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The Cystic Fibrosis Airway Microbiome

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Repeated pulmonary exacerbation and progressive lung function decline characterize cystic fibrosis (CF) disease, and represents one of the leading causes of mortality in this patient population. Recent studies have shown, using culture-independent assays, that multiple microbial species can be detected in airway samples from CF patients. Moreover, specific groups of bacteria within these bacterial communities or microbiota, are highly associated with disease-associated factors such as antibiotic administration. This raises the possibility that, as in other human niches, pathogenic processes in the CF airways represent polymicrobial activities and that microbiome composition and perturbations to these communities define patient pulmonary health status. Airway samples are typically collected through the mouth, and are thus susceptible to contamination by upper airway secretions; hence, caution must be exercised in interpreting these data. Nonetheless, given the continuum of the upper and lower respiratory tract, understanding the contribution of these mixed-species assemblages to airway health is essential to improving CF patient care. This article aims to discuss recent advances in the field of CF airway microbiome research and interpret these findings in the context of CF pulmonary disease.

Cystic fibrosis (CF), an autosomal genetic disorder most prevalent in Caucasian populations, is attributable to mutations within the gene coding for cystic fibrosis transmembrane regulator (CFTR) protein. To date, more than 1600 mutations have been identified (<http://www.genet.sickkids.on.ca/cftr/>), but the functional implications of these mutations and their association with severity of disease symptoms have only been defined for a relatively small number of common CFTR genotypes. Functional consequences of CFTR mutation can be broadly divided into four classes: I, absence of protein synthesis; II, inadequate processing; III, defective regulation; and IV, defective produc-

tion (Collins 1992). For example, the common $\Delta F508$ mutation, which results in deletion of the phenylalanine amino acid residue at position 508, leads to protein misfolding and proteosomal degradation leading to apical epithelia lacking this crucial transporter. The majority of CF patients carry the $\Delta F508$ mutation in at least one copy of the CFTR genes and those homozygous for this mutation show the most severe disease. CFTR plays an established role in chloride transport and more recently has been implicated in transport of thiocyanate (SCN^-), bicarbonate (HCO_3^-), as well as proteins (Quinton 2001; Riordan 2008) across epithelial surfaces. Given the sheer prevalence of epithelial linings in the

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human host, it is no surprise that CF manifests as a systemic disease, characterized by pulmonary, gastrointestinal, and urogenital symptoms, including recurrent airway infection, aberrant nutrient absorption, and reproductive issues.

Disruption of chloride anion transport, one of the key underlying features of CF, leads to altered physiological conditions at epithelial surfaces. From a microbial viewpoint, however, the environment generated by CFTR mutation results in ideal conditions for colonization. For example, in the airways, a site in which microbial colonization is associated with recurrent infection, CFTR mutation results in reduced chloride secretion and increased sodium and water absorption at the epithelial surface, leading to a depleted airway surface liquid layer (ASL). ASL deficiency in turn leads to ciliary dyskinesia and impaired mucociliary clearance, a key component of the host innate immune response to microbes. As a result, mucosal surfaces of the airways show dehydrated mucus and are heavily colonized by microbes that contribute to progressive lung function decline over the lifetime of the patient.

Although it has long been acknowledged that the CF airway represents a permissive environment for microbial colonization, clinical laboratory testing for the presence of specific pathogenic species using culture-based approaches have led to a reductionist view of the microbiology in this niche. As a result, the dogma had been that the diversity of bacterial or fungal species present in CF airways was relatively restricted to a handful of “usual suspects” commonly detected by conventional laboratory culture. However, the last 10 years have witnessed a revolution in our appreciation of the complexity of microbes that exist within and on the human body. The advent of high-resolution molecular approaches, primarily developed in the field of environmental microbial ecology, has dramatically enhanced our ability to interrogate the true diversity of microbes present in a given niche. Application of these tools to human samples has led to the emergence of a new field of “human microbiome” research, focused on defining the types and abundance of microbial species at specific

niches in the human body. The relatively recent establishment of human microbiome research initiatives in the United States, EU, and Asia, underscore the magnitude of global efforts aimed at defining the true microbial complexity resident in the human host and its role in maintenance of health or contribution to disease.

MICROBIOME PROFILING

As a backdrop for discussion of the CF microbiome, it is useful to briefly describe fundamentals of the culture-independent tools used to interrogate human microbiota. Molecular approaches to phylogenetically profile mixed-species members of a given microbial community are typically DNA based and commence with efficient extraction of nucleic acid from all members of the community in any given sample. Following successful extraction, profiling is primarily based on polymerase chain reaction (PCR) amplification of specific biomarker genes known to exist in all members of a specific taxonomic level (e.g., the 16S rRNA gene), ubiquitous to all bacteria or the fungal-specific internal transcribed spacer (ITS) region. Universal primers, specifically designed on conserved regions within a biomarker gene, are used in a PCR reaction to amplify a pool of, for example, 16S ribosomal RNA genes from the various genomes of community members. Although PCR is known to introduce certain biases in the profile (e.g., preferential amplification of specific targets), many researchers have implemented measures to minimize these issues. These include the use of degenerate primers to maximize the diversity-amplified, amplification across a gradient of annealing temperatures for each sample and pooling the amplified products before profiling, as well as minimizing the number of PCR cycles used to amplify the biomarker gene.

A large number of approaches boasting increasing resolution have emerged over the past 20 years to profile the mixed-species biomarker gene amplicons generated by culture-independent approaches. Among the more basic profiling approaches is separation of differential biomarker gene sequences by gel electrophoresis methods (e.g., terminal restriction fragment

length polymorphism [T-RFLP] or denaturing gradient gel electrophoresis [DGGE]), which provide a fingerprint of the most highly abundant members of the community and can be used to show shifts in the dominant community members across time or with treatment. More recently developed approaches include next-generation sequencing platforms, which generate enormous volumes of biomarker gene sequence data, permitting much deeper community coverage and information on the relative abundance of specific groups of microbes in a given community. Building on the recent expansion of microbial biomarker sequence data, groups have also developed phylogenetic microarrays, which, based on hybridization to oligonucleotide probes designed against discriminatory loci on specific biomarker genes, can profile thousands of organisms in a single parallel assay. These tools have more recently been applied to respiratory and other samples collected from CF patients and have, as for many other diseases, shed light on the complexity of the microbial communities present in the airways of patients with chronic inflammatory disease.

THE CF PULMONARY MICROBIOME

Although still in its infancy, some of the most seminal studies of the last decade pertaining to human health have emerged from the field of human microbiome research. For example, pioneering studies by Gordon and colleagues showed a wealth of bacterial diversity present in the gastrointestinal tract of humans (Ley et al. 2005; Turnbaugh et al. 2006). Moreover the investigators revealed that the composition of the bacterial consortia present in lean or obese adults was dramatically different and that characteristic shifts in community composition distinguished healthy and obese states (Ley et al. 2005). Other studies of the gastrointestinal microbiota have identified novel species, whose 16S rRNA gene has not before been sequenced (Eckburg et al. 2005), suggesting that our knowledge of the true diversity of bacteria resident in the human ecosystem is somewhat limited.

These important studies captured the imagination of researchers, garnered international

interest, and certainly paved the way for investigations of other human host niches. However, earlier studies using gel-based separation approaches had already made their way into the public arena several years before the term “human microbiome” had even been coined. In their pioneering culture-independent study of CF patient samples, Rogers and colleagues showed for the first time using adult CF patient bronchoscopic and sputum samples, that multiple distinct 16S rRNA gene PCR products were present in each patient sample, indicating the presence of distinct bacterial phylotypes in this niche. Moreover, subsequent sequencing efforts identified, in addition to *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, multiple other species typically associated with the oral or gastrointestinal cavity including *Prevotella oris*, *Fusobacterium gonidiformans*, and *Bacteroides fragilis* among others, which had not previously been associated with CF airways (Rogers et al. 2003). But the obvious question was whether the diversity of species identified with these culture-independent techniques was viable. The investigators quickly followed up their findings with a subsequent study of bacterial diversity in the airways based on RNA rather than DNA, to profile active members of the CF airway microbiota in adult patients. Total RNA extracted from 71 sputum samples was reverse transcribed and used as template for 16S rRNA gene PCR amplification. Amplicons generated by this approach represent community members who are actively transcribing their 16S rRNA gene and are hence viable members of the community. T-RFLP profiling of the amplicons identified 248 distinct bands, each originating from a viable bacterial member of the CF airway. Across patient samples, bacterial community richness (number of distinct phylotypes detected) was as high as 37 viable phylotypes in one patient, substantially greater than the typical one or two pulmonary pathogens reported through culture-based clinical laboratory testing to colonize CF airways. Follow-up cloning of T-RFLP bands from three patients in the study and sequencing 53 of these clones identified the presence of additional species not previously identified in CF airways including



two *Abiotrophia* species, *Mycoplasma salivarium*, *Ralstonia taiwanensis*, *Rothia mucilaginosa*, *Treponema vincentii*, and *Veillonella atypical* (Rogers et al. 2004). These seminal studies showed that not only were bacterial species not typically associated with the CF airways present in these patients, but that they were evidently viable and thus possessed the potential to contribute to airway disease in this population, having significant implications for cystic fibrosis and other chronic pulmonary diseases.

Data generated by more recent culture-independent studies of CF patient samples, using progressively higher-resolution tools, have clearly showed the presence of even more complex and diverse microbiota in the airways of these patients (Harris et al. 2007; Cox et al. 2010; Klepac-Ceraj et al. 2010). These studies have further expanded the diversity of organisms detected in the airways of CF patients. However, moving beyond sheer description of microbiota and determining relationships between the composition of these assemblages and host health status is necessary to identify key features of the microbiome that contribute to patient health and its decline. In a cross-sectional study of respiratory samples collected from 45 clinically stable CF patients, aged between 9 months and 72 years, who had not received antibiotics for acute pulmonary exacerbation within 2 months of sample collection, Cox and colleagues examined changes in airway microbiota composition within this cohort. They first showed, as expected, a significant negative correlation (-0.48 ; $p < 0.0003$) existed between patient age and pulmonary function, confirming that, compared to the younger population, older CF patients in the cohort showed poorer airway health (Cox et al. 2010). To determine whether this decline in pulmonary health shown by older patients was associated with changes in the airway microbiota, the authors examined whether relationships existed between patient age and gross metrics of bacterial community composition. A significant negative correlation existed between patient age and community richness (number of types of bacteria present), evenness (relative distribution of community members), and diversity (index cal-

culated based on richness and evenness metrics). In addition the communities present in the airways of older CF patients were comprised of phylogenetically related organisms belonging to the family Pseudomonadaceae (Cox et al. 2010). This indicated that substantial shifts in the airway microbiota composition and structure paralleled the decline in pulmonary health shown by the cohort providing the first evidence that microbiota composition was associated with airway function status.

To provide a clearer picture of precisely which members of the community were associated with younger and older CF patients, the authors examined relationships between relative abundance of all community members detected and patient age. Although substantial interpersonal variation has been shown to exist across healthy humans at other sites such as the GI tract (Wu et al. 2011), disease and associated treatments, particularly in the case of chronic illness, appears to act as a strong selective pressure on microbiome composition. Indeed, using simple linear regression, the abundance of >100 taxa were identified as either negatively ($n = 68$) or positively ($n = 45$) correlated with CF patient age. *Haemophilus influenzae* was among the negatively correlated, whereas *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* showed strong and significant positive correlations with patient age, further supporting previous observations that these species are associated with early- or late-stage pathogenic processes, respectively. However, multiple other community members showed equally strong correlations with age, and although correlation does not automatically indicate causality and functional studies are now necessary to confirm their respective roles, at the very least, these species may modify microbiota function and pathogenic processes associated with younger and older CF patients. Further functional analyses of these communities to determine the microbial and host factors that contribute to airway health status are necessary and will further support DNA-based findings of diverse communities in the airways of CF patients. However, collectively, as for other host niches, these studies show that as we interrogate the human microbiome in

distinct niches with more sophisticated tools providing progressively higher-resolution profiles of the species present, the diversity of organisms present increases. This fact was well shown recently by the European Human Microbiome consortium, who showed that when a sequence depth of ~ 4 Gb was used to determine the number of strains common to two gastrointestinal samples, only 1% of strains were deemed common (135 strains). However, when the depth of sequencing was more than doubled to ~ 11 Gb for each of these samples, the number of strains found in common increased by 25% (169 strains [Arumugam et al. 2011]), illustrating that conclusions must be interpreted in the context of the depth of profiling performed on a given sample.

A caveat with all lower airway studies is the need to sample through the oral cavity and upper airway. Goddard and colleagues recently showed, using explanted lungs from CF patients undergoing transplantation, that the diversity of bacteria in the lower airways was substantially less than that described in some recent culture-independent studies and that these communities were characterized by a handful of the usual CF pathogenic suspects (Goddard et al. 2012). By definition, patients undergoing lung transplant have severe airway disease. Examination of microbiome data from comparable adult CF patients with severe disease also shows broad concordance with these findings. The microbiota of these patients show a marked reduction in diversity and possess communities highly enriched for a small number of CF-associated pathogens such as *P. aeruginosa* and *Burkholderia* species (Cox et al. 2010). Indeed, the greatest bacterial diversity has been observed in pediatric airway samples from patients with milder disease and good pulmonary status. Whether this is a function of our inability to obtain clean lower airway samples in this population is a possibility. However, it is also conceivable, particularly in light of recent studies demonstrating a clear association between diminishing airway microbiome diversity in parallel with increased exposure to antibiotic treatments (Zhao et al. 2012), that greater bacterial diversity exists in the airways of younger CF patients compared to those with end-stage disease.

Although the microbiome studies described above have concentrated at the level of species, a further level of strain-based diversity and dynamics exists within these communities. Several studies have shown considerable strain diversity within populations of a given species, not least in *P. aeruginosa* strains isolated from cystic fibrosis airways (Mowat et al. 2011). Mowat and colleagues showed that major changes in the *P. aeruginosa* haplotype were evident in temporal samples from individual CF patients and that these populations were highly dynamic (Mowat et al. 2011). Such strain diversity presumably confers the capacity, within a species population, to house strains capable of withstanding a diversity of insults from both the host and other microbial species, likely contributing to species resilience within these diverse multispecies communities. Little work to date has interrogated the relationship between microbiome composition at the species and subspecies levels. However, with the recent advances in sequence-based capabilities, the ability to interrogate these “worlds within worlds” will improve substantially. Although the obvious need to develop bioinformatic skill sets to cope with such complex datasets is also clear.

ROLE OF MICROBIOME IN MODULATING IMMUNE RESPONSE

Despite the complexity of its microbiome, the human host has evolved a sophisticated immune system to recognize and discriminate commensal from pathogenic microbial species. Evidence from several gastrointestinal microbiota studies (Michail et al. 2011; Walker et al. 2011) has shown that, as in the CF airways, loss of microbiota diversity is a hallmark of chronic inflammatory disease. This suggests that immune homeostasis and colonization resistance (the ability to withstand invasion by pathogenic species) is predicated on appropriate mucosal colonization and that perturbations to the microbiota, particularly those that lead to loss of community diversity and increased abundance of specific immunogenic species, may drive these persistent inflammatory responses.

It is well established that specific pathogens, including key species involved in CF airway pathogenesis such as *P. aeruginosa*, express virulence factors enabling them to evade host immune responses (Kharazmi 1991). However, more recently it has been showed that through induction of host inflammatory responses, some bacterial species gain a significant competitive advantage over other members of the microbiota (Winter et al. 2010; Thiennimitr et al. 2011). For example, host inflammation induced in response to the gastrointestinal pathogen, *Salmonella enterica* serotype *Typhimurium* has been shown to promote the abundance of this species (Winter et al. 2010; Thiennimitr et al. 2011). This phenomenon has more recently been shown to be due, at least in part, to generation (via the interaction of inflammation-derived reactive oxygen species and luminal thiosulfate) of a novel electron acceptor, tetrathionate. *S. typhimurium*, unlike other species, is equipped with the ability to use this novel electron receptor for respiration, providing it with a significant growth advantage over fermentative microbiota in the intestine (Winter et al. 2010), and a mechanistic basis for its increased abundance under inflammatory conditions. Secondary to this growth advantage, host inflammation may also provide such species a competitive advantage by reducing colonization resistance of the host. It is conceivable that reactive oxygen species produced during the inflammatory response from, for example, neutrophils, may impact the viability of strict anaerobic species that colonize mucosal surfaces, particularly in the gastrointestinal tract. Loss of this protective ancillary barrier, which also appears to play a key role in immune homeostasis, may also contribute to pathogen outgrowth in these communities. Although these observations regarding inflammation and microbiome perturbation have, to date, been made in the gastrointestinal tract, an interesting observation in the Cox study supports this hypothesis. Younger CF patients who showed less airway inflammation and better pulmonary function possessed a greater diversity of airway bacterial species, including anaerobic community members, compared with older CF patients.

Factors that perturb the human microbiota have obvious implications for host health. A characteristic of CF patient health management is antibiotic administration. Human microbiome studies have shown dramatic and persistent effects of antibiotic administration on the gastrointestinal microbiota of pediatric subjects. Palmer and colleagues cataloged the fecal microbiota of 11 infants over the first year of life and showed that antimicrobial administration resulted in an acute loss of bacterial burden following treatment (Palmer et al. 2007). However the microbiota rebounded, within a matter of weeks, a community had reassembled that grossly resembled the pretreatment microbiota. Nonetheless, the authors pointed out that specific species present in appreciable abundance pretreatment, were neither detected in samples collected immediately following antimicrobial administration nor over the remainder of the study (in some cases up to 1 year). A recent study of the airway microbiome by Zhao and colleagues performed on samples collected over the course of a decade from CF patients showed a similar phenomenon (Zhao et al. 2012). Despite antimicrobial administration that caused a large perturbation to the assemblages, communities reassembled over time (Zhao et al. 2012). This study also echoed the findings of Cox et al. (2010), demonstrating that patients with progressive disease showed concomitant decreasing diversity. Although counterintuitive in the CF airways, the presence of a diversity of species appears to be associated with better pulmonary health. Given these data, it is tempting to speculate that recurrent antimicrobial administration for pulmonary function management of treatment of acute pulmonary infections, serves to serially select over time a more uneven and less diverse airway microbiota, a feature highly correlated with more severe inflammatory disease in multiple patient populations (Cox et al. 2010; Walker et al. 2011; Zhao et al. 2012).

Overall, this article has identified some of the important advances in microbiome research in relation to the CF airways. Microbiome studies will continue to evolve in parallel with more sophisticated technologies. These advances will clearly benefit expansion of cystic

fibrosis studies on technical, practical, and conceptual levels. Increasingly, we envisage studies that integrate airway microbiome composition and function with therapy, the host immune response, and clinical outcomes. Such a systems-based approach will help shape our understanding of the lung microbiome and how this impacts on the well-being of patients with CF.

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