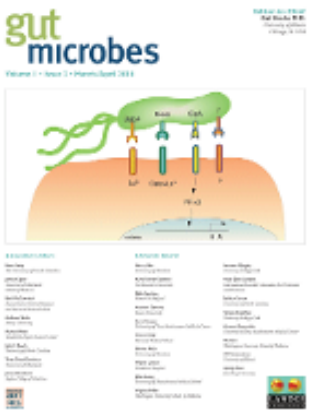

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Comparing the microbiota of the cystic fibrosis lung and human gut

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In recent articles,[1,2] we discussed a fundamental shift in the way in which polymicrobial infections can be viewed. In these articles, we used chronic bacterial infections of the lower airways, and specifically those that occur in cystic fibrosis (CF) patients, as a model system. These infections are of course critical in clinical terms for these patients; respiratory failure due to a combination of these chronic infections with the host immune response that they elicit remains the leading cause of mortality in CF. Given the importance of maintaining lung health in these patients, the CF airways are the focus of a wide range of scientific and clinical studies. In particular, research momentum has built in relation to identifying the microbes present in the CF lung. Already, many important insights have been gained through the application of increasingly sophisticated culture-independent analytical methodologies to identify all microbial nucleic acids in the CF lung,[3-6] and those from viable or metabolically active bacteria.[7,8] In doing so, the data generated have revealed microbial assemblages of far greater diversity and in turn complexity than has previously been recognised in this context. These studies, which have served to highlight the inadequacy of traditional culture-based diagnostic microbiology to fully characterise such infections,[9] have also led to a significant shift in our view of what the word "infection" represents for these and other chronic diseases, with potentially important implications for their optimal treatment. In this article we contrast the information we and others have accrued from chronic lung infections with data generated from studies examining the microbial communities present in the gut. In doing so we highlight parallels between these two contexts and discuss how these commonalities can inform clinical thinking.

Common Beginnings

The process of isolating microbes through enrichment culture was developed in the 19th century, and culture-based strategies remain the basis of diagnostic bacteriology to this day. Whilst there was recognition that certain infections were in fact polymicrobial in nature at the beginning of the 20th century,[10,11] there existed neither the theoretical basis on which to construct models of polymicrobial activity, nor the tools to dissect these systems well.

In a diagnostic context, culture-based microbiology relies on the detection of aetiological agents by providing the conditions that a given species requires for growth in vitro. In many cases, even where the involvement of a specific pathogen is suspected, the provision of such suitable growth conditions can be extremely difficult. This problem is further compounded when diseased tissues contain multiple microbes, challenging the association of a single species with a particular clinical condition. In such circumstances, the size of the pool of uncharacterised species, and the relative distribution of those species within that pool, may not easily be determined using culture-based approaches. To illustrate, culture-independent surveys of phylogenetically-informative ribosomal RNA genes have indicated that more than 75% of the phylotypes detected in the human large intestine do not correspond

closely to cultured species.[12-14] In addition, any analysis based on obtaining species in axenic culture does not afford any insight into the interactions between the microbes present in a tissue, or consequently into the impact that these interactions may have. For these reasons, the rational characterisation of highly diverse microbial systems, and their relationship with disease pathogenesis, using traditional culture-based techniques is severely hampered.

Molecular Advances

The development of polymerase chain-reaction amplification (PCR) provided a basis for assays that exploit differences between species at the nucleic acid level. Here, variation in DNA sequence, particularly the presence of nonconserved genes, can be used to derive species-specific PCR-based assays. These techniques are applied directly to nucleic acids extracted from clinical samples to determine whether particular species are present, often quantitatively using real-time PCR.[15] In all such culture-independent analyses, the first step is the extraction of nucleic acids directly from specimens of interest. As such, these methods avoid many of the biases associated with in vitro culture. However, as with any process however, nucleic acid extraction can introduce other forms of analytical biases, such as through differential lysis of cells of different bacterial species.[16,17] Nevertheless, such species-specific assays enable direct bacterial detection in a wide range of samples, with the popularity of this approach reflected in the ever increasing number of such assays.[18] Nevertheless, whilst conferring advantages in terms of speed, cost and accuracy, these assays share some of the drawbacks associated with the use of selective media in culture-dependent approaches. Again, application of these methods requires a prediction to be made as to which agents are likely to be associated with a particular sample, so that the required reagents (e.g., the appropriate growth medium or species-specific primers) are used. They also share, even with automation, a practical upper limit to the number of species-specific assays that can be performed on one sample.

Alternatives to species-specific approaches are available. In one PCR-based method, primers are used that amplify regions of particular phylogenetically informative genes that are conserved across the Domain Bacteria, in theory producing PCR product for any bacterial species reasonably abundant within a sample. The key to this process is that, between the two conserved regions recognized by the primers, there is sufficient sequence variation among different bacteria to enable species discrimination and identification. As such, this method provides a basis for the characterisation of bacterial species present within a clinical sample without the need for prior prediction of which species may be present, addressing many of the drawbacks of both culture-based assays and species-specific PCR.

By far the most commonly used region in such 'broad-range PCR' strategies is the 16S ribosomal RNA (rRNA) gene.[19] PCR primers are targeted towards regions of the 16S rRNA gene that show conservation across the Domain Bacteria, meaning that a broad range of ribosomal gene products are generated from DNA templates extracted from a mixed bacterial community. To resolve this mixture of gene products and to identify the individual species present in the community, a number of strategies can be applied. These include Single Strand Conformation Polymorphism, Denaturing Gradient Gel Electrophoresis, Temperature Gradient Gel Electrophoresis, Terminal Restriction Fragment Length Polymorphism (T-RFLP) profiling and 16S rRNA gene sequencing (reviewed in refs. 20–22). Each technique exploits the variable internal regions directly or indirectly, with the strategy used selected on the basis of the clinical question and the type of data required.[20,22] Such broad-range strategies are finding increasing use, and in an expanding range of clinical scenarios.[23]

Concepts Emerging from the CF Airways

As highlighted in earlier publications, the application of molecular techniques to the analysis of CF airway infections has significantly changed the way in which such infections are viewed. Traditional culture-based diagnostic microbiology focused on a relatively small number of bacterial species that were both amenable to laboratory cultivation and considered to have important roles in CF lung disease.[24] Of these, the most notable were *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Staphylococcus aureus*, *Haemophilus influenzae, Alcaligenes xylosoxidans* and *Stenotrophomonas maltophilia*.[25,26] However, the use of molecular techniques has shown a far greater range of bacterial species to be present (and abundant) in CF respiratory samples than was previously appreciated,[3-6] and the use of careful culture techniques not commonly used in the clinical arena has corroborated these findings.[27]

Whilst it is difficult to succinctly describe the taxonomy of such a wide diversity of species, to give some insight, the traditionally recognised CF pathogens fall into two phyla; Proteobacteria (*H. influenzae, P. aeruginosa, S. maltophilia, B. cepacia* complex*, A. xylosoxidans*) and Firmicutes (*S. aureus*). Culture-independent studies have also shown CF secretions to be abundant in other members of these phyla, including species of the genera Veillonella, Streptococcus, Abiotrophia and Gemella (Firmicutes), and Neisseria and Acinetobacter (Proteobacteria). Culture-independent studies have further shown however that species belonging to four other phyla are also commonly resolved: Actinobacteria (including Actinomyces spp., Rothia spp.), Bacteroidetes (including Prevotella spp., Porphyromonas spp., Capnocytophaga spp.), Spirochaetes (Treponema spp.) and Fusobacteria (Fusobacterium spp.).

A common but unexpected finding in adult CF respiratory samples has been the high relative representation of species requiring anaerobic conditions for growth, such as those in the genera Veillonella and Prevotella.[9,27] The presence in CF secretions of bacteria with diverse growth requirements is however less surprising once the biology of the CF respiratory tract has been considered. The CF airways have been found to be both chemically and physically diverse, containing complex nutrients and carbon sources.[28] Levels of important factors required for growth by some species, such as iron,[29] and carbon sources such as mucin and alginate,[30,31] and amino acids,[32] vary in concentration across

the airways, as do electron acceptors such as oxygen and nitrogen oxides.[33,34] As such, this heterogeneity provides conditions that are potentially suitable for colonisation by a wide range of microorganisms.

It is important to also recognise that the diversity seen in the CF lung is not only reflected in the number of bacterial species present, but also in their relative abundance. This distribution of bacterial cell numbers between species within the lung may be ecologically important in terms of interactions between different community members. There is also diversity at bacterial sub-species levels, as exemplified by the co-occurrence of multiple strains of *P. aeruginosa*.[35,36] Furthermore, the diversity observed among microbes is not limited to bacteria, but extends, for example, to viruses[37] and to fungi.[38] Whilst of an apparently lesser magnitude, the microbial diversity seen in the CF lung is reminiscent of the microbial communities normally found in other areas of the body, such as the oral cavity or gut. As such, in keeping with the terminology used to describe those polymicrobial communities, the diverse system seen in the CF lung may also be regarded as a "microbiota".

Microbial diversity in turn leads to the potential for "social interactions",[39-43] that may in turn dampen or exaggerate outcomes in relation to pathology and response to therapy. In work examining the bacterial communities that colonise the oral cavity, Jenkinson and Lamont[44] raised the possibility that, through social interactions, a microbial community may itself represent a pathogenic entity. There is mounting evidence that this concept may also be true as applied to the microbes in the CF lung. Sibley et al.[45] demonstrated that the virulence of known CF pathogens, such as *P. aeruginosa,* can be increased significantly by the presence of species previously dismissed as clinically insignificant, such as those derived from the oral cavity. We have therefore proposed that the aggregate microbial content of the CF lung might usefully be considered as a distinct pathogenic entity, whose impact on the host may be greater than the combined impacts of its individual component species alone.[2] In light of this concept, constructing a detailed and comprehensive model of the polymicrobial system in the CF lung is essential if the drive to understand and treat this disease is to be fully effective.

Insights from Studies of the Intestinal Microbiota

Infections in the CF airways were initially selected as a model system for applying molecular techniques to complex microbial communities based on a number of factors. These included the clinical importance of the condition and the assumption that the bacterial assemblages involved would be comparatively simple (as suggested by conventional culture-based analysis). However, as the true complexity of the CF airway microbiota started to emerge, parallels with highly complex microbial systems found in other areas of the body have become increasingly apparent.

The gut microbiota represents a significant difference in scale and complexity compared with that in the CF lung; 16S ribosomal RNA gene analysis has led to estimations that the gastrointestinal (GI) microbiota consists of hundreds of genera and thousands of bacterial species.[46-48] This complexity confounds the use of conventional culture-based analysis, and makes this context an ideal candidate for culture-independent analysis of the community.[48] Over the past decade these techniques have given a substantially broader and more accurate view of the gut microbiota.[49,50] The relatively high costs of large scale 16S rRNA dideoxynucleotide sequencing has limited its use in such systems and, as is the case with the CF airways, studies have utilised community profiling approaches such as T-RFLP[51,52] and denaturing gradient gel electrophoresis (DGGE)[53-55] to ascertain overall community structure and track its dynamics. More recently, the wider applicability of pyrosequencing, combined with the highly abundant data that it is able to provide, has led to an expansion of the use of this approach to the analysis of microbes that colonise the gut.[56-59]

Comparisons of the microbial systems in the gut and the CF lung may help to distinguish factors that underpin the typically mutually beneficial relationships usually found in the GI tract from the injurious relationships in the CF lung. As might be expected when comparing pathogenic and non-pathogenic systems, there are a number of clear differences. In CF, the presence of high densities of bacteria in an area of the body normally free from large-scale microbial colonisation is associated with consequences that can be assumed to be wholly negative. By contrast, the presence of a complex microbiota in the gut has a number of important functions ranging from supply of nutrients to the host, immune system development, angiogenesis and fat storage.[60-67] Rather than representing a pathogenic system, the relationship between the gut microbiota and the host is assumed to be typically symbiotic.

However, the relationship between the host and the microbial community in the CF lung, and that between the host and the normal gut microbiota, may represent opposite ends of a continuum of pathogenicity (**Fig. 1**). Further, this is a spectrum onto which all microbiota at all colonised sites of the body fall. In this scheme, the normally symbiotic relationship between the host and the gut microbiota can occasionally become unbalanced (a phenomenon referred to as dysbiosis), as with the introduction of new pathogens or antibiotics,[68] in some cases resulting in precipitation of disease. Whilst CF airway disease is characterised by acute periods of pulmonary exacerbation in which the relationship between the host and infecting microbial community is considered highly pathogenic, in much of the intervening periods the relationship is of relatively low pathogenicity, referred to as periods of stability, with exacerbations perhaps precipitated by new viral infections or other perturbing factors.[69]

These commonalities between the gut and the CF lung are reflected in a number of significant similarities in the behavior of their microbial systems. One example is the social interaction between microbial community members, exemplified among colonising bacteria by the expression of quorum sensing mediated behaviors such as biofilm formation, which is important for bacterial colonisation in both contexts.[70,71] Other quorum-sensing-mediated behaviors that are also key to infection, such as the expression of virulence factors, have also been shown to be exhibited

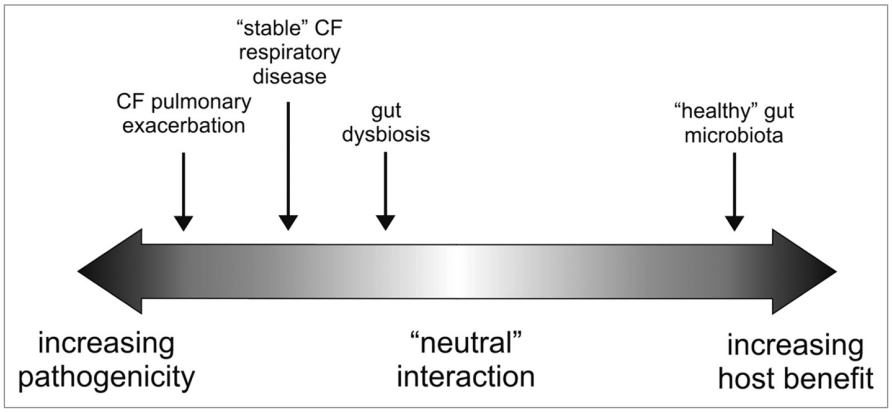

Figure 1. The relative positions of the interactions between the host and the microbial community in stable and exacerbating CF pulmonary disease, and in the healthy and dysbiotic gut, on a conceptual continuum of pathogenicity.

by bacteria in both chronic respiratory infections,[72,73] and the gut.[74,75] Whilst the impact of such coordinated behavior on both virulence and drug impact is well recognised in the CF lung, it has been the focus of less intense study in the gut to date. However, in common with the CF lung, the virulence of clinically important pathogens in the gut has been shown to be modulated by other members of the bacterial community. For example, the synthesis of Shiga toxin by enterohaemorrhagic *Escherichia coli* O157:H7 has been shown to be inhibited by factors secreted by other members of the intestinal microbiota.[76]

The Developing Microbiota

Once established, the microbiota associated with a particular region of the body, such as the gut, is relatively stable in terms of its composition within individuals compared to the levels of variation in microbial constituency among different niches or among the same niches in different individuals.[77] Further, data derived from the study of murine models and humans suggest that, following challenge such as antibiotic treatment, these microbiota will largely revert to their pre-perturbation state,[68,78] provided that perturbation is not too severe.[79,80] Data derived from analysis of the CF lung suggest a comparable situation (data not shown). By comparison, differences that are seen when comparing the same microbiota from body sites in different individuals are likely to reflect a range of factors, including host genetics,[65] niche physiology,[81] as well as the range of environmental microbes that an individual is exposed to.[82,83] Determining the reasons for such differences will require further in depth study; however, some clues may help to elucidate the manner in which these microbiota develop.

The temporal progression of the gut microbiota in neonates has been investigated through metagenomic analysis, with evidence emerging of an ordered progression toward the microbial communities found in the guts of adults.[84,85] Whilst microbial communities varied widely from infant to infant in their composition, the distinct features of the microbial community of each infant were recognizable for intervals of weeks to months. Whilst still distinct, a convergence toward a profile characteristic of the adult gastrointestinal tract was observed by the end of the first year of life.[85] Further analysis of these data by Trosvik et al.[86] suggested the convergence observed is driven, in the main, by simple, phylum-level interactions between colonising species. Further, interactions between the gut microbiota and the host during this process of development may be key to the emergence of the adult intestinal immune system,[87,88] also reviewed by Guarner et al.[89]

While such detailed analysis of the temporal progression of airway colonisation in CF is yet to be performed, recent work has exposed the complexity of microbial communities in the airways of young children with CF, and there is evidence of species succession in this context. Culture-based studies indicate that *Staphylococcus aureus* and non-encapsulated *H. influenzae* are isolated early in life, whereas most CF patients become infected with *P. aeruginosa* over time.[90] In a previous article, we described the use of culture-independent, T-RFLP analysis of bronchoalveolar lavage fluid (BALF) samples from sputum-producing paediatric patients. The data derived showed that there were no significant differences in diversity between the bacterial communities detected among samples from young children with CF and those from CF adults.[91] Further, Harris et al.[92] applied 16S ribsomal RNA gene clone sequence analysis to the characterisation of bacteria in BALF from non-sputum and sputum producing children at single time points. This process identified a total of 65 different bacterial species (including the traditionally recognised CF pathogens) in BALF sampled from 28 children with a mean age of 8 years. These data suggest that, even in the paediatric CF airways, the bacterial diversity is significantly higher than previously recognised. As such, application of further metagenomic analysis will greatly enhance our understanding of the progression of microbial colonisation and infection of the CF airways, the processes that drive this progression, and why some species are able to persist in this niche whilst others are not.

The Influence of Microbiota on Known Pathogens

"Colonisation resistance" is a term used to describe the phenomenon whereby pathogenic species are excluded from a region of the body, such as the gut, by the presence of a microbiota.[93] This exclusion may be due to a range of factors, including changes in oxidation-reduction potential and pH,[94] elaboration of inhibitory substances (e.g., bacteriocins, fatty acids, hydrogen sulfide),[94,95] competition for nutrients,[95] and competition for adhesion sites.[96] Specific examples include the competition for proline with indigenous *Escherichia coli* that has been shown to limit growth of *E. coli* O157:H7 in the intestines of mice,[97] and the age-associated changes in gut microbiota that have been correlated with increased risk of *C. difficile* infection.[98] Interestingly,

the interaction between the host and the gut microbiota can be exploited by certain enteropathogenic bacteria in order to overcome colonisation resistance. The presence of species such as *Citrobacter rodentium* and *Salmonella enterica* in the gut triggers a inflammatory host response. The resulting production of antimicrobial peptides by the host affects the composition of the gut microbial community and suppresses its growth, allowing the pathogenic species to become established.[99-101]

Disruption of gut microbiota through the use of antibiotics can also change its composition, potentially facilitating pathogenic behavior by some bacterial species and infection with new pathogens,[78] and also reviewed by Walk et al.[102] The concepts of clinically relevant interactions between microbial community members, and further complication by antibiotics, have important implications for the CF lung where, once established, clinically significant pathogens are rarely eradicated despite frequent antibiotic treatment. Whilst the CF lung has been the focus of less work in this area, there are some examples of interspecies interactions that influence colonisation by clinically significant pathogens. For instance, as patients acquire *P. aeruginosa, S. aureus* tends to be detected less frequently,[103,104] although both species are commonly co-isolated.[105] A mechanism for this relationship has been suggested involving the production by *P. aeruginosa* of anti-staphylococcal substances, including 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), when co-infecting CF airways with *S. aureus*.[104] However, this compound also protects *S. aureus* during co-culture from commonly used aminoglycoside antibiotics.[105] Furthermore, it has been shown that prolonged growth of *S. aureus* with *P. aeruginosa* selects for typical *S. aureus* small colony variants, which have stable aminoglycoside resistance,[106] and are persistent in chronic infections.[107]

Importantly, however, the presence of a particular species in the polymicrobial community of the CF airways or the gut is not necessarily sufficient to cause clinically evident disease. For example, pathogenic bacteria are able to exist in a carrier state, where the host does not develop disease but harbours an infective organism that may cause disease in others. This phenomenon is exhibited in the GI tract by both *Neisseria meningitidis* and *Vibrio cholerae*, with great clinical significance.[108,109] Similarly, lung infection with nontuberculous mycobacteria can cause substantial decline in lung function in some, but not all, people with CF.[110] An example of a mechanism for such variation in pathogenicity within individual microbial species is the ability of some pathogenic bacteria to turn on or off virulence genes depending on circumstance, as with the production of exotoxins by *Clostridium difficile*.[111] Moreover, where pathogenicity is conferred by the expression of discrete traits, the potential influence of co-infecting species to affect this expression of pathogenesis is clear. In vitro evidence for the potential for clinically relevant polymicrobial interactions in the CF lung comes from Sibley et al.[45] who demonstrated that pathogenically important behaviors of *P. aeruginosa* can be impacted by the presence of bacterial species that are routinely disregarded by most treating clinicians.

Host-Microbiota Interactions

As discussed above, once established, the gut microbiota is typically characterised by a symbiotic relationship with the host. The impacts of this interaction can be far-reaching and profound. The gut microbiota can impact host metabolism and energy storage, with the breakdown of this symbiotic relationship implicated in the development of obesity and type 2 diabetes,[112] as well as conditions such as irritable bowel syndrome (IBS)[113] and inflammatory bowel disease.[114] Further, as discussed by Lyte,[115] microbes both produce and recognise neuroendocrine hormones, potentially allowing for direct signalling with the host. This capacity may be manifested in the suggested association between changes in the composition of gut microbes and changes in the normal functioning of the nervous system,[116] and further, between gut dysbiosis and changes in host behavior.[113,117]

The relationship between the gut microbiota and the host also appears to be bi-directional. Metaproteomic analysis has shown that a significant proportion of the proteins found in faeces is involved in innate immune defense, indicating an extensive effort by the host to regulate the microbial population.[118] The intestinal microbial balance may also be temporarily changed by an alteration in diet,[119] in turn linked to an impact on memory and anxiety-like behavior.[120]

Evidence of such a complex interrelationship between the gut microbiota and the host has implications for the fundamental ways in which we consider CF lung infections. They present the possibility that a microbial community may impact not only the host tissues with which it is in direct contact, but also physically and functionally distant areas of the body. This raises important considerations both for the way in which exacerbations of chronic infections may be triggered, and for the effects of the antimicrobial therapies commonly used in their treatment.

Disruption of the symbiotic relationship between the gut microbiota and the host has been implicated in serious diseases, such as ulcerative colitis, irritable bowel syndrome and *Clostridium difficile* colitis,[113,121,122] and as reviewed by Peterson et al.[114] If the gut model is translated to the CF respiratory tract, a context where any colonising bacterial community is likely abnormal and highly pathogenic, the question can be asked: can a balance between the host and the infective bacterial community in CF be achieved that results in a reduction in pathogenicity? For example, are there bacterial communities that may be associated with minimal pathogenesis? Are there treatments, such as probiotics, nutrients, or other chemicals, that would favor such "beneficial" communities? This concept may be controversial given the presumed highly deleterious impact of airway colonisation. However, in light of the current lack of microbiological explanation for the transition between periods of increased and decreased symptoms, and the common use but only limited efficacy of antimicrobial strategies designed to significantly disrupt this polymicrobial community, this is a possibility that warrants consideration. Further, there is evidence that, as airway disease progresses, primary colonising

species in the CF lung often behave less and less like traditional pathogens. This is illustrated by *P. aeruginosa,* where strains present in advanced CF infections differ substantially from "wild-type" *P. aeruginosa,* with the counterintuitive loss of virulence factors required for acute infection,[66,123] potentially resulting in a reduction in immune response.[66,124] Therefore, the behavior of the bacteria present in the community may be as important as the identification of the species present, rendering the clinical effects of antibiotics less predictable.

Importantly, the polymicrobial nature of the bacterial community infecting the CF lung or the gut microbiota may influence host immunity in a way that affects pathogenesis. For an example from the oral cavity, *Streptococcus cristatus* has been shown to be capable of dampening the IL-8 response induced by infection with *Fusobacterium nucleatum* in epithelial cell culture. Using oral epithelial cells as a model, Mans et al.[125] demonstrated that the degree of complexity of a mixed microbiota influenced the transcriptional response to infection of the epithelium. Thus, both the behavior of the microbiota and the host response can vary depending upon the complexity of the microbiota. In an example of potential relevance for the CF airway, species such as *Streptococcus salivarius* strain K12 have been shown to antagonise *Pseudomonas aeruginosa*-induced IL-8 secretion from human bronchial epithelial cells. This again suggests a role for commensal species in modulating human epithelial cell immune responses in the nasopharynx and, potentially, other parts of the airway.[126]

Elucidating the Relationships Between the Host and Microbiota

Culture-independent techniques are increasingly being used to explore the bacterial content of locations in or on healthy individuals, such as the skin,[77,127,128] the oral cavity,[77,129,130] and the gut.[77,131,132] These microbial habitats do not exist in isolation, but form a microbial landscape interconnecting various niches in each individual.[133] The physical links between the lower airways and areas such as the skin, oral cavity, and gastrointestinal tract provide a ready source of colonising agents for the CF lungs.[134] However, as discussed here, the interrelationships between the microorganisms occupying these different areas of the body may also be mediated indirectly, through their interactions with the host.

The characterisation of the membership and dynamics of the polymicrobial communities colonising areas such as the gut, oral cavity and skin is still in its relative infancy. Much of the complexity of these systems remains to be characterised, but technological advances, such as the development of next generation sequencing technologies, offer an opportunity to make significant gains in our understanding. These endeavours form part of a wider effort to construct an integrated "whole-body" view of the microbial communities associated with healthy people over time.[77] A natural progression of such an approach is to extend these nascent models to include pathogenic polymicrobial communities that exist within chronic infections, such as the CF lung.

There are, however, important methodological considerations to be made when undertaking such work that, if ignored, have the potential to significantly undermine the information that is derived. For example, in order to delineate the links between the microbes that colonise a niche, such as the gut or the CF lung, and parameters of disease, it is essential that we are able not only to identify the species that are present, but also the dynamics of these microbial populations over clinically appropriate timeframes. Culture-independent analysis in these contexts has typically involved the analysis of DNA extracted directly from a sample as a template for PCR amplification. However, such analysis is unable to distinguish between live and dead bacterial cells. Due to the persistence of DNA within clinical samples, such analyses may therefore be unable to detect rapid decreases in bacterial populations,[135] with bias potentially being introduced due to variations between species in the period of retention of amplifiable DNA.[136] One strategy that can be used to limit analyses to viable cells is the use of propidium monoazide (PMA) treatment of samples prior to nucleic acid extraction.[8] PMA intercalates into extracellular DNA or into DNA in cells whose membrane integrity has been lost. In contrast, PMA is excluded from intact cells. Subsequent exposure of a PMA-treated sample to a bright light source causes covalent crosslinks to form, rendering the extracellular or dead cell DNA unable to act as a PCR template. The application of this process has been shown to effectively limit downstream DNA-based processing to signals from viable bacterial cells alone, with its use demonstrated in the metagenomic profiling of the bacterial communities in the CF lung.[8]

Other confounding factors which should be considered when interpreting PCR-based microbial community analyses are the biases introduced from primer selection, variations in nucleic acid extraction efficiencies between species, differential PCR amplification, the number of PCR cycles used, generation of chimeric sequences and the effect of *rrn* operon copy numbers and heterogeneity on diversity estimates.[137-140] In addition, although pyrosequencing has greatly enhanced our ability to monitor microbial communities, this technology throws up different challenges that must be addressed before analysing the data generated.[141] The use of single reads as primary data, in combination with the intrinsic error rate of pyrosequencing, means that diversity estimates are artificially inflated, sometimes by several orders of magnitude.[142,143] On a positive note however, computational methods to remove these artefacts should greatly reduce this problem in the future.[143]

The consideration of these factors will greatly strengthen the data obtained through the metagenomic profiling of microbiota. The challenge that remains for the study both of chronic infections affecting CF airways and of gut microbiota is to construct theoretical frameworks in which the implications of these data can be understood. To this end, studies that examine the behavior, and not just the species constituencies, of polymicrobial communities found in both healthy and diseased contexts, may help to construct such models. Ultimately, however, combining the molecular approaches outlined above with careful epidemiological analysis in longitudinal, well-designed,

translational studies, will be necessary to generate these models. While in vitro models may afford new insights into the interactions between community members and the host, and molecular studies of patient samples can provide a description of the complexity of these communities, the powerful marriage of clinical and laboratory-based approaches will be required to more directly answer the question of how these communities influence human health and disease.

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