NEWS FEATURE

Can microbes keep time for forensic investigators?

Forensic scientists are building a "clock" from the bacteria and other microscopic scavengers that make up the postmortem microbiome. But how reliably will it tick?

Carolyn Beans, *Science Writer*

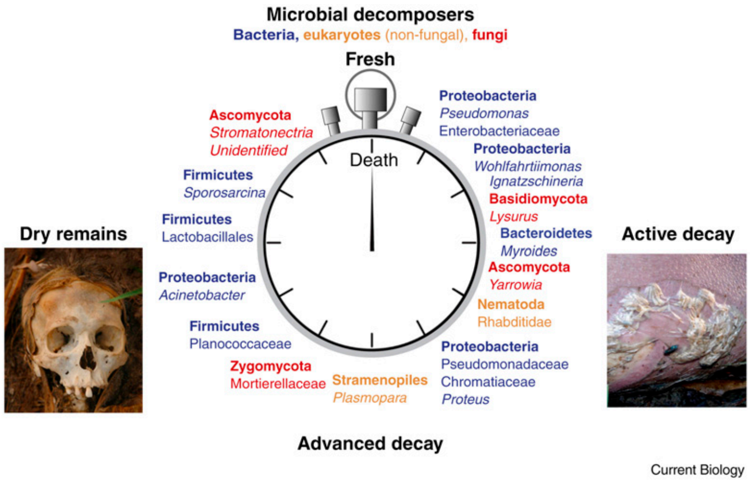
On a small hill outside of Grand Junction, CO, the sun beats on dry earth, and human bodies lie face up amid low sagebrush. Inside each corpse, on it, and beneath it, a rich ecosystem of bacteria, nematodes, and other microbes flourishes. As these tiny forces of nature—constituents of the postmortem microbiome—steadily decompose the remains, scientists document their every move.

At Colorado Mesa University's Forensic Investigation Research Station (FIRS), researchers are studying these bodies—now up to 11—to learn about the waves of microbes that bloom and fade at each stage of human decomposition.

With advances in microbiology and genomics and the declining costs of DNA sequencing, microbes are poised to play a big role in forensics. Researchers have shown that microbes have the potential to alter toxicology results, point to causes of death, and even place a suspect at the scene of a crime (1). If researchers can identify collections of microbes that predictably turn up at specific time points as a body decomposes, then this "microbial clock" could become a powerful tool for crime scene investigators looking to estimate the time and even the circumstances of death.

Recent studies suggest that a microbial clock may well exist. But before this forensic tool can be deployed at crime scenes and in courtrooms, researchers first must prove that the clock ticks reliably whether a body falls in lush forest or dry field, whatever the season.

In a fenced-in outdoor research area just beyond the laboratory at the Forensic Investigation Research Station near Grand Junction, CO, human remains are allowed to decompose so researchers can study the ebb and flow of resident microbes. Close-up photographs are not allowed due to privacy concerns. Image courtesy of Melissa Connor (Colorado Mesa University, Grand Junction, CO).



Jessica Metcalf and her team found that the microbial community in two human cadavers changed with each stage of decomposition in a field at the Applied Anatomical Research Center at Sam Houston State University in Texas. The researchers are now testing whether this microbial clock is repeatable across seasons and geographic areas. Reprinted from ref. 3, copyright 2016, with permission from Elsevier.

Beyond Blow Flies

Investigators rely on estimates of the time since death, known as the postmortem interval, to begin piecing together what happened to a victim and to validate or debunk the alibis of potential culprits. Many factors contribute to the estimate, says Virginia-based criminal defense attorney Betty Layne DesPortes, president of the American Academy of Forensic Sciences. "It can be as simple as the last time someone saw the person alive. Or looking at activity on credit cards," she says. "If you get beyond that, it can be things like the state of decomposition of the body."

"Estimating the time of death is basically an exercise in very broad probabilities," says forensic pathologist, Carl Schmidt of the University of Michigan, who is also the chief medical examiner for Wayne County, MI, which includes Detroit. He knows, for example, that it can take 24 hours for rigidity (the stiffening of muscles) and lividity (the settling of blood) to become fully established. But that metric still leaves ambiguity. "If you have partial rigidity, or lividity isn't fixed, what does that mean? I don't think anybody can say," Schmidt adds.

If a body lies long enough for decomposition to begin, forensic entomologists can make estimates based on a sort of insect clock—the fairly predictable ebb and flow of insect species that colonize a cadaver as it decomposes. Blow flies, for example, are often the first to arrive. The females lay eggs in wounds or natural openings such as the eyes and mouth. Forensic entomologists then use the stage of larvae development to narrow estimates of the postmortem interval to within days for deaths that occurred up to about a month before discovery.

But investigators can't always rely on insects, says entomologist Sibyl Bucheli of Sam Houston State University in Texas. "If somebody dies under questionable circumstances in a really modern high rise apartment," she explains, "the insect biodiversity is going to be highly reduced, if present at all." And insect evidence isn't robust enough to stand alone. "When I work on court cases, if the only piece of information you are relying on to put a person behind bars for murder is entomology, it is a really poor case," Bucheli says.

Outside of TV detective dramas, microbes haven't yet played a major role in criminal investigations. But microbial ecologist and evolutionary biologist Jessica Metcalf of Colorado State University, who leads the FIRS project and coined the term microbial clock, saw their potential in 2010 while working on extremely cold cases. After using ancient DNA to study Ice Age mammals, she wanted to move on to ancient humans. Collaborating with Rob Knight at the University of California, San Diego, who was studying forensic uses for the microbiome profiles of living people (2), Metcalf wanted to find a way to study the feces of ancient humans to understand their gut microbes. Because she was studying remains, she thought she should also understand the microbes involved in decomposition (3).

The Makings of a Microbe Clock

As director of FIRS at Colorado Mesa University, forensic anthropologist Melissa Connor helped design Metcalf's microbial clock study and oversees data collection at the site. But Connor does more than coordinate with researchers. When donors pass away, she fields calls from funeral homes and coordinates with families to receive new bodies.

Much of the knowledge in forensic entomology, as well as forensic taphonomy (the study of fossilization) has been gleaned from donations to FIRS or one of the other five anthropological research facilities, commonly known as "body farms," in the United States. Donors make this bequest for many reasons, says Connor. Some are forensics enthusiasts. Some want to avoid the cost of burial arrangements. Most are looking for some way to be useful even after death. The Forensic Anthropology Center at the University of Tennessee, Knoxville, for example, currently has more than 4,000 registered future donors.

"All remains are used for multiple studies," explains Connor. "All these incredible gifts people give us, we want to make the absolute best use of." For Metcalf's project, Connor's goal is to place 12 bodies in the dry sagebrush field, 3 in each season. With 11 bodies placed, they are nearly finished. At two other research sites, the Tennessee facility and the Applied Anatomical Research Center at Sam Houston State University in Texas where Bucheli works, colleagues are doing the same. In Tennessee, the bodies lie on well-drained clay under a canopy of oak, maple, and hickory. In Texas, they lie on sandy, acidic soils in a forest of loblolly and longleaf pines.

Connor and her team swab the skin on the cheek and the hip bone as well as the surrounding soil when they place a body, then repeat this ritual every day for three weeks. She and the directors at the other sites send their samples to Metcalf for DNA extraction. That DNA then goes to Knight for sequencing, and Metcalf and her team perform a series of genomics analyses that allows them to study entire pools of microbes at once. For example, they use the DNA sequences of a bacterial gene known as 16S rRNA to identify and gauge the relative abundance of species in each sample and a gene known as 18S rRNA to do the same for eukaryotic microbes such as nematodes and amoebas. Pieter Dorrestein of the University of California, San Diego, is now gearing up to conduct a metabolomics analysis on the samples to pick up the chemical signatures of microbes in the process of decomposing remains. "We think that it is very likely that as these microbial communities are going through a succession, the byproducts they're producing are also going to be changing," says Metcalf.

In all this data, Metcalf believes she'll pick up at least one reliable signal of microorganisms keeping time. It probably won't come, she says, in the form of a single metronomic microbe or even a series of individual species that pop up at different time points. She expects the clock will be made from groupings of microbial species that rise and fall in a complex, yet predictable fashion over time. "The power here is that we are not looking at individual species," Metcalf says.

Steady Ticking

The first bodies were placed at the three sites in the spring of 2016, and Metcalf expects to publish results from that season soon. Based on pilot studies and animal work, she has reason to be hopeful.

In 2011, Metcalf followed the microbiomes of decomposing mice over 48 days and found dramatic shifts in the microbial community that occurred at about the same time points for each mouse (4). The results were so consistent that Metcalf could use the microbiome to accurately estimate the time since death to within approximately 3 days, even at 48 days out. "It was a surprise to me even at that point how impressively repeatable the microbial succession during decomposition is across different mice with all of the variables controlled," she says.

In 2012, Metcalf placed mice on different soil types (5). Most of the decomposing microbes came from the soil, she notes, but changing the soil type did not affect the pace of the clock. "That was a real surprise," says Metcalf. "Certainly you could see that the biggest driver of change was time."

Metcalf had also studied four human cadavers at the Texas facility, one pair placed in winter and one pair in spring of 2013 (5). After she controlled for seasonal temperature and how it would affect microbial activity, Metcalf found the microbial successions were remarkably consistent across different human bodies and different seasons. Although humans and mice don't share the exact same postmortem microbial communities, microbes from the same families emerged at similar times in both species. The human microbial clock, it seemed, ticks much like that of the mouse.

In the case of Metcalf's current work in humans, the microbial species that make up the postmortem microbiome appear to vary at least in part by geographic area. But she's betting enough microbial signatures will remain constant. "Not every microbe in a community is equally important in a model," Metcalf says, noting that she employs machine learning to find the steady signals in the noise.

Flies in the Ointment?

To fine-tune models, researchers may also have to account for the diversity of the postmortem microbiome, tracking its multitude of contributors. At least some of these microbes are opportunistic members of the living-human microbiome (5). "Most likely there are some microbes that are just waiting for us to keel over," Metcalf says. Soil microbe populations are geographically diverse, too, potentially affecting which organisms are available to colonize a new corpse (6).

In a previous collaboration with Bucheli, Metcalf found that at least a small percentage of the postmortem microbiome may also be deposited by flies colonizing a body (5). In one study Bucheli designed, she and her team found that the community of microbes on flies visiting recently placed bodies varied in diversity and abundance across different seasons and even across the same seasons in different years.*

Still, Metcalf and Bucheli don't think these many potential sources of variability spell trouble for a microbial clock. First, the clock may keep time without relying on any of the microbes that vary by host, season, geography, or blowfly visitor. And even if these variations do prove to be important, the team expects they could be used to calibrate the clock to specific circumstances, rather than disabling it altogether.

> **"It would be really, really cool to find microbiome patterns that vary according to geographic location."**
> **—Carl Schmidt**

That, says Bucheli, is the approach forensic entomologists have long been honing. Instead of throwing off estimates, variation in insects often adds clues to a case; for example, insect activity might suggest that a body found in one climate actually came from a different climate. "I think we're going to see that with the microbes," says Bucheli. "If we do find changes or variation, it might be able to tell us fine level pieces of information."

True Crime Stories

Despite promising results in the FIRS project and others so far, the odds of finding a forensically useful clock may depend on the time window of interest. The first 48 hours after death are particularly tricky. "There have been no significant reproducible changes in the microbiome within 48 hours in the cases we've seen," says Schmidt.

He has been working with Jen Pechal of Michigan State University to see if she can identify a clock based

*King K, Berry R, Dane D, Lynne A, Bucheli S. Microbiome of forensically significant flies (Diptera) associated with human decomposition. ASM Microbe, June 1–5, 2017, New Orleans, LA.

on bodies entering the medical examiner's office under real-world conditions. The human remains in their study come from a wide range of environments, both outdoors and indoors.

To build their models, Pechal and Schmidt include only bodies with confirmed times of death. Schmidt swabs six regions including the mouth, ears, and rectum. Pechal then identifies the diversity and relative abundance of bacterial species in each sample. Meanwhile, her colleague microbiologist Heather Jordan at Mississippi State University studies which genes are actively being expressed.

Pechal next analyzes whether bodies that have been dead for the same period of time tend to have the same microbial presence and activity. Because she knows the cause of death for many of the bodies, she can also look at whether circumstances such as homicide, suicide, accident, or natural death may shift the microbial clock. For example, Schmidt says, a recent fever might alter a body's microbial profile because temperature affects bacterial proliferation.

The team has sampled about 1,000 bodies and completed genomics analyses on 200 bodies. "We are starting to see that there are key bacteria and key functions that are changing as the decomposition process occurs," says Pechal. The method can at least determine whether more or less than 48 hours has elapsed since the time of death—findings Pechal will publish soon. Whether the manner of death affects the microbiome in any consistent way is still too early to say.

Will It Hold Up in Court?

As a medical examiner, Schmidt sees potential in the microbial clock quest for all kinds of forensically useful discoveries. "It would be really, really cool to find microbiome patterns that vary according to geographic location," Schmidt says. He envisions a scenario in which a body found in Detroit might have a microbiome profile matching, say, Chicago, providing investigators a crucial clue. He's also keen to see if certain microbial markers link to specific medical conditions, such as diabetes or heart disease, and possibly reveal whether a person was recently in a hospital—all windows into preexisting conditions that might help Schmidt determine the cause of death.

But before these insights or a proper clock can be used in real investigations, there will be legal hoops to jump through. "There is enough preliminary data to suggest that microbial signatures could be used to generate a more accurate estimate [of the postmortem interval]," says forensic molecular biologist Tracey Dawson Cruz of Virginia Commonwealth University. "If it is scientifically valid and realistic in a practical setting are two different questions."

Any forensic advances related to DNA analysis, Dawson Cruz explains, must be approved by a group of forensic scientists called the Scientific Working Group on DNA Analysis Methods, which reports findings to the director of the FBI. "The very rigorous system of validation and approval that is required in the forensic community causes a delay in the adoption of new technologies," she says.

Dawson Cruz foresees that the cost of lab work would further delay use of a microbial clock. Most medical examiners' offices don't have molecular support labs so they'd need to contract private labs. "This is not going to be used extensively until the kind of sequencing we need happens faster and more cheaply," agrees Schmidt.

In criminal cases, a judge would also have to determine that the microbial clock meets the standard for admission of expert testimony, says DesPortes. Federal and some state courts follow what is known as the Daubert standard, which requires a judge to consider factors such as whether the technology has a known error rate and is widely accepted by the scientific community (7). It's too soon to know whether the microbial clock will pass scientific and legal muster, says DesPortes.

With her early results in hand, Metcalf believes she's within two to five years of testing her clock in a real crime scene scenario. But she acknowledges that there is "a lot at stake with crime scene investigations." Even so, Metcalf is confident that more data will eventually reveal, with sufficient precision, what makes the microbial clock tick.

1 Carter DO, Tomberlin JK, Benbow ME, Metcalf JL, eds (2017) *Forensic Microbiology* (Wiley, West Sussex, UK).

2 Fierer N, et al. (2010) Forensic identification using skin bacterial communities. *Proc Natl Acad Sci USA* 107:6477–6481.

3 Metcalf JL, Carter DO, Knight R (2016) Microbiology of death. *Curr Biol* 26:R561–R563.

4 Metcalf JL, et al. (2013) A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system. *Elife* 2:e01104.

5 Metcalf JL, et al. (2016) Microbial community assembly and metabolic function during mammalian corpse decomposition. *Science* 351:158–162.

6 Carey J (2016) News feature: Crucial role of belowground biodiversity. *Proc Natl Acad Sci USA* 113:7682–7685.

7 Supreme Court of the United Sates Syllabus. Daubert et ux., individually and as guardians ad litem for Daubert, et al. v. Merrell Dow Pharmaceuticals, Inc. certiorari to the United States Court of Appeals for the Ninth Circuit. No. 92-102. 509 U.S. 579 (1993). https://www.law.cornell.edu/supct/html/92-102.ZS.html.