The airway microbiome in cystic fibrosis: challenges for therapy

Although cystic fibrosis pulmonary infection is polymicrobial, routine laboratory methods focus on the detection of a small number of known pathogens. Recently, the use of strict anaerobic culture techniques and molecular technologies have identified other potential pathogens including anaerobic bacteria. Determining the role of all bacteria in a complex bacterial community and how they interact is extremely important; individual bacteria may affect how the community develops, possess virulence factors, produce quorum-sensing signals, stimulate an immune response or transfer antibiotic resistance genes, which could all contribute to disease progression. There are many challenges to managing cystic fibrosis lung infection but as knowledge about the airway microbiome continues to increase, this may lead to advances in the therapeutic management of the disease.

KEYWORDS: anaerobic bacteria ■ antibiotics ■ cystic fibrosis ■ microbiome ■ polymicrobial

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Cystic fibrosis pulmonary infection: a polymicrobial disease

Over the past 50 years, life expectancy for cystic fibrosis (CF) patients has increased dramatically with one of the most important interventions being antibiotic treatment directed against pulmonary infection. Antibiotic regimens have been developed for prophylaxis, eradication of initial infection and control of chronic infection and exacerbations [201]. Until recently, a small number of bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia* complex were implicated as the predominant pathogens in CF pulmonary disease. For example, between the ages of 24 and 31 years approximately 65% of CF patients are chronically infected with *P. aeruginosa* [201]. As a result, routine diagnostic laboratories focused on detection of these well-characterized CF pathogens [1]. However, evidence now indicates that the bacterial community within the CF lung is highly diverse with aerobes, facultative and obligate anaerobes, including oropharyngeal flora, all present [2–14]. It has also been proposed that the combination of species colonizing the lungs differs between individuals [3,11,12]. CF lung infection is not only chronic but it is also polymicrobial. The presence of bacteria, not identified by culture methods, in a complex polymicrobial community may be a plausible explanation as to why some CF patients fail to respond clinically to standard antibiotic therapy whilst others possess lung inflammation in the absence of the known pathogens [15–17]. In addition, it may also explain why some CF patients improve clinically even though they harbor *P. aeruginosa* isolates that are resistant to the antibiotics administered [18].

It is vital to determine what bacteria contribute directly to the deterioration in clinical status of CF patients; individual community members may not be pathogenic but when part of an array of microorganisms they may interact and contribute to the overall burden of pathogenesis in CF. Furthermore, studying the airway microbiome in CF and how it changes over time is important to determine what is usually present in the CF lung and what contributes to maintenance of clinical stability for the CF patient. Better understanding of the microbiome in the CF lung and how it changes may enable treatment to be optimized.

Characterization of the CF 'cultured' microbiome

Routine culture focuses on detection of the known CF-associated pathogens. However, by using quantitative aerobic and anaerobic culture, studies have attempted to detect the entire culture microbiome [5,8]. The accurate identification of all CF pathogens is important to guide treatment of the infection and understand the occurrence and distribution of the bacteria. There are a number of methods available that can identify bacteria and these can be broadly categorized into two groups:

- Phenotypic identification
- Molecular identification

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Phenotypic identification

Phenotypic identification is dependent on isolation of bacteria on media using the optimum growth conditions to obtain a pure culture. Following growth, colony morphology can be observed, Gram staining performed and various biochemical and physiological properties investigated. Commercial kits and automated technologies have been developed for standardization whilst increasing the ease and reducing the time that is required to carry out these identification tests. Furthermore, pure cultures are essential for antibiotic susceptibility testing, to guide selection of the most appropriate treatment for the patient. Clinical microbiology laboratories that employ phenotypic identification of bacteria encounter several problems when a disease is polymicrobial including:

- Time required to culture and identify all the bacteria;
- Media currently in use may not support growth of all potential pathogens with media continually needing to be developed;
- Organisms capable of rapid growth *in vitro* such as *P. aeruginosa* can mask growth of, or out-compete other less abundant bacteria on the same plate [19]. Certain bacteria are also difficult to identify with newly emerging bacteria absent in databases associated with commercial identification kits. Furthermore, routine culture only enables a semi-quantitative estimation of bacterial load [12].

As an alternative to conventional phenotypic identification, matrix-assisted laser desorption ionization time-of-flight mass spectrometry has been developed to identify bacteria based on production of a unique spectral fingerprint. This spectroscopic technique has been used to identify bacteria cultured from CF respiratory samples including the nonfermenting Gram-negative bacilli such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cenocepacia* [20–22] and to help identify *Prevotella* spp. [23]. Although, matrix-assisted laser desorption ionization time-of-flight mass spectrometry has been reported as being a rapid and reproducible method, novel bacteria will possess a spectrum that will not match those deposited in the database [21,22]. Nevertheless, it has been suggested that expanding the reference spectra should be straightforward [21].

Molecular identification

Molecular methods have been used to identify bacteria grown as pure cultures and also to directly detect bacteria in clinical samples, such as sputum (Table 1). This use of culture-independent approaches in which DNA is extracted directly from patient samples, may result in detection of unculturable bacteria.

Although the success of molecular methods for bacterial detection has been well documented, traditional phenotypic methods using cultured bacteria remain fundamentally important, for example antimicrobial susceptibility testing to determine appropriate treatment of an infection. Therefore, it is likely that a combination of traditional culture and molecular approaches will be more frequently used in the future to detect and identify all CF pathogens.

Characterization of the CF microbiome

Various culture-independent molecular approaches have been developed to enable characterization of bacterial diversity and identification based on DNA extracted directly from the clinical sample. Both cultivation and molecular characterization techniques and analysis can be labor intensive and time consuming. Before the latter could be used in routine clinical practice, laboratories would need to acquire the necessary equipment and expertise.

Molecular characterization techniques used in CF studies

A single study has reported on the use of temporal temperature gradient gel electrophoresis [14]. Temporal temperature gradient gel electrophoresis is a technique based on electrophoretic separation of hypervariable regions, such as V3 or V6 variable regions of the 16S rRNA genes [14]. Following size separation of the DNA mixture, the bacterial species are identified by sequencing [14]. This method is not ideal for routine use as it is expensive, labor intensive and slow. Nevertheless, is more sensitive than conventional microbiological culture [14].

A more popular approach has involved terminal restriction fragment length polymorphism (T-RFLP) and this has provided a useful tool to explore the bacterial diversity in CF samples [3,4,11–13]. This PCR-based fingerprinting technique involves amplification of the 16S rRNA gene followed by digestion of the PCR product with specific restriction endonucleases [11]. A T-RFLP profile is generated after the gene fragments are separated by gel electrophoresis [11]. The length of the terminal restriction fragment can then be assigned to a bacterial species [11]. An advantage of T-RFLP is that the mixed bacterial

Table 1. Summary of molecular methods that have been used to identify bacteria.

Technique	Description	Advantages	Disadvantages	Ref.
16S rRNA PCR and sequencing	Identify bacteria based on comparison to nucleotide sequences deposited in database libraries	More sensitive than biochemical testing, efficient for unusual bacteria	Costly, requires a pure sample	[5]
Gene specific PCR and sequencing	Usually used in conjunction with 16S rRNA sequencing, definitive identification may require another housekeeping gene to be sequenced	Enables species differentiation within the same genus that possess the same/similar 16S rRNA sequences	Costly, requires a pure sample	[89]
Species/genus specific PCR assays	Identify bacteria based on species/genus specific primer-pairs	Culture-independent, quick, useful for clinical microbiological labs	Requires prediction of what bacteria are present	[90]
Multiplex PCR assays	Multiple primer-pairs are used to amplify more than one locus simultaneously in the one reaction	Culture independent, saves time and effort, useful for polymicrobial diseases, useful for clinical microbiological labs	Requires prediction of what bacteria are present, assays can be difficult to optimize	[91]
Real-time PCR	Amplification and quantification of target sequence at the same time	Culture independent, calculate the relative abundance of bacteria present in a sample, useful for clinical microbiological laboratories	Requires prediction of what bacteria are present	[92]
16S rRNA PCR and CE-SSCP	Amplification of 16S rRNA gene regions followed by capillary electrophoresis-single-strand conformation polymorphism	Simple, rapid	Requires a pure sample, primers may not identify target bacteria to species level	[93]
FISH	Specific probes used to detect DNA sequences	Culture-independent	Not as appropriate for high-throughput analysis	[94]

CE-SSCP: Capillary electrophoresis-single-strand conformation polymorphism; FISH: Florescent in situ *hybridization.*

community can be profiled rapidly (12 h) in a single electrophoretic lane [11,12]. However, some T-RFLP fragments can correspond to more than one bacterial species/genus resulting in ambiguity regarding identity [11].

A similar technique is length heterogeneity PCR analysis, which separates amplicons relating to different bacterial isolates based on length [12]. However, T-RFLP profiling has been shown to be superior to length heterogeneity PCR as a technique for analyzing CF samples [12].

Rather than generating electrophoretic patterns for identification, analysis of clone libraries can be performed. The rRNA gene sequences are amplified directly from clinical samples. This amplified DNA can then be cloned and Sanger sequencing can be performed to investigate the diversity of organisms present [10]. Although, clonal Sanger sequencing can identify specific microbes (including newly emerging bacteria) in polymicrobial samples, disadvantages include that it is difficult to analyze, expensive and labor intensive as a large number of clones have to be examined [11]. For example, in one study 760 clones were sequenced from 25 sputum samples (either 24 or 40 clones were sequenced from each sample) to examine bacterial diversity [9]. This study noted that as the number of sequenced clones increased, the mean number of species detected was statistically greater. Although this demonstrates the importance of sequencing a large number of clones, it will significantly increase the cost.

Ribosomal intergenic spacer analysis combined with HPLC has also been used to analyze CF sputum and oropharyngeal samples from children [24]. Ribosomal intergenic spacer analysis involves amplification of the internal transcribed spacer between *rrs* (16S rRNA) and *rrl* (23S rRNA) genes (different microorganisms will have varying internal transcribed spacer lengths) [24]. Ribosomal intergenic spacer analysis PCR products can then be purified and separated by HPLC and the profile analyzed [24]. However, bacteria present in low abundance may not be detected.

Studies that have used these molecular methods to characterize the bacterial community within the CF lung are summarized in Table 2.

Recent advances in culture independent molecular approaches: high-throughput sequencing & 16S rRNA PhyloChip

Compared with the Sanger sequencing method, high-throughput technologies such as the 454 sequencing platform and the Illumina system, increase the depth of sequencing by a factor

of 100–1000 [25,26]. High-throughput sequencing is also advantageous as it eliminates the need for a cloning step and enables direct sequencing following a single DNA amplification step [27]. Recently, high-throughput pyrosequencing has been used in two CF-based studies to investigate the microbiome in a small number of clinical samples [2,28]. Armougom and colleagues used 454 pyrosequencing (Genome Sequencer 20 System, Roche Applied Science), clonal Sanger sequencing and culture to analyze the bacterial community within a CF sputum sample from a 13-month-old child. The *Moraxella* genus was the most commonly detected followed by *Streptococcus*, *Haemophilus*, *Dolosigranulum*, *Granulicatella* and *Pasteurella*. It was reported that pyrosequencing enabled a greater microbial diversity to be detected, but species level identification was hindered by the short reads produced (100 bp) [28]. However, 454 sequencing has been developed further to enhance analysis; the Genome Sequencer FLX (GS FLX) System coupled with the GS FLX Titanium series reagents (Roche Applied Science) produces reads that average 400 bp [202] and more recently the GS FLX+ System (Roche Applied Science) was launched, which enables even longer sequencing reads (up to 1000 bp) [203]. Guss and coworkers performed clonal Sanger sequencing, 454 pyrosequencing of barcoded amplicons (GS FLX System, Roche Applied Science) and traditional bacterial isolation and culture. More than 60 bacterial genera were detected in this study with 19 of these shared between three sputum samples (from three different CF patients) analyzed by pyrosequencing. Only eight of these genera were also detected via clone library analysis and culturing [2]. In comparison to 454 pyrosequencing, the Illumnia system (Solexa) generates more than ten times the number of reads per run enabling bacteria present in lower abundance to be detected [29]. Although the Illumnia system enables greater sequence data, the reads are shorter (approximately 100 bp) [25,29]. However, it is desirable to detect bacteria in low abundance as their pathogenic significance is unknown.

The 16S rRNA PhyloChip™ has also been used more recently to investigate the CF lung

Table 2. Studies that have contributed to knowledge of the microbiome within the cystic fibrosis lung.

Study	Culture-independent molecular method(s)	Key finding	Examples of potential pathogens	Ref.
Kolak *et al.* (2003)	TTGE, shot-gun cloning and pyrosequencing	The bacteria present in sputum samples from 13 adult CF patients were highly diverse	*Streptococcus* spp., *Bifidobacterium* spp., *Actinomyces* spp.	[14]
Rogers *et al.* (2003)	LH-PCR, T-RFLP and clonal Sanger sequencing	T-RFLP revealed distinct bacterial communities in the lungs of 14 adult CF patients (ten sputum and four bronchoscopy samples provided)	*Bacteroides fragilis*, *Prevotella oralis*, *Sarcina ventriculi*	[12]
Rogers *et al.* (2004)	T-RFLP and clonal Sanger sequencing	Identification of 14 bacterial species that had not been previously associated with CF pulmonary infections	*Prevotella melaninogenica, Ralstonia taiwanensis, Bacteroides gracilis*	[11]
Harris *et al.* (2007)	Clonal Sanger sequencing	Bacteria not detected by routine culture were reported in 11 of 28 (39%) children with CF and five of these 11 patients were noted as pathogen negative by culture	*Prevotella denticola, Streptococcus intermedius, Rickettsiales* spp.	[10]
Bittar *et al.* (2008)	Clonal Sanger sequencing	From 25 CF sputum samples 16 of 53 (30%) bacterial species identified were anaerobes	*Porphymonas* spp., *Prevotella melaninogenica, Streptococcus constellatus*	[9]
Sibley *et al.* (2008)	T-RFLP	The *Streptococcus milleri* group are a cause of acute pulmonary exacerbations	*Streptococcus constellatus, Streptococcus intermedius*	[13]
Nazerat *et al.* (2009)	RISA-HPLC	Bacterial species detected in pediatric samples from 16 patients where less diverse compared to outcome of previous studies	*Inquilinus limosus, Pandoraea* spp.	[24]
Stressman *et al.* (2011)	T-RFLP and clonal Sanger sequencing	Eleven of 30 (37%) of the species identified within eight genera were present in adult CF samples from patients in the UK and the US. Two genera were anaerobic	*Veillonella parvula, Prevotella melaninogenica, Parvimonas micra*	[3]
Tunney *et al.* (2011)	T-RFLP	Strict anaerobes were detected in sputum samples before (22 of 26) and after (20 of 25) antibiotic treatment and when patients were stable (nine of 13)	*Veillonella* spp., *Porphymonas* spp., *Prevotella* spp.	[4]

CF: Cystic fibrosis; HPLC: High performance liquid chromatography; LH-PCR: Length heterogeneity PCR; RISA: Ribosomal intergenic spacer analysis; T-RFLP: Terminal restriction fragment length polymorphism; TTGE: Temporal temperature gradient gel electrophoresis.

microbiome. It is a high-density phylogenetic microarray and can also detect bacteria that are present in low abundance (comprise at least 0.01% of the population) [30]. The 16S rRNA PhyloChip has been used by Cox and colleagues to investigate the bacterial diversity in samples provided by 51 clinically stable CF patients. They reported finding 1837 bacterial taxa belonging to 43 phyla with the dominant phyla including Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria [6]. A further study, analyzing 45 CF oropharyngeal swab samples from 45 children, also identified a large number of bacterial taxa (2051 bacterial taxa belonging to 43 phyla) with the three most common phyla being Bacteroidetes, Firmicutes and Proteobacteria [7].

Fingerprinting techniques such as T-RFLP are limited as they only enable detection of the more abundant microorganisms within a community and clonal Sanger sequencing is restricted by its low throughput [31]. Therefore, in the future, it is likely that high-throughput sequencing technologies and phylogenetic microarray analysis will be the methodologies of choice to study the highly diverse CF airway microbiome. These methods enable a much greater depth of community assessment and as a result, offer a more accurate way to determine microbial community composition. This depth of coverage is advantageous as there is no abundance threshold below which microorganisms can be discounted as insignificant [31].

The CF lung bacterial community

A pathogenic community

Rather than individual bacteria being solely responsible for the pathogenesis of CF pulmonary infection, disease progression may also be affected by the overall composition and activity of the airway microbiome [32]. By characterization of the airway microbiome over time, community composition and bacterial abundance can be evaluated at different stages of the disease. The changes in the community structure can be compared with the clinical status of the patient enabling a bacterial community to be assessed for its potential role in infection and inflammation as well as investigating the microbiome during periods of stability. Furthermore, this will provide insight into how the CF airway bacterial community develops. Bacterial communities in other body sites such as the oral cavity, the vagina and the gastrointestinal tract develop via multiple succession and assembly events to form a stable community with changes in the community composition and abundance being related to disease [25]. For example, one study attempted to compare the structure of the normal bacterial community of the vagina and bacterial vaginosis [33]. They reported that the community structure remained stable over time except when patients were positive for bacterial vaginosis.

Loss of bacterial diversity

A number of studies have associated a loss of bacterial diversity with disease. For example, increased incidence of inflammatory bowel disease is associated with a loss of bacterial diversity in the GI tract [34]. Another study investigating the lung microbiome in healthy smokers (no sign of disease or reduced lung function) and those with chronic obstructive pulmonary disease reported that those with reduced lung function possessed a pulmonary microbiome with reduced diversity and that *P. aeruginosa* dominated the community [35]. Similar findings have been reported in CF studies which investigated how bacterial communities differ between age groups [6,7]. Cox *et al.* reported that bacterial diversity was reduced in adult patients when compared with younger patients [6]. Although Klepac-Ceraj and colleagues only obtained samples from children (2–16 years), they also found that there was a loss of bacterial diversity with increased age [7]. This loss of bacterial diversity was associated with reduced lung function and disease progression. Moreover, Cox and coworkers showed that *P. aeruginosa* was one of the most abundant organisms in older CF patients with less bacterial diversity [6]. More studies are required to determine how this loss in diversity specifically correlates to disease progression in both CF and other respiratory diseases characterized by chronic pulmonary infection.

Potentially pathogenic anaerobic bacteria

Although considering the complete airway microbiome as pathogenic is important, it also remains vital to detect potentially pathogenic bacteria that can cause disease on their own, trigger CF exacerbations and have a significant influence on other members of the community in the CF lungs. These key microorganisms can then be tracked over time to determine their influence on community development and/or disease progression.

Traditionally, CF lung infection has been associated with pathogens including *P. aeruginosa*, *S. aureus* and *Haemophilus influenzae*.

However, as bacterial identification methods progressed other aerobic bacterial species emerged as clinically significant including opportunistic pathogens, such as *S. maltophilia*, *A. xylosoxidans*, *B. cepacia* complex, *Ralstonia pickettii* and *Pandoraea* spp., which are rarely associated with other diseases [36]. Furthermore, many other bacteria have been detected but their role is still not clearly understood. For example, in 2002, Coenye and colleagues described a highly mucoid pathogen in CF, *Inquilinus limosus* [37]. Although, patients can become chronically infected with *I. limosus*, its pathogenic role is still to be elucidated.

Potentially pathogenic obligate and facultative anaerobic bacteria have also been detected in CF clinical samples, including sputum and bronchoalveolar lavage fluid using both strict anaerobic culture techniques and molecular methods [2,4,5,9–11]. Previously, their role in disease progression was often overlooked as it was believed they were nonpathogenic as they belonged to the normal commensal flora. However, in 2002, Worlitzsch and colleagues discovered the existence of steep oxygen gradients within the CF lung due to an increase in airway epithelial oxygen consumption coupled with poor oxygen diffusion within the mucus plaques. Moreover, it was found that *P. aeruginosa* could adapt and proliferate within the hypoxic regions and as pulmonary infection persisted, the mucus masses would eventually become fully hypoxic (anaerobic) environments [38]. As anaerobic conditions exist within the CF lung studies sought to detect anaerobic bacteria in CF respiratory samples.

Obligate anaerobic bacteria

A number of studies have specifically cultured obligate anaerobic bacteria from clinical samples and identified them using various methods (Table 3). The majority of these studies detected similar dominant obligate anaerobes and found that their presence in the CF lung persisted over time. For example, in 2011, Tunney and coworkers reported that anaerobic bacteria were cultured from CF sputum samples regardless of the patient status, for example stable, exacerbation or following antibiotic treatment [4]. Furthermore, regardless of the prevailing genera detected, studies agree that obligate anaerobic bacteria are present at all ages [5,8]. Therefore, these bacteria could belong to a core CF airway community and their specific role in CF pulmonary infection and inflammation requires further investigation.

Facultative anaerobic bacteria

The *Streptococcus milleri* group (SMG) was also commonly overlooked as a constituent of the normal commensal flora. SMG is comprised of *Streptococcus constellatus*, *Streptococcus intermedius* and *Streptococcus anginosus* and all are facultative anaerobes [39]. When the epidemiology of SMG isolates was investigated, it was found that patients may become chronically colonized with SMG strains and that the strains are patient specific [40]. Similar to the obligate anaerobes, the SMG have been identified in both children and adult patients [3,5,10]. Thus, they may also form part of a core bacterial community. Recently, SMG has emerged as clinically relevant in chronic CF respiratory infections [13,41]. In a study by Sibley and coworkers, the SMG were detected in 43 of 106 (40.6%) adult patients with clinical deterioration noted when SMG was present in numbers greater than 10^7 cfu/ml [42]. An earlier study by this group demonstrated that antibiotic treatment targeted against SMG not only reduced bacterial load but also improved clinical status [13].

The oral cavity as a source of infection

Most of the obligate anaerobes and the SMG isolated from CF clinical samples have been associated with microbiota of the oropharynx [43,44]. Hence, the oropharynx could act as a reservoir supplying oral bacteria to the lung, where they persist after failure to be removed by the mucociliary clearance defense mechanism. This theory was given more credence after a study focusing on ventilator-associated pneumonia suggested that a potential cause of this respiratory infection was aspiration of bacteria colonizing the dorsal surface of the tongue [45]. Furthermore, the teeth have also been implicated as potential reservoirs of respiratory pathogens [46,47].

Metabolically active cells

Two molecular methods have enabled analysis of the bacterial community to be limited to those that are metabolically active; Reverse transcription T-RFLP, which utilizes RNA [48] and propidium monoazide treated samples coupled with T-RFLP [49]. The former method showed that most of the bacterial species, including the anaerobic bacteria that had been previously detected in the CF lung by DNA-based approaches were indeed metabolically active [48]. The latter method involves treating samples with propidium monoazide prior to DNA extraction [49]. The advantage of this is that all viable

Table 3. Studies that have used strict anaerobic culture techniques to enable detection of obligate anaerobic bacteria from cystic fibrosis respiratory samples.

Strict anaerobic culture	Identification method	Patient (n)	Major finding	Obligate anaerobic genera	Ref.
Tunney *et al.* (2008)	16S rRNA sequencing and RapID Ana II identification system	Adult (50) Child (10)	High numbers (10^4–10^7 cfu/g) of anaerobic bacteria were detected in 42 of 66 (64%) sputum samples from stable patients Similar anaerobic bacteria detected from eight of ten BALF samples but in lower numbers. No bacteria were detected in two of ten samples	*Prevotella, Actinomyces, Veillonella, Propionibacterium*	[5]
Worlitzsch *et al.* (2009)	RapID Ana II identification system	Adult (36), child (9)	In 41 of 45 (91%) patients at least one obligate anaerobe was detected in sputum samples Obligate anaerobes were identified in both children and adult samples (82 and 94%, respectively)	*Staphylococcus, Peptostreptococcus, Actinomyces, Veillonella*	[8]
Field *et al.* (2010)	16S rRNA sequencing	Adult (16)	*Prevotella* spp. detected in sputum from 13 of 16 (81%) patients. Four different *Prevotella* spp. were isolated from two of 13 (15%) patients	*Prevotella, Peptostreptococcus, Veillonella, Propionibacterium*	[19]
Tunney *et al.* (2011)	16S rRNA sequencing	Adult (23)	Anaerobic bacteria were detected in all sputum samples (n = 26) and 23 of 26 (88%) samples before and after antibiotic treatment, respectively	*Prevotella, Veillonella*	[4]

BALF: Bronchoalveolar lavage fluid; cfu: Colony forming units.

cells will be included in analysis including those that have a very low metabolic rate that may be missed by the reverse transcription T-RFLP method [48,49].

The role of obligate anaerobes & SMG in community development & disease progression

Oral contaminants

There have been concerns that obligate anaerobic bacteria and SMG cultured from CF samples including expectorated sputum may be oral contaminants and thus, are not part of the CF airway microbiome. However, using T-RFLP profiling to compare anaerobic bacteria present in both sputum and mouthwash samples from the same adult CF patients, Rogers and colleagues discovered that the terminal restriction fragment bands detected in matched profiles for each patient were different. Furthermore, in 13 of 19 patients the highest relative volume band in the mouthwash sample did not coincide with the corresponding sputum profile. These outcomes imply that as sputum is passed from the lungs to the oral cavity it is not contaminated to a significant degree by anaerobic bacteria normally present in the oropharynx [50]. Moreover, the culture-based study by Tunney and colleagues reported that although anaerobes could be detected in induced sputum from 16 of 20 healthy volunteers, they were present in much lower numbers than in expectorated sputum from CF patients [5]. It has also been reported that when SMG is the dominant pathogen in bronchopulmonary exacerbations, unusual malodorous sputum has been expectorated suggesting that SMG are not oral contaminants [41].

Potential pathogens

In contrast to the known CF-associated pathogens, knowledge about the potential pathogenicity and virulence of the obligate and facultative anaerobes (e.g., *Prevotella* spp., SMG) in CF is limited. However, they possess numerous virulence factors that could contribute to CF lung disease progression. It is known that the SMG can damage host tissue due to the production of extracellular hydrolytic enzymes including hyaluronidase and DNase, which may lead to spread of the infection [51]. Studies have also determined that *Prevotella* spp. could potentially contribute to progression of a disease in a number of ways (Table 4). Furthermore, it has been suggested that enzymes produced by obligate anaerobic bacteria and SMG could work together promoting the spread of infection and tissue damage [52].

Although anaerobic bacteria have been detected in pediatric and adult respiratory samples during periods of stability and exacerbations, the stage at which they potentially contribute to progression of the disease is unknown. In the pathogenesis of other pulmonary diseases (e.g., empyema, lung abscess and pleural effusion) in which anaerobic bacteria are implicated, it has been suggested that they could cause infection by overwhelming the host defense mechanism

in the already diseased lung [53]. Alternatively, anaerobic bacteria may enter the lung first and create a favorable environment that enables the known CF-associated pathogens to colonize and cause infection. This has been suggested by several studies. For example, similar anaerobic species were isolated, from five of eight bronchoalveolar lavage fluid samples from pediatric CF patients in the absence of *P. aeruginosa* [5]. This agrees with the findings of another study, which found that anaerobic bacteria were rarely detected in bronchoalveolar lavage fluid pediatric samples along with known pathogens [10]. Therefore, anaerobes may be vital for development of a bacterial community that enables chronic infection to progress with known pathogens. This progression may be influenced by complex interactions between the microorganisms present, as well as between microbes and the host.

Bacteria within the CF lung may interact synergistically. For example, Guss and coworkers proposed that *Veillonella* spp. could utilize lactate (for anaerobic growth) produced as a fermentation end-product by bacteria, such as *Staphlycoccus* and *Streptococcus* spp. [2]. Various *in vivo* studies have also demonstrated that bacteria in a mixed infection can interact in a synergistic way supporting the hypothesis that anaerobic bacteria may have a significant role in the progression of CF lung disease by affecting the pathogenicity of other bacteria within the community (Table 5).

The ability of bacteria to communicate in a mixed infection may be via the production of signals known as autoinducers, which coordinate gene expression within the microbiome [54]. This cell-to-cell signaling system is known as quorum sensing and allows bacteria to sense when their numbers have reached a critical concentration within the environment [55]. The outcome is stimulation of transcription of quorum sensing controlled genes, including those that code for virulence factors [54,55]. For example, it has been shown that *Prevotella* spp. can produce autoinducer-2, which is able to modify *P. aeruginosa* virulence factor gene expression [19]. Furthermore, Duan and coworkers also detected a high concentration of autoinducer-2 directly in CF sputum samples. They reported that autoinducer-2 produced by oropharyngeal flora bacteria was one signal that was capable of modulating *P. aeruginosa* gene expression [54]. Quorum sensing is also thought to contribute to persistence of the bacterial pathogens in the lower airways because it influences the formation of a biofilm [56].

Polymicrobial communities also offer an excellent opportunity for horizontal gene transfer especially in the presence of biofilms [57]. It is widely known that *P. aeruginosa* infection can become increasingly difficult to treat due to the ease with which this bacterium can acquire resistance mechanisms [58]. This may also be a problem with the obligate anaerobes and SMG. Horizontal gene transfer associated with plasmids, transposons, insertion sequences and integrons can occur between different bacterial genera occupying the same environment. For example, it is thought that different bacterial species that coexist in the intestine transfer genes between themselves [59]. Furthermore, gut-associated bacteria could also exchange genes with bacteria that are passing through the intestine including oropharyneal flora bacteria, which are swallowed [60]. These bacteria as well as gut-associated bacteria could then return to the oral cavity via the fecal–oral route of transmission and potentially enter the CF lung (Figure 1). Gut-associated bacteria, such as *Bacteroides fragilis*, have been detected in CF and chronic obstructive pulmonary disease samples [12,61].

In addition to affecting other colonizing microbes, it is likely that these anaerobic bacteria are likely to elicit an immune response and so contribute to deterioration in lung function even if they are not pathogenic. For example, in one study, focusing on acute exacerbation of chronic bronchitis, elevated antibody levels were detected for two obligate anaerobic bacteria, *Fusobacterium nucleatum* and *P. intermedia* suggesting that they have a role in the inflammatory process [62]. Furthermore, in another study, it was reported that the presence of *P. intermedia* increased airways inflammation *in vivo* as it induced the influx of macrophages and neutrophils to the site [23].

Table 4. Examples of virulence factors associated with *Prevotella* spp.

Virulence factors	Ref.
Production of enzymes (e.g., phospholipases)	[95]
Activation of matrix metalloproteinases	[96]
Degradation of complement factor C3	[97]
Inhibition of phagocytosis	[98]
Degradation of immunoglobulins	[99]
Induction of excessive host inflammatory response due to presence of lipopolysaccarides	[100]

Antibiotic treatment

As CF life expectancy continues to increase, patients may be exposed to increasing doses,

Table 5. *In vivo* studies that support the possibility that microbial interactions exist between the known pathogens and anaerobic bacteria, as well as between the anaerobes themselves, to contribute to the pathogenesis of cystic fibrosis lung disease.

Model	A	B	Outcome when A and B inoculated together	Ref.
Mouse model of acute pneumonia	*Streptococcus constellatus*	*Prevotella intermedia*	Risk of mortality increased by a factor of six compared to either microorganism alone. Lung abscesses and emypema only developed when both organisms were present	[52]
Agar bead rat lung infection model	*Pseudomonas aeruginosa*	Viridans group *Streptococcus*	Enhanced lung damage (greater pulmonary edema and/or inflammation) when a *Streptococcus* strain and *P. aeruginosa* were coinoculated	[54]
Drosophilia as a model of polymicrobial infection	*Pseudomonas aeruginosa*	OF isolates (n = 40)	Some OF isolates were virulent (able to kill flies) and when present with *P. aeruginosa* killing was faster, some were avirulent and in the presence of *P. aeruginosa* killing was not enhanced and others were only pathogenic in the presence of *P. aeruginosa* (reduced fly survival compared to when present alone)	[101]

OF: Oropharyngeal flora.

numbers and combinations of antibiotics, which will increase the risk of multidrug-resistant bacterial species emerging. Therefore, there is a desire to ensure that all CF healthcare professionals possess up-to-date knowledge to ensure that antibiotics are prescribed and administered in a manner that minimizes this risk.

Standard treatment

Standard treatment targets the recognized CF pathogens, which have been detected in clinical samples by routine identification methods. These pathogens are targeted because they are known to contribute to the clinical deterioration of the patient if left untreated. Standard antibiotic therapy is used for:

- Prophylaxis of infection. For example, continuous antistaphylococcal treatment (flucloxacillin) may be started in young children until they are 3 years old [201];
- Eradication of initial infection. For example, the early inhaled tobramycin for eradication (ELITE) trial reported that nebulized tobramyin was effective and well tolerated in the treatment of early *P. aeruginosa* infection [63]. Oral ciprofloxacin and inhaled colistin are also treatment options for initial *P. aeruginosa* infection [201]. However, if more aggressive treatment is necessary intravenous antipseudomonal antibiotics, such as tobramycin, ceftazidime, piperacillin/tazobactam or meropenem, may be necessary [201];
- Suppression of chronic infection. For example, a randomized controlled trial conducted between 2000–2002 confirmed the use of long-term oral azithromycin as an option for those chronically infected with *P. aeruginosa* [64]. Other long term therapies against chronic *P. aeruginosa* infection include nebulized tobramycin or colistin [201];
- Treatment of acute infective exacerbations. For example, two or more intravenous antibiotics administered that have different mechanisms of action. The antibiotics selected depend on the organism identified and the severity of the exacerbation [201].

It has been suggested that failure of the CF patient to respond to standard antibiotic therapy against the known pathogens may be explained by the presence of other potentially pathogenic bacteria, including obligate anaerobic bacteria and SMG, which are not susceptible to the antibiotics administered [5,10]. The poor response of obligate anaerobes and SMG to standard CF treatment has been indicated by:

- *In vitro* antimicrobial susceptibility testing;
- The effect of antibiotic treatment on bacterial abundance;
- Acute exacerbations failing to resolve with standard treatment.

In vitro antimicrobial susceptibility testing

In vitro antimicrobial susceptibility testing has demonstrated that the commonly administered antibiotics may have little effect on obligately anaerobic bacteria and the SMG detected in CF sputum (Tables 6 & 7).

Antibiotic resistance may also be greater in CF isolates compared with those detected in other infections. For example, metronidazole resistance has been rarely reported in Gram-negative anaerobic bacteria but Tunney and colleagues found that 46% of *Prevotella* isolates cultured from CF sputum were metronidazole

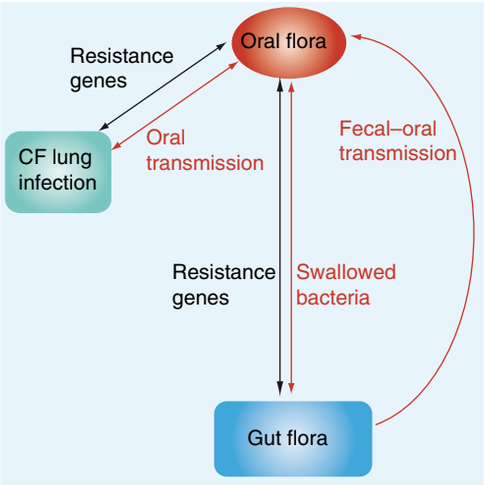

Figure 1. How antibiotic resistance genes could be transferred between bacteria, which contributes to further challenges for therapy. Bacteria in the oral cavity (red) may be swallowed and passed along the GI tract (blue). During their transit they temporarily come into contact with other normal commensal flora of the gut enabling antibiotic resistance genes to be exchanged. Bacteria that are excreted have the opportunity to reach the oral cavity via the fecal–oral route of transmission. Any bacteria here can potentially enter the lungs (green) and contribute to the pulmonary infection after failure to be removed by mucocilliary clearance. Bacteria present in the lungs can also transfer resistance genes especially in the presence of biofilms.
CF: Cystic fibrosis.

resistant [5]. Furthermore, when Grinwis and coworkers compared the antimicrobial susceptibility profiles of SMG isolates cultured from CF sputum and from blood/abscesses associated with invasive infections, resistance to macrolides and clindamycin was higher among the SMG isolated from CF sputum. It was suggested that azithromycin resistance, among SMG isolates, was driven by chronic exposure to the drug; In CF patients who were taking azithromycin long term, 48 of 57 (84.2%) SMG isolates were azithromycin-resistant whereas in those CF patients not receiving chronic azithromycin therapy, only 15 of 61 (24.6%) SMG isolates were azithromycin resistant [65].

The effect of antibiotic treatment on bacterial abundance

Tunney and colleagues investigated the effect of antibiotic treatment, during an exacerbation, on the abundance of the bacteria detected. Quantitative culture results indicated that antibiotic treatment reduced the abundance of aerobes more than anaerobes. Furthermore, a significant reduction in abundance was only detected for *P. aeruginosa*, which may have been a result of patients being administered antipseudomonal antibiotics [4]. This suggests that these antibiotics may have little or no effect on the other bacteria, including the obligate anaerobic pathogens and the SMG, colonizing the lungs.

Acute exacerbations failing to resolve with standard treatment

The SMG have been detected as the numerically dominant microorganism during CF exacerbations and clinical symptoms only resolved when antibiotic treatment was targeted against the group [13]. In one patient, three consecutive acute pulmonary exacerbations were experienced and each time *S. constellatus* was the dominant organism [13]. Complete resolution of exacerbation symptoms was only achieved following intravenous ceftriaxone (no antipseudomonal activity) for 8 days followed by 7 days of ceftriaxone, ceftazidime and tobramycin therapy [13].

Antibiotic resistance

Antibiotic resistance is a major challenge for therapy. Bacteria within the CF airway microbiome may be intrinsically or extrinsically resistant to the standard antibiotics administered to manage CF respiratory infection. Intrinsic resistance is the result of a bacterium being naturally resistant to an antibiotic. For example, obligate anaerobic bacteria are intrinsically resistant to aminoglycosides because uptake of the antibiotic into the cytoplasm requires a functional electron transport system, which anaerobes cannot provide [66]. Thus, tobramycin, which is commonly administered in CF, is ineffective against obligate anaerobes. In contrast, extrinsic resistance

Table 6. Summary of antimicrobial susceptibilities of obligate anaerobic bacteria isolated from cystic fibrosis patients.

Number of isolates	Method	% resistant strains						Ref.
		Ampicillin	*Ceftazidime*†	*Clindamycin*†	*Meropenem*†	*Metronidazole*	*Pip/taz*†	
39	Etest	33	N/A	21	0	47	13	[5]
138	Etest	N/A	51	23	4	49	19	[8]

†*Antibiotics used in the management of cystic fibrosis pulmonary infection* [201].

Table 7. Summary of antimicrobial susceptibilities of the *Streptococcus milleri* group isolated from cystic fibrosis patients.

Number of isolates	Method	% resistant strains						Ref.
		Azithromycin†	*Cefepime*	*Ceftriaxone*	*Clindamycin*†	*Erythromycin*	*Tetracycline*	
118	Disc diffusion	53	1	0	51	52	22	[65]

†*Antibiotics used in the management of cystic fibrosis pulmonary infection* [201].

is acquired by the bacterium and may be associated with horizontal gene transfer. For example, the *cfxA*-type gene encodes for a β-lactamase and is associated with amoxicillin resistance. It has been established that these genes are widely distributed in obligately anaerobic bacteria and closely related sequences of the *cfxA* gene have been identified in different genera including *Bacteroides* and *Prevotella* [67,68]. Furthermore, among the SMG, macrolide resistance has been associated with the *ermB* gene [69]. The *erm* genes encode an erythromycin methyltransferase, which confers resistance to macrolides, lincosamides and streptogrammin B antibiotics [70]. Obligately anaerobic bacteria including *Bacteroides*, *Prevotella* and *Porphymonas* spp., have also been reported as harboring variants of the *erm* gene [71,72].

A further challenge for therapy is biofilm formation within the CF lung, which can contribute significantly to antibiotic resistance for both aerobes and anaerobes [55,73]. It has been reported that the minimal inhibitory concentration and the minimum bactericidal concentration of an antibiotic to bacteria growing in a biofilm is much higher than for bacteria growing planktonic [74,75]. One explanation for this poor antibiotic susceptibility is that bacteria present in biofilms can have a reduced growth rate [76]. The outcome is that antibiotics which are only active against dividing bacterial cells, such as β-lactams, will have poor activity when biofilms are present [77].

Should the anaerobic bacteria detected in the CF airway microbiome be targeted?

It has been demonstrated that, although standard antibiotic regimens fail to specifically target anaerobic bacteria, FEV1 can increase significantly following treatment of an acute exacerbation [4,8]. Therefore, it could be concluded that anaerobic bacteria are not clinically significant and there is no need to modify antibiotic treatment to target them. Although the pathogenicity of anaerobes in CF lung disease, how they specifically interact with known pathogens (e.g., *P. aeruginosa*) and how they affect development of the bacterial community is not clear, anaerobes have been shown to be present in high numbers, to be metabolically active and to produce virulence factors. To obtain the required evidence base for inclusion of antibiotics that specifically target anaerobic pathogens in standard treatment regimens, a randomized control trial targeting these bacteria would need to be conducted. CF patients could be split into two groups: patients in one group would receive antibiotics targeting both the known CF pathogens and anaerobic pathogens; whereas patients in the second group (control) would only receive antibiotics targeting the recognized CF pathogens. Clinical outcomes for CF patients in both groups could then be compared. Furthermore, it would also be necessary to determine when antibiotic treatment should be targeted against anaerobic bacteria: at initial colonization, chronic colonization, during an acute exacerbation or during all of these stages.

Treatment of anaerobic infections

Antibiotics commonly used to treat obligate anaerobic infections include metronidazole, clindamycin and β-lactams [78]. Treatment of SMG infection has included trimethoprim-sulfamethoxazole, clindamycin and ceftriaxone [13]. However, resistance is emerging to some of these antibiotics. Therefore, the antimicrobial susceptibility of anaerobic bacteria can be difficult to predict and as a result *in vitro* susceptibility testing will need to be performed to guide selection of antibiotics to target anaerobes isolated from individual patients.

However, during an acute exacerbation, it may not be appropriate to wait for the antimicrobial susceptibility results for the anaerobic bacteria as this may take several days. As a result empiric therapy to target anaerobic bacteria may be necessary. For example, in managing chronic osteomyelitis, which can also involve polymicrobial infection, empiric antibiotic therapy is often initiated [79]. This can involve broad-spectrum antibiotics, such as meropenem and vancomycin [79]. Once antimicrobial susceptibility testing has

been completed the most appropriate narrow-spectrum antibiotics can be selected [79]. This approach is known as de-escalation therapy and is widely used in the treatment of other conditions, such as noscomial pneumonia [80]. One antibiotic that has demonstrated good activity against obligate anaerobes isolated from CF pulmonary samples is meropenem (TABLE 6) [5,8]. In one study, two CF patients were categorized as *Prevotella*-negative having previously been positive for this genus [19]. It was proposed that one reason for this change was that meropenem was administered to these patients to treat an acute exacerbation. Furthermore, ceftriaxone has been successful in treating exacerbations associated with SMG [13].

As CF is a polymicrobial disease all aerobic and anaerobic pathogens may need to be targeted. In a review of primary and secondary lung abscesses, Patradoon-Ho and Fitzgerald recorded that previously first-line therapy was monotherapy with penicillin due to its ability to target both aerobes and anaerobes. However, due to the widespread production of β-lactamases by anaerobes, some centers have added metronidazole or clindamycin [81]. Further studies would be required to determine the most effective combination therapy in CF patients.

Treatment of the entire microbiome

It has been suggested that disease progression may be affected by the existence of a pathogenic community in the absence of an apparent exacerbation [35]. Therefore, future CF therapies may need to consider treatment of the entire microbiome to decrease the pathogenicity of the bacterial community as well as treatment of individual pathogens. This approach presents numerous challenges including how to determine the effect of the bacterial community on disease progression. It has been proposed that a longitudinal study would be required especially with those CF patients who have yet to acquire infection with *P. aeruginosa* [7]. This would allow the bacterial community within the lung to be observed over time, with changes being related to worsening disease. Perhaps, a better understanding of how the airway bacterial community develops to cause chronic infection would enable measures to be implemented to prevent chronic infection from becoming established [32]. Another challenge is that flexible treatment protocols will need to be developed so that they can be modified to treat different combinations of bacterial species in different CF patients throughout their life [7]. As knowledge of how bacterial species interact with each other increases, therapy could also be targeted towards preventing these interactions and reducing bacterial activity. Therefore, it could be hypothesized that any treatment that disrupts or alters the community structure could prevent or reduce the rate of disease progression and result in clinical improvement for the patient.

A novel treatment in CF: probiotics

Probiotics are nonpathogenic bacteria that are taken to promote health [82]. Probiotics are available as an oral preparation and they have been shown to be capable of modifying the intestinal microflora, immunity and inflammatory response in the GI tract [83]. *In vivo* studies have also indicated that probiotics can have an immunoregulatory effect outside this area. For example, when *Lactobacillus plantarum* was injected locally into the burns of a mouse model of infection the pathogenicity of *P. aeruginosa* was affected including a significant increase in *P. aeruginosa* phagocytosis [84]. In a mouse model of pneumonia, it was reported that oral administration of *Lactobacillus pentosus* strain b240 reduced both the carriage of *Streptococcus pneumoniae* and the secretion of inflammatory cytokines in the lungs as compared with saline-treated mice [85]. Histopathological examination of the lung tissue revealed less tissue damage in the probiotic treated mice.

Two pilot studies have been conducted to examine the effect of probiotics on the incidence of CF pulmonary exacerbations [86,87]. In one of these studies, 38 children (group A, n = 19; group B, n = 19) colonized with *P. aeruginosa* completed a crossover study [86]. As an adjunct to standard therapy, patients in group A took *Lactobacillus* GG dissolved in oral rehydration solution for 6 months followed by another 6 months of oral rehydration solution alone. Patients in group B were administered the same solutions but in the reverse order to enable a comparison to be made. Significant findings included a reduction in the number of exacerbations recorded during *Lactobacillus* GG administration, greater weight gain after *Lactobacillus* GG administration and increased IgG levels during oral rehydration solution administration [86]. These findings suggest that probiotics may have a beneficial effect in CF pulmonary infection. The other study also discovered that a commercially available probiotic mix (*Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium bifidum* and *Streptococcus thermophilus*) significantly

reduced the number of pulmonary exacerbations in ten patients during the 6 months of probiotic treatment when compared with the previous 2 years and to 6 months after treatment [87]. Although these two small studies have provided promising results, a case report in 2010 detailed a patient who developed necrotizing pancreatitis with pseudocysts induced by the commensal bacteria, *Veillonella* and *Bifidobacterium* (also marketed as a probiotic) species [88]. While this is not a common phenomenon, the authors state that care should be taken when using probiotics in those with a prolonged illness or compromised immune system as these bacteria could be pathogenic. Probiotics may be a promising CF therapy for the future but more research is required to determine any potential risks in these patients.

Conclusion

A complex bacterial community exists in the airways of CF patients. The known CF pathogens form only a small proportion of this community and as strict anaerobic culture techniques and molecular characterization tools continue to be used in research, more clinically relevant pathogens will be identified. It will be important to determine if a core microbiome exists in all patients and the role of these bacteria in disease pathogenesis and clinical deterioration. All bacteria detected may not necessarily trigger exacerbations but they may contribute to pathogenesis in other ways including influencing the virulence of other microorganisms. Furthermore, understanding how the bacterial community develops and changes during an exacerbation may enable better prediction of disease progression and more appropriate treatment on an ongoing basis.

Future perspective

New molecular technologies are continually emerging to enable a greater bacterial diversity to be detected and enhance the knowledge of disease progression. Advances in the therapeutic management of CF pulmonary disease may arise from treatment of the entire CF lung microbiome.

Executive summary

Cystic fibrosis pulmonary infection: a polymicrobial disease

- A polymicrobial community exists in the cystic fibrosis (CF) lungs with aerobes, facultative and obligate anaerobes, and normal commensal flora all present and the combination of bacteria present differs between individuals.

Characterization of the CF 'cultured' microbiome

- By using strict anaerobic culture techniques an attempt has been made to detect the entire culture microbiome.
- Phenotypic identification of fastidious pathogens can be difficult and bacteria that cannot be cultured will be missed. In contrast, certain molecular methods can be exploited to identify bacteria from DNA extracted directly from the clinical sample enabling unculturable bacteria to be detected.

Characterization of the CF microbiome

- Molecular tools continue to be developed to enable greater characterization of the CF microbiome with the most recent being high-throughput sequencing and the 16S rRNA PhyloChip. These techniques enable bacteria in low abundance to be detected.

The CF lung bacterial community

- Disease progression may be affected by the overall make-up of the bacterial community within the CF lung.
- Loss of bacterial diversity and changes in bacterial abundance, have been associated with reduced lung function and disease progression.

Potentially pathogenic anaerobic bacteria

- Obligate anaerobic bacteria persist in the lungs of CF patients and are present at all ages. The *Streptococcus milleri* group (SMG) may also be chronic colonizers and have been identified as a cause of pulmonary exacerbation.
- The source of these anaerobic bacteria may be the oral cavity.

The role of obligate anaerobes & SMG in community development & disease progression

- A number of the obligate anaerobes, such as *Prevotella* spp., and SMG possess virulence factors, may interact synergistically with other bacteria, transfer antibiotic resistance genes or stimulate the host immune system. They may contribute to disease progression.
- Anaerobic bacteria could be involved in creating a favorable environment for more virulent bacteria to infect the lungs. Therefore, their role in development of the bacterial community needs to be investigated.

Antibiotic treatment

- Antibiotic therapy may need to target the anaerobic bacteria. It is a challenge that antibiotic resistance to many of the antibiotics used to treat anaerobic infections, such as metronidazole and clindamycin, is emerging.

Treatment of the entire microbiome

- Treatment to disrupt the development of the bacterial community before infection becomes chronic may be a promising therapy in the future.

A novel treatment in CF: probiotics

- Two pilot studies have investigated the effect of probiotics on pulmonary exacerbations. Results are promising with significant decreases in exacerbations reported in those patients taking the probiotic.

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