Changes in Cystic Fibrosis Airway Microbiota at Pulmonary Exacerbation

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Abstract

Rationale: In persons with cystic fibrosis (CF), repeated exacerbations of pulmonary symptoms are associated with a progressive decline in lung function. Changes in the airway microbiota around the time of exacerbations are not well understood.

Objectives: To characterize changes in airway bacterial communities around the time of exacerbations and to identify predictors for these changes.

Methods: DNA prepared from 68 paired baseline and exacerbation sputum samples collected from 28 patients with CF were subjected to barcoded 16S rRNA gene pyrosequencing. Bacterial density was calculated by quantitative PCR.

Measurements and Main Results: Overall, significant differences in bacterial community diversity and bacterial density between baseline and exacerbation samples were not observed. However, considerable changes in community structures were observed in a subset of patients. In these patients, the dominant taxa and initial level of community diversity were significant predictors of the magnitude of community structure changes at exacerbation. *Pseudomonas*-dominant communities became more diverse at exacerbation compared with communities with other or no dominant species. The relative abundance of *Gemella* increased in 24 (83%) of 29 samples at exacerbation and was found to be the most discriminative genus between baseline and exacerbation samples.

Conclusions: The magnitude of changes in the CF lung microbiota around the time of exacerbation was found to be largely dependent on community diversity and composition at baseline. Certain genera appear to play important roles in driving change in airway bacterial community composition at exacerbation. *Gemella* might play a direct role in and/or be a biomarker for pulmonary exacerbation.

Keywords: lung microbiome; cystic fibrosis exacerbation; *Pseudomonas*; *Gemella*

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Respiratory failure due to recalcitrant airway infection and inflammation is the predominant cause of morbidity and mortality in persons with cystic fibrosis (CF). Recent studies using culture-independent microbial detection have revealed that CF airway bacterial communities are more diverse than previously appreciated (1–5) and vary across short- and long-term scales (5, 6). It is not clear, however, how changes in the airway microbiota relate to lung disease progression. More specifically, it is not known whether significant changes in airway bacterial communities occur around the time of pulmonary exacerbations, events that are well known to be associated with a progressive and irreversible decline in lung function, increased hospitalization and

Table 1. Patients and samples

Variable	n
Patients (n = 28)	
Sex	14 female, 14 male
CFTR genotype, n (%)	
ΔF508/ΔF508	14 (50)
ΔF508/other	13 (46)
Other/other	1 (4)
Specimens (n = 34 pairs)	
Age at baseline sample, yr (mean ± SD)	27.9 ± 11.9
Baseline $\%FEV_1$, mean ± SD	55.6 ± 19.4
Baseline disease stage, n (%)	
Early ($\%FEV_1 > 70$)	10 (29)
Intermediate ($70 \geqslant \%FEV_1 \geqslant 40$)	15 (44)
Advanced ($\%FEV_1 < 40$)	9 (27)
Days between paired samples (mean ± SD)	84 ± 46

antibiotic use, decreased quality of life, and increased mortality rates (7–12). Studies addressing this important question have provided valuable insight but have been limited by cross-sectional design, small numbers of paired baseline–exacerbation samples from the same individual, and a focus on relatively narrow measures of bacterial community change (e.g., total bacterial density or community diversity) (5, 6, 13–15).

Here, we examine changes in airway microbial communities around the time of exacerbation events by analysis of airway communities in paired sputum samples collected before and at the onset of respiratory symptoms but before the administration of antibiotic therapy. In addition to assessing total bacterial density and community diversity, we investigate changes in community structure and in the abundance of individual taxa to identify features associated with exacerbation.

Methods

Patients, Sputum Specimens, and Medical Record Review

Sputum sample collection and medical record review were approved by the University of Michigan Institutional Review Board. Sputum specimens, collected during the course of routine medical care, were obtained from the University of Michigan Health System clinical microbiology laboratory after processing for bacterial culture and maintained at −80°C in 0.5-ml aliquots.

Medical records from approximately 400 patients with CF from whom sputum samples had been obtained were reviewed to identify samples recovered around the time of pulmonary exacerbations. Briefly, an exacerbation was identified when called such by the treating physician and/or when an increase in two or more patient-reported outcomes resulted in the prescription of antibiotics (5). Sputum specimens were categorized by the patient's clinical state at the time of sample collection as previously described (5): *1*) baseline, *2*) onset of pulmonary exacerbation (before antibiotic administration), *3*) treatment of pulmonary exacerbation, or *4*) recovering from pulmonary exacerbation (within 21 d after cessation of antibiotics). Specimens were further categorized by disease stage (5) based on $\%FEV_1$ values at the time of sample collection: early ($\%FEV_1 > 70$), intermediate ($\%FEV_1 \leqslant 70, \geqslant 40$), or advanced ($\%FEV_1 < 40$). All antibiotic use was recorded from the medical record.

In total, 34 pairs (68 samples) of baseline–exacerbation samples, obtained from 28 patients, were identified. A single pair of isolates was obtained from each of 23 patients; two pairs were obtained from each of four patients, and three pairs were obtained from the remaining patient. Patient demographics are provided in Table 1. With the exception of maintenance antibiotic therapy (inhaled tobramycin, inhaled colistimethate, and/or oral azithromycin), no antibiotics were administered, to our knowledge, between samples. The average patient age at baseline sample collection was 27.9 years (range, 9.9–53.3 yr). Among the 34 baseline samples, 15 (44%) were obtained from patients with intermediate disease, whereas 10 (29%) and 9 (27%) were collected when patients were at early or advanced disease stages, respectively. The median number of days between baseline and exacerbation samples was 84 (mean, 84 d; range, 11–230 d).

DNA Extraction and Quantitative PCR

DNA was prepared from frozen sputum as previously described (5). Briefly, samples were treated with Sputolysin (EMD Chemicals, Gibbstown, NJ) and subjected to bead beating before DNA extraction by the MagNA Pure nucleic acid purification platform (Roche Diagnostics Corp., Indianapolis, IN). Total bacterial load was measured by quantitative PCR using a universal primer/probe set targeting the bacterial 16S rRNA gene as previously described (5, 16). The abundance of specific operational taxonomic units (OTUs) was calculated by multiplying the total 16S copy number by the OTU's relative abundance.

DNA Sequencing and Data Analyses

Pyrosequencing of the V3 to V5 hypervariable region of the 16S rRNA gene was performed by the Human Genome

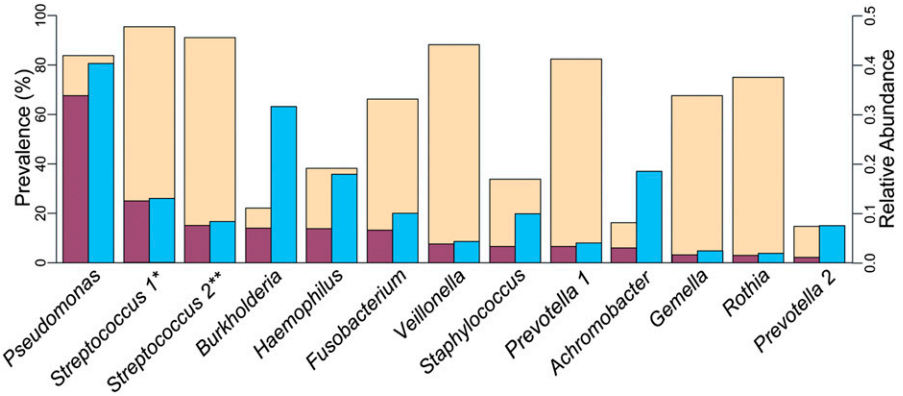

Figure 1. Prevalence, average total relative abundance (RA), and average RA when present for 13 top operational taxonomic units calculated from subsampled data. *Tan bars*, prevalence; *burgundy bars*, average total RA (n = 68); *blue bars*, average RA (when present). **S. mitis* group, ***S. salivarius* group.

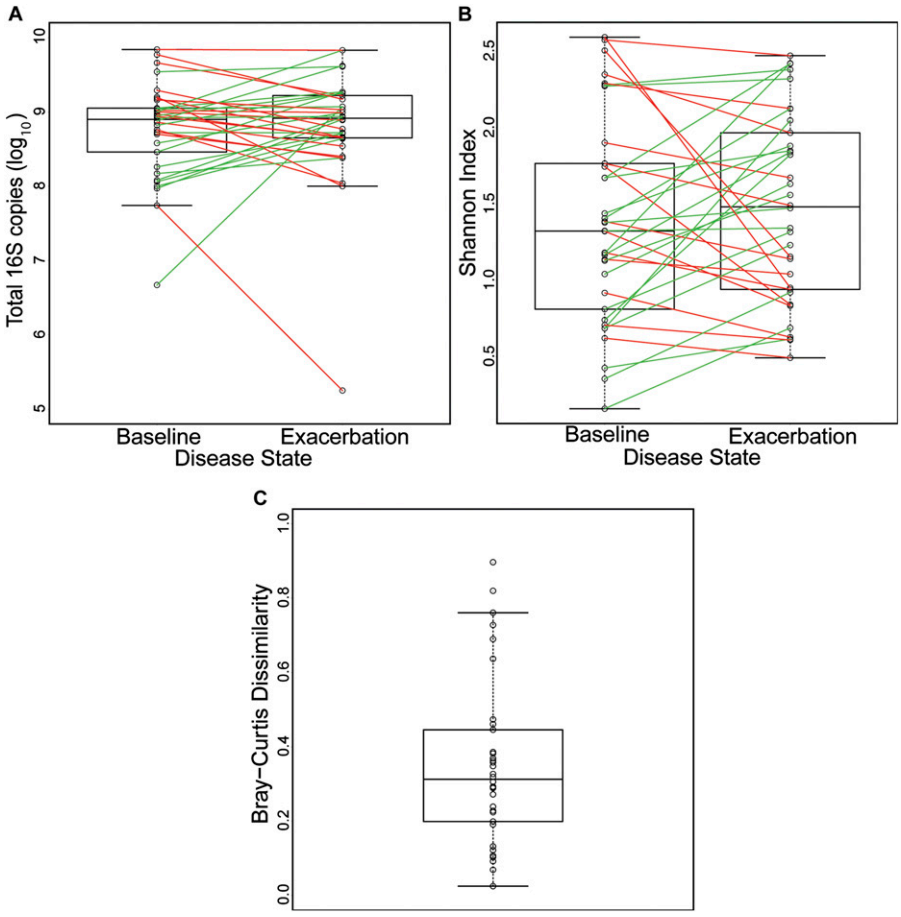

Figure 2. Changes in global community measures, including total bacterial load (total 16S rDNA copies) (*A*), community diversity (Shannon index) (*B*), and community structure (Bray-Curtis dissimilarity) (*C*). *Black lines* connect pairs of samples in *A* and *B*, *green lines* indicate increasing values, and *red lines* indicate decreasing values. The top and bottom boundaries of each box indicate 75th and 25th quartile values, respectively; *black lines* inside each box represent the median values. Ends of the whiskers mark the lowest and highest values within 1.5 times the interquartile range. Outliers are defined as samples with bacterial density lower than 1.5 times the interquartile range and samples with Bray-Curtis dissimilarity greater than 1.5 times the interquartile range in *A* and *C*, respectively.

Sequencing Center at Baylor College of Medicine using Roche 454-based sequencing protocols developed for the Human Microbiome Project (http://www.hmpdacc.org/tools_protocols.php) as previously described (5). The mothur (v.1.24) software package was used to process sequences as described elsewhere (17, 18). A total of 278,811 sequences (average per sample, 4,100; range, 726–9,948) were obtained from 68 sputum samples. The total number of reads for each community was randomly subsampled or rarefied to 726, the smallest number of reads obtained in the sample set, to control for differences in sequencing depth before α (nonparametric Shannon index [19]), and β diversity measures were calculated. Subsampling was performed by randomly sampling 726 sequences from each sample 1,000 times and calculating the mean value. Bray-Curtis (BC) dissimilarity was used to measure the difference in community structures between paired samples (20).

Statistical analyses were performed with IBM SPSS 19. Linear mixed models were used to control for the patient random effect because several patients contributed more than one sample set to the analysis. Paired *t* tests were used to compare measures between sample pairs. When assumptions of normality were not met, Wilcoxon signed-rank tests were used. For single comparisons, a P value < 0.05 was considered statistically significant. For analyses with multiple comparisons, a Bonferroni-adjusted α value (0.05/number of comparisons) was used. Analysis of molecular variance was used to compare community structures by clinical state and dominant OTU (21). Random Forest analysis was performed using the randomForest package in R with 500 trees and default settings (22).

Results

DNA Sequencing Analysis

A total of 151 OTUs were detected, with an average of 16.8 OTUs (range, 3–34 OTUs) per sample. Thirteen OTUs, hereafter referred to as "top" OTUs, were present in the set of 68 samples with an average relative abundance of greater than 1% (Figure 1; *see* Table E1 in the online supplement). On average, an OTU that affiliated with the genus *Pseudomonas* was present with the greatest relative abundance; this taxon also had a high prevalence, being detected in 84% of samples. Although genera such as *Streptococcus*, *Fusobacterium*, *Veillonella*, *Prevotella*, *Gemella*, and *Rothia* were detected at relatively low abundances (2–14%), they had a high prevalence, being found in 66 to 96% of samples. Taxa such as *Burkholderia* and *Achromobacter* were detected with low prevalence in this sample set; however, they had a relatively high abundance when present. Good's coverage was at least 97% for each community.

Changes in Bacterial Communities at Exacerbation

No significant overall difference in bacterial load was detected between baseline and exacerbation samples (mean, 1.23×10^9 and 1.35×10^9 copies/ml, respectively) (Figure 2A). Relative to baseline, bacterial load increased at exacerbation in 18 pairs and decreased in 16 pairs. To investigate whether specific variables might predict an increase or decrease in bacterial density, we used a multivariate linear mixed model with the difference between $\log_{10}$ total 16S rRNA copies at baseline and exacerbation as the outcome variable. We included demographic variables (patient age and sex) that have been used previously for predicting exacerbation or survival in CF (8, 23, 24), variables that describe baseline microbial community composition (Shannon diversity and dominant OTU), and variables that we have previously

reported to affect bacterial communities, including disease stage and maintenance antibiotic use (5). We also included the number of days between corresponding baseline and exacerbation samples to control for the unbalanced sampling interval. A dominant OTU was defined as the most abundant OTU having at least twice the relative abundance of the next most abundant OTU in a sample; 23 of 34 baseline samples had a dominant OTU. *Pseudomonas* was the dominant genus in 13 samples, with relative abundances ranging from 0.449 to 0.975. *Burkholderia*, *Fusobacterium*, *Haemophilus*, and *Streptococcus* were dominant in two samples each, whereas *Achromobacter* and *Staphylococcus* were each dominant in a single sample. The relative abundance of the dominant OTU among these samples ranged from 0.403 to 0.859. Given this uneven distribution of dominant genera, samples were categorized as *Pseudomonas* dominant (n = 13), as non–*Pseudomonas* dominant (n = 10), or as having no dominant OTU (n = 11). Patients were considered to be receiving maintenance antibiotics if thrice weekly azithromycin, inhaled tobramycin, and/or inhaled colistimethate were part of the patient's routine care. None of these factors was found to have a significant influence on change in bacterial density at exacerbation (Table E2).

To investigate changes in bacterial communities that may have occurred with exacerbation, the Shannon diversity index was calculated for all samples. Relative to the corresponding baseline sample, the Shannon index increased in 19 exacerbation samples and decreased in 15 exacerbation samples (Figure 2B). No significant difference in community diversity was observed by pairwise comparisons of all baseline–exacerbation sets ($P > 0.10$; paired t test).

Although the Shannon index measures community complexity, taking into account richness (number of OTUs) and evenness (relative abundance of OTUs), it does not reflect community composition. Two communities could have the same Shannon index but vastly different community structures. Therefore, Bray-Curtis (BC) distances were calculated to evaluate changes in community structures (membership and relative abundance) between baseline and exacerbation samples. Although the average BC distance between the 34 baseline–exacerbation pairs was 0.351, the range was quite large (0.025–0.897) (Figure 2C), suggesting that although community structures changed little in some patients, considerable changes occurred in others.

Factors Predicting Changes in Community Structures at Exacerbation

Given the range of community structure changes observed between baseline–exacerbation pairs, we sought to identify factors that correlate with the magnitude of change (i.e., dissimilarity) between baseline and exacerbation communities. We again used a multivariate linear mixed model, which included the same variables used in the analysis of changes in bacterial density, to determine the influence of these variables on BC distance between baseline and exacerbation samples. Baseline diversity and baseline dominant OTU were the strongest predictors of community dissimilarity (BC distance) between paired baseline–exacerbation samples ($P < 0.05$) (Table 2). More specifically, there was a significant positive correlation between baseline Shannon diversity and BC distance; greater baseline community diversity was associated with greater dissimilarity between baseline and exacerbation samples ($P = 0.029$). Furthermore, *Pseudomonas*-dominant samples were more dissimilar between baseline and exacerbation compared with pairs of samples with no dominant OTU ($P = 0.031$). The model also revealed a significant interaction between baseline diversity and dominant OTU ($P < 0.05$), which we investigated further by examining subsets of sample pairs differentiated by baseline-dominant OTUs. In communities dominated by a non-*Pseudomonas* OTU or with no dominant OTU, we still observed significant positive correlations between baseline diversity and BC distance between baseline and exacerbation samples. In contrast, this correlation was not observed in *Pseudomonas*-dominant communities (Figure 3). That is, *Pseudomonas*-dominant samples changed at exacerbation regardless of degree of baseline diversity. Baseline

Table 2. Fixed effect coefficient estimates from a multivariate linear mixed model of patient and bacterial community attributes' influence on community structure change at exacerbation

Variables	Estimate	*P* Value	95% CI
Intercept	−0.229	0.557	−1.030 to 0.571
Sex			
Female	−0.005	0.961	−0.235 to 0.224
Male	0.000		
Inhaled tobramycin			
Yes	0.015	0.887	−0.200 to 0.230
No	0.000		
Azithromycin			
Yes	−0.093	0.385	−0.310 to 0.125
No	0.000		
Inhaled colistimethate			
Yes	−0.012	0.953	−0.420 to 0.396
No	0.000		
Disease stage			
Early	0.003	0.984	−0.280 to 0.285
Intermediate	0.067	0.525	−0.149 to 0.283
Advanced	0.000		
Dominant OTU			
Pseudomonas	0.812	0.031	0.084 to 1.540
Other	0.411	0.307	−0.407 to 1.230
None	0.000		
Shannon index	0.365	0.029	0.042 to 0.688
Age	−0.007	0.138	−0.016 to 0.002
Days between samples	0.001	0.474	−0.001 to 0.003
Dominant OTU × Shannon index			
Dominant OTU = *Pseudomonas*	−0.408	0.049	−0.814 to −0.002
Dominant OTU = other	−0.153	0.541	−0.666 to 0.360
Dominant OTU = none	0.000		

Definition of abbreviations: CI = confidence interval; OTU = operational taxonomic unit.

diversity was not significantly different between *Pseudomonas*-dominant and non–*Pseudomonas*-dominant communities ($P > 0.10$; ANOVA with Tukey HSD *post hoc* test). In *Pseudomonas*-dominant samples, bacterial community diversities were significantly higher in exacerbation samples compared with baseline samples ($P < 0.05$; paired *t* test). In contrast, in samples with a non–*Pseudomonas*-dominant OTU or with no dominant OTU, no significant differences in community diversity between baseline and exacerbation samples were found ($P > 0.10$).

Changes in Individual OTUs at Exacerbation

After characterizing bacterial changes at the community level, we examined individual OTUs to determine if predictable genus-level signatures occurred at exacerbation. Comparison of the abundance of the top OTUs (Figure 1; Table E1) between all pairs of baseline and exacerbation samples revealed that the average absolute abundance ($\log_{10}$ total 16S rRNA copies) of *Gemella* was significantly higher in exacerbation samples compared with baseline samples ($P = 0.001$; Wilcoxon signed-rank test with Bonferroni adjustment). *Gemella* average relative abundance was also higher at exacerbation than at baseline, although this result failed to reach statistical significance with multiple testing correction ($P = 0.008$; Wilcoxon signed-rank test with Bonferroni adjustment) (Figure 4A). The Random Forest machine-learning algorithm also identified *Gemella* relative abundance as the most predictive among the top OTUs to differentiate baseline from exacerbation samples (Figure 4B). There were no significant differences between baseline and exacerbation sample pairs in average relative or total abundance for any other top OTUs ($P > 0.004$; Wilcoxon signed-rank test with Bonferroni adjustment).

Multivariate analysis of community structure measures based only on the top OTUs also showed a statistically significant distinction between baseline and exacerbation samples for the subset of *Pseudomonas*-dominant communities ($P < 0.05$; analysis of molecular variance). These changes were analyzed using principal coordinates analysis, a method that reduces the dimensions of a complex data set so that relationships within the data can be visualized (Figure 5). At exacerbation, these communities shifted right along the first principal coordinate (PCo1) in the principal coordinates analysis plot. Correlation coefficients between each of the top OTUs and the two axes were calculated and plotted as vectors to visualize the impact of individual genera on the movement of samples in the ordination space. *Pseudomonas* shifted communities to the left along PCo1, whereas multiple genera shifted communities to the right along PCo1. No significant distinction between clinical states was found for communities with a non–*Pseudomonas*-dominant OTU or with no dominant OTU.

As predicted by the multivariate analysis, *Pseudomonas*, *Streptococcus* 1 (representing *Streptococcus mitis* group species), and *Streptococcus* 2 (representing *Streptococcus salivarius* group species) appeared to have the greatest impact on changes in community structure at exacerbation for the *Pseudomonas*-dominant subgroup. The average relative and total abundances of *Pseudomonas* were significantly higher at baseline than at exacerbation ($P < 0.017$; Wilcoxon signed-rank test with Bonferroni adjustment). No significant differences in abundance between baseline and exacerbation samples were found in this subgroup for either of the *Streptococcus* OTUs ($P > 0.017$). In the non–*Pseudomonas*-dominant samples, the relative abundance of dominant OTUs was not significantly different between baseline and exacerbation ($P > 0.10$).

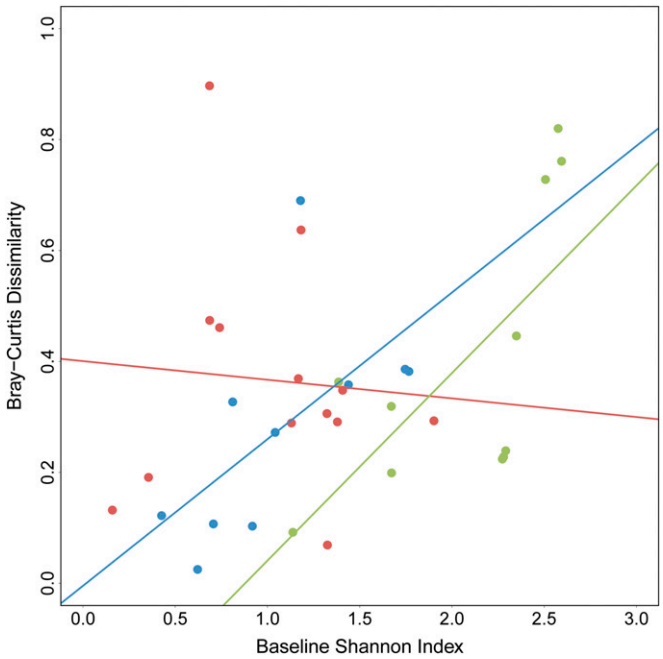

Figure 3. Baseline community diversity compared with dissimilarity between baseline and exacerbation samples. Increasing diversity is positively correlated with increasing community structure dissimilarity between baseline and exacerbation states for communities with a dominant operational taxonomic unit other than *Pseudomonas* (*blue*) or no dominant operational taxonomic unit (*green*) but not for *Pseudomonas*-dominant communities (*red*). Regression lines were calculated using the adjusted regression model based on the standardized sample profile (44% chance of being female; 50% chance of taking inhaled tobramycin; 65% chance of taking oral azithromycin; 6% chance of taking inhaled colistimethate; 29, 44, and 27% chance of being early, intermediate, or advanced disease stage, respectively; average number of days between samples = 84; and average age at baseline sample = 27.9).

Discussion

Studying the dynamics of the CF lung microbiota at the time of exacerbation is of particular interest in efforts to better understand the pathologic underpinnings of these events and, ultimately, to develop new strategies to prevent or mitigate their occurrence. A challenge in this regard has been the paucity of serial samples obtained from the same patients before and at the

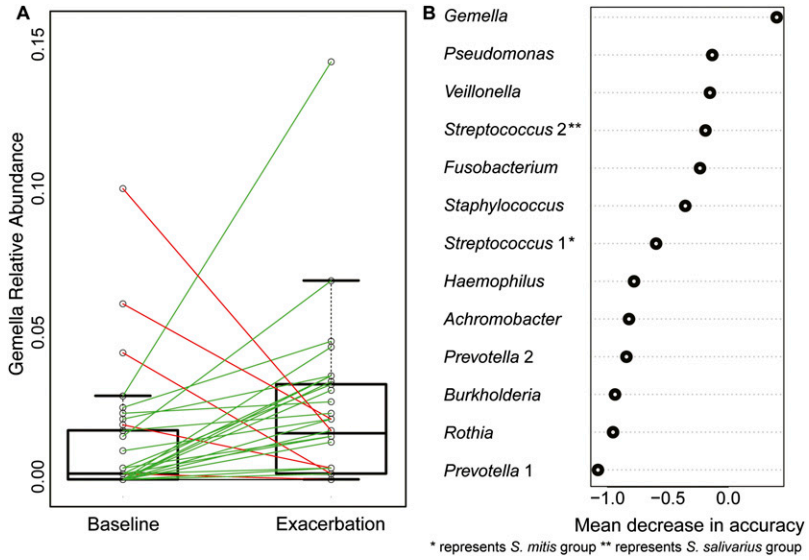

Figure 4. Changes in individual operational taxonomic units at exacerbation. (*A*) Relative abundance of *Gemella* at baseline and exacerbation states. Pairs of samples are connected with lines: *green lines* indicate increasing abundance, *red lines* indicate decreasing abundance, and *black lines* indicate no change in abundance. The top and bottom boundaries of each box indicate 75th and 25th quartile values, respectively; *black lines* inside each box represent the median values. Ends of the whiskers mark the lowest and highest relative abundance of *Gemella* within 1.5 times the interquartile range. Outliers are defined as samples with relative abundance of *Gemella* greater than 1.5 times the interquartile range. (*B*) Random Forest variable importance plot. Random Forest classification shows the importance that the relative abundances of different taxa have in predicting exacerbation. The mean decrease in accuracy for each taxon is the normalized difference between the classification accuracy when data for that taxon are included as observed and when the data for that taxon are randomly permutated. Taxa with higher values of mean decrease in accuracy are stronger predictors of exacerbation.

time of exacerbation but before the administration of antibiotic therapy, which may have a significant impact on bacterial community structure (5, 13).

We identified 68 paired baseline–exacerbation samples from a large collection of CF respiratory samples. The selection of adjacent baseline–exacerbation samples from the same individual reduced confounding effects due to interindividual differences, age-dependent changes, and study period effects that limit cross-sectional studies. Medical record review showed that no antibiotics for the treatment of exacerbation were administered to patients before the exacerbation sample. Consistent with previous reports by us and others (5, 15), we found no significant differences between baseline and exacerbation samples regarding overall bacterial density and no patient or community specific factors that could predict whether density would increase or decrease at exacerbation.

Also consistent with our previous work (5), we observed no significant differences between baseline and exacerbation samples regarding overall community diversity. However, although diversity provides a measure of community complexity based on the number (richness) and relative abundances (evenness) of the species present, it does not necessarily reflect changes in community structure, including changes in the presence or relative abundance of specific taxa. When we assessed community structures to describe the degree of change occurring in the airway microbiota at exacerbation, we found that pairs of baseline and exacerbation samples varied widely in their levels of dissimilarity, as reflected in the large range of BC distances found between these pairs. In other words, although some communities appeared to change little between baseline and exacerbation, others showed considerable movement, suggesting different "types" of exacerbations, only some of which involve substantial changes in bacterial community structures.

We also found an overall positive correlation between baseline community diversity and degree of change from baseline to exacerbation. Stratification of samples by baseline-dominant OTU, however, revealed that this trend was not significant for *Pseudomonas*-dominant communities, which showed greater overall dissimilarity between baseline and exacerbation samples compared with communities with no dominant OTU and fairly high degrees of dissimilarity regardless of baseline diversity. That is, although greater dissimilarity was found between baseline and exacerbation samples in *Pseudomonas*-dominant samples, there was no correlation between the level of baseline diversity and the degree of community movement at exacerbation in these samples.

We noted that *Pseudomonas*-dominant communities differed from other communities regarding global measures of diversity. In these sets, diversity increased at exacerbation, a reflection of increasing community evenness due to a decrease in the absolute and relative abundance of *Pseudomonas* at exacerbation, with an increase in the relative abundance of a group of other species. These results suggest that fluxes in the density of *Pseudomonas* may play an important role in shaping community structures around the time of exacerbation in those communities wherein this genus dominates. In non–*Pseudomonas*-dominant communities, global changes in community structures at exacerbation appear to be much more modest. In a recent longitudinal study, Fodor and colleagues (25) compared CF airway microbiota when patients were stable and experiencing an exacerbation. They observed *Pseudomonas* trending toward higher sequence abundance at stable compared with exacerbation time points, although this result failed to reach statistical significance.

Communities with a preponderance of *Pseudomonas* were also found to behave differently than communities predominantly comprised of other species when analyzing paired preexacerbation baseline and postexacerbation baseline samples (*see* online supplement). We found that *Pseudomonas*-rich communities were significantly more similar to each other at baseline before and after exacerbation than were communities in which another OTU was most abundant (*see* Figure E1 in the online supplement). This suggests that *Pseudomonas*-rich communities were more resilient, returning to a community structure closely resembling

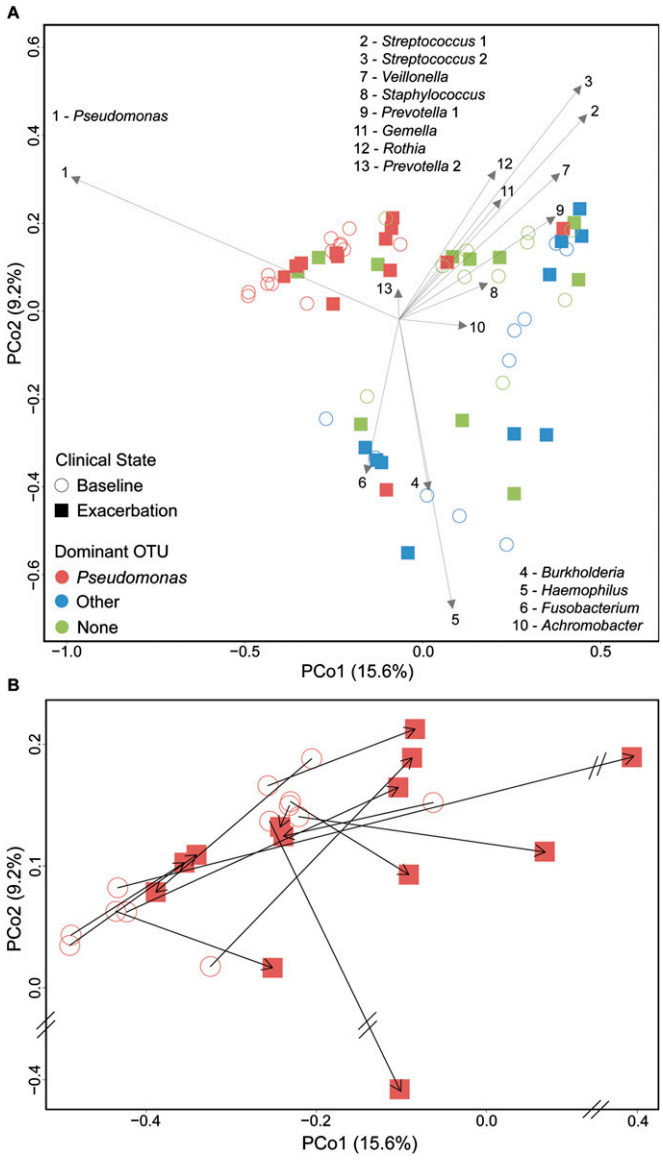

Figure 5. Principal coordinates analysis of baseline and exacerbation samples. Principal coordinates analysis reduces high-dimension data by assigning each item a location on a low-dimensional space based on a distance matrix. The first principal coordinate (PCo1) accounts for as much of the variability in the data as possible, and each succeeding coordinate accounts for as much of the remaining variability as possible. The proximity of points (samples) within the ordination space indicates similarity between communities; more similar communities are closer together on the plot. The *arrows* indicate the correlation of the relative abundance of the top operational taxonomic units (OTUs) with the two axes. (*A*) Community structures of 68 samples. *Arrows* show the influence of the top 13 OTUs on position of samples in the ordination space. (*B*) *Pseudomonas*-dominant subset of samples. Pairs of samples are connected by *arrows* pointing to exacerbation sample. PCo2 = the second principal coordinate.

the preexacerbation state, or more resistant to change in structure with exacerbation and antibiotic perturbation.

In contrast to a report by Sibley and colleagues (3) suggesting a role for *Streptococcus milleri* group species in exacerbation, we did not find *S. milleri* group species to be dominant members of the airway communities in our 28 patients. In other studies, additional facultative or obligate anaerobic species have been detected in CF respiratory specimens at densities comparable to those of aerobic species (26, 27). However, the role these species play in disease progression remains uncertain. In our study, an OTU representing *Gemella* was the most discriminative OTU between baseline and exacerbation samples, increasing in 83% of pairs when present at baseline and/or exacerbation (n = 29). We did not identify any distinctive patient-or community-specific factors among the five pairs of samples where *Gemella* decreased at exacerbation. Although the relative abundance of *Gemella* was generally low (1.6% overall and 2.4% when present), its prevalence in all samples included in this study was high (67.6%). Recently, the keystone-pathogen hypothesis has been supported by multiple reports (28, 29). This hypothesis underscores the important role of low-abundance pathogens in orchestrating inflammatory disease by remodeling normally benign microbiota into a dysbiotic community (29). Our data are consistent with this model, suggesting that the low abundant facultative anaerobe *Gemella* may be involved in triggering pulmonary exacerbation, possibly by remodeling the airway microbiota.

Our observation that baseline community diversity was a significant predictor of community structure change at exacerbation for non–*Pseudomonas*-dominant communities might be expected in that greater community diversity may provide for greater opportunity for changes in community structure. This presumption is supported by our analysis of community structure changes between paired exacerbation–treatment samples (*see* online supplement). In this analysis, community diversity at exacerbation (before antibiotic therapy) was the most important predictor of community structure changes with antibiotic therapy (Figure E2). Recently, Fodor and colleagues (25) similarly showed that low-diversity communities were generally resistant to change during antibiotic treatment. In our study, this was true even when the antibiotic load was relatively high, suggesting that communities lose the ability to respond to disturbance as diversity declines (Figure E2).

Consistent with previous reports by us and others (5, 25), we found that community diversity at baseline decreased significantly with disease stage (Figure E3). This decrease in diversity was primarily the result of a reduction in the numbers of species present rather than changes in the relative abundances (evenness) of species.

We did not find, however, that disease stage was a significant predictor of community structure change at exacerbation (Table 2).

There are a number of well-described technical caveats associated with the use of next-generation sequencing to analyze bacterial community diversity. Artifacts due to DNA preparation, amplification, and sequencing methods, as well as the choice of data analytic methods, present important limitations (18, 30, 31). The approach that has been used for deep-sequencing in most human microbiomic studies does not differentiate DNA derived from live versus dead bacteria. Although the exclusion of DNA from nonviable bacterial cells with the use of reagents such as propidium monoazide has been proposed (32–34), there is no clear consensus among Human Microbiome Project investigators as to the best approach to deal with this issue. There are reasonable concerns that essentially any step in DNA processing presents an opportunity for introducing bias (e.g., propidium reagents do not interact with all cell types in the same way). One could also argue that bacterial DNA is not biologically inert and should not be excluded. In previous work we have shown that antibiotic treatment significantly reduces bacterial community diversity in CF sputum (5). In an ongoing study, we found that some species present in relatively high abundance in sputum become undetectable with deep sequencing immediately after antibiotic therapy, suggesting that DNA from lysed bacteria does not contribute significantly to pyrosequencing measures of community structure (unpublished data). Most studies of microbial community dynamics in CF lung by other groups have not attempted to inhibit the sequencing of DNA from lysed bacteria (2–4, 14, 26, 35).

In this study, we carefully reviewed medical records for evidence of episodic antibiotic use between baseline and exacerbation samples. Although we found none, we cannot be certain that no patient received antibiotics "off the record." Another limitation inherent to a retrospective study such as this is that we did not have measures of lung function that would have allowed us to more fully assess the relationship between exacerbation severity and changes in specific microbiota. Furthermore, we did not have access to samples that would have allowed an assessment of daily fluctuations in measures of airway microbiota. A prospective study with more frequent collection of samples and relevant metadata, including antibiotic use, lung function measures, and clinical outcomes, is necessary to address these questions.

In summary, we found that changes in airway bacterial community structures varied greatly upon exacerbation; although little change occurred in some patients, dramatic changes were observed in others. Baseline community diversity and dominant OTU were significant predictors of community structure changes at exacerbation. In communities dominated by *Pseudomonas*, exacerbations were associated with a decrease in the absolute and relative abundance of *Pseudomonas*. This increase in community evenness is reflected in an increase in diversity at exacerbation in these communities. We also found that exacerbations were associated with increases in the relative abundance of *Gemella*, suggesting a pathogenic role for this genus in disease progression in CF. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1 Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 2004;42: 5176–5183.

2 Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, Kaess H, Deterding RR, Accurso FJ, Pace NR. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proc Natl Acad Sci USA* 2007;104: 20529–20533.

3 Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc Natl Acad Sci USA* 2008;105:15070–15075.

4 Guss AM, Roeselers G, Newton IL, Young CR, Klepac-Ceraj V, Lory S, Cavanaugh CM. Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis. *ISME J* 2011;5:20–29.

5 Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, *et al.* Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci USA* 2012;109:5809–5814.

6 Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TW, Patel N, Forbes B, *et al.* Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. *Thorax* 2012;67:867–873.

7 Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax* 2007;62:360–367.

8 Liou TG, Adler FR, Fitzsimmons SC, Cahill BC, Hibbs JR, Marshall BC. Predictive 5-year survivorship model of cystic fibrosis. *Am J Epidemiol* 2001;153:345–352.

9 Lieu TA, Ray GT, Farmer G, Shay GF. The cost of medical care for patients with cystic fibrosis in a health maintenance organization. *Pediatrics* 1999;103:e72.

10 Robson M, Abbott J, Webb K, Dodd M, Walsworth-Bell J. A cost description of an adult cystic fibrosis unit and cost analyses of different categories of patients. *Thorax* 1992;47:684–689.

11 Britto MT, Kotagal UR, Hornung RW, Atherton HD, Tsevat J, Wilmott RW. Impact of recent pulmonary exacerbations on quality of life in patients with cystic fibrosis. *Chest* 2002;121:64–72.

12 Sanders DB, Bittner RC, Rosenfeld M, Redding GJ, Goss CH. Pulmonary exacerbations are associated with subsequent FEV1 decline in both adults and children with cystic fibrosis. *Pediatr Pulmonol* 2011;46:393–400.

13 Daniels TW, Rogers GB, Stressmann FA, van der Gast CJ, Bruce KD, Jones GR, Connett GJ, Legg JP, Carroll MP. Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. *J Cyst Fibros* 2013;12:22–28.

14 Tunney MM, Klem ER, Fodor AA, Gilpin DF, Moriarty TF, McGrath SJ, Muhlebach MS, Boucher RC, Cardwell C, Doering G, *et al.* Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 2011;66:579–584.

15 Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TW, Carroll MP, Hoffman LR, Jones G, Allen CE, Patel N, *et al.* Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J Cyst Fibros* 2011;10:357–365.

16 Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002;148:257–266.

17 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–7541.
18 Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS ONE* 2011;6:e27310.
19 Chao A, Shen T-J. Nonparametric estimation of shannon's index of diversity when there are unseen species in sample. *Environ Ecol Stat* 2003;10:429–443.
20 Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr* 1957;27:326–349.
21 Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;26:32–46.
22 Liaw A, Wiener M. Classification and regression by randomforest. *R News* 2002;2:18–22.
23 Nick JA, Chacon CS, Brayshaw SJ, Jones MC, Barboa CM, St Clair CG, Young RL, Nichols DP, Janssen JS, Huitt GA, *et al.* Effects of gender and age at diagnosis on disease progression in long-term survivors of cystic fibrosis. *Am J Respir Crit Care Med* 2010;182: 614–626.
24 Ellaffi M, Vinsonneau C, Coste J, Hubert D, Burgel PR, Dhainaut JF, Dusser D. One-year outcome after severe pulmonary exacerbation in adults with cystic fibrosis. *Am J Respir Crit Care Med* 2005;171: 158–164.
25 Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, Wolfgang MC. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. *PLoS ONE* 2012;7:e45001.
26 Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, Wolfgang MC, Boucher R, Gilpin DF, McDowell A, *et al.* Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 2008;177: 995–1001.
27 Field TR, Sibley CD, Parkins MD, Rabin HR, Surette MG. The genus *Prevotella* in cystic fibrosis airways. *Anaerobe* 2010;16:337–344.
28 Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. *Nat Rev Microbiol* 2012;10:717–725.
29 Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD, *et al.* Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe* 2011;10:497–506.
30 Zhou J, Wu L, Deng Y, Zhi X, Jiang YH, Tu Q, Xie J, Van Nostrand JD, He Z, Yang Y. Reproducibility and quantitation of amplicon sequencing-based detection. *ISME J* 2011;5:1303–1313.
31 Zhao J, Carmody LA, Kalikin LM, Li J, Petrosino JF, Schloss PD, Young VB, LiPuma JJ. Impact of enhanced Staphylococcus DNA extraction on microbial community measures in cystic fibrosis sputum. *PLoS ONE* 2012;7:e33127.
32 Rogers GB, Stressmann FA, Koller G, Daniels T, Carroll MP, Bruce KD. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. *Diagn Microbiol Infect Dis* 2008;62: 133–141.
33 Rogers GB, Marsh PA, Stressmann AF, Allen CE, Daniels TV, Carroll MP, Bruce KD. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. *Clin Microbiol Infect* 2010;16:1656–1658.
34 Rogers GB, Cuthbertson L, Hoffman LR, Wing PA, Pope C, Hooftman DA, Lilley AK, Oliver A, Carroll MP, Bruce KD, *et al.* Reducing bias in bacterial community analysis of lower respiratory infections. *ISME J* 2013;7:697–706.
35 Zemanick ET, Wagner BD, Sagel SD, Stevens MJ, Accurso FJ, Harris JK. Reliability of quantitative real-time PCR for bacterial detection in cystic fibrosis airway specimens. *PLoS ONE* 2010;5:e15101.