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Understanding the role of the microbiome in COPD: Principles, Challenges and Future Directions

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Abstract

In the past several years, advances in sequencing platforms and bioinformatics have transformed our understanding of the relationship between microbial ecology and human health. Both the normal and diseased lung are host to hundreds of bacterial genera, blurring the lines between “colonization” and “infection”. However whereas in health the respiratory microbiome is determined primarily by the dynamic balance of immigration and elimination, in chronic disease conditions become much more favorable for the reproduction of resident bacteria. Recent studies demonstrate that the microbiota of the COPD lung differ from the healthy lung although significant intra- and inter-subject heterogeneity are still present with variation impacted by factors such as disease stage and inhaled medications. Changes in the relative abundance of specific bacterial taxa during COPD exacerbations have also been noted although further longitudinal analyses are needed to ascertain the malleability and resilience of this ecological system and its role in the occurrence and frequency of exacerbations. Whether patients with a “frequent exacerbator” phenotype possess specific or greater alterations in their airway microbiome that predispose them to recurrent exacerbations as compared to non-frequent exacerbators needs to be determined. While recent data suggest that the presence of bacteria has the potential to influence the host immune response, a key challenge in the next few years will be to continue to move beyond descriptive studies to define the clinical relevance of differences in lung microbiota associated with COPD.

Keywords

Airway disease; bacteria; 16S rRNA; emphysema; exacerbation

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Introduction

In the past five years, knowledge regarding the human lung microbiome has exploded. Advances in sequencing platforms and bioinformatics have transformed our understanding of the relationship between microbial ecology and human health. Countering prior beliefs that the normal human lung is sterile, culture-independent studies using advanced molecular techniques to identify bacterial sequences have shown that both the normal and diseased lung are host to hundreds of bacterial genera, blurring the lines between “colonization” and “infection”. Studies to date have demonstrated differences in lung microbial communities between health and lung diseases including cystic fibrosis, asthma and chronic obstructive pulmonary disease (COPD) (1). Yet as we move into a “modern age” of understanding the relationship between humans and their resident microbiome, the key challenge for clinicians and researchers studying COPD will be to move beyond descriptive studies to define the clinical relevance of differences in lung microbiota associated with stable COPD as well as during exacerbations of COPD. Here we review studies that have been performed to date in COPD and discuss the future of microbiome research in COPD.

Modern Methods for Studying the Human Lung Microbiome

The usage of conserved genes to reconstruct bacterial phylogenies was pioneered over thirty years ago by the work of Carl Woese. This work used housekeeping genes, specifically the genes for the ribosomal RNA, to build bacterial phylogenies and demonstrate that Archaea were phylogenetically distinct from Bacteria (2-4). Norman Pace and colleagues subsequently built molecular tools for assessing the ecology of microorganisms (5, 6). Since these early initial studies, more than 100 bacterial phyla have been identified, most of which do not include culturable representatives (7). The majority of culture-independent techniques are based upon the PCR amplification of the 16S rRNA gene. This gene possesses both regions that are highly conserved in all bacteria and nine variable regions that have varied throughout bacterial evolution and are therefore useful for taxonomic identification (8). The conserved sequence stretches within the 16S rRNA gene have allowed for the design of so-called “universal” primers with broad recognition of the 16S rRNA gene throughout the bacterial kingdom. Using these primers, pools of 16S rRNA amplicons can be generated from a mixed population of bacteria and the relative abundances of the individual bacteria within the population estimated based on the relative abundance of individual 16S rRNA sequences.

Prior to next-generation sequencing, high-throughput methods of community estimation were performed using molecular fingerprinting techniques such as denaturing or temperature gradient gel electrophoresis (DGGE and TGGE, respectively) and terminal restriction fragment length polymorphisms (T-RFLP) (9). T-RFLP involves amplification of 16S rRNA using a fluorescently labeled primer with subsequent digestion of the amplicons using a restriction enzyme, followed by separation of the digested fragments on a capillary DNA sequencer. Electropherograms showing the relative abundance of the terminal restriction fragments (TRFs) could then be created (10, 11). Changes in the community were detected by the loss or gain of TRFs from the profile. While various multi-dimensional statistics could be applied to these datasets, definitive taxonomic assignments of TRFs were usually

not accurate, due to machine-to-machine variation. To obtain taxonomic identities of 16S sequence pools, it was necessary to construct libraries of 16S rRNA by cloning amplicons into a bacterial expression vector, growing the bacteria, selecting individual transformants, growing the clones to sufficient density to purify the plasmid containing the 16S gene and sequencing the insert using traditional Sanger sequencing. Sequences of the clones were compared using either classifier-based or operational taxonomic unit (OTU)-based algorithms, to existing taxonomic databases, e.g., the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/). The construction and analysis of these clone libraries to study a bacterial community sample is a robust technique that offers almost full length 16S sequencing information, but suffers from the limitations of low throughput, high cost, and low depth of sampling (~96 isolates at a time).

Next generation sequencing platforms and tools

With the advent of next-generation sequencing, the landscape of culture-independent microbiology was irrevocably changed. These machines are capable of throughput, in some cases, of billions of bases, addressing all of the limitations of previous methods. It also created a very particular market: the need for high-throughput, but with long reads. One of the earliest platforms to be adopted was the 454-pyrosequencing platform, which offered sequencing depths of 500,000 reads and read lengths of ~400 base-pairs. The longer reads assured that multiple variable regions could be sequenced at once. By sample barcoding, the addition of unique short (6-8) bp of DNA, multiple samples could be multiplexed for sequencing at an affordable price (12-14). However, sequencing technology has rapidly advanced with higher-throughput technologies that have increased their read-lengths, most notably platforms from Illumina® (e.g. MiSeq or HiSeq; Illumina Corporation, San Diego, CA). The MiSeq, with read depths of millions of reads, coupled with dual-indexing strategies (i.e. sample barcoding), has reduced the cost of 16S rRNA sequencing to the point where often times the most expensive steps are DNA extraction and analysis (15). Whether 3rd and 4th generation sequencing technologies now available might compete with MiSeq is unclear. The specialization of Pac Bio systems (Pacific Bioscenses™, Menlo Park, CA) for very long reads might seem attractive for full length 16S rRNA analysis; however, the much higher cost-to-throughput ratio makes this technology substantially less attractive for this application. Ion Torrent (a 4th generation technology; ThermoFisher, Inc.) has been used for 16S analysis; however, the technology has been shown to be more error prone than light-based technologies (16). Oxford Nanopore's MinIon (also a 4th generation technology) may find a role in 16S analysis not simply because of the portable design of the sequencer, but also because of a unique technology that has the potential to allow the instrument to sequence only what the user desires from a pool of DNA sequences (17). Thus, this technology potentially eliminates the need for PCR amplification before sequencing.

Finally, it is now possible to take full advantage of the tremendous sequencing depth of modern sequencers to sequence everything, all of the genomes, in a given sample. This technique is known as metagenomics. The advantages of this technique are that one: (a) does not need to rely solely on a single gene to identify what organisms are present; (b) can identify non-bacterial community members; (c) can gain an understanding of the genetic capacity of the community members. The disadvantages are that, in the case of low biomass

samples like the lung, one wastes a lot of reads sequencing the host genome, and that one must either have an idea of what organisms are present or a very large genome databases to assemble the genomes against (such as MG-RAST (18)). Another recent solution, Metaphlan, aligns only the reads that align to clade-specific markers (contained in a marker database) to identify the organisms present (19). Identifying the members of a microbial community in this way uses much less in terms of computational resources.

Bioinformatics and data analysis

The ability to generate a huge amount of molecular data has been accompanied by an equal, if not larger, need for tools to process raw sequence data, organize the information into useful units for analysis, and perform robust analyses integrating such data with biological and clinical variables (i.e. metadata) of interest. Fortunately, many tools and pipelines have been developed over the past decade to address this need (20), which in part grew out of large research consortiums such as the NIH Human Microbiome Project. Although a detailed discussion of available tools is beyond the scope of this review, we highlight the following. First, most tools are freely available for public use including the popular sequence processing and analysis pipelines, QIIME and *mothur* (21, 22). Secondly, the same is true of methods to perform statistical analysis for associations between biological variables and sequence data (the latter organized, for example, into OTUs based on >97% homology of 16S rRNA sequences). Many such methods are integrated into QIIME and *mothur*, but can also be accessed through the open-source R statistical environment and various R analysis packages available through Bioconductor (www.bioconductor.org). The latter allows the end-user to directly manipulate analysis parameters as desired. Finally, these and other tools are increasingly user-friendly for biologists and clinical scientists. However, consultation with bioinformaticists or statisticians well-versed in these tools is recommended because nuances of sequence data processing and analysis-related tuning parameters could significantly impact both the inputs and outputs of analyses.

Challenges of a Low-Biomass Environment

The application of these methodologies to the respiratory tract has come with unique challenges. Unlike the GI tract where bacteria can number in the trillions, the lungs are a lower microbial biomass environment. Accordingly, special attention needs to be paid to the analysis methodologies for accurate results. This process begins with generation of the 16S rRNA amplicon library. The standard amplification protocols will often fail to amplify 16S efficiently when the level of bacterial DNA is extremely small and/or the level of non-bacterial DNA is log orders greater than the bacterial DNA. Solutions for this problem have included over-amplification (running PCR reaction for more than 40 cycles); nesting (using a pre-amplification step followed by standard amplification); and touchdown PCR (starting the reaction at temperatures that favor more stringent primer binding, and gradually lowering the temperature (for greater efficiency) as more amplicons are generated. Of these methods, the last is the least affected by PCR-induced bias and has been well published as effective (23, 24). A second challenge is that within the context of low biomass, DNA contamination from reagents can have a disproportionately large effect on the results (25). Because of this, extracting DNA from different sample groups using different kits or at different times can,

just through the act of sequencing, introduce an inherent difference between the groups. When not carefully controlled for (by including multiple reagent controls, extraction controls, blanks, etc.), these biases present a fundamental flaw that can obfuscate primary study findings. However, while these concerns are particular to studies involving low biomass, they are tractable with some thought to the experimental design.

Microbial Ecology of the Human Respiratory Tract

In retrospect, the longstanding belief that “the normal lung is free of bacteria” (26) was anatomically, physiologically and ecologically naïve. The lungs and airways are topologically outside of the body, separated from the microbially dense pharynx by only inches of mucosa. The lungs are also under constant bombardment by bacteria from the inhalation of 8,000 liters of non-sterile air a day as well as subclinical microaspiration of pharyngeal secretions (27-29). Decades before the advent of 16S rRNA-based community sequencing, we knew that even in health, the lungs are subject to a constant immigration of pharyngeal and environmental bacteria. We also knew that this microbial immigration was counterbalanced by the respiratory system's processes of microbial elimination: cough, mucociliary clearance, and the innate and adaptive immune systems(30).

Confusion first arose due to a misguided and incomplete comparison between the lungs and the lower gastrointestinal tract, which harbors a resident and relatively stable community of organ-specific bacteria. The perception arose that in order for the lung microbiome to be “real,” it must likewise have a community structure shaped by environmental pressures on the relative growth rates of its reproducing members. This belief ignored the countless examples of dynamic communities in nature that are defined by a balance of immigration and elimination of community members, with little or no contribution from reproducing residents (31). A useful analogy is a tide pool: its community is determined exclusively by immigration and elimination from and to its source, the ocean. Few or no resident species can survive the starkly different conditions of high and low tide; thus little or no site-specific selective pressure on reproduction is present. Yet tide pools are by no means devoid of life, and the presence of common creatures in a tide pool and the ocean that fills it is not evidence of “contamination.”

The past five years of culture-independent study have revealed how apt this analogy is. Just as a tide pool is subject to constant immigration from the ocean, the lungs are subject to constant immigration from the oropharynx (32-34). Whether a bronchoscope is inserted via the mouth or the nose, lung microbial communities resemble those of the former and bear no resemblance to the latter, indirect empirical evidence against the influence of pharyngeal contamination on bronchoscopic specimens (24, 30). Ecological modeling has shown that there is little evidence of site-specific reproduction of community members in the healthy lung (35), consistent with a constant and taxonomically neutral immigration of oropharyngeal bacteria via subclinical microaspiration.

In the last five years, the field has answered key questions about the viability, variability and immunogenicity of lung microbiota in health. Though in isolation, DNA-dependent techniques used in microbiome studies cannot determine the viability of detected bacteria,

advanced culture techniques using a variety of growth media and environmental conditions have revealed that that the majority (61%) of bacteria detected in bronchoalveolar lavage fluid can indeed be recovered by cultivation (35). Some spatial variation can be detected in lung microbiota when multiple sites are sampled within the same subject. Intra-subject variation is significantly less than that of inter-subject variation (33); the microbiota within a given healthy subject's right middle lobe more closely resembles that of her own left upper lobe than it does another subject's right middle lobe. The more similar a healthy subject's lung microbiota are to oral microbiota, the more robust the inflammatory signal detected in concurrently collected BAL fluid (34). Thus even in health, the lung microbiome is viable, host-specific, and certainly not invisible to the host.

These ecological pressures change dramatically in chronic lung disease such as COPD. Whereas in health the respiratory microbiome is determined primarily by the dynamic balance of immigration and elimination, in chronic disease conditions become much more favorable for the reproduction of resident bacteria. Nutrient density, quite sparse in healthy airways and alveoli, becomes replete with the introduction of mucus and vascular leak (30). Key environmental factors such as temperature, oxygen tension and the local concentration of pathogen-favoring host stress molecules all change dramatically (36, 37). As opposed to the dynamic conditions of health, in illness the microbiome grows dominated by a select spectrum of familiar bacteria that are especially adapted to reproducing in the lower respiratory tract (27). In turn, shifts in community composition of the respiratory microbiome change the identity and activation state of host inflammatory cells and further change environmental growth conditions for bacteria. Thus, a cycle of dysbiosis and inflammation can perpetuate injury to the host. In chronic lung disease, alterations in the lung microbiome likely are both cause and effect.

Microbiome of the COPD Lung

COPD is a disease of inflammation that frequently results from chronic inhalational exposures with tobacco smoke being the most important risk factor in developed countries. COPD is characterized by airflow obstruction that is not completely reversible (38), yet this disorder is also very heterogeneous with significant variation in disease presentation, rates of progression, histologic abnormality and exacerbation frequency (39). One mystery surrounding COPD is that frequently the inflammatory response persists despite smoking cessation, making bacteria a plausible explanation for the persistent inflammation. However at the same time, one of the enormous challenges to understanding the role of the microbiome in COPD is that COPD phenotypes and their associated endotypes are still not well defined and patient heterogeneity within and across studies of the COPD microbiome adds to the difficulty of deciphering this relationship.

The role of bacteria as a potentially pathogenic and etiologic factor in COPD has been a topic of debate for many years with the pendulum of thought having swung back and forth numerous times. To provide some perspective, the original British hypothesis put forth chronic colonization of the lower respiratory tract with bacteria was the cause of chronic bronchitis (40, 41). However, by the mid 1970's this hypothesis had fallen out of favor as there appeared to be “no support for a role of respiratory infections experienced in adult life

in the progressive obstructive airway disease found in some patients...(42).” However, by the early 21st century, there was mounting evidence that there are more culturable organisms present in stable COPD than healthy individuals (43), yet whether those bacteria play a causal role in the development of airflow obstruction still remains unclear.

The advent of modern, non-culture based methods to detect bacteria have led us to begin thinking about the role of the microbial community of the lungs, as opposed to a single culturable organism, in both health and disease. One of the earliest studies to examine the lung microbiome in COPD using 16S rRNA sequencing to identify the bacteria was by Hilty et al., published in 2010 (1). This analysis used bronchoscopically obtained airway brushings to compare the microbiota of five patients with COPD to eleven subjects with asthma and eight healthy controls. Pathogenic Proteobacteria, particularly *Haemophilus* were more frequent in asthmatics and COPD than controls. Conversely, Bacteroidetes, particularly *Prevotella* were less frequent in asthmatics and COPD. A subsequent study by Erb-Downward, et al. compared bronchoalveolar lavage (BAL) fluid from a small cohort of healthy subjects, smokers without airflow obstruction and subjects with obstruction. These data demonstrated comparable concentrations of 16S rRNA genes across their groups, suggesting a similar range of bacterial burdens across groups but decreased diversity in individuals with the most severe airflow obstruction. Common genera among individuals with COPD included Pseudomonas, Streptococcus, Prevotella and Haemophilus. Tissue brushings from several COPD explants were also examined revealing marked regional heterogeneity of lung microbiota within a given lung.

Sze et al. used surgically acquired lung tissue samples to demonstrate low but comparable levels of bacteria were present in COPD tissue versus controls; distinct community differences were also demonstrated in COPD subjects versus smoking and non-smoking controls (44). However, this study demonstrated no significant difference in microbial diversity between COPD lung and controls, although the COPD lungs were considerably more diverse than patients with cystic fibrosis also included in this study. Although both the study by Erb-Downward et al. and Sze et al. focused on individuals with advanced, even end-stage COPD, a difference between these two studies is in the lung compartment examined. Sze et al. used bulk tissue including alveolar parenchyma, whereas Erb-Downward utilized airway brushes and BAL. In another small study of expectorated sputum, bronchial aspirate, BAL and bronchial mucosal biopsies in eight moderate to severe COPD patients, Cabrera-Rubio et al. demonstrated similar genera to prior studies including Streptococcus, Prevotella, Moraxella and Haemophilus although diversity by disease stage was not examined.

It is likely that differences in inhaled medications also influence the microbial communities present in the lung. In another study by Pragman et al., BAL specimens from 22 COPD patients and 10 controls demonstrated that the microbiota of BAL samples clustered by subject exposure to inhaled bronchodilators and/or inhaled corticosteroids (45). This study also found that older age as opposed to disease severity was associated with increased microbial diversity. In sum, these studies suggest the microbial communities of the COPD lung to be different from that of healthy lungs, although the microbial membership varies based on sample location and type. Other host factors including medications, age and

disease severity also likely influence microbial community membership. Larger studies of patients with more detailed clinical and radiographic characterizations and longitudinal follow-up will be required to understand the relationship between the lung microbiome, disease phenotype and disease progression. A summary of studies using 16S-based sequencing methods to describe the microbiome in COPD can be found in Table 1.

COPD exacerbations

The role of bacteria in acute exacerbations of COPD (AECOPD) requires special consideration. AECOPD are characterized by an increase in symptoms that deviate from normal day-to-day variation and warrant a change in usual treatment (46). They are also associated with worse health status, increased airway and systemic inflammation and more rapid lung function decline (47). It has been believed that bacteria are an important etiology of AECOPD based on the ability to culture bacteria during these events. These culture-based studies have focused on the role of single species with known respiratory pathogenic potential such as Haemophilus influenzae, Moraxella catarrhalis, Streptococcus pneumoniae, and in more severe COPD, *Pseudomonas aeruginosa*. However, many COPD patients during clinical stability exhibit evidence of chronic airway colonization by these and other species, suggesting a pathogenic role for bacteria in the exacerbated state may be more complex than simply presence of bacteria. This point also echoes decades-old but ongoing discussions about definitions of “infection”, “colonization” and “pathogen” (48). In fact, this has led to calls for updated conceptual frameworks about microbial pathogenesis that (1) focus on the *outcomes* of microbial-host interactions, which can be highly variable in terms of illness manifestations, and (2) accommodate new knowledge about microbiota and the role of commensal microbial communities in shaping these outcomes (49-51).

Microbiome investigations of AECOPD

Insights from recent studies of the airway microbiome during AECOPD highlight the microbial complexity associated with these events (52-56). An important and consistent observation across studies to date is significant heterogeneity between COPD patients in not only their baseline airway bacterial community composition but also the compositional changes that occur with AECOPD. For example, some individuals exhibit marked increases in the relative abundance of *H. influenzae* or *M. catarrhalis* at the onset of AECOPD, while others manifest more subtle changes (53, 54). These inter-patient differences mirror findings from studies in other obstructive airway diseases, like exacerbations of cystic fibrosis (57).

The pathogenesis of AECOPD therefore very likely reflects the outcome of complex interactions among the established community of airway microbiota, innate and adaptive immune responses, and the ability of new organisms (e.g. viruses or new bacterial strains) to disrupt the relative homeostasis of the airway ecosystem that characterizes clinical stability. This framework is useful for conceptualizing how new microbial exposures and shifts in the balance of microbial composition can impact interactions within the microbial community, as well as between the microbiota and host. For example, experimental rhinovirus infection of COPD patients resulted in increased airway bacterial burden, which correlated with increases in sputum neutrophil counts and neutrophil elastase levels (54). Expansion in

Proteobacteria phylum members, predominantly *H. influenzae*-assigned taxa presumably pre-existing in the community, was seen after rhinovirus infection in some, but not all, COPD subjects. Studies have shown that new strains of pre-existing species play a role in AECOPD (58). However, these data suggest the additional possibility that bacteria involved in acute exacerbations could be pre-existing strains whose selective outgrowth is newly favored in a perturbed microbial ecosystem. More detailed genetic analysis of detected strains would be necessary to dissect this, which is not feasible to discern by 16S rRNA sequence-based methods alone.

Temporal studies of airway microbiome in AECOPD

Longitudinal studies are clearly needed to better understand the dynamics of microbiota-host interactions in relation to AECOPD. In a study of twelve COPD subjects enrolled in a longitudinal study of AECOPD, Huang et al. analyzed temporally-collected sputum samples from before, at the onset of, and after exacerbation events (53). They demonstrated significant changes in the relative abundance of specific bacterial taxa at the onset of AECOPD. Also, given findings from gut microbiome studies that specific organisms present in a niche can promote community enrichment for related species (termed the “like will to like” phenomenon) (59), the investigators additionally examined co-occurrence relationships between *H. influenzae* (as well as *P. aeruginosa* or *M. catarrhalis*) and all other identified sputum bacterial communities during exacerbations. Results of these analyses demonstrated significant positive correlations between the abundance of *H. influenzae* and many other phylogenetically-related bacteria, whereas negative relationships were seen with more distantly related bacteria (**Figure 1**). These observations suggest that similar “like will to like” phenomena occur in the airway microbiome. In other words, the intrusion-success of an extrinsic bacterial species or strain into an established ecosystem may be related to the abundance of closely related bacteria, already present in the ecosystem. It is important to recognize, however, that it is not possible using 16S rRNA-based techniques to discern differences in strains of bacterial species present in a sample, and therefore determine if they are newly introduced or pre-existing in the airway ecosystem. Further longitudinal analyses will help ascertain the malleability and resilience of this ecological system and its role in the occurrence and frequency of AECOPD amongst patients.

The effects of commonly prescribed treatment classes for AECOPD may have long-term impacts on the airway microbiome, even after completion of therapy and clinical resolution. Microbiome analysis of sputum samples collected after completion of treatment for AECOPD, in some cases several weeks to months later, revealed sustained microbial alterations after antibiotic and systemic steroid treatments (53). Interestingly, antibiotics appear to exert prolonged suppressive effects, while steroids result in increased relative abundance of many taxa, in particular members of the Proteobacteria phylum. One could speculate that the cumulative effects of AECOPD treatments on the airway microbiome could, over months to years, result in sustained alterations in airway microbial composition, phenotype and expressed functions that contribute to the severity or frequency of exacerbations in certain patients. The effects of long-term antibiotic administration such as azithromycin, which has been demonstrated to reduce COPD exacerbation frequency on the lung microbiome (60), are also unknown. It is unclear whether azithromycin's effect is due

to anti-inflammatory properties, anti-microbial properties or both. A small study of five patients with moderate to severe asthma demonstrated that azithromycin therapy was associated with decreased bacterial richness and altered airway microbiota (61). Not surprisingly, *Pseudomonas*, *Haemophilus* and *Staphylococcus* (three pathogenic genera associated with airway disease) were all reduced. However, more data are needed to understand the impact of azithromycin on factors such as airway mucus secretion and neutrophil accumulation and whether these are due to direct anti-inflammatory effects or indirect antimicrobial effects.

Microbiome and Host Inflammation

While the majority of microbiome studies in COPD thus far have been largely descriptive, Sze et al. combined bacterial sequencing with micro-CT, quantitative histology and host gene expression analyses (23). This analysis was performed on control and GOLD IV surgically resected lung explants. A decline in microbial diversity was associated with emphysematous destruction and remodeling of the bronchiolar and alveolar tissue by CD4+ T cells. These data support the hypothesis that the microbial diversity of the lungs decrease as the geographic diversity of the lungs decrease with advancing emphysema. They also demonstrated that both Proteobacteria and to a lesser extent the Actinobacteria increased in COPD compared to controls, whereas the relative abundance of Firmicutes and Bacteroidetes decrease as the alveolar surface is being destroyed by emphysema in lungs affected by COPD. The gene expression data also demonstrated that specific genes are up- or down-regulated in association with changes in Firmicutes and Proteobacteria, suggesting a specific host immune response to the microorganisms present. These data suggest that the presence of bacteria has the potential to influence the host immune response and that this interplay may vary depending on the type of histologic abnormality present.

Work has also been ongoing to develop animal models to further understand the relationship between microbiome and host response in airway disease, although primarily centered on allergic inflammation. In particular, since the seminal discovery that the community structure of the gut microbiome shapes host immunity systemically (62, 63), there has been considerable interest in developing animal models to test tenets of the hygiene hypothesis. Germ-free mice were shown to develop increased lung inflammation in the ovalbumin (OVA) model of allergic airway inflammation, relative to specific pathogen-free (SPF) mice (64). Differences were seen in lung eosinophils, basophils, lymphocytes and dendritic cells and were accompanied by increases in IgE and lung type 2 (T2) cytokines, but in this study, not in CD4+, CD25+, FOXP3+ T cells. Importantly, this susceptible phenotype could be reversed by co-housing germ-free mice with SPF mice. This study provided the initial evidence that the bacterial microbiome contributed to the regulation of T2 immunity, but left unanswered many questions about the responsible mechanism. Subsequent understanding has been gained rapidly, but to date, remains almost entirely related to allergic inflammation and the contribution of the gut microbiome. There is urgent need for animal studies relevant to COPD development in response to tobacco-smoke or biomass fuel exposures, as well as animal studies using culture-independent techniques to analyze manipulations of the lung microbiome in animal models.

Future Directions and Unanswered Questions

In just the past few years, there have been significant advances in our understanding of the lung microbiome, its characteristics in COPD and its relationships to clinical outcomes. However, much more remains to be gleaned if we are to successfully translate knowledge about the microbiome into useful strategies for COPD management. Towards that end, we next highlight topics for further investigative focus pertinent not only to COPD, but also other lung diseases characterized by respiratory dysbiosis. These topics include dissecting (1) functions expressed by the lung microbiome, (2) relationships between the lung microbiome and COPD phenotype, (3) potential roles of non-bacterial respiratory microbiota in COPD, and (4) potential influences of the gut microbiome in COPD.

Functions imparted by lung microbiota

The young field of lung microbiome investigation has been immersed in establishing foundational knowledge about the composition of microbial communities found in the respiratory tract, in both health and disease. Much less is known about the collective functional potential of bacterial consortia, and more importantly, what functional components most influence disease pathogenesis or prognosis. In considering these issues, it is necessary to move past the single-species framework of conceptualizing the role of bacteria in complex inflammatory diseases, like COPD. Microbes do not exist in isolation but respond to cues in their environment. Through quorum-sensing mechanisms, bacteria also shape the behaviors and functions of their neighbors, as has been shown for interactions between *Haemophilus influenzae* and *Moraxella catarrhalis* (65). In writing about the role of microbiota in disease, Byrd and Segre recently emphasized that the “criteria for disease causation must take microbial interactions into account” (51).

Elucidating the functional potential of respiratory microbiota poses several methodological challenges from clinical samples. Tools include shotgun DNA sequencing to perform pan-metagenomic studies or RNA-sequencing. However, the high ratio of human-to-microbial sequence reads generated by such studies, magnified in certain sample types like airway brushings or bronchoalveolar lavage fluid, means that often deep sequencing (i.e. high number of total sequence reads) is needed to obtain a useful representation of microbial sequences for analysis. In the absence of direct methods to clearly separate bacterial cells from host cells, bioinformatic methods can be used that focus on non-human prokaryotic sequences reads such as the MG-RAST pipeline (18). Few studies to date have applied meta-genomics or meta-transcriptomics to study the respiratory microbiome (66-68), but this situation is likely to change.

Relationships between the lung microbiome and COPD phenotypes

Studies to date have reported differences in lung bacterial community composition depending on the severity of COPD, as well as inflammatory and immune features in explanted COPD lungs (1, 44, 45, 52-55, 69-75). However, COPD is heterogeneous, and it is unclear if specific patterns of lung dysbiosis are more likely to be associated with particular phenotypes, such as patients with more airway disease versus emphysema. Moreover, two studies have now shown that treatment of exacerbations with antibiotics or steroids impact

microbiota composition differently, and the effects may last beyond the treatment period (53, 56). These considerations also raise the question of whether patients with a “frequent exacerbator” phenotype possess specific or greater alterations in their airway microbiome that predispose them to recurrent exacerbations, compared to non-frequent exacerbators.

Potential roles of non-bacterial respiratory microbiota in COPD

Bacterial communities have been the predominant focus of lung microbiome studies to date. The reasons involve greater accessibility, relative to non-bacterial microbiota, to fairly well-established pipelines and databases for bacterial community profiling, preceded by decades of work in the environmental ecology field. There is increasing interest in identifying the spectrum of fungal microbiota present in the airways, but studies of fungi are, in general, more difficult. There is limited culture-based data on the types of fungi that might be important in COPD, as fungal culture methods in clinical microbiology laboratories are sub-optimal. More recently, various investigative groups with specific expertise in fungal sequencing have reported on the range of fungal species potentially harbored in the respiratory tract. In contrast to leveraging knowledge about the broadly conserved 16S rRNA gene to profile bacteria, a straightforward corollary does not exist in the fungal kingdom. The internal transcribed spacer (ITS) region has been the most widely sequenced DNA region in molecular ecology studies of fungi, but there is selectivity in species detection depending on the region chosen (76). Moreover, reference databases for fungal sequences and genomes are not as mature, which can limit species identification.

In light of these challenges to pan-fungal sequencing studies, it seems reasonable to suggest thoughtful consideration of the goals of such studies and the utility of the information yielded. A recent analysis of fungal communities in cystic fibrosis sputum observed greater fluctuations in fungal richness, which contrasted with that for bacterial richness (77). Though chronic treatments with antibiotics during this period might have influenced the findings, the investigators nonetheless concluded that fungal elements detected in CF sputum are predominantly transient and likely related to inhaled sources.

Potential influences of the gut microbiome in COPD

A final aspect to consider for future investigation is the gut-lung axis in COPD. COPD can be viewed as a primary lung disease with systemic consequences, and non-lung comorbidities are common. Cachexia is also feature in some patients, thought to relate to systemic inflammation. Given increasing evidence that the gut microbiome can shape manifestations of inflammatory airway diseases (either in animal models or in association studies of pediatric asthma and CF patients), it is possible that the gut-lung axis also plays a role in adults with COPD. However, studies are lacking in human adult patients. Moreover, COPD primarily affects older individuals, and aging is known to be associated with changes in gut microbiota and immune function (78). Therefore, studies of the gut microbiome in COPD patients seems particularly relevant and may reveal additional mechanisms that play a role in the pathogenesis of COPD.

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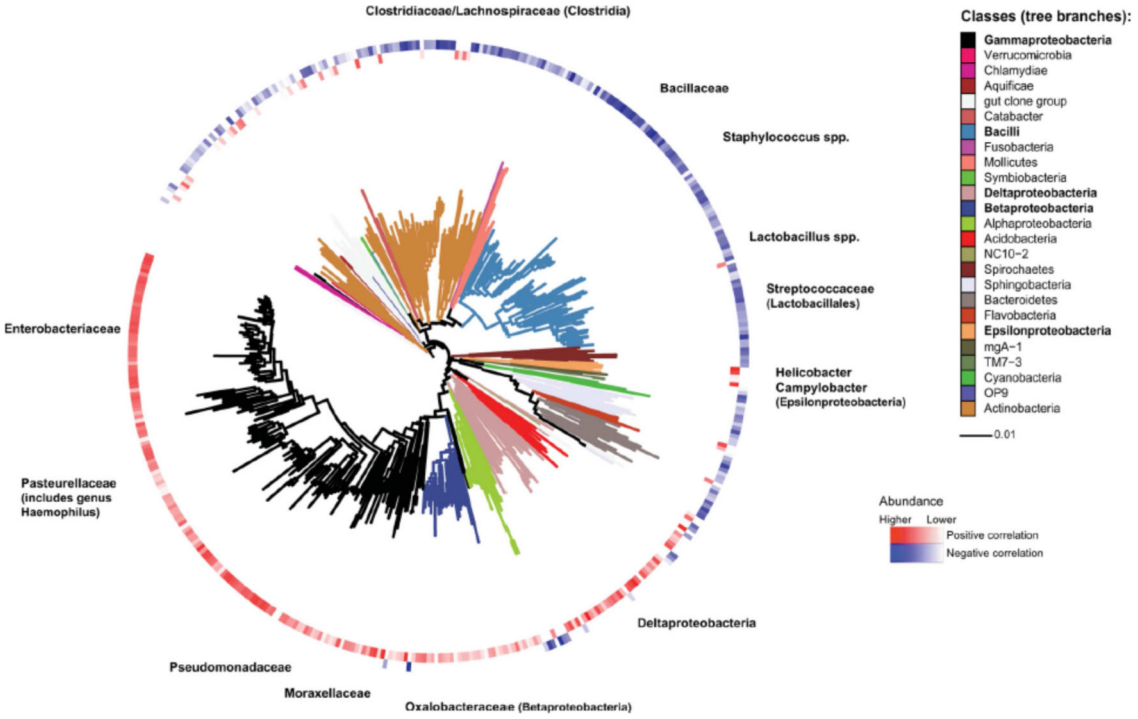

Figure 1.

Correlations between the relative abundance of H. influenzae (a member of the Pasteurellaceae/Gammaproteobacteria) and that of all other identified taxa. The analysis was performed using data from all 60 samples in this study. Significant positive and negative correlations are shown (Pearson $R \geq 0.5$, Benjamini-Hochberg-adjusted $P < 0.05$). Positive correlations (red) occur predominantly with members of Pasteurellaceae or closely related bacterial families and classes of Proteobacteria. Negative correlations (blue) occur with bacterial families and classes that phylogenetically are more distant to H. influenzae/ Pasteurellaceae. Tree branches are color coded by bacterial class in the key on the right.

Table 1

Summary of studies utilizing 16S sequencing to describe the microbiome in COPD

Author	Year	Type and Number of Patients	Type of Sample	Significant Findings
Hilty, et al.(1)	2010	5 COPD, 11 asthma, 8 controls	BAL	Pathogenic Proteobacteria, particularly *Haemophilus* were more frequent in asthmatic and COPD subjects than healthy controls. Bacteroidetes, particularly *Prevotella* spp., were more frequent in controls than asthmatic or COPD patients.
Huang, et al.(52)	2010	8 COPD patients requiring mechanical ventilation for COPD exacerbation	Minibronchoalveolar lavage	Core community of 75 taxa was detected in all patients. Bacterial community composition influenced by duration of intubation.
Erb-Downward, et al.(69)	2011	Group 1: 6 COPD subjects with severe disease Group 2: 3 non-smokers with normal spirometry; 7 smokers with normal spirometry, 4 subjects with COPD	Group 1: Excised airway tissue samples from lung explants Group 2: BAL	Significant micro-anatomic differences in bacterial communities within the same lung of subjects with advanced COPD.
Cabreera-Rubio, et al.(70)	2012	8 COPD patients	Sputum, bronchial aspirate, BAL and bronchial mucosal biopsy	Sputum samples showed significantly lower diversity than the other three sample types. Lower-bronchial-tree samples showed similar bacterial compositions in contrast to sputum and bronchial aspirate samples.
Sze, et al.(44)	2012	8 non-smokers, 8 smokers, 4 COPD, 6 cystic Fibrosis	Surgically obtained lung tissue samples	In COPD as compared to smoking and non-smoking controls, comparable abundance of Proteobacteria and Bacteroides but increased Firmicutes as compared to controls. Bulk of analyzed tissue is likely alveolar parenchyma which distinguishes this study from other analyses.
Pragman, et al.(45)	2012	22 COPD and 22 controls	BAL	Principal coordinate analyses demonstrated separation of control and COPD samples, but samples did not cluster based on disease severity. However, samples did cluster based on the use of inhaled corticosteroids and inhaled bronchodilators.
Molyneaux, et al.(54)	2013	14 COPD subjects and 17 healthy controls	Induced sputum collected before and after rhinovirus infection	After rhinovirus infection, a significant increase in bacterial burden and a significant outgrowth of *Haemophilus influenzae* from the existing microbiota of subjects with COPD was seen. This is not observed in healthy individuals.
Huang, et al.(53)	2014	12 COPD subjects including current and former smokers	Sputum collected before, during and after exacerbations.	Shifts in the abundance of many taxa at exacerbation and after were observed, with different effects seen with antibiotic versus corticosteroid treatments. Significant positive correlations between the abundance of *H. influenzae* and many other phylogenetically-related bacteria were seen, whereas negative correlations existed with more distantly related bacteria. Findings suggest that the intrusion-success of an extrinsic species or strain into an established ecosystem may depend in part on the abundance of closely related bacteria already present in the ecosystem.
Millares, et al.(55)	2014	16 COPD subjects	Sputum collected before and during exacerbation	5/16 patients demonstrated chronic colonization with *P. aeruginosa* based on culture. Exacerbation in severe COPD patients showed the same microbial pattern assessed via 16S rRNA analysis, independently of previous colonization by *P. aeruginosa.*
Galiana, et al.(72)	2014	9 subjects mild or moderate COPD and 10 subjects with severe or very severe COPD	Sputum	Those with moderate disease were seen to exhibit greater bacterial biodiversity. The absolute quantity of bacteria is greater in more severe disease. The composition of bacterial genera in samples from severe patients differed more among themselves than as compared to the samples from the other group.

Author	Year	Type and Number of Patients	Type of Sample	Significant Findings
Garcia-Nunez, et al.(73)	2014	17 subjects with COPD	Sputum collected during disease stability	A greater microbial diversity was found in patients with moderate-to-severe disease, and alpha diversity showed a statistically significant decrease in patients with advanced disease.
Millares, et al.(66)	2015	8 severe COPD subjects	Sputum collected before and during exacerbation	The bronchial microbiome as a whole is not significantly modified when exacerbation symptoms appear in severe COPD patients, but predicted functional capabilities of microbiota show significant changes in several pathways.
Sze et al.(23)	2015	5 subjects with COPD and 4 controls	Surgically excised lung tissue	Decline in microbial diversity associated with emphysematous destruction. Specific OTUs were also associated with neutrophils, eosinophils, and B-cell infiltration. The expression profiles of 859 genes and 235 genes were associated with either enrichment or reductions of Firmicutes and Proteobacteria.
Aguirre, et al.(74)	2015	19 subjects with COPD	Expectorated sputum	Using conventional culture, 3 phyla and 20 bacterial genera were identified, whereas the pyrosequencing approach detected 9 phyla and 43 genera. Enterobacteriaceae, detected frequently in conventional culture, were not significantly detected with pyrosequencing methods. Haemophilus and Moraxella were detected more frequently by 454-pyrosequencing.
Su, et al.(75)	2015	6 subjects hospitalized with acute exacerbation of COPD	Expectorated sputum	*Acinetobacter, Prevotella, Neisseria, Rothia, Lactobacillus, Leptotrichia, Streptococcus, Veillonella*, and *Actinomyces* were the most commonly identified genera. The fungal population was typically dominated by *Candida, Phialosimplex, Aspergillus, Penicillium, Cladosporium* and *Eutypella.*
Wang, et al.(56)	2016	87 subjects with COPD	Sputum samples collected during stable state, exacerbation, 2 and 6 weeks post-therapy	Dynamic lung microbiota where changes appeared to be associated with exacerbation events and indicative of specific exacerbation phenotypes. Antibiotic and steroid treatments appear to have differential effects on the lung microbiome.