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## 16S rRNA GENE SEQUENCING REVEALS SITE-SPECIFIC SIGNATURES OF THE UPPER AND LOWER AIRWAYS OF CYSTIC FIBROSIS PATIENTS

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### Abstract

**Background**—Metastasis of upper airway microbiota may have significant implications in the development of chronic lung disease. Here, we compare bacterial communities of matched sinus and lung mucus samples from cystic fibrosis (CF) subjects undergoing endoscopic surgery for treatment of chronic sinusitis.

**Methods**—Mucus from one maxillary sinus and expectorated sputum were collected from twelve patients. 16S rRNA gene sequencing was then performed on sample pairs to compare the structure and function of CF airway microbiota.

**Results**—Bacterial diversity was comparable between airway sites, though sinuses harbored a higher prevalence of dominant microorganisms. Ordination analyses revealed that samples clustered more consistently by airway niche rather than by individual. Finally, predicted metagenomes suggested that anaerobiosis was enriched in the lung.

**Conclusions**—Our findings indicate that while the lung may be seeded by individual sinus pathogens, airway microenvironments harbor distinct bacterial communities that should be considered in selecting antimicrobial therapies.

### Keywords

Chronic sinusitis; microbiome; microbial ecology; mucus

## INTRODUCTION

Defective CFTR ion transport at the sinus epithelium leads to decreased mucociliary clearance and obstruction of sinus ostia [1]. Secondary events such as impairment of host

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defenses and inflammatory processes render this niche susceptible to bacterial colonization and chronic rhinosinusitis (CRS)[1,2]. Notably, a striking incidence of CRS exists among CF subjects (71–100%[3]) relative to the general population (~16%[4]). This is particularly evident in patients with class I–III mutations, who, based on radiological evidence, have a CRS incidence rate of ~100% [1].

Culture-based studies have revealed that CF-associated CRS (CF-CRS) patients harbor distinct microbiota, including *Staphylococcus aureus* and *Haemophilus influenzae* in pediatric patients, followed by *Pseudomonas aeruginosa* and other pathogens as patients age [5]. This dynamic generally follows the same succession of CF lung microbiota [6] and several groups have demonstrated similarities in upper and lower airway bacteriology in CF subjects [7,8]. In fact, evidence has implicated the sinuses as infection foci for lung pathogens, where they first adapt to the host before descending into the lungs [9–15]. Genotypic analyses suggest a direct exchange between sites; *P. aeruginosa* isolates cultured simultaneously from sinuses and lungs were genetically identical in 38 of 40 subjects [10]. These data are supported by studies of CF lung transplants, where recipient allografts were re-colonized by the same *P. aeruginosa* clones found prior to transplantation [11,12]. Altogether, these observations support the notion of a sinus pathogen reservoir and a unified airway, in which treatment of CRS could have profound benefits for CF lung disease management.

While *P. aeruginosa* and *S. aureus* are commonly isolated from the CF sinuses [9], culture-independent studies also suggest colonization by anaerobes (e.g. *Propionibacterium*) and other non-canonical pathogens [13]. These data are consistent with microbiome profiles of CF lung disease, which is recognized to have a polymicrobial etiology [6,16]. Recent sequencing studies have evaluated relationships between oral, nasal and lung microbiota in adult CF patients [17,18], but to our knowledge, molecular approaches have not been used to assess relationships between sinuses and lungs at a microbial community level. From a clinical perspective, these data could be of considerable therapeutic benefit; sinus culture can be invasive and time consuming, and to date, sputum culture-guided therapies for CRS have been largely ineffective, with many subjects ultimately requiring surgical intervention [19,20]. Therefore, a deeper understanding of microbiological relationships between the sinuses and lungs may not only improve upon CF-CRS management, but may also motivate the use of less-invasive sinonasal sampling to help inform patient therapy [8,9,21].

Here, we used 16S rRNA gene sequencing to compare the bacterial composition of upper (sinus) and lower (lung) airways in CF patients undergoing endoscopic sinus surgery. The primary objective was to compare bacterial community diversity between sites, though we also performed predictive metagenomic profiling to assess differences in bacterial phenotypes throughout the airways. We discuss these findings in the context of using sputum versus sinus microbiota composition to steer targeted therapies for upper airway infection.

## MATERIALS AND METHODS

### Patient cohort and specimen collection

Twelve participants with CF undergoing functional endoscopic sinus surgery (FESS) were recruited at the University of Minnesota Department of Otolaryngology. Informed consent was obtained from all subjects. Prior to FESS, each patient spit to discard saliva, followed by sputum expectoration into a collection tube. All samples were assessed macroscopically for salivary contamination, and none were rejected. Sinus secretions were obtained from a single maxillary sinus (middle meatal region) under endoscopic visualization by suction into mucus traps (Cardinal Health, Dublin, OH). Clinical data were also obtained (Table S1), including CFTR genotype, FESS procedures, clinical cultures, spirometry (FEV1%) and sinonasal outcome test (SNOT-22) scores. The UMN Institutional Review Board approved these studies (#1403M49021).

### Quantitative PCR

Bacterial burden was estimated by quantifying 16S copy number from DNA extracted from clinical specimens using quantitative PCR. Reactions were prepared in triplicate using QuantiTect SYBR Green (Qiagen) and Universal 16S rRNA qPCR primers [22]. Details can be found in the supplemental data.

### DNA sequencing and analysis

Genomic DNA was extracted from 300  $\mu$ L of mucus using Powersoil Kits (MoBio, Carlsbad, CA) and was submitted to the UMN Genomics Center (UMGC) for 16S library preparation using a two-step PCR protocol [23]. The V4 region was amplified and sequenced using Illumina MiSeq TruSeq 2 $\times$ 300 paired-end technology. Water and reagent control samples were also submitted, but were below detection thresholds. Raw sequence files were deposited in the NCBI Sequence Read Archive (accession #PRJNA374847). Data were analyzed using a pipeline developed by the UMN Informatics Institute in collaboration with UMG. Details are provided in the supplemental data.

### Metagenomic prediction

Metagenomes were inferred from 16S rRNA data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[24]. PICRUSt uses marker gene survey data to predict metagenome content through ancestral state reconstruction of quality-filtered 16S sequence data. We also used BugBase [25] to summarize predicted metagenomes by bacterial phenotype. BugBase scripts were run with default settings using filtered sequence data used in PICRUSt. Details are provided in the supplemental data.

### Statistical analyses

Analyses were performed in GraphPad Prism 6.0 unless stated otherwise. Significance was assessed at the  $\alpha=0.05$  level. Hypothesis testing was conducted assuming a paired sample study design. Student's t-tests were used where data passed the Kolmogorov-Smirnov test

for normality. Statistics implemented in beta diversity and BugBase analyses are documented in the supplemental data.

## RESULTS

### Patient Cohort

Twelve CF adults with CRS undergoing functional endoscopic sinus surgery (FESS) each provided an expectorated sputum sample. Sinus mucus was collected at the beginning of FESS. These samples are herein referred to as “sinus” and “lung” samples, denoting their anatomical origin. Clinical data, including CF genotype, microbiology cultures, prior FESS procedures, spirometry and sino-nasal outcome test (SNOT-22) scores were also collected (Table S1). Six patients were homozygous  $\Delta$ F508, five were heterozygous  $\Delta$ F508, and two had heterozygous non- $\Delta$ F508 mutations. Sinus culture data were available for 11 of 12 sinus samples. Of these, 6 were positive for *P. aeruginosa*, and 5 were positive for *S. aureus*. Predominant lung pathogens detected by sputum culture were also recorded (Table S2).

### Bacterial load

qPCR of 16S ribosomal RNA (rRNA) gene copy number was used to estimate bacterial load (Fig. 1). On average, sinus specimens contained  $2.72 \times 10^3$  (IQR= $2.69 \times 10^2$ – $1.72 \times 10^3$ ) 16S gene copies per ng genomic DNA, while lung sputum harbored  $5.33 \times 10^4$  (IQR= $6.18 \times 10^2$ – $5.02 \times 10^4$ ). These data suggest a modest difference in 16S gene abundance between sample types (Fig. 1A,  $P=0.0637$ ). Interestingly, patient age was positively associated with 16S gene abundance in sinus samples, but this relationship was not observed for the lung (Fig. 1B). We also assessed the relationship between bacterial load and two clinical metrics, FEV1% and SNOT-22, neither of which significantly correlated with 16S copy number in either sample group (Fig. 1B). Taken together, these data suggest that the specific composition of each bacterial community rather than bacterial abundance contributes to disease states in both sinus and lung niches.

### Bacterial community membership varies with location

Bacterial composition was profiled using 16S rRNA gene sequencing. After filtering for quality and subsampling to an even depth, 51 genera were identified across samples (Fig. 2). To investigate genera that accounted for the majority of sequences, we adopted the definition of a dominant genus (the most abundant genus with over twice the abundance of the second most abundant genus)[16]. A dominant genus was present in 100% of sinus samples but only 33% of lung samples. *Pseudomonas* and *Staphylococcus* were dominant genera in five sinus samples each (42%), while *Streptococcus* and *Burkholderia* were each dominant in a single sample (Fig. 2). As expected, only *P. aeruginosa* and *S. aureus* were dominant lung pathogens. Species-level identification for abundant pathogens was determined by culture data, when available (Table S2). The median relative abundance of the predominant genus was 0.88 (IQR=0.75–0.99) in each sinus sample, and 0.42 (IQR=0.34–0.78) for lung samples. The most abundant sinus OTUs were assigned to *Pseudomonas*, *Staphylococcus*, and *Streptococcus*. By contrast, lung samples harbored an abundance of *Pseudomonas*, *Veillonella*, and *Prevotella*, consistent with previous studies [6,16].

Interestingly, although many taxa were shared between sample pairs, the absence of a pathogen (e.g. *Pseudomonas*, *Achromobacter*) in one sample was not predictive of its absence in its paired sample sequence data. For example, subjects 5 and 12 harbored *Achromobacter* in their upper airways, despite being undetectable in sputum. This was also true of *Staphylococcus* in subject 6. Conversely, subjects 1 and 11 harbored an abundance of known CF pathogens (*Pseudomonas* and *Haemophilus*, respectively) in the lung that were not associated with upper airway infection. These examples demonstrate that infections at either site can be perpetuated by different pathogen within an individual, and that sputum cultures and 16S rRNA sequence data are not always representative of upper airway infection.

Based on these examples, we then determined the extent to which genera were shared between sites. Spearman correlations between genera in matched pairs revealed that within-patient similarities allowed for significant positive correlation between sites in ten of twelve sample pairs (average Spearman  $\rho=0.45$ )(Table S3). However, a group-wise comparison of paired samples showed a weaker correlation (Spearman  $\rho=0.32$ ,  $P=0.001$ ). These results highlight the potential for bacterial communities of the upper and lower airways to be similar within a given patient, yet the group correlation underlines the general dissimilarity in microbiota between sinus and lung microenvironment.

### Bacterial diversity varies between upper and lower airways

As described above, few taxa dominated most sequences in each sample. To investigate this further, two alpha diversity metrics, Observed operational taxonomic units (OTUs) and Shannon diversity, were used as measures of richness (biodiversity) and evenness (equitability)[26]. Observed OTUs in lung samples were greater than in sinuses, indicating greater richness, though the difference was not significant (Fig. 3A). Using the Shannon diversity index, sinuses were characterized by greater unevenness relative to lung samples, consistent with the high prevalence of dominant sinus genera (Fig. 3B). When ordered by rank, an average of 10 and 20 genera accounted for 99% of sequences in sinus and lung samples, respectively (Fig. 3C). These data indicate that the lung bacterial community is greater in both richness and evenness relative to the sinuses.

Spearman correlations were then calculated to assess relationships between bacterial load, alpha diversity and patient clinical data (Fig. 3D). These data revealed a significant inverse correlation between Shannon diversity in the sinus and lung ( $\rho=-0.664$ ,  $P=0.022$ ). Because the data show a similar richness between sites, and both sinus and lung Shannon diversity revealed positive relationships with observed OTUs (sinus  $\rho=0.881$ ,  $P=3.35\times 10^{-4}$ ; lung,  $\rho=0.774$ ,  $P=0.007$ ), we infer that diversity differences are driven by evenness in these niches. Lung Shannon diversity was also positively correlated with lung 16S rRNA copy number ( $\rho=0.678$ ,  $P=0.019$ ), and these data reiterate the positive correlation between sinus 16S rRNA gene copy number and patient age ( $\rho=0.624$ ,  $P=0.033$ ; Fig. 1B). There was no correlation found between alpha diversity metrics, or relative abundance of *Staphylococcus*/*Pseudomonas* and antibiotics prescribed three days prior to surgery for both sample types (Table S4). Altogether, these results demonstrate that bacterial diversity differs between

sinus and lung niches, and that a decrease in diversity at either site is associated with the decrease in even distribution of bacterial taxa.

### Airway location as a descriptor of phylogenetic variance

Beta diversity of airway microbiota was then visualized using ordination. Of interest was whether bacterial communities would cluster more closely by patient or sampling site. Previous studies characterizing upper and lower airway microbiota revealed shared taxa between sites, but also inter-individual variation [8,17]. Evidence also suggests that bacterial metastasis between the sinuses and lung is commonplace [9]. Based on these previous studies, we hypothesized that samples would cluster more closely by patient, rather than by sampling site.

To address this hypothesis, we compared samples utilizing weighted Unifrac, an abundance-sensitive, phylogenetically-relevant diversity metric [28]. This metric was calculated to determine phylogenetic pairwise distances between each sample, then plotted using principal coordinates analysis (PCoA). Contrary to our hypothesis, within-patient sample pairs did not cluster together nearly as strongly as they did by sampling site (Fig. 4A), which rejected the null hypothesis the groupings (sinuses and lungs) had the same centroid (PERMANOVA,  $P=0.019$ ). Lung samples also demonstrated considerable phylogenetic variation when compared to sinus samples (Fig. 4A). When relative abundances from dominant taxa (*Pseudomonas* and *Staphylococcus*) were overlaid, there was a strong association with the PCoA sample distribution (Fig. 4B). These analyses demonstrate that overall community structure is largely driven by dominant genera, and that upper and lower airways select for unique bacterial community structures, despite sharing individual taxa.

### Predicted metagenomes show phenotypic conservation between sites

To gain insight into bacterial community function, PICRUSt[23] was implemented to infer bacterial metagenomes based on 16S rRNA gene content. Sequences derived from each sample had a low Nearest Sequenced Taxon Index average of 0.017, indicating a high relatedness between bacteria found in the samples to sequenced genomes, and a high prediction accuracy for the dataset. Twenty unique KEGG pathways were represented among inferred metagenomes, and showed striking similarity between sinus and lung samples (Fig. S1). This suggests that the functional capacity of lung and sinus bacterial communities are relatively similar, despite taxonomic differences.

To further summarize PICRUSt output, we utilized BugBase, a bioinformatics tool that infers community-wide phenotypes from predicted metagenomes[24]. BugBase identified that gene functions associated with anaerobiosis were enriched in lung samples ( $P=0.01$ ), and could be attributed to three genera: *Veillonella*, *Prevotella*, and *Porphyromonas* (Fig. 5A). Gram-negative ultrastructure and biofilm formation are two bacterial phenotypes often associated with airway pathogenicity, however, our analysis showed that these phenotypes did not differ significantly between sites. Gram-negative genera contributing to this phenotype were more varied in lung samples and included *Veillonella*, *Prevotella*, *Neisseria*, *Stenotrophomonas*, supporting the increased richness observed in these samples. Biofilm-forming bacteria were observed in both sinus and lung predicted metagenomes. This



phenotype was influenced by the presence of *Pseudomonas* in both airway sites, but *Burkholderia* and *Achromobacter* both differentiated sinus from lung samples, while *Neisseria* and *Stenotrophomonas* were more highly represented in lung samples. Altogether, these data point towards the lung environment being a more anaerobic niche, but that other phenotypes classically linked to bacterial pathogenicity, such as biofilm formation, are similar between sites.

## DISCUSSION

It is poorly understood how complex bacterial communities promote the development of sinus disease. It is also not known how upper airway microbiota contributes to lower airway infections that are a primary cause of CF patient morbidity. The sinuses have been proposed as a reservoir in which individual pathogens adapt to the host prior to lung colonization, but it is unclear whether polymicrobial community composition of the CF lung can be a reasonable surrogate for upper airway microbiota and treatment of CF-CRS. We therefore took an ecological approach towards exploring bacterial diversity throughout the airways.

Our cohort showed striking variability in bacterial community membership between subjects. Despite this variability, we found that lung samples harbored an increased diversity (evenness) relative to sinuses which were dominated by either *Staphylococcus* or *Pseudomonas*. The abundances of these two canonical pathogens are commonly revealed by sinus culture [9], though many other taxa identified in our study are not. For example, when compared to summarized culture data comparing the upper and lower CF airways [8], the most striking difference between analyses was the identification of obligate anaerobes. These differences highlight the utility of molecular-based methods in detecting fastidious and anaerobic bacterial taxa. Given the emerging evidence of anaerobes being causative agents of pulmonary disease [29], increased use of culture-independent diagnostics to detect these bacteria throughout the airways is warranted.

Our most intriguing data were that several sinus samples harbored canonical CF pathogens, yet these bacteria were absent in the paired lung samples. Conversely, the lungs of several subjects were colonized by a dominant pathogen that went undetected in the upper airways. This study outcome has significant clinical implications; respiratory cultures and sequence data from the two sites are not necessarily interchangeable for the determination of antibiotic therapy. These findings are consistent with those of Muhlebach [30] who showed that bronchoalveolar lavage and oropharyngeal cultures are poor predictors (40–50% accuracy) of sinus bacteriology in children. Together, these observations may partially explain why patient response to therapy is poor, and suggest that the expanded use of non-invasive, sequence-based sinonasal sampling measures could be beneficial in steering patient therapies.

Often in microbiome surveys, bacterial membership may differ dramatically between samples, but functional capabilities remain conserved [31]. Here, we highlight this relationship in the airways. Contrasting differences in bacterial diversity and community composition between sinuses and lungs, we found that the predicted functional capacity in both niches was similar. In the context of bacterial contribution to CF disease, it is plausible

that there are many similarities in the microenvironments of the upper and lower airways that may contribute, or even result from these conserved functions.

This exploratory study reveals many opportunities for future research. Notably, this work focuses on a small adult cohort with lung function lower than 70% predicted (mean). While the inter-individual variability reported is not unfounded, it highlights the need for increased enrollment for assessing clinical and sequencing variables. Moreover, future work should extend to both pediatric and adult subjects across the spectrum of disease severity, as our conclusions may not be generalizable to all subjects. For example, it is possible that in patients where lung-adapted strains differ in prominence, the influence of bacterial trafficking from the upper airways on bacterial structure also varies. Likewise, longitudinal studies are warranted to investigate microbiota trafficking dynamics over time as patients age. Our work also does not account for airway microenvironments harboring distinct microbiomes. For example, a single maxillary sinus may not capture an infectious agent present in another sinus, whereas sputum may reflect multiple lower airway niches. Prior studies have shown that the middle meatus is a valid representation of the sinus cavity [33], though multiple samples that define the biogeography of the upper airways will be informative for infection management. Finally, a common concern in sputum-based studies is the potential for salivary contamination. Though we cannot rule out the contribution of oral flora to our analyses, samples from lung explants and bronchoalveolar lavage have clearly demonstrated the presence of oral-associated anaerobes in the lung [33,34]. Moreover, others suggest that contamination of sputum is limited during transit through the oral cavity [33,35]. Moving forward, oropharyngeal swabs should be included in analyses to help define the site of sequence origin, particularly for diagnostic purposes.

Despite these limitations, our data shed important light on microbial community relationships throughout the CF airways. Though airway niche spaces differ in diversity, shared taxa between the sample pairs reflects the interconnectedness of the airway and does not discount the sinuses as a source of lower airway colonization. However, the distinct bacterial community structures suggest sinus and lung microenvironments play a critical role in governing the prevalence and abundance of canonical CF pathogens. These data can be translated to the clinic by informing caregivers to utilize respiratory specimens originating from the site of infection. In the context of our findings, we advocate for the use of 16S rRNA gene sequences in the clinical setting to supplement culture data in the assessment and informed therapeutic approach towards the management of CF-associated CRS infections.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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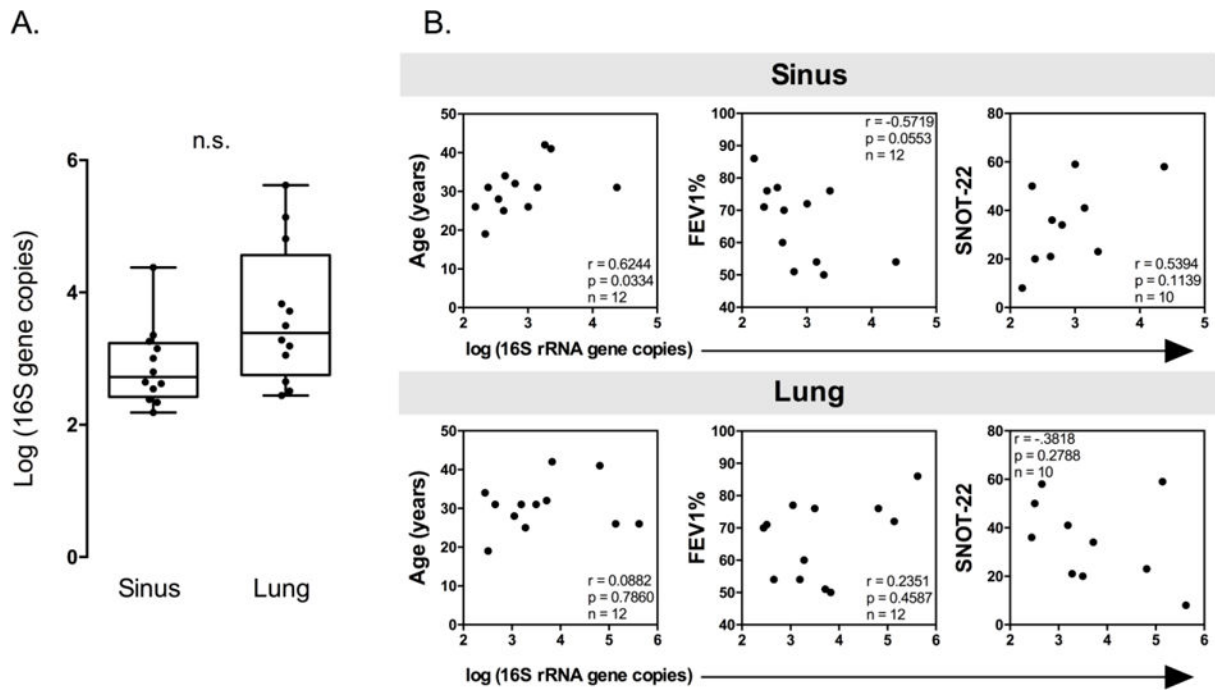
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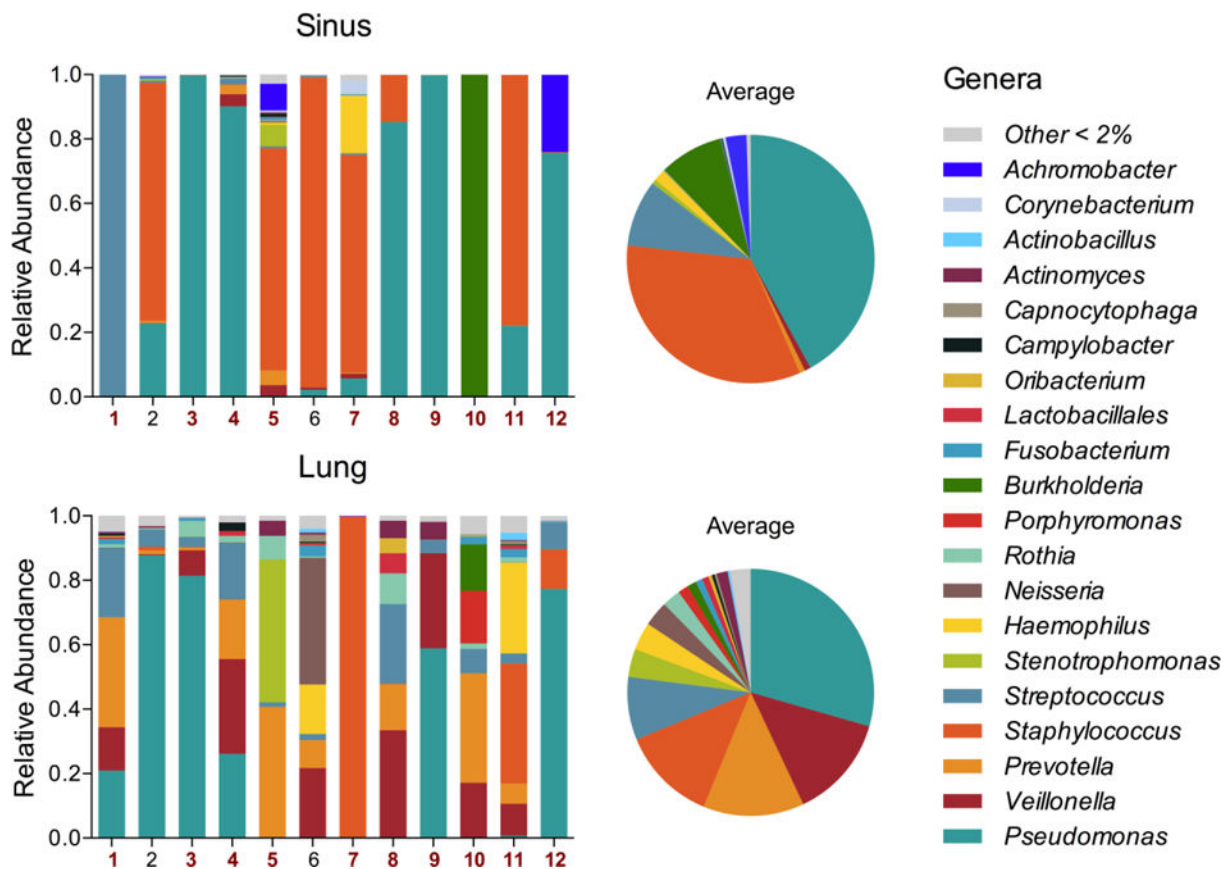
### Highlights

- 16S rRNA sequencing reveals a complex CF-associated sinus microbiota
- Comparison of upper and lower airway communities demonstrates a discordance between sites
- Community composition clusters more closely by sampling site than by patient.
- It is not known whether this relationship holds across the spectrum of disease severity or patient age.
- Metagenome prediction reveals that anaerobiosis is more strongly associated with the lung.



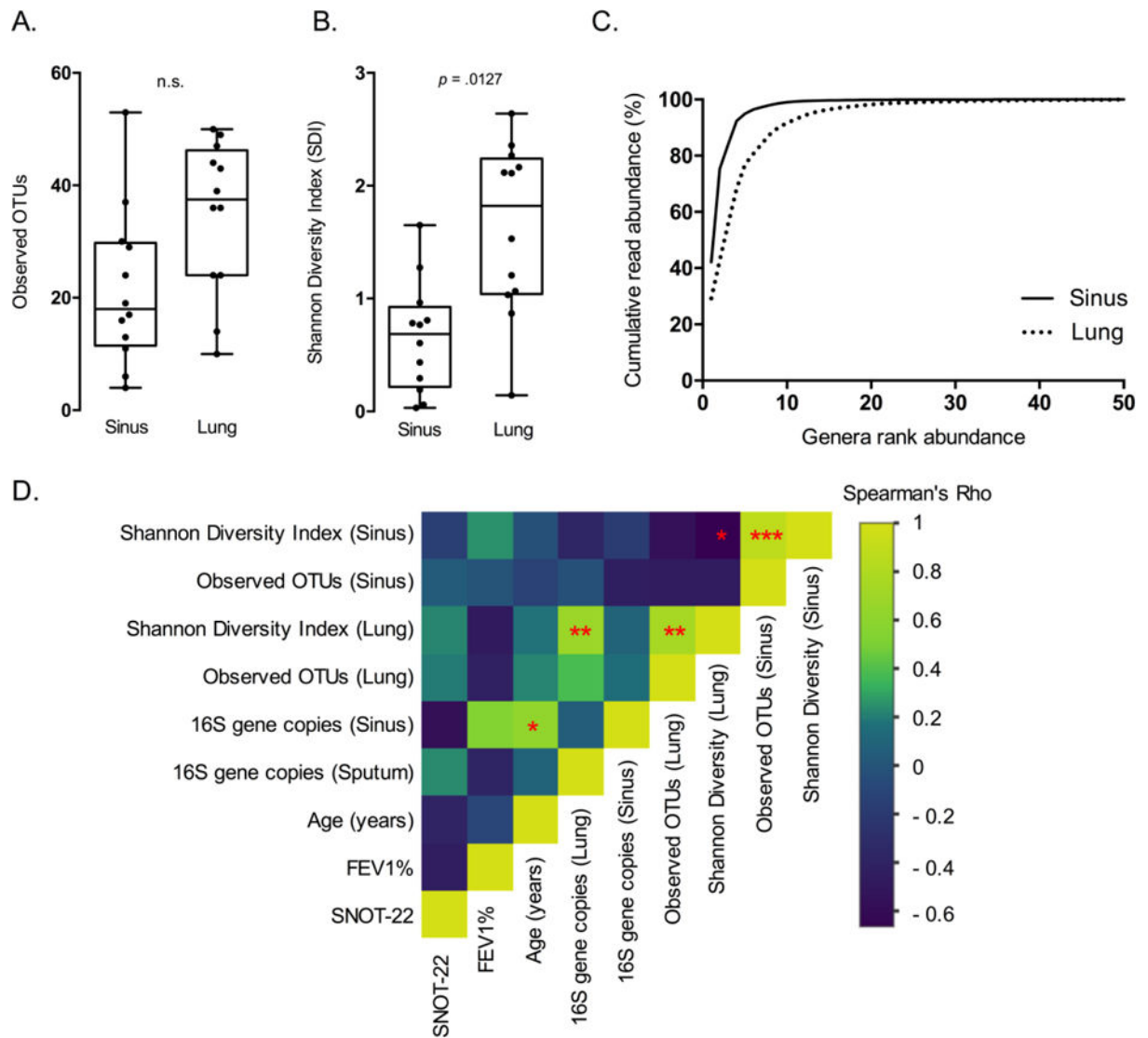
**Figure 1. 16S gene copies are greater in CF lung sputum compared to sinus samples, and correlate with patient age**

Quantitative PCR was used to enumerate 16S rRNA gene copies ng of genomic DNA isolated from sinus and lung samples. **A.** Comparison of 16S rRNA gene copies in sinus and lung sample pairs by qPCR. (Paired Student's t-test  $P=0.0637$ ). **B.** Spearman correlations with 16S rRNA gene copies and patient clinical data. In sinus samples, 16S copies are positively and significantly correlated with patient age.



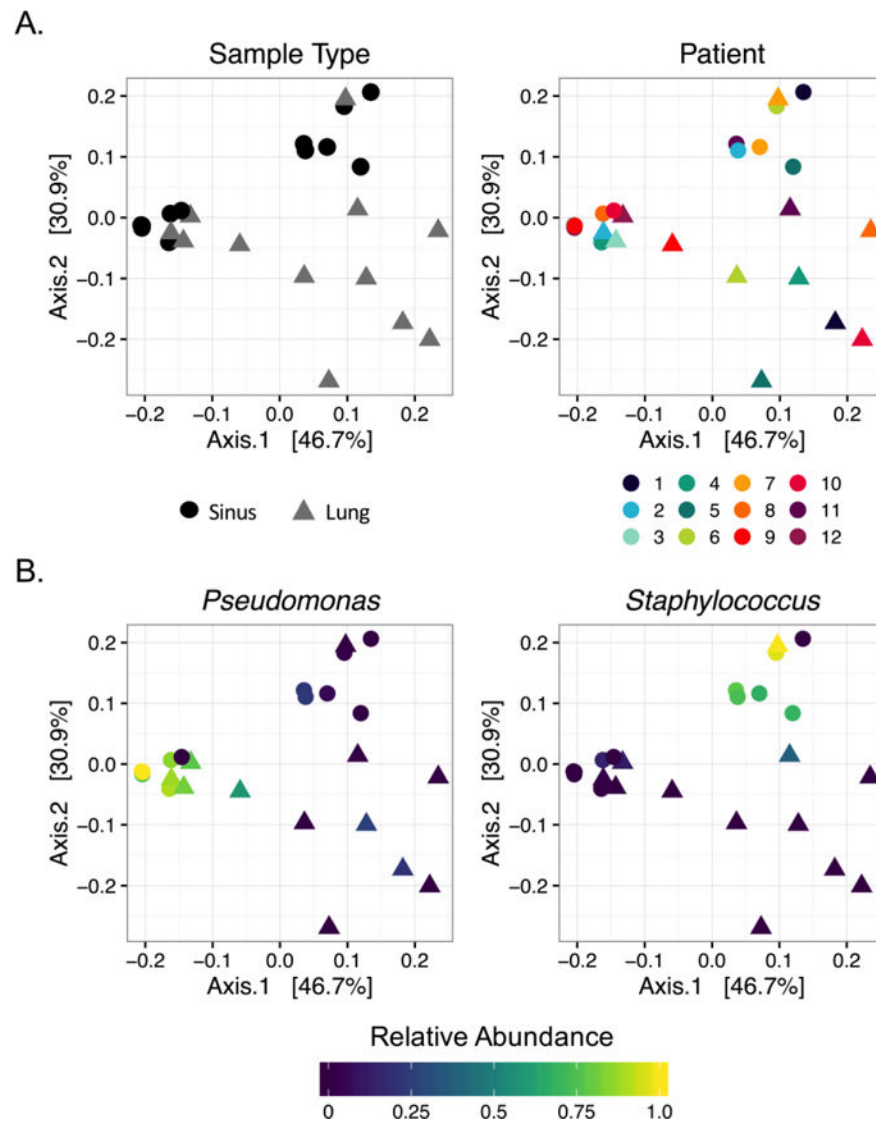
**Figure 2. Bacterial community composition of the upper and lower airways**  
 Stacked bar plots of relative abundances of genera in paired sinus and lung samples demonstrate dominance of *Pseudomonas*, *Staphylococcus*, and *Streptococcus* genera. Red patient numbers indicate samples where bacterial membership was significantly correlated within pairs. Data for these relationships is presented in Table S2. Pie charts show the average abundance of genera for each sample type across the patient cohort.





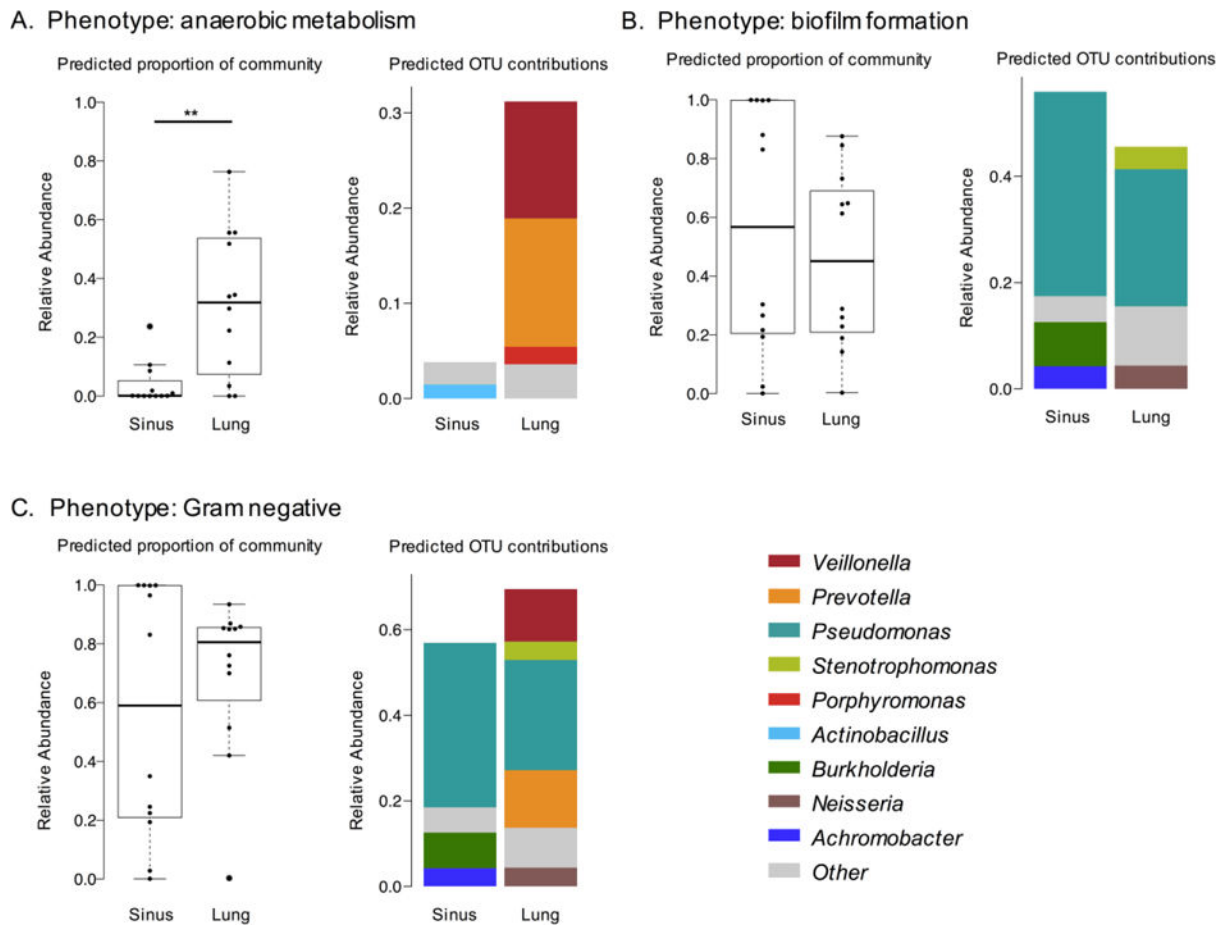
**Figure 3. Alpha diversity of CF maxillary sinuses differs from CF lung sputum**

**A.** Observed OTUs are modestly greater in lung samples compared to the sinuses. Lung samples display significantly greater evenness in OTU distribution relative to sinus samples (Paired Student's *t*-test,  $P=0.0127$ ). **B.** Rank abundance curves reveal that both sinus and lung bacterial communities are dominated by a few organisms. 10 and 20 genera represent 99% of the sequences for sinus and lung samples, respectively. **C.** Spearman correlation heatmap shows association of bacterial diversity (Observed OTUs, Shannon), bacterial 16S gene abundance, and clinical factors. (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).



**Figure 4. Ordination of weighted Unifrac distances shows clustering by sample type and dominant organism**

**A.** Samples show more similarity by sample type rather than sampled individual (PERMANOVA,  $P=0.019$ ). Color and shape denote patient and sample type, respectively. Sinus and lung samples do not cluster by patient, but do show clustering by sample type. **B.** PCoA colored by relative abundance of dominant organisms (defined in text), shows sinus sample grouping is highly dependent on relative abundance of *Pseudomonas* and *Staphylococcus*.



**Figure 5. BugBase analysis of PICRUSt-predicted metagenomes**

**A.** Anaerobic metabolism is significantly enriched in lung samples (Wilcoxon signed-rank test,  $P=0.01$ ). **B.** Biofilm formation does not differ significantly with sample type (Wilcoxon signed-rank test,  $P=0.57$ ). **C.** Gram-negative phenotype is driven by presence of *Veillonella*, *Prevotella* and *Pseudomonas* in lung samples (Wilcoxon signed-rank test,  $P=0.47$ ). (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ )