Refining the Application of Microbial Lipids as Tracers of *Staphylococcus aureus* Growth Rates in Cystic Fibrosis Sputum

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ABSTRACT Chronic lung infections in cystic fibrosis (CF) could be treated more effectively if the effects of antimicrobials on pathogens *in situ* were known. Here, we compared changes in the microbial community composition and pathogen growth rates in longitudinal studies of seven pediatric CF patients undergoing intravenous antibiotic administration during pulmonary exacerbations. The microbial community composition was determined by counting rRNA with NanoString DNA analysis, and growth rates were obtained by incubating CF sputum with heavy water and tracing incorporation of deuterium into two branched-chain ("anteiso") fatty acids (a-C$_{15:0}$ and a-C$_{17:0}$) using gas chromatography-mass spectrometry (GC/MS). Prior to this study, both lipids were thought to be specific for *Staphylococcaceae*; hence, their isotopic enrichment was interpreted as a growth proxy for *Staphylococcus aureus*. Our experiments revealed, however, that *Prevotella* is also a relevant microbial producer of a-C$_{17:0}$ fatty acid in some CF patients; thus, deuterium incorporation into these lipids is better interpreted as a more general pathogen growth rate proxy. Even accounting for a small nonmicrobial background source detected in some patient samples, a-C$_{15:0}$ fatty acid still appears to be a relatively robust proxy for CF pathogens, revealing a median generation time of ~1.5 days, similar to prior observations. Contrary to our expectation, pathogen growth rates remained relatively stable throughout exacerbation treatment. We suggest two straightforward "best practices" for application of stable-isotope probing to CF sputum metabolites: (i) parallel determination of microbial community composition in CF sputum using culture-independent tools and (ii) assessing background levels of the diagnostic metabolite.

IMPORTANCE In chronic lung infections, populations of microbial pathogens change and mature in ways that are often unknown, which makes it challenging to identify appropriate treatment options. A promising tool to better understand the physiology of microorganisms in a patient is stable-isotope probing, which we previously developed to estimate the growth rates of *S. aureus* in cystic fibrosis (CF) sputum. Here, we tracked microbial communities in a cohort of CF patients and found that anteiso fatty acids can also originate from other sources in CF sputum. This awareness led us to develop a new workflow for the application of stable-isotope probing in this context, improving our ability to estimate pathogen generation times in clinical samples.

KEYWORDS microbial growth rate, stable-isotope probing, heavy water, CF sputum, *Staphylococcus aureus*, *Prevotella*, respiratory pathogens

Cystic fibrosis (CF) is a genetic disease that affects more than 30,000 individuals in North America, with a median predicted survival age of 41.6 years in the United States (1). This disease is caused by mutations in the CF transmembrane conductance regulator (CFTR) protein, which functions as an apical epithelial chloride channel. Reduced or absent chloride channel function leads to dehydrated, viscous secretions that cause dysfunction in the lungs, pancreas, and other organs. As CF progresses, bacteria colonize the lung, resulting in chronic polymicrobial infections and inflammation (2). These infections are notoriously hard to treat because species, strains within a species, and genomic variants within a strain differ between individuals and discrete regions of the lung. As CF progresses, microbial populations likely adapt to and shape microenvironments in the lung, making infections ever more resistant to drug treatment (3, 4). Understanding which pathogens are present can inform the choice of appropriate treatment strategies (5) and improve lung function, in tandem with restoration of CFTR activity by CFTR potentiator drugs such as ivacaftor (6).

Staphylococcus aureus is one of the earliest bacteria detected in infants and children with CF (7). In the United States, more than one-half of CF patients had at least one culture positive for methicillin-sensitive *S. aureus* (MSSA) in 2016 (1). The development of chronic polymicrobial infections in the CF lung involves many contributing factors. Over the past decade, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) has increased in CF patients in the United States. Compared to CF patients with MSSA infections, CF patients with MRSA have lower lung function, increased likelihood of hospitalization, and lack of responsiveness to antibiotic treatment (8). Persistent MRSA infection in CF patients between 8 and 21 years is associated with more rapid lung function decline (9).

CF patients experience episodes of acute worsening of respiratory symptoms called pulmonary exacerbations. Pulmonary exacerbations are treated with antibiotics and airway clearance therapies (10). For decades, antibiotics have been the cornerstone of CF care, and in recent years, there has been a clinical focus on the aggressive management of pulmonary exacerbations because 25% of CF patients do not recover their baseline lung function after intravenous antibiotic treatment for a pulmonary exacerbation (11). A higher risk of failing to recover to baseline lung function was associated with several factors, including persistent infection with *Pseudomonas aeruginosa* or MRSA (11).

A largely unresolved question is how quickly CF pathogens proliferate in the lung during antibiotic therapy (12–15). It is conceivable that the ability of infections to persist in the lung is connected in intricate ways to low growth rates. Slow bacterial growth, for example in biofilms, can be beneficial for survival partly because many drugs have their greatest effect on rapidly dividing cells (16). Nevertheless, for a microbial population to persist in the lung, a certain amount of growth is necessary to counteract loss of cells due to attack by the immune system or mucociliary clearance. As chronic infections have a highly complex and heterogeneous composition, it is challenging to determine *in situ* growth rates of CF pathogens.

Recently, two advances in microbial ecology have enabled measurement of microbial growth rates *in situ*. The first is based on DNA sequencing and exploits the observation that growing cells yield more sequencing reads at genomic regions near the origin of replication (17, 18). This difference between nongrowing and rapidly growing cells is relatively small (a maximum 2-fold increase when assuming duplication of origin of replication in growing cells). To derive quantitative information from this signal, metagenomic data must have high sequence coverage, assumptions must be made about chromosome copy number in growing cells, and care must be taken to avoid biases introduced by preferential DNA sequencing from certain members of the population. The second approach uses isotopic labeling and mass spectrometry to quantify the incorporation of isotopes into biomass, which provides a more direct measurement of anabolic activity (14, 19). Stable-isotope probing requires incubation of the sample with isotopic label and relies on the identification of diagnostic metabolites. Because the natural abundance of rare isotopes such as deuterium is small and

the number of metabolite molecules per cell is large, the sensitive detection of isotope incorporation into metabolites by mass spectrometry can have a greater dynamic range than DNA sequencing. Thus, stable-isotope probing may be better suited for studies that attempt to gain growth information for microbes in a sample where microbes would be expected to be growing slowly and the DNA of the target organism(s) might make up only a small portion of the total DNA. While DNA sequencing and stable-isotope probing provide new opportunities to study *in situ* growth rates, both methods provide information by analyzing bulk extracts and cannot resolve spatial organization and heterogeneity (14), though techniques that permit microbial metabolic activity to be measured at the single-cell level are emerging (20–22).

Utilizing a bulk stable-isotope labeling approach, we previously attempted to quantify *S. aureus* growth rates in CF sputum; we found that its growth rate was lowest in acutely sick patients, despite extensive growth rate heterogeneity at the single-cell level (14). Freshly expectorated sputum was incubated with a small percentage of heavy water (D_2O) for 1 h at 37°C, and subsequently the incorporation of deuterium into diagnostic fatty acids was measured. While sample incubation *ex vivo* prior to a measurement always runs the risk of altering the *in vivo* state of what is being measured, to the best of our knowledge, the incubation period used in these experiments was short enough to not significantly perturb the physiology of the microbes under study (14, 23). These fatty acids, anteiso-$C_{15:0}$ (*a*-$C_{15:0}$) and anteiso-$C_{17:0}$ (*a*-$C_{17:0}$), are the dominant fatty acids of *S. aureus*, and their biosynthesis rate was used as a proxy for growth of the pathogen. The two main findings of the previous study (14) were that (i) *S. aureus* had a median *in situ* generation time of ~2 days, far slower than previous estimates of CF pathogen growth rates, and (ii) growth rates were lowest in patients during early treatment of pulmonary exacerbations (i.e., the first few days). The low growth rates that we measured support the hypothesis that *S. aureus* populations experience environmental constraints within the lung, which could facilitate tolerance to conventional antibiotics. Based on the observed cross-sectional growth trends (14), we hypothesized that during early hospitalization, treatment with antistaphylococcal antibiotics may have suppressed *S. aureus* growth rates but as other members of the microbial community also succumbed to antibiotic treatment, *S. aureus* could occupy these abandoned niches and grow more quickly.

This study was designed to test the hypothesis by performing a longitudinal study. We correlated changes of microbial community composition with *S. aureus* growth rate in a cohort of seven CF patients that were treated with intravenous antibiotics due to pulmonary exacerbations. Our results did not support our hypothesis but led to important refinements in our approach to measuring *in situ* growth rates in clinical samples.

RESULTS

We collected and analyzed eight time series of sputum samples during pulmonary exacerbations from seven CF patients (aged 9 to 19 years). Three or more longitudinal samples were acquired from all patients during their hospitalization. When possible, the first sputum sample was collected on admission and the last sputum sample was collected during a routine CF clinic visit 3 to 4 weeks following hospitalization, when the patient's condition had stabilized. All enrolled patients were positive for *S. aureus* based on clinical microbiology culturing procedures. Two patients had severe lung disease (patients 1 and 7), and the remaining longitudinal series were collected from patients with mild to moderate lung disease. All patients recovered to their spirometric baseline forced expiratory volume in 1 s (FEV1 percent) following treatment. Clinical and demographic information for study participants is summarized in Table 1 (see also Tables S1 and S2 in the supplemental material).

The individual time series revealed dramatic changes in the microbial populations based on counting rRNA within total RNA extracted from sputum using NanoString technology (Fig. 1). Though for this study we specifically recruited CF patients with *S. aureus* infections on the basis of clinical cultures, polymicrobial infections were the

TABLE 1 Clinical summary of patients in this study[a]

Patient ID	Age (yr)	Clinical summary	Sputum culture	Inpatient antibiotics	Outpatient antibiotic(s)
1	9	Severe lung disease on BPAP. Two hospitalizations, 1 mo apart	MSSA, *P. aeruginosa*	Cefe, Tob	Azi, Tob (inhaled)
2	19	Mild lung disease	MSSA, *Achromobacter*	P-T, Col (inhaled)	Azi, Tob (inhaled)
3	12	Mild lung disease	MSSA	Oxa, Cefe, Tob	Tob (inhaled)
4	14	Mild lung disease. *Mycobacterium abscessus* was detected on culture	MSSA, mucoid *P. aeruginosa*	P-T, Tob	Tob (inhaled)
5	17	Mild lung disease. Failed outpatient antibiotics (amoxicillin-clavulanic acid). Initial *P. aeruginosa* detected on culture. *M. abscessus* was detected on culture. Treated at home with intravenous antibiotics from day 6	MSSA, *P. aeruginosa*	P-T, Tob	None
6	14	Mild lung disease. Allergic bronchopulmonary aspergillosis	MRSA, mucoid *P. aeruginosa*	Ceft, Tob, Van	Azi, Azt (inhaled), Tob (inhaled)
7	17	Severe lung disease on BPAP. CF-related diabetes. *Mycobacterium avium* was detected on culture, not treated. Required prolonged antibiotics for 19 days due to poor initial response	MSSA, mucoid *P. aeruginosa*	Ami, Mero, Cipro	Azt, Tob (both inhaled)
8	17	Moderate lung disease. CF-related diabetes. *M. abscessus* was detected on culture	Rare *P. aeruginosa*	NA	Azi, Ami (inhaled)
9	22	Mild lung disease	Few MSSA, rare mucoid *P. aeruginosa*	Cefe, Tob	Azi, Tob (inhaled), Azt (inhaled)
10	17	Severe lung disease on BPAP. CF liver disease following liver transplant	Few *Achromobacter* sp. organisms	NA	Azt, Col (both inhaled)

[a]Abbreviations: BPAP, bilevel positive airway pressure; Ami, amikacin; Azi, azithromycin; Azt, aztreonam; Cefe, cefepime; Ceft, ceftazidime; Cipro, ciprofloxacin; Col, colistin; Mero, meropenem; Oxa, oxacillin; P-T, piperacillin-tazobactam; Tob, tobramycin; Van, vancomycin; MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; NA, not applicable; ID, identification. Lung disease severity was based on FEV1: mild, FEV1 $\geq$ 70%; moderate, FEV1 = 40% to 69%; severe, FEV1 < 40%.

norm, as expected (24). Microbial community composition varied by patient (see Fig. S1 in the supplemental material). Overall, patient ID contributed significantly ($P < 0.001$; permutational multivariate analysis of variance [PERMANOVA]) to the observed variation and accounted for 82% of the observed variation. In three patients, *S. aureus* was
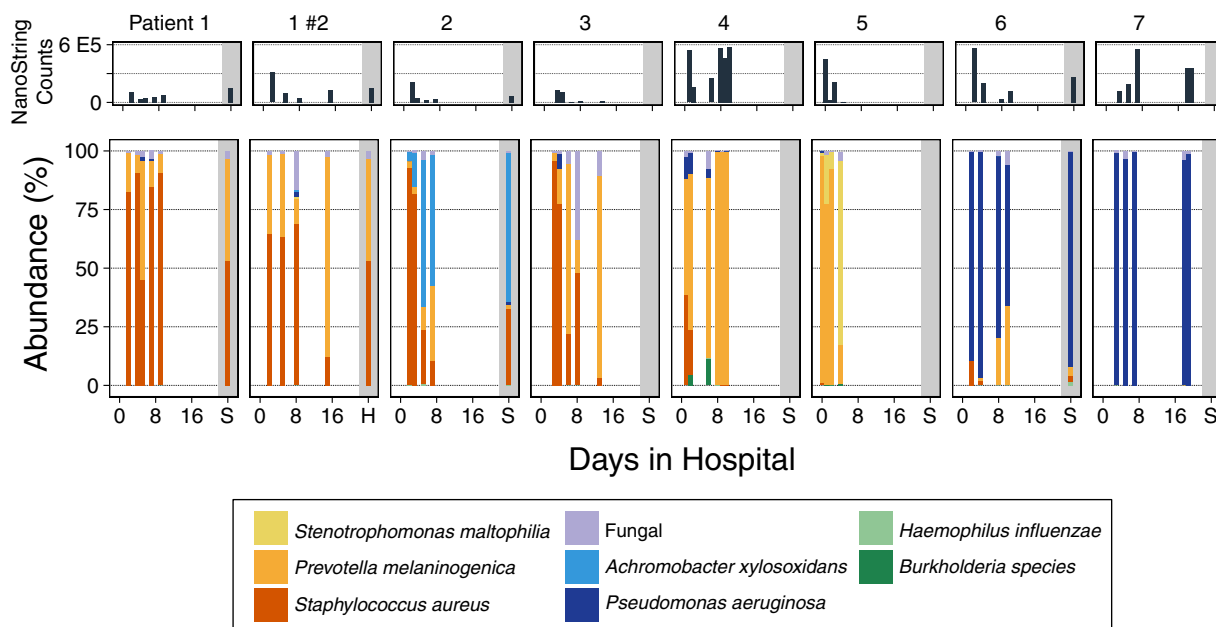

FIG 1 Microbial community composition of expectorated CF sputum during pulmonary exacerbations. Expectorated CF sputum samples from eight time courses were used to isolate RNA for counting of rRNA molecules by NanoString, using CF-specific probes (see Materials and Methods). Patients were hospitalized and treated with intravenous antibiotics. For each longitudinal series, the top panel shows the sum of microbial rRNA counts, an indicator of microbial abundance. The bottom panel shows the relative abundance of common CF-associated microorganisms. Producers of anteiso fatty acids are colored yellow to red. S, control sample ("stable") taken at a routine visit to the clinic after treatment.
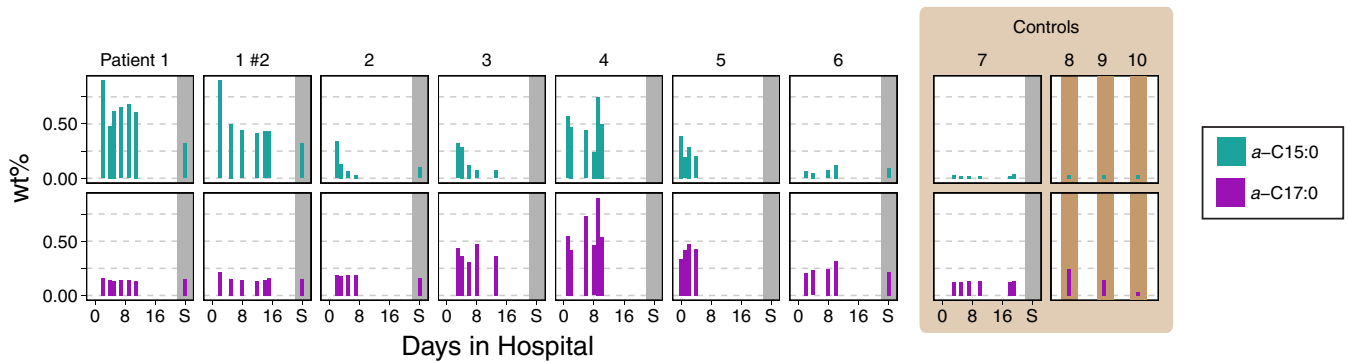
FIG 2 Abundance of anteiso fatty acids in expectorated CF sputum. The wt% abundances of a-$C_{15:0}$ and a-$C_{17:0}$ in extracts of saturated fatty acids are shown. Patient 7, whose sputum did not contain common microbial producers of anteiso fatty acids and whose microbial community composition was distinct (Fig. 1 and S1), can be interpreted as a control. Patients 8, 9, and 10 were three individuals whose clinical data did not indicate the presence of *S. aureus*.

the most abundant member of the microbial community at the beginning of treatment (patients 1, 2, and 3). In some patients, *Prevotella melaninogenica* (patients 4 and 5) or *Pseudomonas aeruginosa* (patients 6 and 7) was most abundant. For patient 7, *P. aeruginosa* accounted for almost all (>96%) of the NanoString counts, while *S. aureus* was detected only at the beginning of hospitalization (0.4% abundance). During treatment, the relative abundance of *S. aureus* decreased in most patients as assessed by rRNA levels. Patient 1, however, who had severe lung disease, showed a varied response. Based on total NanoString counts, we can infer microbial population size (Fig. 1) (25). Several patients showed a large decrease in microbial population size throughout their hospital stay (patients 2, 3, 5, and 6), whereas in patient 1, microbial population size did not decrease, and it even increased in patients 4 and 7. These time series illustrate that CF microbial infections change and mature differently in individuals during antimicrobial therapy. Accordingly, we might expect that differential microbial growth activities during drug treatment in any given patient would correlate with the rise or fall of particular members of the microbial community.

To determine whether this was the case, we focused on measuring the growth activity of *S. aureus* by tracking anteiso fatty acids previously thought to be diagnostic for this bacterium in the context of CF microbial communities, comparing its specific growth rates to changes in the microbial community composition. Although *S. aureus* was detected in initial sputum cultures of all patients included in the longitudinal study, our analysis of microbial populations showed that patient 7 samples contained almost exclusively *P. aeruginosa*. This bacterium does not produce anteiso fatty acids, so we did not expect to detect a-$C_{15:0}$ and a-$C_{17:0}$ analytes in samples from patient 7. Nevertheless, a-$C_{17:0}$ occurred at abundances of >0.15% (weight percent [wt%] of saturated fatty acids), which is similar to the levels in sputum from patients that had dominant *S. aureus* infections (e.g., patient 1) (Fig. 2). For comparison, we analyzed sputum samples from three CF patients whose clinical data did not indicate the presence of *S. aureus*. Two of these controls also showed more than 0.15% a-$C_{17:0}$. Interestingly, a-$C_{15:0}$ was detected at lower abundances (<0.05%) in patient 7 and the three controls. This suggested that sources other than *S. aureus* can contribute anteiso fatty acids, especially a-$C_{17:0}$, in expectorated sputum.

Whether nonstaphylococcal sources of anteiso fatty acids were sufficiently abundant to affect the interpretation of our D_2O-labeling experiments became the next question. Our prior work had taught us that *Stenotrophomonas* species can also produce anteiso fatty acids and can occur in CF sputum (14). In some samples from patient 5, *Stenotrophomonas maltophilia* constituted the majority of microorganisms. The fatty acid analysis of these sputum samples also revealed an increased amount of *iso*-$C_{15:0}$, which had been observed for the pure strain (14). In all other patients, *S. maltophilia* occurred at low abundances (<1%), likely too low to account for the high levels of

TABLE 2 Concentrations of anteiso fatty acids in various sources[a]

Source	Concn of anteiso fatty acid (wt% of fatty acids)		Reference or source
	a-C$_{15:0}$	a-C$_{17:0}$	
S. maltophilia	20	<1	Kopf et al. (14)
S. aureus	45	20	Kopf et al. (14)
P. melaninogenica	43	4	Wu et al. (26)
S. epidermidis	28	20	This study
CFBE	0.05	0.3	This study
Human promyelocytic leukemia cells (HL-60)	0.04	0.1	This study
PMN	0.07	0.1	This study
FBS	0.04	0.3	This study
Diet			
Beef	0.23	0.54	Ran-Ressler et al. (32)
Milk	0.56	0.61	Ran-Ressler et al. (32)

[a]CFBE, CF bronchial epithelial cells; PMN, polymorphonuclear neutrophils; FBS, fetal bovine serum.

a-C$_{17:0}$ that we measured. For example, in patient 4, neither *S. aureus* nor *S. maltophilia* was detected, yet a-C$_{15:0}$ and a-C$_{17:0}$ were both present at up to 0.8% of saturated fatty acids. A likely source of anteiso fatty acids in sputum from patient 4 and samples from other patients in our cohort is *Prevotella melaninogenica*. This species can produce anteiso fatty acids, but it was not previously recognized as a relevant source of anteiso fatty acids in CF sputum (14, 26). *P. melaninogenica* is an anaerobe and part of the oral flora. It was isolated from CF sputum and in bronchoalveolar lavage fluid from infants with CF (27, 28), yet the clinical significance of *Prevotella* in CF lung disease is still debated (29).

It is possible that microbial communities in CF sputum contain additional bacteria that produce anteiso fatty acids. This may be particularly relevant for older patients, a subset of which sometimes contain strains not represented in our cohort (27). We surveyed the literature for information about the fatty acid content of microbial strains from the genera *Acinetobacter*, *Mycobacterium*, *Veillonella*, *Ralstonia*, and *Rothia*. Strains of the genus *Rothia* can contain high abundances of anteiso fatty acids (30). *Rothia* was not present (in amounts detectable by NanoString) in the patients participating in this study. Regardless, *Rothia* could be a relevant source of a-C$_{15:0}$ and a-C$_{17:0}$ in addition to *Prevotella* in other CF patients.

Sputum from patient 7 had elevated a-C$_{17:0}$ but did not contain *Prevotella* (Fig. 2). This raised the new concern that perhaps additional input of anteiso fatty acids might emanate from the human host itself. Anteiso fatty acids are rare in human tissues but can accumulate in skin and fat cells (31). Might human cells such as neutrophils and epithelial cells contribute to the anteiso fatty acid pool in expectorated CF sputum? To answer this question, we analyzed the fatty acid composition of two human cell lines (Table 2). CF bronchial epithelial cells (CFBE) and polymorphonuclear neutrophils (PMN) indeed contained anteiso fatty acids on the order of 0.1 wt% of total fatty acids. It is possible that some of these anteiso lipids in cell cultures originate from the growth substrate. Fetal bovine serum (FBS), the only component of the growth medium that contains lipids, contained a similar percentage of anteiso fatty acids; hence, diet could also be a contributing factor. For humans, dietary intake of anteiso fatty acids in the United States is largely via milk and meat products (32). We concluded that an additional source of anteiso fatty acids in expectorated sputum likely stems from human cells or dietary intake. The impact of this contribution on the interpretation of D$_2$O-labeling experiments will depend on the relative contribution of human cells to the pool of anteiso fatty acids in a given sample.

Given these new findings, what can we say with confidence about the growth rate of any particular pathogen in sputum? The deuterium labeling of anteiso fatty acids, interpreted at face value, yielded a median generation time of *S. aureus* of 2.0 days
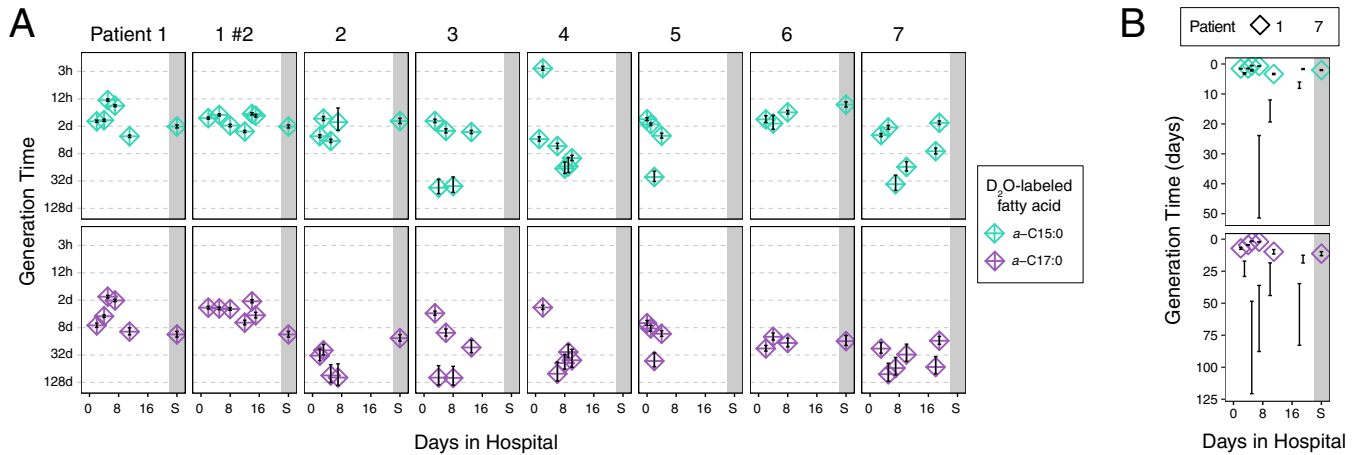
FIG 3 Generation times based on deuterium incorporation into anteiso fatty acids. (A) Generation time estimates obtained for the eight time series. Error bars represent ±1 standard deviation (SD). (B) Data for patient 1, who had abundant microbial producers of anteiso fatty acids, and patient 7, who had no detectable microbial producers of anteiso fatty acids, are plotted on a linear scale for better comparison.

when a-C$_{15:0}$ was used as proxy for microbial growth. The longer anteiso fatty acid, a-C$_{17:0}$, yielded systematically slower generation times, with a median of 14.6 days. The individual time series in our data set reveal a large variation between patients when deuterium incorporation is interpreted without taking microbial abundance and community composition into account (Fig. 3). For example, the second time series for patient 1 (1 #2), who had two hospitalizations 1 month apart, showed a lower growth rate during hospitalization followed by a rebound increase in growth rate. This patient had severe lung disease (baseline FEV1, 34%) requiring noninvasive ventilation during sleep and treatment for chronic airway *P. aeruginosa* colonization with cycled nebulized tobramycin. However, a similar growth rate trend was not observed in the same patient during the first hospitalization (patient 1 #1) and in other patients. It is possible that these discrepancies were due to stochastic differences in sputum composition with respect to the proportion of human to microbial cells at these different time points. Whether or not this was the case, the majority of our longitudinal time series did not show an obvious connection between the duration of intravenous antibiotic treatment and deuterium labeling of anteiso fatty acids.

Most samples in our longitudinal data set contain a-C$_{15:0}$ at concentrations that are significantly higher (10- to 50-fold) than those of patient 7, in which *S. aureus* is absent (Fig. 2). Accordingly, a-C$_{15:0}$ in these samples is likely providing information about the growth of other CF-associated microbes that produce a-C$_{15:0}$, namely, *S. aureus*, *P. melaninogenica*, and *S. maltophilia*. Whether isotope incorporation yields reliable growth rates for any microbe also depends on the labeling rates of anteiso fatty acids from nonmicrobial origins. Nonmicrobial sources appear to contribute mainly a-C$_{17:0}$, which in CF sputum generally shows slower label incorporation than a-C$_{15:0}$ (14). Accordingly, contributions of nonmicrobial anteiso fatty acids tend to decrease growth rate estimates.

To better constrain how nonmicrobial anteiso fatty acids might affect our ability to infer microbial growth rates, we analyzed their potential impact using modeling. To a first approximation, we assume that the pool of anteiso fatty acids is a mixture of two end members at the beginning of the labeling experiment: a portion of biosynthetically active microbial lipids (x) and a portion of inactive dietary lipids ($1 - x$). Assuming that bacterial fatty acids are produced exponentially at a constant generation time (GT) and dietary fatty acids remain unlabeled and constant during incubation with D$_2$O, we can calculate the apparent generation time (y) using equation 1:

$$y = \frac{t}{\log_2\left[1 + x\left(2^{t/\text{GT}} - 1\right)\right]} \quad (1)$$
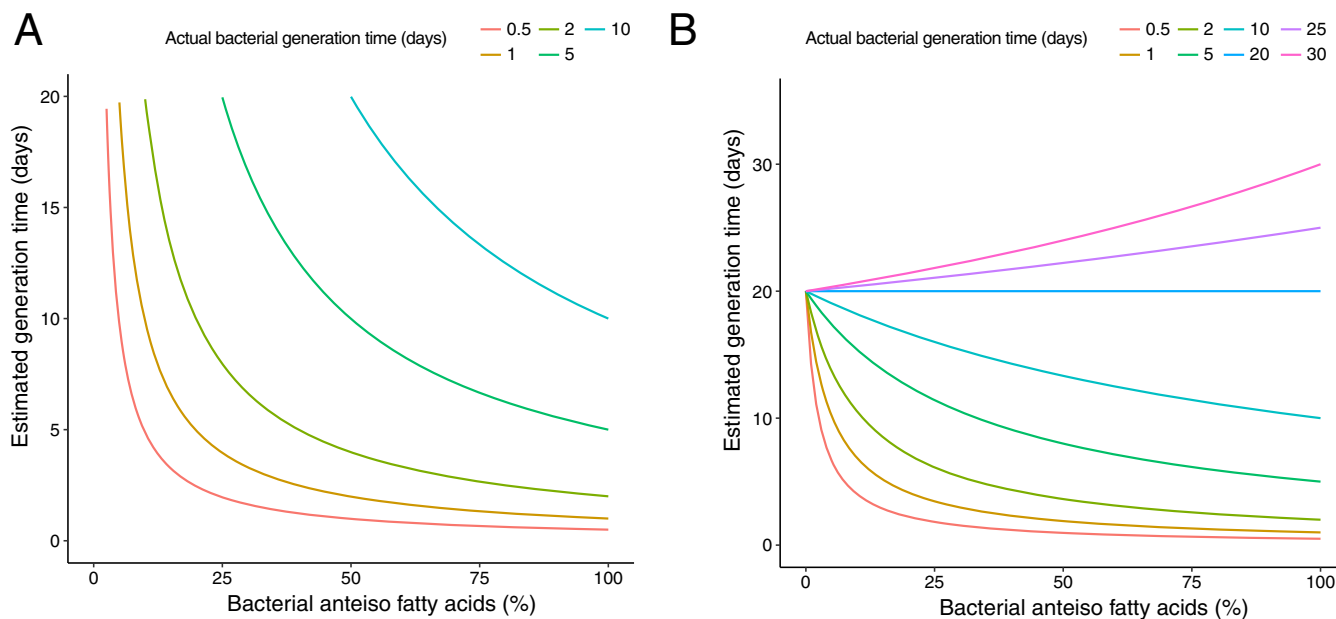
FIG 4 Effects of nonmicrobial sources of anteiso fatty acids on bacterial growth rate estimates. (A) The sensitivity analysis assumes exponential production of isotopically labeled anteiso lipids for different doubling times (colored lines) and zero isotope incorporation into anteiso fatty acids from other sources, e.g., diet. Equation 1 was used to calculate generation times. (B) Same as in first panel, but assuming that the nonmicrobial fraction of the anteiso fatty acid pool has isotope incorporation with a doubling time of 20 days.

The effect of nonmicrobial sources of anteiso fatty acids on bacterial growth rate estimates from this basic model is shown in Fig. 4A. For example, if the microbes have an actual generation time of 2 days and 90% of the anteiso fatty acids originated are of microbial origin and the remaining 10% dietary, the apparent generation time after 1 h of incubation would be 2.2 days, i.e., only slightly slower than the actual doubling every 2 days.

We can extend this basic model to include scenarios in which nonbacterial fatty acids can become isotopically labeled, e.g., due to *de novo* biosynthesis in human cells. If we assume a constant generation time GT_b for the bacterial fraction (x) and GT_n for the nonbacterial fraction ($1 - x$), the apparent generation time y is given by equation 2:

$$y = \frac{t}{\log_2\left[x \times 2^{t/GT_b} + (1 - x) \times 2^{t/GT_n}\right]} \tag{2}$$

Figure 4B shows the effect of nonbacterial growth with a generation time of 20 days (GT_n), which is on the order of generation times measured for a-$C_{17:0}$ in samples from patient 7 (presumed to be nonbacterial in origin). In this extended model, the nonbacterial fatty acids have a relatively low but nonzero isotope incorporation; as a result, the deviations between actual and apparent microbial growth rates tend to be smaller than in the simpler model.

This sensitivity analysis suggests that a-$C_{15:0}$ can be interpreted as a robust proxy for bacterial growth in CF infections, provided most of the analyte originates from bacteria. In sputum from patient 7, a-$C_{15:0}$ did not derive from any known microbial sources and was detected at a mean concentration of 0.025 wt%. Using 4-fold this value (0.1 wt%) as a threshold, we filtered our longitudinal data to identify samples for which microbial growth rates in sputum could be obtained reliably from deuterium labeling of a-$C_{15:0}$. The median growth rates estimated for these 23 samples is 1.9 days, which represents a mixture of growth rates for all three anteiso fatty acid-producing microbial strains (Table 3). As expected, the amount of anteiso fatty acids detected in CF sputum has a moderately positive correlation with the amount of microbial producers (see Fig. S2 in the supplemental material) (Pearson correlation coefficient, +0.52). This analysis suggests that *P. melaninogenica* and *S. aureus* are the predominant microbial producers of

TABLE 3 Summary of samples for which growth rates can be reliably inferred from a-$C_{15:0}$

Patient ID	Day	a-$C_{15:0}$ (wt%)	GT (days)	*S. aureus* (%)	*Stenotrophomonas* (%)	*Prevotella* (%)
1	2	0.90	1.6	82.5	0	16.8
1	4	0.48	1.5	90.5	0	7.8
1	5	0.62	0.5	45.0	0	50.8
1	7	0.65	0.7	84.7	0	11.1
1	Stable	0.32	2.0	53.4	0	43.5
1 #2[a]	2	0.90	1.3	64.5	0	34.0
1 #2	5	0.50	1.1	63.1	0	35.7
1 #2	8	0.44	1.9	69.1	0.9	10.5
1 #2	15	0.43	1.2	12.2	0	85.6
2	2	0.34	3.3	92.8	0	2.8
2	3	0.13	1.4	81.6	0	3.3
3	3	0.32	1.5	95.8	0	3.6
3	4	0.29	45	77.5	0	15.0
3	6	0.12	2.5	22.1	0	72.4
4	2	0.47	0.1	19.2	0	66.6
4	6	0.44	5.5	0.4	0	76.4
4	8	0.24	17.1	0.1	0	99.6
4	9	0.75	15.3	0	0	99.5
4	10	0.50	10.1	0.1	0	99.7
5	0	0.39	1.4	0.9	1.5	96.6
5	1	0.19	1.8	0	21	77.2
5	2	0.29	26.2	0.4	7.6	91.9
5	4	0.20	3.2	0	78.2	16.7

[a]1 #2, patient 1, 2nd hospitalization.

anteiso fatty acids in most expectorated CF sputum samples in our cohort. When we compared the abundances of microbial strains with the generation times in the 23 samples with high a-$C_{15:0}$ content (Table 3), we found that samples that were dominated by *P. melaninogenica* (median, 2.5 days; 11 samples) and *S. maltophilia* (3.2 days; 1 sample) had slower generation times than those that contained >50% of *S. aureus* (1.5 days; 11 samples). Based on this refined analysis of anteiso fatty acids, we did not observe a consistent decrease of bacterial growth rates during pulmonary exacerbation treatment with intravenous antibiotics in individual patients. The only *S. aureus*-rich sample that had a much slower growth was from patient 3 (generation time, 45 days; day 4). By the following time point (day 6), the bacterial community size had dropped by 94% and the relative abundance of *S. aureus* had decreased from 78% to 22%, while growth recovered to a generation time of 2.5 days. This indicates that low levels of label incorporation into a-$C_{15:0}$ can coincide with recent population decline. Killed *S. aureus*, including intact phospholipids, may transiently remain in sputum and cause low apparent growth rates independent of the growth rate of surviving bacteria. In summary, *S. aureus* generation time in our cohort was typically on the order of 1 to 2 days and did not consistently decrease during antibiotic treatment of pulmonary exacerbations.

DISCUSSION

Our comparison of CF microbiome composition with the abundance of fatty acids indicated that anteiso fatty acids in expectorated sputum can originate from previously unrecognized sources, such as *Prevotella* and human cells. Compared to a-$C_{17:0}$, smaller amounts of a-$C_{15:0}$ seem to be introduced by nonmicrobial sources, making a-$C_{15:0}$ a more reliable biomarker for microbial activity. We now view incorporation of deuterium into a-$C_{15:0}$ as a likely biomarker for the anabolic activity of *P. melaninogenica* and *S. maltophilia* in addition to *S. aureus*. Fatty acids from human cells or from diet are expected to yield little to no isotope incorporation. From a practical standpoint, best practices for application of deuterium stable-isotope probing to CF sputum samples require knowledge of the microbial community composition and amounts of a-$C_{15:0}$ analyte in excess of 0.1 wt% (to be confident that measurements are well above human background levels).

Much of the variability that we observed in deuterium labeling data was eliminated when focusing our analysis on samples that had high levels of a-C15:0. These samples generally yielded generation times between 0.5 and 2.5 days, with a median of 1.9 days (Table 3). Samples dominated by *S. aureus* (>50%) had a median generation time of 1.5 days. Overall, these estimates for generation times are in the range measured for a-$C_{15:0}$ in our previous data set (14). Because isotopic labeling provides only an average growth estimation for the microbial population, we cannot exclude the possibility that a subpopulation of cells grows significantly slower or faster than at the median generation times we observe. To resolve heterogeneity at this level, single-cell growth rate visualization is necessary (21, 22).

Nevertheless, the comprehensive data set collected for this longitudinal study allows us to refine our interpretation of whether there are any reproducible patterns in how pathogen growth rates change over the course of antibiotic treatment during a pulmonary exacerbation. After filtering for samples that allow confident estimation of pathogen growth rates (Table 3), we find that growth rate estimates generally remained stable during treatment. We note that samples with low levels of a-$C_{15:0}$-producing microorganisms do not yield reliable pathogen growth estimates. However, samples containing high levels of a-$C_{15:0}$ and low deuterium incorporation values likely indicate that an antibiotic is inhibiting pathogen growth (patient 3; day 4). It is interesting that opportunist growth remained stable through exacerbation treatment. This is also supported by other studies looking at microbiome composition and the abundance of *P. aeruginosa* through exacerbation (33, 34). In sum, stable-isotope probing in combination with community analysis did not support our previous hypothesis that the *S. aureus* population undergoes niche expansion during antibiotic treatment during an exacerbation. On the contrary, our NanoString data showed that all patients had lower relative abundance of *S. aureus* at the end of treatment than at the beginning.

In conclusion, stable-isotope probing is likely to be most useful in tracking pathogen growth dynamics in response to antibiotic treatment early during pulmonary exacerbations, when pathogenic load is high. Stable-isotope probing also has the potential to enable a better understanding of how various factors affect microbial physiology *in situ*. For example, with expectorated CF sputum, microbial microcosm incubations could be used to test how the growth rates of pathogens are affected by nutrient availability and drug treatments. With future development, rapid analysis of incubations of CF sputum with different drugs might eventually help identify the most promising treatment options. Currently, we are developing a method to quantify deuterium label in intact lipids with liquid chromatography-mass spectrometry (LC/MS) (35). This approach promises to increase the utility of stable-isotope probing to assess growth of diverse pathogens.

MATERIALS AND METHODS

Study population. Children and young adults with CF hospitalized for a pulmonary exacerbation were included in this study. Inclusion criteria were a positive diagnosis of cystic fibrosis, presence of *S. aureus* in CF sputum culture, hospitalization for a pulmonary exacerbation, and ability to expectorate sputum. Pulmonary exacerbations were defined as acute worsening of respiratory symptoms. These included clinical features such as increased cough, increased sputum production, shortness of breath, chest pain, loss of appetite, loss of weight, and lung function decline (10). In addition, sputum samples were collected from three CF patients without the presence of *S. aureus* on sputum culture to analyze their fatty acid composition. The study was approved by the Institutional Review Board at Children's Hospital Los Angeles (IRB number CCI-13-00211). All patients were recruited from Children's Hospital Los Angeles, and informed consent or assent was obtained from all study participants or from a parent/legal guardian.

The medical records of enrolled subjects were reviewed for age, gender, body mass index, CFTR mutations, duration of hospitalization, results of CF sputum culture, mycobacterial culture, antibiotics used, and results of pulmonary function tests. This information is presented in Table 1 and in Tables S1 and S2 in the supplemental material.

Sputum sampling and D$_2$O labeling. Fresh expectorated sputum from children and young adults admitted with a CF pulmonary exacerbation was collected on admission, throughout hospitalization, and after return to baseline health. All hospitalized patients received intravenous antibiotics and airway clearance therapies as directed by their treating physician. Expectorated sputum was sampled and treated as described by Kopf et al. (14). Within 10 min of expectoration, sputum samples were segregated into two parts. One part was flash frozen and stored at −80°C for RNA analysis. The second part (weight, >0.6

g) was incubated at 37°C for 1 h with an equivalent weight of prewarmed phosphate-buffered saline (PBS) solution containing 5% (wt/wt) D_2O. The D_2O content in isotope-labeled sputum samples was determined on a DLT-100 liquid water isotope analyzer (Los Gatos Research) (14). To measure the D_2O content, 150 μl supernatant liquid from the incubated sputum sample was filter sterilized using a centrifuge tube filter (Costar Spin-X; Corning) and diluted with water of known natural isotopic composition. The remaining sample was flash frozen and stored at −80°C for fatty acid analysis.

RNA isolation and analysis. For sputum community profiles, RNA was prepared and analyzed as described by Grahl et al. (25). Measurements were made using a NanoString nCounter (NanoString Technologies) (36). We used the custom-designed Nanostring CodeSet described in reference 25, which enables the simultaneous detection of rRNA for 38 clinically relevant CF-related bacterial and fungal species or genera. Raw counts were corrected by subtracting 5 times the maximum count for internal negative controls (25). PERMANOVA and nonmetric multidimensional scaling analysis (NMDS) of microbial community composition was performed using the R Package "vegan" (37). Weighted species abundances (fourth root of the percentage value) were used to calculate Bray-Curtis dissimilarity.

Fatty acid extraction, gas chromatography, and isotope ratio measurement. Methyl esters of the two target analytes, 12-methyl-tetradecanoic acid (a-$C_{15:0}$) and 14-methyl-hexadecanoic acid (a-$C_{17:0}$), were prepared from sputum samples by transesterification of phospholipids in the presence of a base catalyst (0.5 M NaOH in anhydrous methanol), extraction into hexane, and cleanup using solid-phase extraction (14). Two internal standards (10 μg) were added before extraction [PC(21:0/21:0)] or solid-phase extraction ($C_{22:0}$ methyl ester). The extraction yielded about 10 μg saturated fatty acid methyl esters per 1 mg dry sputum. The fraction containing saturated fatty acid methyl esters (FAMEs) was analyzed by gas chromatography/mass spectrometry (GC/MS) on a Trace DSQ instrument (Thermo Fisher Scientific) with a ZB-5ms column (30 m by 0.25 mm; film thickness, 0.25 μm) (14). Peaks were identified by comparison of mass spectra and retention times to authentic standards and library data.

Analytical controls to assess contributions of a-$C_{15:0}$ and a-$C_{17:0}$ from sources other than *S. aureus* were performed by GC/MS. About 20 mg of lyophilized biomass was transesterified by acid hydrolysis at 100°C (10 min) in the presence of 20:1 (vol/vol) anhydrous methanol-acetyl chloride and hexane (38). Materials tested were *Streptococcus epidermidis*, CF bronchial epithelial (CFBE) cells (39), fetal bovine serum (FBS), and polymorphonuclear neutrophils (PMN; differentiated from HL-60 cells).

Deuterium enrichments were quantified in replicates on a Delta$^+$XP instrument (Thermo Fisher Scientific) by GC pyrolysis isotope ratio mass spectrometry (GC/P/IRMS) as described by Kopf et al. (14) with the following optimization. The GC program was adjusted for a shorter run time and better peak separation of target analytes a-$C_{15:0}$ and a-$C_{17:0}$. The GC oven was held at 80°C for 0.5 min, temperature then was increased at 30°C/min to 170°C, held at 170°C for 45 min, and increased again at 30°C/min to 190°C, followed by a final increase of 30°C/min to 320°C (held for 10 min). Chromatographic peaks were identified in comparison to GC/MS runs by retention order and signal intensity. Reported δD values are corrected for the hydrogen atoms in FAME originating from methanol. Values are stated in the conventional notation versus the VSMOW (Vienna Standard Mean Ocean Water) standard (40). Experimental and analytical errors were estimated as described by Kopf et al. (14), and we used a Monte Carlo simulation to propagate these uncertainties when calculating growth rate estimates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00365-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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