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Microbiomics: The Germ Theory of Everything

Fast, cheap DNA sequencing technology now allows scientists to study unculturable microbes; the results are challenging some of biology’s most fundamental notions. By Alan Dove

In the 19th century, some biologists began espousing an apparently absurd theory: that diseases were caused not by poor hygiene and foul vapors, as everyone knew they were, but by organisms too small to see with the naked eye. Pioneering researchers working on this strange idea developed sterile culture techniques, improved microscopes, and created other cutting-edge tools. Gradually, their results convinced their colleagues that the new germ theory of disease, odd as it seemed, was true.

In the 21st century, some biologists have begun espousing an even more absurd theory: that humans and other macroorganisms are not individual entities, as everyone knows they are, but complete ecosystems dependent on billions of microbes. Pioneering researchers working on this unusual idea have developed novel sampling strategies, powerful new gene sequencing and data analysis techniques, and other innovative technologies. Gradually, their results are convincing a new generation of scientists that the microbiomic theory of life, odd as it seems, may be true.

“There are more microbial genomes within us than we have human cells. We’re a walking ecosystem. That’s a pretty profound reality,” says Timothy Harkins, director of research and development at Life Technologies in Carlsbad, California.

The Uncultured Majority

Microbiologists have long known that there are many bacteria, fungi, protozoans, and viruses that won’t grow in the lab with current culturing techniques. Now the plummeting prices and skyrocketing sensitivity of next generation DNA sequencing technologies are finally letting researchers study this unculturable majority.

Most studies in this emerging field consist of sampling an environment, sequencing as much of the DNA in the sample as possible, and using the sequence information to identify the organisms in it and possibly their ecological functions. The results can be surprising. For example, microbiome analyses of the human gut have revealed that each person’s large intestine carries a unique mix of bacterial species, and that perturbations of this intestinal ecosystem may cause severe illness and even starvation.

Though DNA sequencing forms the backbone of microbiomics, even the best sequencing protocols are useless without careful sampling and experimental design. “Sequencing is exciting and it’s very interesting, and people have frequently … said ‘let’s just sequence everything and sort it out later,’” says Jonathan Eisen, professor of evolution and ecology at the University of California in Davis, California. Eisen argues that this approach glosses over some crucial questions: “Do you want living cells? Do you want dead cells too? It’s a pretty coarse tool to just say, ‘I’m going to look at DNA.’”

Besides adding confounding factors such as dead cells and host DNA, poor sampling can destroy some of the genomes researchers wanted to find in the first place. “When [an anaerobic] bacterium is exposed to an oxygen environment it goes into apoptosis and kills itself, and shreds its genome, so how do you characterize something like that?” asks Harkins.

Once they’ve determined how to collect a useful sample, investigators need to decide exactly what questions they intend to ask, and how they want to frame the answers. Fortunately, zoologists and botanists have been studying ways to characterize ecosystems for decades. Unfortunately, they haven’t reached much agreement. continued>

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To classify the organisms, biologists can take either a taxonomic approach, making a list of the species that are present and sorting them by their characteristics and the niches they occupy, or focus on constructing phylogenetic trees based on evolutionary relationships. Both methods have adherents and detractors. “People have been arguing about this for a hundred years,” says Eisen, a member of the phylogenetic camp.

Quantifying the diversity in ecosystems is somewhat more straightforward. Ecologists generally measure three types of diversity: alpha diversity, based on the number of species or phylogenetic groups in a specific area; beta diversity, which compares diversity between different areas; and gamma diversity, which uses alpha and beta to account for the total biodiversity of a large ecosystem. In medical microbiomics, researchers often measure the alpha diversity within a single person’s microbial sample, and calculate beta diversity between the microbiomes from different people.

Covering the Bases

After settling on an experimental design, microbiomics researchers move to the sequencing phase, where they face another major choice: sequencing ribosomal RNA (rRNA) or sequencing random snippets of whole genomes.

In rRNA sequencing, investigators use primers designed to amplify only the genes for 16S ribosomal RNA, a molecule that has changed very slowly throughout evolution. The number of different rRNA sequences in a sample is a good proxy for the number of species, and public databases of such sequences can be used to identify many organisms. Shotgun or metagenomic sequencing, in contrast, involves sequencing short, random pieces of all of the genomes in a sample, then trying to piece them together afterward.

Each method has advantages and drawbacks. “There are folks who like the shotgun approach I think because overall it can be easier, [but] the one thing the ribosomal RNA approach offers is it’s effectively an enrichment technique,” says Todd Arnold, head of research and development at 454 Life Sciences, a Roche Company in Branford, Connecticut. Ribosomal RNA sequencing is particularly useful for samples from human microbiomes, as the technique makes it relatively easy to ignore the enormous background of host DNA and focus only on the microbial components. Researchers in the field also regard rRNA sequencing as the more mature technology, with better-defined procedures and clearer equipment choices.

However, metagenomic sequencing can identify a much wider range of variations across entire genomes, and may eventually enable scientists to sequence whole genomes in mixed samples. “I think there are aspects of metagenomic analysis which are becoming more routine, more off-the-shelf, [and] there’s a lot more that could be learned from metagenomic data,” says David Relman, professor of microbiology and immunology at Stanford University in Stanford, California.

Fortunately, sequencing equipment makers show no signs of resting on their laurels, and several companies are vying to increase their machines’ performance for both types of efforts. Though many microbiomics researchers have settled recently on Illumina’s HiSeq system for rRNA projects, Eisen is quick to point out that sequencing technology is still changing rapidly. “I don’t think it’s reached a plateau,” he says, adding that genome sequencing for metagenomics studies is particularly ripe for new breakthroughs.

Regardless of the platform they choose, biologists can expect the technology to be relatively user-friendly. “Sequencing is no longer seen as a skill for those who are very, very good at sequencing, it’s no longer in core labs,” says Arnold. Instead, modern high throughput sequencing machines are highly automated and include software that analyzes the raw data and performs initial quality control checks on it.

As a few early applications of microbial sequencing have reached the heavily regulated world of medicine and drug development, some gear makers have taken the automation a step further. For example, the Applied Biosystems Microseq platform performs traditional Sanger sequencing to identify bacterial and fungal contaminants in pharmaceutical manufacturing facilities. The system streamlines sequencing to detect a handful of specific organisms quickly and accurately, rather than probing the entire microbiome for all of the species present.

Down the Data Mine

Streamlined data and simple answers are the opposite of what basic researchers get from microbiome samples, though. “[Microbiomic] studies involve deep sequencing and are data-intensive—for both data storage and data analysis,” says Susan Knowles, senior marketing manager for microbiology at Illumina in San Diego, California. With the field still in its infancy, most of the software for analyzing those data comes from the investigators themselves. “There is a range of open source tools that researchers use for [microbiomics],” says Knowles, adding that “most of these data analysis tools require some bioinformatics skills.”

For scientists who are new to high throughput sequencing, the sheer quantity of information coming out of a sequencing machine can be a shock. 454’s Arnold says that the deluge of data often flummoxes newcomers.

In projects that focus on rRNA, investigators can take advantage of the simplified pool of possible sequences and large databases of known
rRNA genes. Because this technique is more established than shotgun microbiome sequencing, the software for analyzing rRNA is also somewhat easier to use. Depending on the information experimenters hope to find, they may be able to complete a simple rRNA project without having to hire—or become—bioinformaticians.

Shotgun sequencing is a different story. “The methods to do that with metagenomics are much more complex,” says Eisen. In a metagenomics study, scientists have to identify putative genes from fragmentary sequences, determine what families those genes belong to, and try to identify what organisms they come from. Each step presents unique and serious challenges.

Microbiome data analysis also raises a question that has vexed biologists for centuries: Exactly what is a species? Botanists and zoologists have reached some tentative definitions, but in bacteria, promiscuous gene swapping and rapid evolution make the concept trickier. For viruses, it may not apply at all. Worse, microbiomics itself could undermine traditional views of species distinctions. If each organism’s microbial ecosystem drives crucial parts of its biology, where does one individual end and the next begin?

To avoid being bogged down in philosophy, many microbiome researchers have settled on the idea of operational taxonomic units (OTUs), a practical, gene sequence-based analog of the species concept. Sequences that diverge beyond a certain threshold fall into distinct OTUs.

The European-funded MetaHIT project has recently used another approach: characterizing the human intestinal microbiome based on the putative functions of the different gene families in the sample, rather than by the species carrying those genes. Eisen explains that “they were some of the first people to do beta diversity and alpha diversity of function,” just as biologists have done with taxa. In this view, the organisms’ contributions to the ecosystem are what matter, not their identities or evolutionary origins.

After deciding how they want to view an ecosystem, researchers need to account for the inherent biases in their data. “There is no unbiased approach. The real challenge and important goal is to understand how biases arise in data, and what [one can] do to either minimize them or control for them,” says Relman. As examples, exposing samples to oxygen will eliminate obligate anaerobes, sequencing DNA will ignore RNA-containing viruses, and gentle extraction techniques may fail to lyse durable fungal or bacterial spores.

**Going Long**

While early pioneers in microbiomics continue sorting out the best ways to ask the scientific questions, engineers and equipment makers are trying to address some of the remaining technical needs. One of the top items on the agenda is longer sequence reads. “The longer the read the better,” says Harkin. He adds that “300 bases seems to be a good sweet spot, 300–350, and then the next sweet spot is when you get up to say 600, 800 base pairs.” Those lengths allow researchers to map microbial diversity at distinct levels of resolution, with greater lengths providing finer-grained separations between species.

Longer rRNA sequence reads allow researchers to distinguish organisms more clearly on a phylogenetic tree or taxonomic list, and longer metagenomic reads make it easier to assemble larger portions of each organism’s genome. Companies are also trying to make their sequencing systems more efficient at handling multiple samples, which is especially important for large clinical studies that try to identify variations in microbiomes across a human population.

Scientists are also trying to define clear methods and controls to ensure reproducible results. That’s turned out to be a thorny problem. “People have not tested a lot of the methods that are being used, they push a button and they run them, and we do the same thing,” says Eisen. Highly automated sequencing systems make it easy to produce results, but without clear guidelines for data analysis it’s unclear what those results mean. To try to establish a reference point, Eisen and his colleagues recently created a completely artificial microbiome by mixing known bacterial species that would never encounter each other in nature. “We shotgun sequenced them with different methods, and we tested how much we could actually figure out about a system [for which] we knew the answer. Even in that relatively simple artificial system some parts were very hard,” he says.

Classical microbiology may be able to help. Relman says that the new sequencing technologies, plus ongoing efforts to characterize bacterial environments in more detail, are enabling investigators to determine the culturing requirements for previously unculturable organisms. Growing these microbes in the lab makes it much easier to study them.

More exploratory surveys of microbiomes should also clarify some of the field’s boundaries, especially in medicine. For example, an ongoing project to study the lung microbiome has identified wide microbial population variations in the lungs of healthy individuals. “What is a healthy microbiome? We don’t know yet really, we’re just scratching the surface,” says Harkin.

Meanwhile, microbiome sequencing and data analysis continue getting simpler and cheaper, so that even undergraduates and nonscientists can now study microfauna almost as easily as fauna. “You’d be amazed at how many people are just starting to do this with ribosomal RNA. It’s a little bit different than what you might do with a set of binoculars and a bird book, but they get it,” says Eisen.

*Alan Dove is a science writer and editor based in Massachusetts.*
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