ORIGINAL ARTICLE

Host Response to the Lung Microbiome in Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: The relatively sparse but diverse microbiome in human lungs may become less diverse in chronic obstructive pulmonary disease (COPD). This article examines the relationship of this microbiome to emphysematous tissue destruction, number of terminal bronchioles, infiltrating inflammatory cells, and host gene expression.

Methods: Culture-independent pyrosequencing microbiome analysis was used to examine the V3–V5 regions of bacterial 16S ribosomal DNA in 40 samples of lung from 5 patients with COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stage 4) and 28 samples from 4 donors (controls). A second protocol based on the V1–V3 regions was used to verify the bacterial microbiome results. Within lung tissue samples the microbiome was compared with results of micro–computed tomography, infiltrating inflammatory cells measured by quantitative histology, and host gene expression.

Measurements and Main Results: Ten operational taxonomic units (OTUs) was found sufficient to discriminate between control and GOLD stage 4 lung tissue, which included known pathogens such as *Haemophilus influenzae*. We also observed a decline in microbial diversity that was associated with emphysematous destruction, remodeling of the bronchiolar and alveolar tissue, and the infiltration of the tissue by $CD4^+$ T cells. Specific OTUs were also associated with neutrophils, eosinophils, and B-cell infiltration ($P < 0.05$). The expression profiles of 859 genes and 235 genes were associated with either enrichment or reductions of Firmicutes and Proteobacteria, respectively, at a false discovery rate cutoff of less than 0.1.

Conclusions: These results support the hypothesis that there is a host immune response to microorganisms within the lung microbiome that appears to contribute to the pathogenesis of COPD.

Keywords: COPD; microbiome; inflammation; bacteria

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At a Glance Commentary

Scientific Knowledge on the Subject: The bacterial microbiome within lung tissue is different between control individuals and those with GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage 4 chronic obstructive pulmonary disease.

What This Study Adds to the Field: This study provides the first analysis of the host response to the bacterial microbiome during both emphysematous destruction and loss of terminal bronchioles within the lung tissue.

Chronic obstructive pulmonary disease (COPD) is a progressive, debilitating lung disease with multiple comorbidities that affects more than 200 million people worldwide and is responsible for approximately 3 million deaths each year (1). Although the pathogenesis of small airway obstruction and emphysematous destruction responsible for the progressive airflow limitation in COPD has been associated with the host innate and adaptive inflammatory immune response (2–4), the antigens that drive this response remain poorly understood. The British hypothesis, that smoking compromises the host response, allowing colonization and infection of the lower respiratory tract by organisms that cause chronic bronchitis and a decline in FEV_1, has been rejected on the basis of a prospective longitudinal study conducted by Fletcher and associates (5). This study showed that many people with chronic bronchitis never developed airflow limitations and that many others developed severe airway obstruction in the absence of chronic bronchitis (5, 6). Sethi, Murphy, and colleagues reawakened interest in the possible role of bacteria in the pathogenesis of COPD by showing that acute exacerbations of COPD were commonly associated with the emergence of new bacterial strains that could be isolated from the sputum and protected bronchial brushings (7). Moreover, Hurst and associates extended these observations by showing that frequent exacerbations of COPD within the same individual are associated with an accelerated rate of decline in lung function leading to COPD (8).

The application of culture-independent techniques to the identification and community analysis of bacteria led to the discovery that the human gastrointestinal and genitourinary tracts, as well as the skin, mouth, and upper airways, host relatively large and complex microbiomes that live commensally within the host (9–12). In contrast, the long-held view that the lung was sterile below the larynx persisted until Hilty and associates (13) used these techniques to challenge this hypothesis by analyzing the microbiome in bronchial brushings and washings from human lungs. Their new data suggested that lower airways of patients with asthma and COPD contained a microbiome that became less diverse and was associated with the emergence of potential pathogens (13). These results were criticized as artifact produced by contamination of the bronchial brushings and washing as they passed through the upper airways. This criticism was refuted by Erb-Downward and colleagues (14) and Sze and colleagues (15) in studies that demonstrated a human lung microbiome in samples obtained either by brushing the airways of explanted lungs where the upper airways were absent (14) or by rapidly freezing the explanted lung solid to allow peripheral lung samples to be removed without disturbing the central airways (15). The present report extends these observations by examining the microbiome in relation to emphysematous destruction of the lung surface and providing preliminary evidence that this destruction is associated with the development of a host immune response to this microbiome. Some of the results of these studies have been previously reported in the form of abstracts (16–19).

Methods

Informed Consent

Informed consent was obtained either directly from patients undergoing lung transplantation as treatment for very severe COPD or from the next of kin of organ donors, who agreed that the lungs could be released to serve as controls if considered unsuitable for transplantation. The conditions under which consent was obtained were approved by the appropriate committees at each of the participating institutions (20, 21), and the shipment of specimens between institutions was compliant with the U.S. Health Insurance Portability and Accountability Act.

Specimen Preparation

Specimen preparation has been described in detail in previous publications (20–22) and in the online supplement. Briefly, five explanted lungs from patients with GOLD stage 4 COPD and the four donor (control) lungs were fully inflated with air to a transpulmonary pressure of 30 cm H_2O and then deflated and held at a transpulmonary pressure of 10 cm H_2O while frozen solid in liquid nitrogen vapor. These lung specimens were kept frozen on dry ice while a volumetric multidetector computed tomography scan was performed and while the specimen was cut into contiguous 2-cm-thick transverse slices from lung apex to base. A cluster of four cores of lung tissue was removed from each slice for each of the investigations outlined below.

Microbiome Analysis

The pipeline for protocol 1 is fully described by Ward, Schloss, and colleagues (23, 24) and was developed on the basis of touchdown PCR amplification of the V3–V5 region of the bacterial 16S ribosomal RNA (rRNA) gene with pyrotag sequencing of the amplified DNA, done at the University of Michigan (Ann Arbor, MI) microbiome sequencing facility according to a low-biomass protocol (14, 25–27). The pipeline for protocol 2, used to analyze bacterial 16S ribosomal DNA, is fully described in our previous publications (4, 15, 20, 21) and the online supplement. Protocol 2 was developed in Vancouver, Canada and is based on nested PCR amplification of the V1–V3 region of the bacterial 16S rRNA gene and pyrotag sequencing of the amplified DNA by Genome Quebec (Montreal, QC, Canada) (15). It was used as an independent method to confirm microbiome results obtained from protocol 1.

Microbial diversity. Microbial diversity was assessed as $H = E_H \times \ln S$, where H is the Shannon diversity index, E_H represents the evenness of the community of OTUs in the sample, and ln S represents the natural log of OTU richness (or numbers of different OTUs). Differences between the bacterial community composition in control and COPD lung samples were visualized by principal component analysis (PCA) of pair-wise Bray–Curtis

dissimilarities and tested by permutational multivariate analysis of variance (PERMANOVA) (28). Raw data used in the microbiome analysis can be found on Data Dryad under the following DOI: 10.5061/dryad.2.p66n.

Emphysematous destruction. Emphysematous destruction was assessed in each lung sample as $SA = 4 \times V/Lm$, where SA is the internal surface area of the core of lung tissue removed at each of the sampled sites, V is the total volume of lung in the tissue core removed from the lung, and Lm is the mean linear intercept.

Infiltration of inflammatory immune cells. Infiltration of inflammatory immune cells into tissue was estimated by point counting the volume fraction (Vv) of bronchiolar and alveolar tissues occupied by polymorphonuclear leukocytes; macrophages; and $CD4^+$, $CD8^+$, and B lymphocytes on appropriately stained histological sections from companion cores of tissue and comparing the results with those of micro–computed tomography (microCT) of lungs from patients treated by lung transplantation and their controls.

Gene Expression Profiling

Detailed methods can be found in the online supplement and in a previously published article (22). These gene expression data are available through the Gene Expression Omnibus (GEO) under accession number GSE27597.

Statistics

A linear mixed-effects model was used to compare OTU richness to emphysematous destruction assessed from measurements of the lung surface area, as well as the host response to this tissue destruction. These were obtained by Vv of the tissue occupied by inflammatory immune cells or gene expression profiling studies conducted on the RNA isolated from histological sections cut in close proximity to those examined by histology. The linear mixed-effects model allowed correction for the effect of lung height and position of samples within each lung slice (22). Gene expression pathways were further analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery; 29).

Only the phyla and families that achieved significant correlations with at least one of the Vv or microCT measurements were compared with host gene expression. If a phylum or family was undetected in more than 30% of the samples the data were converted to a categorical variable (positive or negative) and then analyzed using the linear mixed-effects model. To identify the OTUs that were most likely driving the correlations with phyla, the data were separated on the basis of the average value of the host measurement of interest and high and low groups were created. If an OTU was significantly different between these two groups and matched the direction of correlation found in the phyla analysis, it was considered a potentially important OTU. In addition, OTUs identified by Boruta feature selection (30) after random forest analysis, as discriminative for control and GOLD stage 4, were also analyzed using linear mixed-effect models and compared with microCT, quantitative histology, and gene expression data. Gene set enrichment analysis (GSEA) was used to compare similarity in the overall gene expression data sets. Further details on the full data analysis are provided in the online supplement.

Results

Table 1 and Tables E1–E3 in the online supplement summarize the data concerning age, sex, smoking history, lung function, number of tissue samples used for each analysis, and the number of reads per sample on all the subjects in this study.

Microbial diversity as measured by OTU richness declined as emphysematous destruction increased (Figure 1), and there was a linear correlation ($R^2 = 0.27$) between OTU richness and alveolar surface area. This was confirmed after applying a second independent protocol to assess the microbiome (Figure E1). Furthermore, the PCA showed differences in the bacterial communities between GOLD stage 4 lung tissue samples and the control lungs (Figure 1B; $P = 0.001$), based on PERMANOVA (31). This difference was also found using the alternative protocol (Figure E1B) ($P < 0.01$). Although there was a trend for Shannon diversity to be lower in samples of lung from patients with GOLD stage 4 COPD, this difference became statistically significant ($P < 0.05$) only in samples from position 6 (Figure 1C) (1 = apex, 12 = base). The Shannon diversity index for the control, GOLD stage 4, and negative water control tissue samples was 3.40 ± 0.24, 2.25 ± 0.69, and 1.6 ± 0.1, respectively (mean $\pm$ SD).

Relative abundances of bacterial phyla differed ($P < 0.05$) between GOLD stage 4 and control lungs (Figure 2A). On the basis of Bonferroni *post hoc* testing, expansion of the Proteobacteria phylum was the most significant driver of the difference between control and GOLD stage 4 ($P < 0.05$). Overall, the greatest differences between the two groups were seen between the Proteobacteria (controls, $46 \pm 16\%$; GOLD stage 4, $66.0 \pm 1.6\%$), Firmicutes (controls, $17.7 \pm 19.6\%$; GOLD stage 4, $7.04 \pm 0.87\%$), and Bacteroidetes (controls, $31.7 \pm 11.3\%$; GOLD stage 4, $21.1 \pm 4.1\%$). The proteobacterium *Haemophilus influenzae* was among the 10 OTUs that were important for discriminating between the control and GOLD stage 4 bacterial microbiomes (Figure 2B) according to Boruta feature selection with random forest analysis. Although the majority of these bacterial species decreased in abundance in GOLD stage 4 COPD lung tissue, a notable exception was *Elizabethkingia meningoseptica* (Figure 2C). The similarity and differences between the two methods for the important OTUs can be found in the online supplement (Table E4).

Table 1. Patient Demographics

	Control Subjects ($n = 4$)	GOLD Stage 4 ($n = 5$)
Age, yr	53.8 ± 4.3	60.0 ± 1.6
Sex, M:F:unknown	4:0:0	3:2:0
FEV_1/FVC	N/A	0.31 ± 0.07
FEV_1% predicted	N/A	17.89 ± 5.47
Samples per individual (n)	8 (3), 5 (1)	8 (5)

Definition of abbreviations: GOLD = Global Initiative for Chronic Obstructive Lung Disease; N/A = not available.

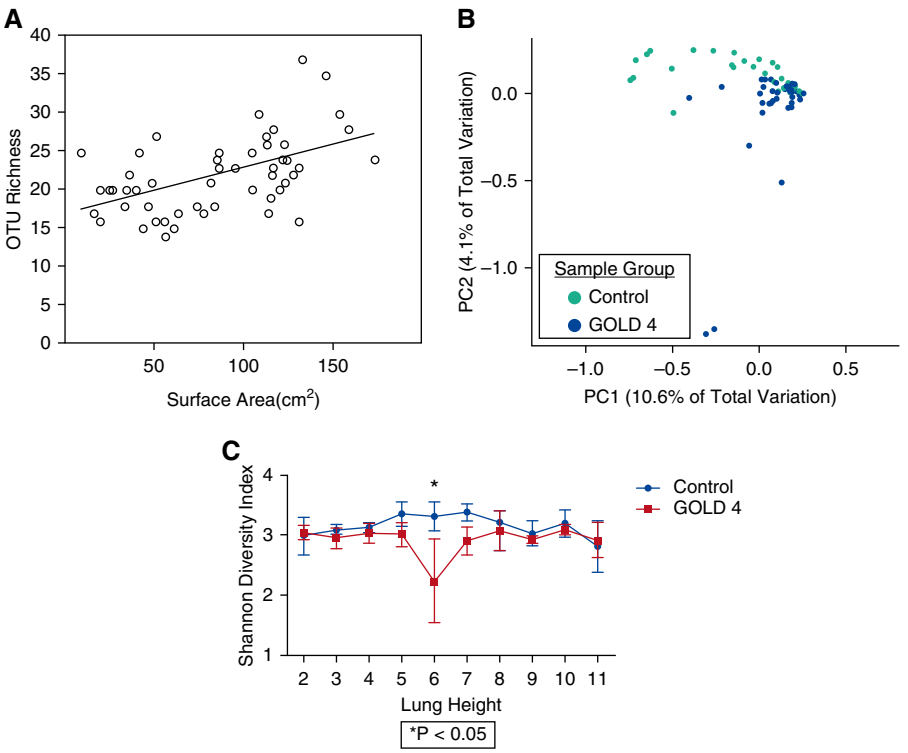


Figure 1. (*A*) Operational taxonomic unit (OTU) richness as a function of alveolar surface area. (*B*) Ordination of samples based on Bray–Curtis dissimilarity of microbiomes. GOLD = Global Initiative for Chronic Obstructive Lung Disease; PC1 and PC2 = principal components 1 and 2, respectively. (*C*) Shannon diversity versus lung height between control and GOLD stage 4. (*A*) Alveolar surface area values and OTU richness were determined from spatially adjacent cores ($R^2 = 0.27$, $P < 0.05$). (*B*) Dissimilarity was calculated by the same approach as in *C*. The two groups were significantly different (permutational multivariate analysis of variance; pseudo-$F = 6.58$; $P = 0.001$). (*C*) Lower lung height values represent lung tissue taken closer to the apex; higher lung height values represent lung tissue taken closer to the base. There was a significant difference between control and GOLD stage 4 ($P < 0.05$) at the relative middle of the lung.

To determine contamination, negative water controls were assessed for the important OTUs identified by Boruta feature selection with random forest analysis for both protocols 1 and 2 (Figures E2 and E3). Except for *Streptococcus* in protocol 1, all OTUs that were identified as discriminative for control and GOLD stage 4 lung tissue contained low square root relative abundances, or they were not identified at all in the negative control samples (Figures E2 and E3).

Table 2 summarizes the results obtained by comparing the microbiome data at the phylum level with the host response measured in terms of the Vv of lung tissue occupied by infiltrating inflammatory immune cells. These comparisons show that Shannon diversity is negatively related to $CD4^+$ lymphocyte infiltration and also that Shannon diversity was positively correlated to lung surface area. OTU richness was also negatively correlated with $CD4^+$ lymphocyte infiltration. Further analysis shows that neutrophil infiltration was negatively associated with the presence of Proteobacteria, Comamonadaceae, *Pseudomonas*, and Betaproteobacteria OTUs (Tables 2 and 3). In addition, it also shows that eosinophil infiltration and elastin content were positively associated with Actinobacteria OTUs and B-cell infiltration with *Propionibacterium*, Micrococcaceae, and *Atopobium* OTUs.

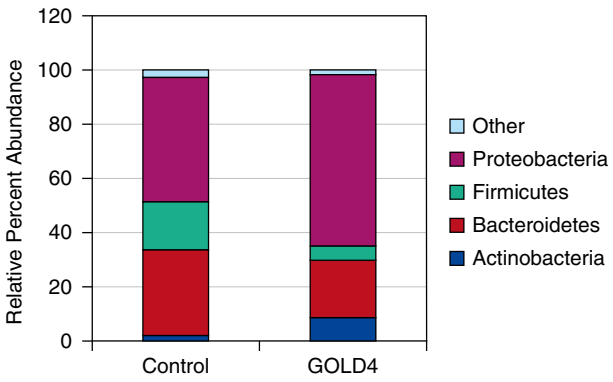
The data in Table 4 summarize the relationships between predictive OTUs selected by random forest analysis and the results obtained by quantitative histology and microCT. These data show that the Vv of neutrophil infiltration was positively correlated with *Dialister* (false discovery rate [FDR] = 0.0001), Bacteroidales (FDR = 0.03), *Streptococcus* species (FDR = 0.06), and *H. influenzae* (FDR = 0.06). The number of terminal bronchioles per milliliter was positively correlated with both *H. influenzae* and *Dialister* species (FDR < 0.05). *E. meningoseptica* was positively correlated with the Vv of elastin, $CD4^+$ T cells, and Lm (FDR < 0.1) and negatively correlated with total alveolar collagen (FDR = 0.09). *Flavobacterium succinicans* was negatively correlated with $CD4^+$ T cells, and *Flavobacterium gelidilacus* was positively correlated with alveolar surface area (FDR < 0.06).

The changes in microbiome composition were associated with a number of host gene expression differences. We identified 859 genes whose expression was associated with the presence of bacteria from the Firmicutes phylum at an FDR cutoff of less than 0.1 (Table E6). DAVID analysis indicated that the down-regulated genes were involved mostly with zinc finger domain regions (FDR $< 2.5 \times 10^{-10}$), whereas the up-regulated genes were involved in pathways with disulfide bonding, signal peptides, membrane, and defense response (FDR $< 2 \times 10^{-3}$). This finding does not change if an FDR cutoff of 0.05 is used instead of 0.10 (data not shown). In addition, Proteobacteria were associated with 235 genes below an FDR of 0.1 (Table E6). No pathways were identified from the down-regulated genes, but the up-regulated genes were involved in pathways for splicing, cilium, cell projection, and cell–cell junctions (FDR < 0.1). When the most important predictive bacterial OTUs were analyzed for a correlation with human host gene expression only *H. influenzae* was associated with a single gene below an FDR cutoff of 0.1 (C21orf51, FDR = 0.05). The GSEA analysis comparing host gene expression versus the microbiome from protocol 2 with those reported here from protocol 1 shows that the same genes were up- and down-regulated in relation to Shannon diversity and OTU richness (Tables E5 and E7, and Figures E4 and E5).

Discussion

The present results confirm earlier reports showing that adult human lungs contain a sparse, yet relatively complex microbiome that maintains density but becomes less diverse in the lungs of patients with COPD

A



B

Important Species
Prevotella oralis
Streptococcus
Prevotella oris
Porphyromonas
Flavobacterium succinicans
Haemophilus influenzae
Bacteroidales
Elizabethkingia meningoseptica
Dialister
Flavobacterium gelidilacus

C

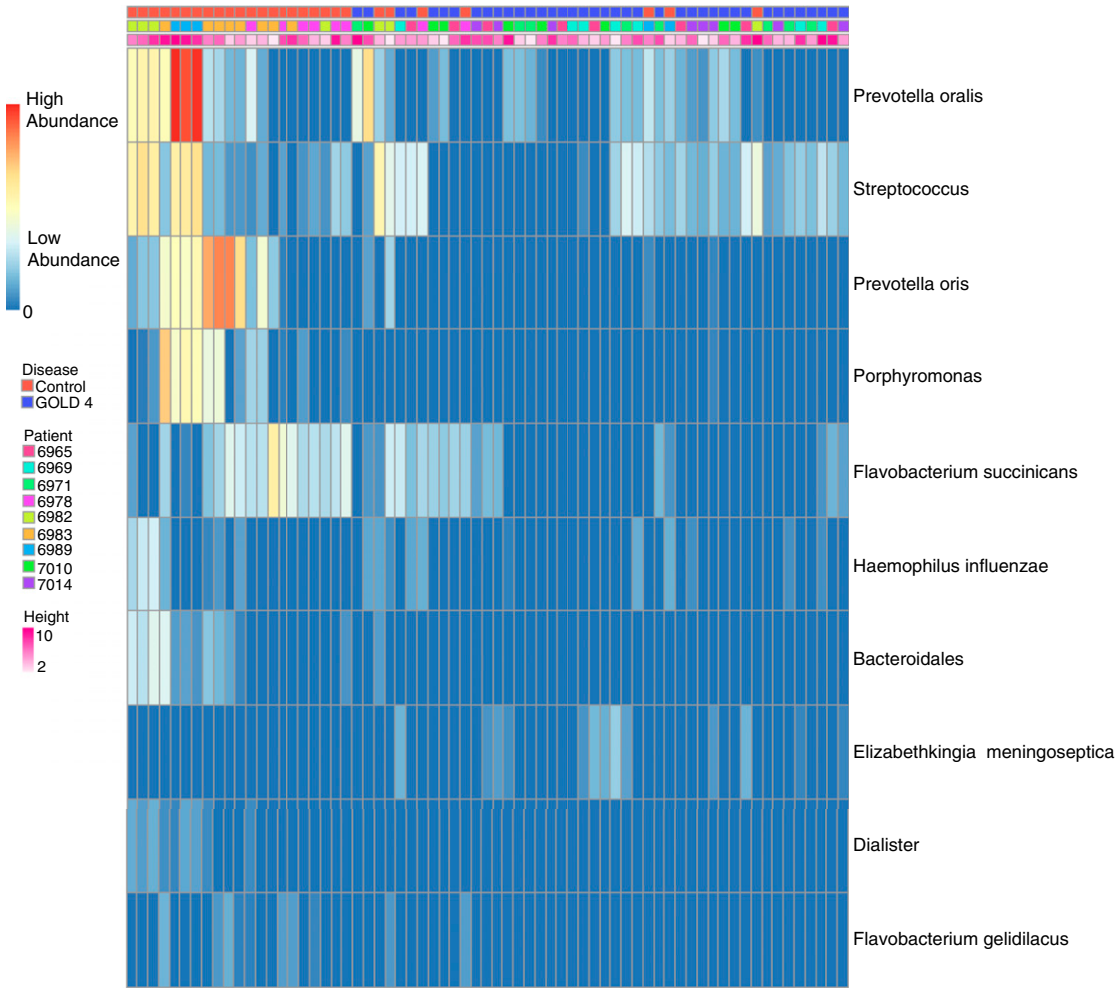


Figure 2. (*A*) Phylum relative percent abundances in control and GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage 4 lung tissue. (*B*) Most important species for discriminating control and GOLD stage 4 microbiomes, using random forest analysis with Boruta feature selection. (*C*) Heat map of the important bacterial species and relative abundance in each tissue sample. (*A*) The distribution of phyla was significantly different between control and GOLD stage 4 ($P < 0.05$), and this was driven by Proteobacteria ($P < 0.05$). (*B*) The average (±SD) 10-fold cross-validated error rate was 17 ± 2% with a per-class error rate of 34 ± 4% for the controls and 6 ± 3% for the GOLD stage 4 group. (*C*) Samples were clustered by similarity, and the three *color-coded bars* at the top represent control (*red*) or chronic obstructive pulmonary disease GOLD stage 4 group (*blue*), patient (*nine distinct colors*), and lung height (shades of *pink*, with *dark pink* being closer to the base and *light pink* closer to the apex). *Dark red* represents high abundance, *pale blue* represents low abundance, and *dark blue* represents undetectable values of the respective operational taxonomic unit.

Table 2. Summary of Phyla and Diversity Correlations

Significant Result	Coefficient	*P* Value	FDR
Shannon diversity vs. alveolar CD4 T cells	−6.99	0.0042	0.05
Shannon diversity vs. surface area	0.12	0.014	0.09
OTU richness vs. CD4 T cells	−183	0.006	0.06
Proteobacteria vs. neutrophils	−1.63	0.011	0.09
Actinobacteria vs. eosinophils	8.63	2.2×10^{-5}	0.0005
Actinobacteria vs. alveolar B cells	2.11	0.0025	0.037
Actinobacteria vs. elastin	0.13	0.0054	0.067

Definition of abbreviations: FDR = false discovery rate; OTU = operational taxonomic unit.

(15, 26, 32). They also extend these observations by showing that both a touchdown PCR (protocol 1) used to amplify the V3–V5 region and nested PCR (protocol 2) used to amplify the V1–V3 region of the same bacterial 16S rRNA gene showed a decline in OTU richness in association with emphysematous destruction of the lung surface (Figure 1A and Figure E1A). Both methods also showed differences in the microbial community composition between control lung tissue and tissue from patients with GOLD stage 4 COPD (Figure 1B and Figure E1B). In addition, they confirm and extend earlier reports (13) by showing (Figure 2) that both the Proteobacteria and to a lesser extent the Actinobacteria expand in COPD compared with controls whereas the Firmicutes and Bacteroidetes phyla contract as the alveolar surface is being destroyed by emphysema in lungs affected by COPD. Most importantly they show that these changes produce a measureable host response in lung tissue.

A study by Salter and colleagues (33) has highlighted the fact that sample contamination is an important source of error in the analysis of sparse yet relatively diverse microbiomes, such as the lung. Therefore it is a concern that some of the OTUs identified as important by the random forest analysis in this study (noticeably *Flavobacterium* and *Streptococcus*) also do not align to genera identified as potential contaminants (33). Even though the negative controls included with our samples showed these same OTUs were either absent or greatly reduced in our negative control samples (Figures E2 and E3), we cannot conclusively rule out contamination as playing a role in some of the bacteria identified (e.g., *Flavobacterium*, *E. meningoseptica*, and *Dialister*). Therefore these findings need to be interpreted with caution until more precise methods of ruling out contaminating organisms are developed.

A random forest analysis showed (Figure 2B) that the OTUs best able to distinguish between lung tissue from control subjects and patients with severe GOLD stage 4 COPD had both positive and negative effects. For example, *H. influenzae* was observed to be virtually absent in very severe GOLD stage 4 COPD and to increase in association with the numbers of terminal bronchioles seen in the milder forms of COPD. This could suggest a protective phenomenon that has been previously demonstrated in mice, where simultaneous inoculation of *H. influenzae* and *Streptococcus pneumoniae* onto the upper respiratory mucosal surface showed that *H. influenzae* out-competes *S. pneumoniae* for the mucosal surface by inducing a host response that brings in neutrophils to destroy the *S. pneumoniae* (34). These observations suggest the hypothesis that *H. influenzae* is capable of causing infection and producing acute exacerbations in the early stages of COPD (7). Moreover, it is also consistent with the hypothesis that the decline in terminal bronchioles and increase in emphysematous destruction associated with progression of COPD destroys the habitat that favored the emergence of *H. influenzae* and allows a different set of microbes to emerge, colonize, and infect lung tissue in late-stage COPD. In addition, the tissue vacated by *H. influenzae* might provide a niche for certain exotic bacteria, such as *E. meningoseptica*, that correlate with the inflammatory immune cell infiltration and tissue remodeling that correlate with progression of COPD in this study. However, additional studies that take into account all of the reported corrections for contamination will need to be performed to get the best description of the host response to the microbiome in COPD.

Table 3. Summary of Significant Genus and Species Results

Significant Result	Direction	*P* Value	FDR
Comamonadaceae (OTU2) and neutrophils	Negative	0.005	0.06
Comamonadaceae (OTU49) and neutrophils	Negative	4.5×10^{-4}	0.019
Pseudomonas (OTU42) and neutrophils	Negative	0.0042	0.06
Betaproteobacteria (OTU83) and neutrophils	Negative	0.0058	0.06
Propionibacterium acnes (OTU22) and B cells	Positive	0.015	0.086
Micrococcaceae (OTU41) and B cells	Positive	0.026	0.087
Atopobium (OTU98) and B cells	Positive	0.023	0.057

Definition of abbreviation: FDR = false discovery rate; OTU = operational taxonomic unit.

The relative expansion of Proteobacteria, and to a lesser extent Actinobacteria, that occurred in relation to the contraction of the Firmicutes and Bacteroidetes phyla, in this study, is consistent with a competition for space on the reduced alveolar surface created by emphysematous destruction. For example, the expanded Proteobacteria phylum (Figure 2) contributed all five of the individual OTUs associated with neutrophil infiltration and one of four of the OTUs associated with B-cell infiltration (Tables 2 and 3). The smaller expansion of the Actinobacteria phylum contributed three of four OTUs associated with B-cell infiltration as well as a strong association with eosinophil infiltration. In contrast, the Firmicutes phylum did not contain any OTUs associated with specific responses, and the Bacteroidetes phylum contained only *E. meningoseptica* that helped separate the control from the GOLD stage 4 COPD cases. Collectively, these data suggest that OTUs located within the phyla that expand as the

Table 4. Relationships between Predictive Operational Taxonomic Units Selected by Random Forest Analysis and Results Obtained by Quantitative Histology and Micro–Computerized Tomography

Comparison	Coefficient	*P* Value	FDR
Dialister and Vv of neutrophils	0.32	6.93×10^{-7}	9.01×10^{-5}
Elizabethkingia meningoseptica and Vv of elastin	0.23	1.58×10^{-4}	0.01
Haemophilus influenzae and number of terminal bronchioles	0.01	7.0×10^{-4}	0.02
Flavobacterium gelidilacus and surface area	3.0×10^{-4}	6.32×10^{-4}	0.02
Bacteroidales and Vv of neutrophils	0.62	1.24×10^{-3}	0.03
Dialister and number of terminal bronchioles	2.7×10^{-3}	2.49×10^{-3}	0.05
Streptococcus and Vv of neutrophils	1.35	3.65×10^{-3}	0.06
Flavobacterium succinicans and Vv of CD4 T cells	−2.58	3.96×10^{-3}	0.06
H. influenzae and Vv of neutrophils	0.48	4.35×10^{-3}	0.06
E. meningoseptica and Lm	0.02	4.40×10^{-3}	0.06
E. meningoseptica and Vv of CD4 T cells	1.00	5.03×10^{-3}	0.06
E. meningoseptica and Vv of total collagen	−0.08	8.00×10^{-3}	0.09

Definition of abbreviations: FDR = false discovery rate; Lm = mean linear intercept; Vv = volume fraction.

alveolar surface is destroyed stimulate the host response to a greater degree than OTUs in the phyla that contract. Moreover, they suggest the hypothesis that the organisms that compete successfully for the contracting bronchiolar and alveolar surface are recognized by the host immune surveillance system, which normally does not respond to the bacterial microbiome of the lung.

The gene expression profiling data provide additional evidence in support of a robust host response to changes in the composition of the bacterial microbiome, by showing that 859 and 235 genes whose expression was either up- or down-regulated in association with the presence of bacteria from the Firmicutes or Proteobacteria phylum, respectively, at an FDR cutoff less than 0.1 (Table E6). Moreover, the GSEA analysis showed that many of the bacteria associated with changes in host genes were directionally the same using both protocol 1 and protocol 2. In addition, the analysis of protocol 2 based on DAVID showed that Shannon diversity was positively associated with genes in the dynein, coiled coil, cilium, and microtubule motor activity pathways (FDR < 0.0004) required to clear the mucosal surface. On the other hand, a negative association existed between Shannon diversity and gene expression involving genes in the immunoglobulin, glycoprotein, and Fc γ receptor III pathways (Table E7), which are related to the immune response.

In 1996, Fredricks and Relman (35) upgraded Koch's postulates for situations in which identification of microorganisms is based on sequencing technology. These revised criteria include the following: (*1*) the nucleic acid sequence of the putative pathogen must be preferentially found in organs or anatomic sites within organs known to be diseased; (*2*) fewer or no copies of that sequence should be found in nondiseased regions of affected organ; (*3*) resolution of the disease should be associated with a decrease in copy number and relapse with increased copy numbers of the putative pathogen; (*4*) a causal relationship is more likely if sequence detection predates disease and increases in copy number in association with disease progression; (*5*) the nature of the microorganism inferred from the available sequence data is consistent with the known biological characteristics of that group of organisms thought to be responsible; (*6*) *in situ* hybridization techniques should be used to demonstrate the relationship between organism and disease at the cellular level; and (*7*) all of the sequence-based forms of evidence for microbial causation should be reproducible.

Although the present results do not satisfy all of these criteria they provide preliminary data showing that OTUs within the expanding Proteobacteria and Actinobacteria phyla account for all the associations observed between individual OTUs and infiltrating inflammatory immune cells. On the basis of these findings we postulate that the persistent low-level inflammatory immune response that has been associated with the progression of COPD (4) is primarily driven by OTUs from within the phyla that expand on a diminishing bronchiolar and alveolar surface with progression of COPD (21). Furthermore, we suggest that the milieu created by these changes allows particular OTUs from within these expanding phyla to punctuate this progressive decline with acute exacerbations of COPD.

An important limitation of this study is that a relatively large number of samples needed to be studied from a small number of individuals, to observe the progression of disease within individuals of the same genetic background. The heterogeneity of the disease within individuals and the observation that terminal bronchioles are destroyed before the onset of emphysematous destruction make it possible to assess the response at different levels of tissue destruction (21), but future studies of larger numbers of cases that include better methods of assessing the host response to specific microbial antigens are needed to confirm the present results. Despite this obvious shortcoming the experimental approach described here provides preliminary evidence in support of the hypothesis that there is a host response to the microbiome in COPD and that it is primarily directed at OTUs within the expanding Proteobacteria and Actinobacteria phyla that have successfully competed for space on a reduced alveolar surface. Furthermore, even though none of the patients receiving a transplant had an exacerbation at the time of transplantation, we postulate that the milieu present within the lung microbiome might encourage the emergence of strains from within the expanding Proteobacteria phylum, which is known to contribute many of the organisms that produce acute exacerbations of COPD (36, 37). ■

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