

## PARSING UNKNOWNNS

**Sarah Webb explores techniques researchers are using to elucidate biofilm structure and characterize the makeup of microbial communities.**

Credit: Knut Drescher

The human gut and skin, the world's oceans and soils, even the surfaces of plant leaves and their seeds—all play host to tiny communities of unseen microbes. In some instances these microbial populations consist of a single species of bacteria; in other cases they comprise many different species living together. The effects of these bacterial collections can range from harmful to helpful. But for researchers trying to tease out the range of intrinsic and environmental factors governing community structure and function, even dealing with a single species can be difficult.

“There are many unknown unknowns,” says Janet Jansson of Pacific Northwest National Laboratory (PNNL). Jansson should know—she has spent the last 30 years researching the compositions and relationships within various types of microbial communities in nature.

Scientists are making headway though—techniques such as next-generation sequencing (NGS), mass spectrometry (MS), and microscopy, alongside creative interdisciplinary solutions involving biologists, chemists, and engineers, are allowing researchers to slowly chip away at understanding the fundamental composition and behavior of these systems. Much of this work is currently focused on basic structure–function relationships, such as those that govern biofilm behavior.

### Life in the biofilm

Biofilms are multicellular, surface-attached microbial communities encased in a matrix of extracellular polysaccharide polymers. Within these communities, the bacterial inhabitants can take on different roles as they experience different chemical and physical environments.

Karin Sauer of Binghamton University notes that the cells in biofilms can also create wildly different structures, like a city with water channels and skyscrapers, mushroom shapes, or thick layers peppered with pores.

Sauer has applied a variety of molecular genetics techniques to studying biofilm development as well as proteomics and, more recently, transcriptomics techniques to look for genes supporting processes such as tolerance to antibiotics or dispersion from a biofilm. She and her colleagues have demonstrated that *Pseudomonas aeruginosa* biofilms show a form of reversible development. (*P. aeruginosa* is an opportunistic infection that often occurs in the lungs of immunocompromised patients with diseases such as HIV or cystic fibrosis. It is also a popular model system for studying biofilms.) Unlike embryonic development, if the right environmental conditions aren't available to support a *P. aeruginosa* biofilm's continued development and dispersion, the cells will revert back to an earlier developmental stage. The cues that the bacteria use to sense environment changes are another major focus of investigation for biofilm researchers.

### Chemical snapshots

“We know that there are all these metabolites that have been released—some of them are signals, some of them are nutrients. But how, exactly, are they distributed within a biofilm?” says Lars Dietrich, a microbiologist at Columbia University. “It's very difficult to visualize this.”

To understand chemical signaling in biofilms, one needs to pinpoint and analyze the spatial distribution chemicals within these structures. Because of chemical and bacterial heterogeneity, simple chemical analysis isn't sufficient for probing

biofilms, even those made up of a single species; therefore, researchers need tools to examine the spatial and even temporal relationships between cells.

A number of researchers are using MS to develop chemical pictures within biofilms. University of California, San Diego researcher Pieter Dorrestein was one of the pioneers of imaging mass spectrometry (IMS) in microbial systems, adapting a technique used in pathology labs to study and map the chemistry on the surface of tissue samples.

The fundamental idea emerged after thinking about the way that Alexander Fleming discovered penicillin as a compound in the bacterial environment, Dorrestein says. Fleming noticed zones of bacterial inhibition that coincided with fungal contamination on the surface of Petri dishes coated with *Staphylococcus aureus*. In IMS, researchers raster across the surface of a biofilm, collecting chemical data and assembling a two-dimensional picture. Dorrestein has used the technique to examine interactions between microbial species in a biofilm. For example, IMS experiments with one microbe that doesn't form a biofilm placed next to one that does can reveal the presence of a secreted molecule that suppresses biofilm formation.

According to Dorrestein, roughly 98% of the molecules secreted by bacterial communities have not been characterized. He and his colleagues have been trying to map this complex chemistry using their MS techniques. They've undertaken various projects to examine the chemical environment of microbes from the ocean, human lungs, plant seed surfaces, and human skin. For their skin project, his team found an estimated 50,000 different molecules that came from perhaps a million different MS signals. To identify the compounds found on skin, the team analyzed the chemistry of personal care products, skin cells, and the microbes themselves to begin tracking the origin of and some of the modifications to compounds they found in skin microbial communities. They assembled the connections in an approach called molecular networking, a method of grouping and annotating MS data

for analyzing complex, heterogeneous mixtures.

When designing an IMS experiment for a biofilm, one critical consideration is the molecular weight range of interest, says Jonathan Sweedler of the University of Illinois. Sweedler and his colleagues have been particularly interested in quinolones, quorum sensing molecules produced within *P. aeruginosa* biofilms. They use secondary ion MS (SIMS) to detect these molecules, both because the technique produces high spatial resolution and because the researchers are able to see a full range of quinolone chemical fragments with this ionization technique. "We don't just see just one quinolone," he says. "We can actually detect the 20 or so that are known, and we see mass peaks that we're still identifying that look like there's an additional 20 quinolones," he explains. But much of this choice depends on the chemistry of interest—a team looking for larger molecules, peptides or even small proteins would need to use laser desorption techniques to see their chemical fragments of interest.

Though IMS is powerful and relatively versatile, it does have its disadvantages.

The approach requires some sample preparation, and it is a destructive technique. So, even though IMS can give a detailed chemical and spatial snapshot of a biofilm, it typically can't be used to follow that biofilm over time.

For that reason, Sweedler and his colleagues often collaborate with other groups that use different techniques. Vibrational spectroscopy, particularly Raman spectroscopy, has allowed them to look at the chemistry of biofilm polymers, he says. And, unlike MS, samples aren't damaged by vibrational spectroscopy. Sweedler's team has also been able to pair IMS with fluorescence microscopy.

## Chips with redox potential

Collaborations between biologists and engineers at Columbia University have produced another tool for studying biofilms: an electrochemical chip.

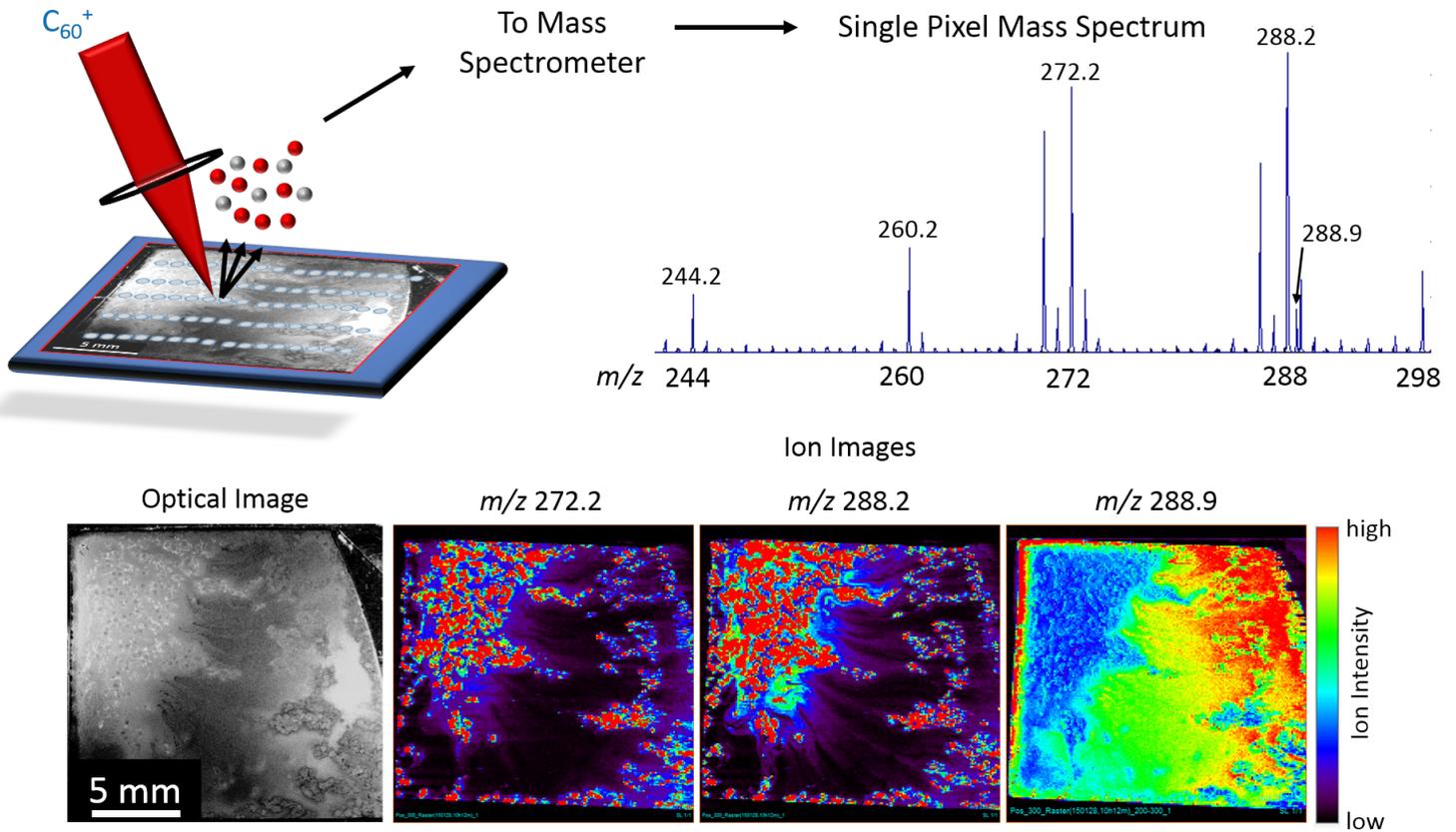
Microbiologist Lars Dietrich, who studies *P. aeruginosa*, is particularly interested in the connections between chemistry and cell morphology. Previous research showed that redox-active small molecules known as phenazines act as molecular snorkels, facilitating the movement of oxygen deep within a biofilm. In *P. aeruginosa* biofilms that can't produce these molecules, cells are hyperwrinkled rather than smooth. But researchers didn't know how phenazines were actually distributed within a biofilm.

Although IMS could provide access to some of the information that they were seeking, Dietrich was interested in determining whether the phenazines were in an oxidized or a reduced state, and he wondered whether it might be possible to map the location of these molecules in a biofilm based on their electrochemical signals.

To solve this problem, Dietrich teamed up with Columbia University engineer Ken Shepard. Shepard and his team were convinced they could build a chip to measure changes in redox potential across a surface. While one of Shepard's graduate students focused on chip development, one of



**Karen Sauer from Binghamton University uses a variety of molecular genetics techniques to study the structure and function of biofilms.** Credit: J. Cohen/Binghamton University.



In imaging mass spectrometry (IMS), a focused microprobe sequentially scans across the sample surface. Here, an ion beam (red arrow) generates chemical fragments (red, gray balls) from a *P. aeruginosa* biofilm. At each position, the fragments produce a mass spectrum (top right). The intensity of the individual ions is then plotted on a false color map to show their location and abundance on the biofilm's surface. Credit: Sage Dunham/Nameera Baig/Jonathan Sweedler.

Dietrich's graduate students figured out how to coat its surface with agar.

The team now has a chip with 1800 electrodes positioned across 64 mm<sup>2</sup> that can take measurements every 3 minutes. In a recent paper in the journal *Proceedings of the National Academy of Sciences*, the researchers used the chip to track the transport of a redox-active phenazine via an efflux pump, documenting its role in the gene expression and development of *P. aeruginosa* biofilms.

## Single-cell microscopy

As with other cellular systems, fluorescence microscopy is widely used to study biofilm structure, typically through the use of staining or fluorescent reporters to follow the expression of genes of interest. Recently, Bonnie Bassler's group at Princeton University took microscopy another step forward when they followed *V. cholerae* biofilms at single-cell resolution from inception to maturity, at sizes up to 10,000 cells.

The ability to observe communities at this level of detail required a few tweaks to confocal microscopy, says



Janet Jansson is using next-generation sequencing techniques to study microbial populations in complex mixtures. Credit: Pacific Northwest National Laboratory.

Knut Drescher, lead author on the study, who is now an investigator at the Max Planck Institute for Terrestrial Microbiology in Marburg, Germany. While they used spinning disk confocal microscopy to minimize photobleaching, the team also had to modify the beam path immediately before and after the spinning disk to improve the depth resolution in the z direction, allowing them to examine cells up to 35 microns deep—a 10-fold improvement over previous microscopy methods. In the *V. cholerae* system, that depth of resolution is sufficient to image the full biofilm lifecycle, before bacteria break off and colonize at other locations. The team was able to describe four stages in *V. cholerae* biofilm development: an initial stage where cells grow in one dimension, a second stage where they spread as a 2-D colony attached to a substrate before eventually buckling into the third stage, a disordered 3-D structure that eventually organizes into the fourth stage, a highly ordered structure wherein cells are aligned, and daughter cells form at the outer, more nutrient-rich edges.

## Sequencing and ‘meta-omics’

Visualizing biofilm development is one thing – but researchers also want to learn about the genes and proteins at work within these bacterial communities.

In recent years, Sauer has shifted from using proteomics to screen for proteins of interest to NGS to look at transcription in her *P. aeruginosa* studies. “It’s amazing how much data you can get in a short period of time,” she says.

NGS is also a powerful tool for examining highly heterogeneous natural microbial communities. According to Jansson, experimental technologies have improved exponentially since she started in the field, especially sequencing. “Now sequencing isn’t the bottleneck,” she says. “It’s interpretation of the data.”

Assembling mountains of NGS data into meaningful pictures remains challenging. Because of sample diversity, it’s hard to get good gene assemblies, particularly in soil, Jansson says. The vast majority—an estimated 99%—of microbial species haven’t been isolated, and fewer species have fully sequenced genomes available. Jansson, however, also works on human microbial communities, such as those in the gut, an area that is farther along—in part because those communities are less diverse. But the biggest advantage to working on the human gut, she says, is the greater number of reference microbes available.

According to Jansson, the ability to “bin” entire genomes will be a “gamechanger.” Her team used an approach to bin microbial genomes out of complex mixtures of fragmented DNA sequences from soil. Each bin represents the genome of a specific microbial population. To improve assembly and facilitate binning, Jansson and her colleagues have been using long-read technologies, in their case Illumina’s Moleculo, that allow them to get thousands of longer contiguous sequences (>10 kb). Those sequences serve as scaffolds to assemble much smaller, but higher resolution, NGS sequences of just a few hundred bases. In a recent paper, her team obtained hundreds of genome bins that represented soil microbes that have never been cultivated and for which there is no existing information about their potential functions in soil.

Still, DNA only tells part of the tale. “DNA only looks at potential—at least in soil, a lot is inactive at a given time,” notes David Myrold of Oregon State who stud-

ies carbon and nitrogen cycling in the soil microbiome. “It shows what could happen, as opposed to what is happening.”

As researchers try to understand function, they can marry metagenomics with a traditional functional assay that looks at an activity of interest. For nitrogen cycle processing, Myrold and his colleagues used metagenomics as a way to see how frequently known genes show up in a sample as a potential measure of how many different organisms can carry out a chemical process, such as the oxidation of ammonia. But to measure ammonia oxidation within a soil sample, they turned to a sensitive colorimetric enzymatic assay.

Jansson and her colleagues are integrating metagenomics with additional “-omics” approaches to understand more about the activities of specific microbes in these complex communities. Metatranscriptomics is a natural extension of metagenomics that provides information about gene expression in active microbes. In that same study of soil microbes, Jansson and her colleagues used metatranscriptomics of the mRNA to look at which genomic bins represented microbes that were active.

Metaproteomics and metabolomics are two additional areas with strong potential for assessing biofilm function. But here again, the lack of reference data complicates experiments; with so few databases of genes, proteins, and metabolites, it can be hard to make meaningful assignments. Right now, metaproteomics can only access a few thousand proteins from a soil sample, compared to millions of genes, Jansson says. But efforts are underway to improve separation technologies and dynamic yields. According to Myrold, in the next few years, it could be metaproteomics and metabolomics where the microbiome field sees the greatest technical advances, in part because they’re the areas where scientists know the least right now.

## Moving toward control

Researchers are just beginning to scratch the surface of the many questions about microbial communities that they’d like to address. “I would like to be able to puzzle together all of the different metabolic path-



**Knut Drescher, working with Bonnie Bassler, was able to explore the stages of biofilm development using confocal microscopy.** Credit: Z. Donnell.

ways that are carried out, for example, by a soil microbial community,” says Jansson. A better understanding of those pathways could be critical for producing better models to predict the effects of global climate change, for example. As the earth warms, soil microbes in permafrost become more active, and some of these organisms degrade organic carbon and release greenhouse gases such as methane.

From a chemical perspective, there isn’t an iron-clad definition of what molecular components must be present in a biofilm, Dorrestein says. And although his team is pursuing that question, it’s complicated enough that he’s not sure that there’s a definitive answer. Or at the very least, there might not be an answer that applies across all microbial species.

Ultimately, according to Dorrestein, understanding the chemistry that drives microbial communities will be critical to understanding which groups of organisms are healthy and which are harmful in an ecosystem—whether within a human, in agriculture or in the ocean. “Then you can start to think about controlling and moving that unhealthy microbial community into the healthy realm.”

Written by Sarah Webb, Ph.D. 

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