Pamala Coker of Louisiana State University, Baton Rouge, studies how anthrax disperses from a bison carcass in the Northwest Territories, Canada.

CREDIT: CHRISTY WICKENHEISER

Using that growing arsenal should make it possible to distinguish almost any isolate from any other, says Keim. Even within a conservative bug like *B. anthracis*, changes occur from generation to generation, he says, and some of the new markers could pick those changes up. The number of mutations may even provide an estimate of the number of generations between the original sample and one used in an attack.

That is exactly the kind of resolution needed to help solve a bioterrorist crime. Keim won't say whether his analysis has helped investigators much, except to repeat the scant information issued so far by Homeland Security director Tom Ridge: that the isolates from New York, Washington, and Florida are the same, and that they all belong to the so-called Ames strain.

But other researchers speculate that the FBI may learn far more from Keim's lab than they are letting on. Over the past 2 decades, the U.S. Army Medical Research Institute of Infectious Diseases in Fort Detrick, Maryland, sent the Ames strain to several research labs. And as it was passed around and grown in different labs, it may well have accumulated minute new changes. "The Ames strain can be many different things," says Hugh-Jones. "A very detailed fingerprint could reveal very minor variations."

That's why comparing the strain used in the anthrax attacks to those stored in freezers around the United States could well pinpoint the lab that the spores came from, says Keim. "So far, I haven't heard that any cultures have been subpoenaed," he says. "But that would be a logical next step."

A genetic fingerprint may also form part of the evidence if researchers ever apprehend suspects in the case—which both Keim and Hugh-Jones are convinced is just a matter of time—and anthrax spores are found in their home or possessions (see sidebar on p. 1811).

Meanwhile, anthrax researchers are grappling with the unsettling possibility that the microbes used in the attacks could have come from their labs. "I've given that a lot of thought recently," says Keim, who thinks the prospect is highly unlikely. Hugh-Jones, too, says he could think of no one fitting the bill when federal agents questioned him recently. But he did post the FBI's psychological profile of the perpetrator on ProMED, believing that some subscribers might have more information. "It's a small world," says Hugh-Jones. "I'm sure I know somebody who knows him."

–MARTIN ENSERINK