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Getting Our Fingers on the Pulse of Slow-Growing Bacteria in Hard-To-Reach Places

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ABSTRACT Chronic infections with slow-growing pathogens have plagued humans throughout history. However, assessing the identities and growth rates of bacteria in an infection has remained an elusive goal. Neubauer et al. (J. Bacteriol. 200:e00365-18, 2018, https://doi.org/10.1128/JB.00365-18) combine two cutting-edge approaches to make progress on both fronts: probing specific RNA molecules to assess the identity of actively transcribing microbes and measuring growth rates through incorporation of stable isotope labels. They found that growth rates of pathogens were relatively stable during antibacterial therapy. The article delves into a basic and unanswered question that gets to the heart of understanding infection: what are the microbial growth rates?

KEYWORDS *Staphylococcus aureus*, cystic fibrosis, fatty acids, growth modeling, stable isotope

Measuring the growth rates of infecting bacteria has always been challenging. Standard clinical microbiology approaches are biased by nutrient-rich, aerobic culture conditions used to isolate bacteria from clinical samples, favoring the growth of bacteria suited to those conditions (1). Improvements were sought using culture-independent DNA sequencing-based approaches (2), but many of these studies are stymied by misrepresentation of the community composition. For example, DNA sequencing approaches include the extracellular DNA that is produced by biofilm-forming bacteria (3) and do not distinguish DNA from actively dividing microbes. Furthermore, physiologically active bacteria may not be actively dividing and would thus have abundances so low that DNA sequencing-based approaches would not be useful for determining their role in infection. Hence, the basic and central question goes unanswered: what is the growth rate of infecting bacteria *in situ*?

The inability to discriminate between physiologically active and dead bacteria limits our understanding of the role of bacterial members in a polymicrobial community, including in airway infections (4). In "Refining the application of microbial lipids as tracers of *Staphylococcus aureus* growth rates in cystic fibrosis sputum" (5), Neubauer et al. build on previous work using deuterium isotope labeling to assess cystic fibrosis (CF) pathogen growth rates *in situ*. Specifically, the authors looked at pathogenic growth rates surrounding treatment for pulmonary exacerbations, periods of worsened symptoms endured by CF patients throughout their lives. Pulmonary exacerbations cause irreversible and life-shortening lung damage (6). Understanding whether and how microbes are involved in triggering exacerbations is a central mystery in the world of CF microbiology, and yet culturing, quantitative PCR (qPCR), and amplicon sequencing of sputum samples have so far largely failed to yield clear signatures associated with exacerbations (7). In fact, there is no measurable increase in abundance of bacteria in general or the common CF pathogen *Pseudomonas* spp. specifically, as measured by culturing or qPCR (8, 9). There are two clearly physiologically distinct populations of bacteria colonizing the airways of CF patients (10): the opportunistic pathogens, including *Pseudomonas* spp. and other Gram negatives that

come to dominate the infections as the disease progresses, and the anaerobes that are likely derived from the oral cavity and may themselves be important indicators or even triggers of some exacerbations (11–16). Moving toward active measurements of bacterial metabolic output in the CF airway environment is an exciting and important step toward understanding the conditions surrounding exacerbations, to enable earlier and more specific diagnosis and treatment.

USING STABLE ISOTOPE TRACKING AND RNA COMMUNITY PROFILING TO UNDERSTAND INFECTION DYNAMICS

The methods Neubauer et al. use to measure growth rates of bacteria include rRNA measurements with the NanoString nCounter and *in situ* stable-isotope probing of expectorated sputum from CF patients (17). They look at the distribution of isotopically labeled fatty acids after incubation of sputum samples with heavy water (D_2O) to measure microbial growth rates in sputum from CF patients undergoing treatment for pulmonary exacerbations. When a labeled compound that can be produced only by a specific bacterium is detected, this gives important insight into the growth rate of a pathogen in the infection environment. They specifically look at anteiso fatty acids produced by a subset of microbes, including *S. aureus*: 12-methyl-tetradecanoic acid and 14-methyl-hexadecanoic acid (referred to as a-$C_{15:0}$ and a-$C_{17:0}$, respectively). Anteiso fatty acids are not recycled or catabolized in microbial metabolism, an important feature that justifies using their abundances as measurement of bacterial replication. Neubauer et al. use Nanostring to probe RNA and even use the total RNA counts as a proxy for population size. While there are limitations with measuring rRNA during very slow growth and subsequently using it as a proxy for growth rate, Neubauer et al. were able to overcome these limitations by coupling rRNA counts with stable-isotope profiling to more accurately assess growth dynamics *in situ*.

STABLE ISOTOPES: FROM PROBING HUMAN HEALTH TO EXPLORING THE SPECTACULAR DIVERSITY OF MICROBIAL METABOLISM

Stable isotopes—nonradioactive forms of atoms with the same number of protons but a different number of neutrons—were first discovered in the early 20th century. Natural abundances of stable isotopes vary geographically and biologically, and both natural and spiked isotope-enriched tracers have since been used to probe growth, nutrition, and metabolism in diverse contexts (18). The very early applications of isotope tracers followed the fate of a specific labeled substrate in animals in the 1930s (19, 20).

In microbial ecology, stable isotopes have been used to track the flow of a labeled metabolite through an ecosystem. For example, stable-isotope probing (SIP) involves administering an isotope-labeled substrate *in situ* and utilizing ultracentrifugation to separate and sequence light (nonlabeled) nucleic acids and heavy (labeled) nucleic acids (21). Radajewski et al. first used DNA-based SIP to identify soil bacteria that metabolize labeled methanol by sequencing the 16S rRNA amplicons from the heavy DNA fractions (21). Stable isotopes have since proven to be a powerful tool for exploring the central tenets of microbial ecology, that microbes are the most numerous and diverse entities on the planet and that most molecules can be used by microbes. This idea is also especially important in the context of human health, where bacterial metabolites may play an underappreciated role in disease progression. McLean et al. used a combination of RNA- and DNA-based SIP to identify oral microbes that metabolize carbohydrates at a low pH, potentially important drivers of dental plaque formation (22). Another example, the urea breath test, tracks hydrolysis of ^{13}C-labeled urea into ^{13}C carbon dioxide to diagnose gut infections by *Helicobacter pylori* (23).

In addition to specifically ^{13}C- or ^{15}N-labeled compounds, a variety of isotope labels are available, including heavy water containing deuterium, which will be universally incorporated in virtually any metabolism. Focusing on heavy water incorporation into a specific, known metabolite such as the membrane components described by Neubauer et al. yields powerful data that can be connected to growth rate. However, there

are enormous numbers of microbial metabolites in the human body—as many as one-half of the many thousands of molecules in a drop of blood are thought to be produced or modified by microbial metabolism—leaving an immense and undiscovered frontier in terms of understanding the impact of microbial metabolism on human health (24–27). Stable-isotope approaches for following active microbial metabolism can be designed to track specific compounds or to globally follow metabolism with mass spectroscopy and Raman spectroscopy (28).

UNEXPECTED ORIGINS OF ANTEISO FATTY ACIDS AND SURPRISINGLY STABLE PATHOGEN GROWTH RATES

Neubauer et al. hypothesize that antistaphylococcal antibiotics suppress growth rates of S. aureus and other members of the microbial community but over time, S. aureus will overcome the antibiotics and occupy the niches where the other microbes have succumbed to the antibiotics. A signature they expected to see was an increased growth rate of S. aureus based on anteiso fatty acid production. However, they were surprised that the generation time of S. aureus did not significantly or consistently decrease during antibiotic treatment for pulmonary exacerbations: the average growth rates were 1.9 days (usually ranging from 0.5 to 2.5 days, and without significant connection to the time of exacerbation or antibiotic treatment in this cohort of seven CF patients). The authors also found that the molecule believed to be a marker specific for S. aureus growth was still present when there was a low abundance of S. aureus, suggesting another source of anteiso fatty acids. They then take a wonderful tour of the potential sources of the fatty acids of interest, from the diet and human metabolism (which do not take up deuterium at significant rates) to microbial metabolisms. Based on their previous work (29), the literature (30), and a subset of their samples from patients in this study, it was determined that Stenotrophomonas maltophilia and Prevotella melaninogenica were also producing anteiso fatty acids. This conclusion was made possible by the combination of microbial community profiling of rRNA using Nanostring and isotope-enriched metabolite detection (anteiso fatty acids).

THE OBSERVED LOW GROWTH RATES LEAD TO ANOTHER QUESTION: WHAT IS LIMITING MICROBIAL GROWTH?

The CF airway environment is rich in nutrients, including carbon and nitrogen sources (31). However, access to oxygen and other electron acceptors may be quite limiting (32, 33) and may be responsible for the low growth rates observed in this study and others, with doubling times averaging days to weeks. Furthermore, local gradients of pH (32) and toxic molecules of human immune (34) or microbial origin may also alter growth (35–38). Creating realistic culture conditions for clinical microbiology research—beyond recapitulating the nutrient composition in artificial sputum medium recipes that are based in part on metabolite analysis of sputum—should also involve representing the physiological conditions that result in realistic growth rates. Manipulating oxygen access or growing samples under conditions that enable the formation of pH and oxygen gradients may be an important step forward (39, 40).

CAVEATS IN THE CONTEXT OF CF MICROBIOLOGY

Sputum samples are local representatives of a heterogeneous airway environment, which is a continuous system with contributions from the upper and lower airways (41). Any individual sputum sample cannot globally represent the heterogeneous lung. In the future, breath testing may allow for a more global sampling of the airways and the microbial metabolites being produced there (15, 42).

On the opposite end of the spectrum, any bulk method using a heterogeneous sputum sample will not yield spatial resolution at the smaller scales that microbes occupy, or at larger scales, in terms of where in the lung the microbes are located. Approaches for clarifying and imaging microbes from human samples (43) and then resolving the location of the different bacterial species with specific probes are also becoming more feasible (44).

Another layer of complexity is the heterogeneity in single-cellular metabolism that can arise from genomic variants or the complicated microenvironments found in CF sputum. In fact, Neubauer et al. noted that single-cell growth rate visualization methods could elucidate whether the observed low growth rates are arising from a subpopulation. In previous work, the authors showed that *S. aureus* replication varied at a single-cell level in a chemostat (29, 45).

The approach Neubauer et al. use involved brief incubation of the sputum samples with heavy water and therefore may enable assessment of *in situ* growth. The oxygen, pH, and metabolite gradients found within CF sputum make it difficult to recapitulate all relevant conditions that dictate bacterial metabolism in an experiment. The growth of a strict anaerobe (*P. melaninogenica*) in these experiments supports the maintenance of *in situ* conditions during this brief 1-h incubation. Sputum is characterized by steep oxygen gradients (32, 46), but the role of anaerobes in CF infection is an active area of debate (16). Nonetheless, the nutrient-rich, aerobic conditions used in research and clinical microbiology labs may select for an unrepresentative subset of the community. Using both anaerobic and aerobic conditions may help in identifying bacterial contributors of CF disease.

CONCLUSIONS AND RECOMMENDATIONS

Growth is an ultimate measure of bacterial activity. Assessing growth rates of bacteria in their natural environment is essential for understanding natural microbial interactions and physiology. Stable isotopes have been used for nearly a century to track cellular activity, and in the work done by Neubauer et al., they were used to determine the growth rates of CF microbes *in situ* surrounding antibiotic treatment of pulmonary exacerbation events. Specifically, Neubauer et al. used heavy water to measure production of anteiso fatty acids as a biomarker of growth in *S. aureus*-infected sputum. They also found that *P. melaninogenica* and *S. maltophilia* produced anteiso fatty acids in sputum from their patient cohort. The team did not confirm their original expectations, i.e., that they would be able to observe changes in pathogen growth rates during antibiotic treatment. However, the valuable combination of isotope incorporation as a proxy for growth rate and RNA profiling with Nanostring to link the identity of the bacteria to the active metabolite production has already been informative in the context of CF. Together the team makes two main recommendations based on their results: (i) to perform parallel microbial community profiling in combination with isotope label tracking and (ii) to determine the background levels of a new diagnostic compound. Specifically, the authors analyzed CF sputum samples with a minimum a-$C_{15:0}$ concentration of 0.1 weight percent of saturated fatty acids to ensure that measurements were above background contributions from nonbacterial sources.

This study and a few others show that the bacteria in an infection are growing slowly, which may result in reduced efficacy of antibacterial treatments (29); this may explain the lack of change in growth rate during antibiotic treatment seen here. Insight into *in vivo* bacterial activity not only affects our clinical measurements but also translates to better representative *in vitro* model systems. The use of poorly representative lab models is particularly detrimental to the assessment of antibiotic susceptibility. In the case of CF and other infections, treatment often involves antibiotics that target replication processes. As a result, slower-replicating bacteria in infection are better able to tolerate antibiotic treatment. The study underlines a need for improved treatment strategies and *in vitro* model systems that are more representative of the low *in vivo* growth rates of cystic fibrosis bacteria. As our knowledge of *in vivo* bacterial growth and metabolism expands, clinical decision-making can also evolve to tackle these slow-growing, hard-to-reach bacterial infections.

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