



# 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

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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  $\alpha$ -C<sub>15:0</sub> and  $\alpha$ -C<sub>17:0</sub>, 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  $\alpha$ -C<sub>15:0</sub> 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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## REFERENCES

- Lagier J-C, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D. 2015. Current and past strategies for bacterial culture in clinical microbiology. *Clin Microbiol Rev* 28:208–236. <https://doi.org/10.1128/CMR.00110-14>.
- Lim YW, Haynes M, Furlan M, Robertson CE, Harris JK, Rohwer F. 2014. Purifying the impure: sequencing metagenomes and metatranscriptomes from complex animal-associated samples. *J Vis Exp* <https://doi.org/10.3791/52117>.
- Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295:1487–1487. <https://doi.org/10.1126/science.295.5559.1487>.
- Bergkessel M, Basta DW, Newman DK. 2016. The physiology of growth arrest: uniting molecular and environmental microbiology. *Nat Rev Microbiol* 14:549–562. <https://doi.org/10.1038/nrmicro.2016.107>.
- Neubauer C, Kasi AS, Grahl N, Sessions AL, Kopf SH, Kato R, Hogan DA, Newman DK. Refining the application of microbial lipids as tracers of *Staphylococcus aureus* growth rates in cystic fibrosis sputum. *J Bacteriol* 200:e00365-18. <https://doi.org/10.1128/JB.00365-18>.
- Sanders DB, Bittner RCL, Rosenfeld M, Hoffman LR, Redding GJ, Goss CH. 2010. Failure to recover to baseline pulmonary function after cystic fibrosis pulmonary exacerbation. *Am J Respir Crit Care Med* 182:627–632. <https://doi.org/10.1164/rccm.200909-1421OC>.
- Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TWV, Patel N, Forbes B, Bruce KD. 2012. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. *Thorax* 67:867–873. <https://doi.org/10.1136/thoraxjnl-2011-200932>.
- Lam JC, Somayaji R, Surette MG, Rabin HR, Parkins MD. 2015. Reduction in *Pseudomonas aeruginosa* sputum density during a cystic fibrosis pulmonary exacerbation does not predict clinical response. *BMC Infect Dis* 15:145. <https://doi.org/10.1186/s12879-015-0856-5>.
- Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TWV, Carroll MP, Hoffman LR, Jones G, Allen CE, Patel N, Forbes B, Tuck A, Bruce KD. 2011. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J Cyst Fibros* 10:357–365. <https://doi.org/10.1016/j.jcf.2011.05.002>.
- Whiteson KL, Bailey B, Bergkessel M, Conrad D, Delhaes L, Felts B, Harris JK, Hunter R, Lim YW, Maughan H, Quinn R, Salamon P, Sullivan J, Wagner BD, Rainey PB. 2014. The upper respiratory tract as a microbial source for pulmonary infections in cystic fibrosis. Parallels from island biogeography. *Am J Respir Crit Care Med* 189:1309–1315. <https://doi.org/10.1164/rccm.201312-2129PP>.
- Carmody LA, Zhao J, Schloss PD, Petrosino JF, Murray S, Young VB, Li JZ, LiPuma JJ. 2013. Changes in cystic fibrosis airway microbiota at pulmonary exacerbation. *Ann Am Thorac Soc* 10:179–187. <https://doi.org/10.1513/AnnalsATS.201211-107OC>.
- Caverly LJ, LiPuma JJ. 2018. Cystic fibrosis respiratory microbiota: unraveling complexity to inform clinical practice. *Expert Rev Respir Med* 12:857–865. <https://doi.org/10.1080/17476348.2018.1513331>.
- Quinn RA, Whiteson K, Lim YW, Zhao J, Conrad D, LiPuma JJ, Rohwer F, Widder S. 2016. Ecological networking of cystic fibrosis lung infections. *Npj Biofilms Microbiomes* 2:4. <https://doi.org/10.1038/s41522-016-0002-1>.
- Twomey KB, Alston M, An S-Q, O'Connell OJ, McCarthy Y, Swarbrick D, Febrer M, Dow JM, Plant BJ, Ryan RP. 2013. Microbiota and metabolite profiling reveal specific alterations in bacterial community structure and environment in the cystic fibrosis airway during exacerbation. *PLoS One* 8:e82432. <https://doi.org/10.1371/journal.pone.0082432>.
- Whiteson KL, Meinardi S, Lim YW, Schmieder R, Maughan H, Quinn R, Blake DR, Conrad D, Rohwer F. 2014. Breath gas metabolites and bacterial metagenomes from cystic fibrosis airways indicate active pH neutral 2,3-butanedione fermentation. *ISME J* 8:1247–1258. <https://doi.org/10.1038/ismej.2013.229>.
- Caverly LJ, LiPuma JJ. 2018. Good cop, bad cop: anaerobes in cystic fibrosis airways. *Eur Respir J* 52:1801146. <https://doi.org/10.1183/13993003.01146-2018>.
- Grahl N, Dolben EL, Filkins LM, Crocker AW, Willger SD, Morrison HG, Sogin ML, Ashare A, Gifford AH, Jacobs NJ, Schwartzman JD, Hogan DA. 2018. Profiling of bacterial and fungal microbial communities in cystic fibrosis sputum using RNA. *mSphere* 3:e00292-18. <https://doi.org/10.1128/mSphere.00292-18>.
- Wilkinson DJ. 2018. Historical and contemporary stable isotope tracer approaches to studying mammalian protein metabolism. *Mass Spectrom Rev* 37:57–80. <https://doi.org/10.1002/mas.21507>.
- Schoenheimer R, Rittenberg D. 1935. Deuterium as an indicator in the study of intermediary metabolism. *I. J Biol Chem* 111:163–168.
- Schoenheimer R, Rittenberg D. 1935. Deuterium as an indicator in the study of intermediary metabolism. *Science* 82:156–157. <https://doi.org/10.1126/science.82.2120.156>.
- Radajewski S, Ineson P, Parekh NR, Murrell JC. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* 403:646–649. <https://doi.org/10.1038/35001054>.
- McLean JS, Fansler SJ, Majors PD, McAteer K, Allen LZ, Shirliff ME, Lux R, Shi W. 2012. Identifying low pH active and lactate-utilizing taxa within oral microbiome communities from healthy children using stable isotope probing techniques. *PLoS One* 7:e32219. <https://doi.org/10.1371/journal.pone.0032219>.
- Atherton JC, Spiller RC. 1994. The urea breath test for *Helicobacter pylori*. *Gut* 35:723–725. <https://doi.org/10.1136/gut.35.6.723>.
- Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci U S A* 106:3698–3703. <https://doi.org/10.1073/pnas.0812874106>.
- Aksenov AA, da Silva R, Knight R, Lopes NP, Dorrestein PC. 2017. Global chemical analysis of biology by mass spectrometry. *Nat Rev Chem* 1:0054. <https://doi.org/10.1038/s41570-017-0054>.
- Dorrestein PC, Mazmanian SK, Knight R. 2014. Finding the missing links among metabolites, microbes, and the host. *Immunity* 40:824–832. <https://doi.org/10.1016/j.immuni.2014.05.015>.
- Patterson AD, Turnbaugh PJ. 2014. Microbial determinants of biochemical individuality and their impact on toxicology and pharmacology. *Cell Metab* 20:761–768. <https://doi.org/10.1016/j.cmet.2014.07.002>.
- Berry D, Loy A. 2018. Stable-isotope probing of human and animal microbiome function. *Trends Microbiol* <https://doi.org/10.1016/j.tim.2018.06.004>.
- Kopf SH, Sessions AL, Cowley ES, Reyes C, Van Sambeek L, Hu Y, Orphan VJ, Kato R, Newman DK. 2016. Trace incorporation of heavy water reveals slow and heterogeneous pathogen growth rates in cystic fibrosis sputum. *Proc Natl Acad Sci U S A* 113:E110–E116. <https://doi.org/10.1073/pnas.1512057112>.
- Wu C-C, Johnson JL, Moore WEC, Moore LVH. 1992. Emended descriptions of *Prevotella denticola*, *Prevotella loeschei*, *Prevotella veroralis*, and *Prevotella melaninogenica*. *Int J Syst Bacteriol* 42:536–541. <https://doi.org/10.1099/00207713-42-4-536>.
- Palmer KL, Mashburn LM, Singh PK, Whiteley M. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* 187:5267–5277. <https://doi.org/10.1128/JB.187.15.5267-5277.2005>.
- Cowley ES, Kopf SH, LaRiviere A, Ziebis W, Newman DK. 2015. Pediatric cystic fibrosis sputum can be chemically dynamic, anoxic, and extremely reduced due to hydrogen sulfide formation. *mBio* 6:e00767-15. <https://doi.org/10.1128/mBio.00767-15>.
- Basta DW, Bergkessel M, Newman DK. 2017. Identification of fitness determinants during energy-limited growth arrest in *Pseudomonas aeruginosa*. *mBio* 8:e01170-17. <https://doi.org/10.1128/mBio.01170-17>.
- Sagel SD, Wagner BD, Anthony MM, Emmett P, Zemanick ET. 2012. Sputum biomarkers of inflammation and lung function decline in children with cystic fibrosis. *Am J Respir Crit Care Med* 186:857–865. <https://doi.org/10.1164/rccm.201203-0507OC>.
- Phan J, Gallagher T, Oliver A, England WE, Whiteson K. 2018. Fermentation products in the cystic fibrosis airways induce aggregation and dormancy-associated expression profiles in a CF clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 365(10). <https://doi.org/10.1093/femsle/fny082>.
- Chen AI, Dolben EF, Okegbe C, Harty CE, Golub Y, Thao S, Ha DG, Willger SD, O'Toole GA, Harwood CS, Dietrich LEP, Hogan DA. 2014. *Candida albicans* ethanol stimulates *Pseudomonas aeruginosa* WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* 10:e1004480. <https://doi.org/10.1371/journal.ppat.1004480>.

37. Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK. 2012. Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. *Am J Respir Cell Mol Biol* 47:738–745. <https://doi.org/10.1165/rcmb.2012-0088OC>.
38. Flynn JM, Niccum D, Dunitz JM, Hunter RC. 2016. Evidence and role for bacterial mucin degradation in cystic fibrosis airway disease. *PLoS Pathog* 12:e1005846. <https://doi.org/10.1371/journal.ppat.1005846>.
39. Sønderholm M, Kragh KN, Koren K, Jakobsen TH, Darch S, Alhede M, Jensen PØ, Whiteley M, Kühl M, Bjarnsholt T. 2017. Aggregate formation of *Pseudomonas aeruginosa* in an alginate bead model system exhibits in vivo like characteristics. *Appl Environ Microbiol* 83:e00113-17. <https://doi.org/10.1128/AEM.00113-17>.
40. Quinn RA, Whiteson K, Lim Y-W, Salamon P, Bailey B, Mienardi S, Sanchez SE, Blake D, Conrad D, Rohwer F. 2015. A Winogradsky-based culture system shows an association between microbial fermentation and cystic fibrosis exacerbation. *ISME J* 9:1024–1038. <https://doi.org/10.1038/ismej.2014.234>.
41. Dickson RP, Huffnagle GB. 2015. The lung microbiome: new principles for respiratory bacteriology in health and disease. *PLoS Pathog* 11:e1004923. <https://doi.org/10.1371/journal.ppat.1004923>.
42. Bos LD, Sterk PJ, Fowler SJ. 2016. Breathomics in the setting of asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 138:970–976. <https://doi.org/10.1016/j.jaci.2016.08.004>.
43. DePas WH, Starwalt-Lee R, Sambek LV, Kumar SR, Gradinaru V, Newman DK. 2016. Exposing the three-dimensional biogeography and metabolic states of pathogens in cystic fibrosis sputum via hydrogel embedding, clearing, and rRNA labeling. *mBio* 7:e00796-16. <https://doi.org/10.1128/mBio.00796-16>.
44. Welch JLM, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. 2016. Biogeography of a human oral microbiome at the micron scale. *Proc Natl Acad Sci U S A* 113:E791–E800. <https://doi.org/10.1073/pnas.1522149113>.
45. Kopf SH, McGlynn SE, Green-Saxena A, Guan Y, Newman DK, Orphan VJ. 2015. Heavy water and <sup>15</sup>N labelling with NanoSIMS analysis reveals growth rate-dependent metabolic heterogeneity in chemostats. *Environ Microbiol* 17:2542–2556. <https://doi.org/10.1111/1462-2920.12752>.
46. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G. 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 109:317–325. <https://doi.org/10.1172/JCI0213870>.