Perspective

Recapitulation of polymicrobial communities associated with cystic fibrosis airway infections: a perspective

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The airways of persons with cystic fibrosis are prone to infection by a diverse and dynamic polymicrobial consortium. Currently, no models exist that permit recapitulation of this consortium within the laboratory. Such microbial ecosystems likely have a network of interspecies interactions, serving to modulate metabolic pathways and impact upon disease severity. The contribution of less abundant/fastidious microbial species on this cross-talk has often been neglected due to lack of experimental tractability. Here, we critically assess the existing models for studying polymicrobial infections. Particular attention is paid to 3Rs-compliant *in vitro* and *in silico* infection models, offering significant advantages over mammalian infection models. We outline why these models will likely become the 'go to' approaches when recapitulating polymicrobial cystic fibrosis infection.

Cystic fibrosis (CF) is the most common life-limiting genetic disease within the Caucasian population and is estimated to affect 70,000 people worldwide [1]. It results from an autosomal recessive defect within the cystic fibrosis transmembrane conductance regulator (CFTR) gene [2] and a myriad of different mutations have been described that bring about the onset of CF [3]. Dysfunctional CFTR activity leads to complications in multiple organs, but perhaps the most striking presentation of CF is the overproduction of a nutrient rich, viscous mucus in the CF-airways [4]. Defective mucociliary clearance mechanisms further contribute toward airway obstruction which results in the CF airways being a highly heterogenous environment, typically characterized by steep oxygen gradients, lowered pH and an abundance of mucin, amino acids, nitrate and iron [5–10]. This unique environmental niche is prone to chronic microbial colonization, and such infections contribute toward the death of 80–95% of CF patients [11–15] through triggering bouts of excessive inflammation, termed acute pulmonary exacerbations (APEs). Cumulatively, these lead to tissue destruction and a steady decline in lung function [14,15].

Traditionally, culture-based microbiological investigation of sputum samples expectorated from CF-patients have been used to establish which microbial species are associated with chronic infection of the CF airways. It was suggested, in line with Koch's postulates, that single-microbial species were the primary cause of infection [16], with *Staphylococcus aureus* being prevalent in the early stages of life, before being outcompeted as *Pseudomonas aeruginosa* (PA) becomes the dominant pathogen alongside occasional co-infections from other 'keystone' respiratory pathogens [17–19]. More recently, culture-independent molecular profiling techniques, for example, sequencing of the hypervariable bacterial 16S rDNA and fungal ITS regions, suggest that a previously unanticipated diverse polymicrobial population of both bacteria and fungi are associated with CF-airway infections [14,15,18–22]. However, this notion has been strongly contended by the recent work of Jorth *et al.*, who sampled lavage fluid directly from the lungs of CF-children displaying stable lung function. These authors suggest that the previously reported diversity of the CF-associated microbiome may arise from the sampling of oral contaminants. This notwithstanding, Jorth *et al.* did suggest that a core population of 'nonconventional' bacterial species are found alongside the traditional CF-pathogens [23].

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With the CF airways containing a polymicrobial ecosystem, it could be hypothesised that members of this community interact with one another either through quorum sensing, the recognition of cell surface proteins and the secretion of other small metabolites. Indeed, it has been demonstrated that the co-culture of different bacterial species leads to large alterations in their gene expression profile, both *in vivo* and *in vitro* [24]. Such changes can be synergistic or antagonistic in nature [25,26] and may cause members of the community to adopt different lifestyles and activate distinct metabolic pathways. These changes could alter the expression of virulence factors and influence the chemical environment, causing deviations from behaviors observed when studying single species in isolation [21,25,27–30]. For example PA is able to sense the presence of peptidoglycan shed from Gram-positive bacteria. This stimulates the production of extracellular factors that are lytic against both prokaryotic and eukaryotic cells [31]. Ultimately, polymicrobial infections display altered responses to therapeutic interventions. Such interventions are often aimed at decreasing the microbial load of a principal pathogen, and differentially impact disease severity between patients [32].

The microbial consortium present within the CF airway is thought to consist of both stable and disturbed states [25], yet little is known about what triggers the switch between the two. A better understanding of the extent of interactions occurring within the CF airway, and of how the architecture of polymicrobial communities adapts over time and responds to clinical intervention, may lead to the identification of novel therapeutic targets. It is possible that less abundant and poorly characterized species, typically viewed as avirulent when studied in isolation, could trigger the adoption of a more pathogenic lifestyle by known pathogens or in species classically thought to be nonpathogenic or nonconventional. Through targeting the pathways involved, and the chemical changes inducing the onset of a decline in lung function, it may be possible to delay or prevent key CF pathogens from dominating the patient's airways.

Despite the likely role played by interspecies interactions on the severity of disease within CF and other polymicrobial infections, relatively little is understood about this, or about how interspecies interactions impinge upon adaptation in the airways. This lack of understanding is, at least partially, attributable to the complexity of mixed-species interactions, and the paucity of polymicrobial models available for studying such interactions. Although research focus is now gradually moving away from studying single species in isolation, and moving more toward cocultivating the major members of mixed populations, these studies often ignore the presence of less abundant and hard-to-cultivate members of the community, thereby neglecting their impact as drivers of population change.

It would be of enormous benefit for models to be developed within the laboratory that enable the stable recapitulation of the whole polymicrobial community derived from the airways of CF-patients, in other words, those associated with sputum or bronchoalveolar lavage fluid (BALF) samples. These models would better represent the nature of the CF airway community and shed light on how both key pathogens and less represented species might be influenced by external perturbations. Such a model system would allow for a multitude of biological questions to be addressed relating to interspecies interactions and evolution within mixed microbial populations. The community-wide impact of external perturbations, either through antimicrobial treatment or through the introduction of new species/strain variants, could all be studied in a robust and reproducible manner.

This commentary aims to provide a critical overview of the existing models used to study microbial infection in relation to CF, and we hope to clarify how such approaches might be utilized to develop true polymicrobial infection models utilizing the microbial communities derived from CF sputum. Close attention is paid to the development of *in vitro* and *in silico* infection models that comply with the 3Rs guidelines for the reduction, replacement and refinement for the more humane use of animal models within research. Additionally, we highlight the need to consider CF as a mixed-species infection scenario, in order to identify novel targets for therapeutic intervention and understand the progression of disease within a more physiologically relevant context.

Mammalian CF infection models

Animals have long served in the field of comparative biology as a proxy for studying human genetic disease, and CF is no exception to this trend. In 1992, just 3 years after the identification and initial characterization of the CFTR gene, the first CF-mouse model was generated through gene targeting of embryonic stem cells to abolish CFTR activity [33]. In recent years, countless advances in gene editing techniques have led to the generation of a wealth of CF-animal models aimed at mimicking the mutations associated with the disease in humans, with varying success. However, some of these CF-animal models have been pivotal in furthering our understanding of the underlying mechanisms of CF pathology and the development of therapeutics now approved for the treatment of patients

carrying a distinct subset of CFTR mutations. This notwithstanding, our aim here is to provide an overview into how models can be used to study CF-associated infections, and as such we will not review further the utility of animal models for developing gene therapy treatments [34–36].

The comparatively low cost, rapid reproduction rate and ease of genetic manipulation ensure that mice are usually the first point of call when developing transgenic mammalian models. To date at least 14 different CF-mouse models have been reported, with differing CFTR mutations and genetic backgrounds available [37]. Despite this impressive selection of models at hand, numerous issues have plagued the use of mice for studying CF-associated microbial infections. The biggest issue seems to arise from differences in the spatial structure of murine airways and an inability to spontaneously develop airway infection [33], even with CF-mice demonstrating impaired mucociliary clearance. The CFTR protein is known to play a key role in innate lung immunity and several studies have since been undertaken to determine why even the 'β-NaEC' mouse [38] and other backgrounds lacking residual CFTR expression fail to develop infection [39,40].

Lack of an existing microbial community could be considered a 'clean-slate' for inoculating CF-mice with polymicrobial communities derived from patients. However, the introduction of pathogens into these mice leads to rapid microbial clearance [41], and repeat infections cause death [42]. Infection timescales using murine hosts can be considered semi-chronic at best, with successful infection (when achieved) typically lasting no more than a few days and reliant upon immobilizing the bacteria (PA) within agarose/alginate/agar beads that are directly instilled into the lung [41–50]. Using specific hypermucoid strains (e.g., NH57388A) it has been possible to maintain strain specific PA infections of up to 3 months within a host that bears the hallmarks of chronic CF infection [44,50]. An alternative inoculation approach that avoids the mechanical immobilization of microbial species and attempts to better represent a natural route of infection is the intranasal inoculation of mouse models. A persistent infection lasting 28 days could be established in non-CF mice following the respiratory inhalation of the transmissible Liverpool epidemic strain (LES) of PA [51]. Bacteria were found to be harbored in the nasopharynx throughout the experiment, and no PA could be detected in the lungs even by day 14. However, on day 28, PA could once again be isolated at low levels from the lung (26 CF per lung). These findings demonstrate that persistence in the nasopharynx allows reseeding of the lower airways and support the hypothesis that the upper respiratory tract provides a 'silent reservoir' able to harbor CF-associated species that can then be aspirated in to the lungs of CF patients. It would certainly be interesting to compare how a CF-mouse model responds to the respiratory inhalation route of infection and if multiple microbial species could be maintained within the nasopharynx. The intranasal inoculation of PA isolates recovered longitudinally over the course of a patient's life into CF-mice revealed that only late-stage 'CF-adapted' isolates could establish persistent infection [45,49]. It has also been suggested that older CF-mice may be more predisposed to microbial infection, but little work has been carried out to determine if such mice have an expanded range of infective capabilities [47,52].

Co-infection studies have been trialed in recent years, but again these have been limited to an acute infection timescale (often just 18 h) until the clearance of non-PA species occurs. Importantly, these studies have revealed that co-infection of PA with *S. aureus* or *Burkholderia cenocepacia* enhances the murine immune response and upregulates virulence factor production in the lungs [43,48,53]. Furthermore, *in vivo* coinfection studies of PA and *S. aureus* found that 'late-stage' PA isolates had undergone phenotypic adaptation associated with enhanced persistence in the CF lung, and demonstrated a reduced capacity to outcompete *S. aureus* during co-culture [48]. Interestingly, immobilizing *B. cenocepacia* in PA-derived alginate caused increased microbial persistence, inflammation and mortality rates [46]. Such studies provide clear evidence that interspecies interactions drive modulations in microbial lifestyles, and that these changes have a major impact upon disease severity.

Mouse infection models aim to provide an insight into the mechanisms underlying host-responses to infection but are severely limited by the small subset of PA isolates proven to be able to establish an infection once physically immobilized. Further research is required into how CF mice rapidly clear airway infections; this may lead to the generation of novel transgenic models with an improved propensity for sustaining mixed species infections. Until this is achieved, CF mice need to be considered with caution as models for polymicrobial CF infection. The impact of this is that researchers need to consider critically the impact that other members within a mixed community have on driving PA adaptation and host-immune responses in these systems, due to concerns regarding the physiological relevance of the model.

The inability of CF mice to spontaneously develop lung disease comparable to that observed in human patients led researchers to develop CF models within larger mammals that share a higher degree of airway similarity with humans. CF models have now been reported in ferrets [54–56], pigs [57–60] and more recently sheep [61], all with

higher levels of CFTR homology with the human gene (92, 91 and 91% respectively, and in contrast to just 78% in mice). Crucially, these larger mammals do develop spontaneous airway infections, demonstrating a reliance on functioning CFTR protein for effective airway clearance. Despite their apparent predisposition for microbial infection, these models are not without limitations and multiple issues hamper their use in studying polymicrobial CF-associated infections.

Soon after birth, CF ferrets develop spontaneous bacterial infection and severe lung pathologies mirroring those observed in humans. Examination of BALF established that members of the *Streptococcus*, *Staphylococcus*, *Enterococcus* and *Pseudomonas* genera were present at low levels and could not be eradicated from the airways [53–55]. This presence of a diverse airway microbiota suggests CF ferrets are permissive for microbial colonization, yet they are limited in their suitability for the inoculation of human-derived polymicrobial communities. Abnormal inflammation is believed to begin *in utero*, before being excessively amplified upon bacterial exposure at birth [55]. Single-species intratracheal microbial challenges with PA and *B. cenocepacia* in CF ferrets led to a hyperinflammatory response from the host macrophages and increased mortality rates [40], supporting the notion of bacterially driven immune modulation. The extreme severity of bacteria-associated lung pathologies result in neonatal CF ferrets relying on antimicrobial therapy to survive weaning [62], limiting their use in studying chronic infection. No studies have challenged a ferret model with a microbial co-culture, although given the spontaneous development of neonatal infection, it may be possible to develop acute infection models with polymicrobial communities derived from CF patients. It would be interesting to compare whether there are any similarities between the hyperinflammatory response(s) observed in CF ferrets with those observed during APEs in CF patients. In this regard, the acute ferret infection model could potentially provide a physiologically relevant framework for understanding better the mechanisms of host-microbe responses leading up to APEs.

Gut-corrected, humanized CF pig models provide an *in vivo* system that parallels human CF lung pathophysiology remarkably well. CF pigs develop spontaneous airway infections with diverse and varied microbial populations a few months after birth, and fail to eradicate bacteria as effectively as wild-type pigs [57–60]. The microbiota of older CF pigs mostly resembles that of adolescent human patients, with *S. aureus* being the most commonly isolated pathogen [20,57,59]. Infection studies have not yet been carried out on CF pigs, although introducing PA isolates into the established microbial population *in vivo* would provide unique insight into how this key CF pathogen initially adapts within the CF airways. Alternatively, a polymicrobial community derived from CF patients could be introduced into CF pigs to examine the host-microbe response and interspecies interactions that occur within an environment that is close to being physiologically representative of the human CF lung. However, the high costs and requirement for specialized expertise and dedicated lab space for conducting research using porcine models has restricted experimental sample size, widespread uptake, and length of studies; more so than many other *in vivo* models.

Plant & invertebrate infection models

With key differences in physiology being apparent between plants/invertebrates and the human airways, the use of such hosts cannot (strictly speaking) be considered CF-infection models. However, their low cost, basic equipment requirements and minimal technical skill requirements, as well as a lack of ethical concerns, means such models provide a highly attractive, high-throughput approach to screen mutant libraries for genes essential for virulence and growth *in vivo.*

Limited similarities exist between the immune response of mammals and such simple host systems, yet they do enable some insights into the host-response to infection. How closely invertebrates approximate mammalian models is still cause for debate. These models are primarily limited by which microbial species have the capability to establish an infection, and there is little chemical or spatial similarity in the infection environment compared with human hosts. The small size of invertebrate models means very low microbial inoculums are rapidly fatal [63] and ensures that in general only acute, single-species infection studies can be readily undertaken. Mutant knockout libraries of 'rare' or less abundant CF-associated microbial species simply do not currently exist, and cannot be subject to systematic screens or included within co-infection studies, in spite of the likely role(s) played by these species in modulating pathogenesis during CF infection. The result of library screens using plant and insect hosts must be viewed with caution as essential genes for infection within mammalian models may have been missed, yet such high-throughput screens are certainly useful in the identification of potential virulence genes that can be further characterized *in vitro* or in larger *in vivo* model systems.

Multiple plant-based infection assays have been reported, including: *Arabidopsis thaliana* [63], lettuce stems [64] and mung bean seedlings [65]. With a limited range of human pathogens able to establish infections within these models, the majority of studies simply focus on PA isolates and mutants. Infection assays often compare the extent of destruction within stem or leaf infiltration assays or compare the growth of seedlings infected with wild-type or mutant strains, enabling a rough quantitative measure of the contribution that specific genes have toward causing virulence *in vivo*. However, as several mutants can be introduced into the same model, for example, multiple mutant strains inoculated within a single lettuce stem [64], plants provide an extremely rapid fitness screening system for a limited number of microbial species.

Caenorhabditis elegans is a very well-characterized, well-established laboratory model organism and is susceptible to infection by a range of both bacterial and fungal species [66,67], including some that have been isolated from the CF-lung and are associated with worsened patient prognosis. Microbial species are introduced as a top lawn or bacterial suspension containing the nematodes during feeding, limiting infection studies to species that can be successfully cultivated *in vitro*. In order to establish a co-culture within *C. elegans* both species must first be successfully grown together without one outcompeting the other and is something which may not be possible for the polymicrobial communities associated with CF. Vega and Gore describe the successful co-culture of two members of the Enterobacteriaceae in *C. elegans* [68]. However, they also report the dominance of one species over the other within the intestine during 'slow colonization' further demonstrating the limitation of nematodes as a vehicle for maintaining both the rare and the dominant microbial species associated with CF. When grown on nutrient-rich media, PA isolates cause 'fast-killing' of nematodes due to the production of hydrogen cyanide and phenazine compounds [69], yet when grown on minimal media PA replicated within the digestive tract to cause 'slow-killing' [70]. Such results demonstrate that nematodes are susceptible to death from secreted metabolites and that the growth media used in the screening of mutant libraries must be carefully considered.

Galleria mellonella (waxmoth larvae) are an alternative invertebrate model used for studying key CF-pathogens. Although waxmoths are infected by a limited range of pathogens, they are susceptible to infection from PA and some fungal species associated with the CF-airway [71]. Comparative studies have established that the waxmoth infection model shows good correlation of antimicrobial efficacy, pharmacokinetics [72] and essential virulence requirements with mouse models [70], suggesting this system is more representative of mammalian infection than the plant or nematode models. However, waxmoths are extremely susceptible to infection, with as little as ten bacterial cells being fatal, thus limiting use of this model to acute single-species infection only. This notwithstanding, serial passage of microbial populations through waxmoths could be used to represent a more chronic infection, and reports suggest the phenotypic adaptation of PA isolates does reflect limited elements of evolution within the CF airways [73].

Drosophila melanogaster presents the most plausible invertebrate model for studying polymicrobial infections. Not only are there a large number of genetic tools available for the generation of mutant flies (enabling detailed genetic dissection of host-microbe interactions occurring during infection), but successful *S. aureus*-PA coinfection models have been reported [31,74]. These studies build upon previously described single-species feeding models which demonstrate that bacteria replicate within the fly-crop and cause host mortality. Using this invertebrate co-infection model it was found that PA can sense peptidoglycan shed from the walls of Gram-positive species, triggering an enhancement in toxin and antimicrobial production [31]. This not only causes a 1000-fold decrease in the Gram-positive flora, it also enhanced host mortality. A mutant PA strain, PA601, which lacks the peptidoglycan receptor, did not increase mortality rates upon co-infection with *S. aureus*, and these results were verified using a murine infection model [75]. Such co-infection studies not only provide strong evidence for the need to understand interspecies interactions in a suitable host model, but also provide clear insights into the mechanisms underpinning these. The *Drosophila* model also relies on feeding bacteria to the host, again severely limiting which species can be studied and this model from being utilized for inoculation with patient-derived polymicrobial samples.

As with all infection studies, it can be difficult to pinpoint which of the multifarious biological interactions that occur within a host model are crucial for driving changes in human disease severity. A reductionist approach could be taken to systematically screen mutant libraries within an invertebrate co-infection model to identify key genes for subsequent study in more advanced model systems. *In vivo* models provide a system for studying host-microbe interactions, but they are ill-suited to analyse interspecies microbe–microbe interactions. This is because the added complexity of an immune response strongly limits the likelihood of sustaining a polymicrobial model mimicking the chronic infections often associated with CF patients. With emerging evidence suggesting that co-infection

drives changes in microbial lifestyles, that in turn impact upon disease severity, models that enable the real-time tracking of microbial populations are urgently required.

In vitro & *ex vivo* models of CF-infection

Emerging evidence suggests that chemical, not spatio-temporal, challenges have the greatest impact on driving changes in microbial lifestyles [76,77]. Inherent natural variation between *in vivo* models leads to local variations in the chemical environment and immune responses, adding an additional layer of variability. There is clear need for a robust and reproducible model recapitulating a physiologically relevant environment that allows the chemical and genetic changes within a mixed population to be dissected. *In vitro* models present a defined and easily perturbable environment, that can potentially provide simple, tractable and high-throughput systems for studying mixed species populations derived from CF patient sputa. The real-time tracking of a model microbial population would allow a number of fundamental biological questions to be addressed, making it possible to identify novel therapeutic avenues that might be missed in *in vivo* infection models.

A number of microbial species associated with CF-airway infections are known to have a narrow host-range of infection, for example, *Haemophilus influenza* does not infect nonhuman hosts. Therefore, it may be impossible to develop certain *in vivo* polymicrobial infection models. Conversely, several species have been identified in *in vivo* CF-models that are not known to infect humans, adding an extra layer of complexity when trying to recapitulate human infection-associated microbial consortia *in vivo*. Rearing animal models under gnotobiotic conditions can potentially reduce the presence of animal-specific species, although this does add significantly to the cost of maintaining the model.

A combination of targeted and untargeted culture-independent molecular approaches can be used to follow the composition of a polymicrobial community over time in an *in vitro* infection model. Culture-independent molecular analyses allow the influence of less abundant and hard-to-isolate species to be tracked effectively within models representative of chronic infections. The utility of untargeted molecular approaches, such as sequencing the hypervariable 16S rDNA and ITS regions of bacteria and fungi (respectively), has been reviewed by Rogers *et al.* [21]. Moreover, the development of revolutionary, high-throughput and sensitive targeted gene assays such as the NanoString nCounter system allows the expression of up to 800 different genes to be studied in a single reaction [78]. Utilizing probes designed against known 16S rDNA and ITS regions alongside key virulence genes has allowed accurate determination of the microbial consortium associated with CF-sputum samples [79], alongside tracking the composition and transcriptome profiles of complex, mock microbial communities [80]. Probes targeting mRNA molecules provide a more sensitive indication of the metabolically active members of a community, identifying the key drivers that potentially influence community adaptation and responses to environmental challenges.

Artificial sputum medium (ASM) or synthetic cystic fibrosis sputum (SCFM), is a highly defined synthetic growth medium that closely mimics the nutritional composition of sputum found in the CF-airway [76,81–85]. Comparison of the essential genome of PA isolates following growth in patient-derived sputum or ASM found virtually no difference between the two [83], providing compelling evidence that ASM is a physiologically relevant *in vitro* culture medium to support the growth of CF isolates. This notwithstanding, Cornforth *et al.* recently showed that machine learning approaches can discriminate PA grown *in vivo* from PA grown *in vitro* in ASM based on transcriptional profiling. However, little account was taken in that study of possible differences in the *in vivo/in vitro* transcriptome data arising from the presence of less abundant non-PA species in the human samples [86].

ASM induces PA to grow as microcolonies closely associated with the mucin, strongly resembling the growth observed in CF-sputum [81]. However, numerous modified recipes for ASM have been proposed, each of which is associated with distinct changes in microbial growth phenotype [82,87], thus demonstrating the need to standardise this complex media to ensure reliability and reproducibility between *in vitro* experiments. *In vitro* models allow facile experimental tuning of growth conditions to mimic different disease states; something that is simply not possible using *in vivo* models. Using ASM, the long-term maintenance of both PA and *S. aureus* in mixed species biofilms has been made possible [84], something previously not attainable using conventional laboratory media [88] despite the co-isolation of both species from the airways of ~31% of CF-patients [89]. Although no published studies have yet attempted the cultivation of a CF-associated polymicrobial community within ASM, similarities in nutrient availability allow us to postulate that in these conditions, the microbiota might be experimentally maintained *in vitro*. Direct inoculation of the 'complete' microbial community contained within sputum samples would permit inter-species cross-feeding to occur between cohabiting organisms. This may permit the growth of fastidious and auxotrophic strains known to be associated with the CF-airways [90].

In addition to the ease with which *in vitro* models can be experimentally perturbed, such systems are free from the temporal limitations imposed by animal infection studies. Longitudinal sampling of an *in vitro* model inoculated with a diverse polymicrobial community would reveal the chain of evolutionary events preceding the latest sampling point, essentially allowing a direct experimental analysis of Gould's speculations about 'rewinding the tape of evolution'. Whether parallel, identically inoculated multi-species populations that are subject to the same (intensely competitive) selection pressures can demonstrably follow the same independent evolutionary trajectory becomes an experimentally tractable problem. Such studies that examine compositional and genetic changes within communities can also be supported with chemical analyses to link how these changes affect the chemical environment of the airways. A novel Winogradsky-based culture model (WinCF) using a number of different chemical indicators has been used to monitor the growth of sputum samples in ASM. The study revealed a 2 unit reduction in the pH and 30% increase in gas production due to the increased abundance of fermentative anaerobes present in the sputum prior to the onset of APEs [76]. We also note that sequential sampling the secretome of a polymicrobial community is possible within an *in vitro* model, allowing a direct analysis of the impact of inter-species interactions on virulence factor secretion.

Introducing sputum samples taken from disease states within the same CF patient, in other words, before, during and after APEs, into an *in vitro* system would allow differences in the polymicrobial composition to be studied and could shed light into how subtle differences within these complex communities contribute to a decline in lung function. Sousa *et al.* utilized ASM to develop a long-term (10 days) *in vitro* culture system mimicking the physiological conditions of the airways to determine how clinically used antibiotics affected the phenotypic diversification of PA isolates [85]. That study found that sub-inhibitory concentrations of ciprofloxacin drove diversification in CF isolates but not in laboratory reference strains. Similar studies using polymicrobial cocultures could be undertaken to examine how therapeutic intervention drives phenotypic changes within the total microbial consortium associated with CF airways.

New species or strain variants, including 'keystone' CF pathogens such as PA, could be introduced into an established steady-state *in vitro* polymicrobial community to simulate the events following initial introduction of such variants in the patient airways. Such events are very difficult indeed to capture through direct analysis of patient sputa. Furthermore, such an *in vitro* model provides the unparalleled opportunity to study the genetic and phenotypic changes that occur in both the 'invading' species and in the endogenous polymicrobial population. Similarly, hypermutator strains, for example, *mutS* mutants [91], could be introduced into CF derived polymicrobial populations to examine the impact of what happens to all the species present when one of them is allowed to 'step on the evolutionary gas pedal'. A knowledge of the order of succession of major CF pathogens in a polymicrobial community may reveal clinically relevant insights into the types of interspecies signaling pathways involved in maintaining stable community architectures [92].

Biofilm formation may be the biggest contributor toward enhancing infection persistence, decreasing antimicrobial susceptibility, and elevating mutation rates within the CF airways [26,29,84,93–96]. Unsurprisingly the formation of biofilms has been extensively studied *in vitro*, with a focus in recent years on moving away from mono-species biofilms in favor of co-cultures more representative of real infection scenarios [53,77,84,94–98]. Using mixed-species biofilm models it has been found that species co-isolated from the CF lung can either increase or decrease the antimicrobial susceptibility [77,95] and the production of biomass [52,84,95] by other species present, and that strain variants provide cross-protection against different host-generated antimicrobials [98]. Furthermore, *in vitro* co-cultures of PA–*S. aureus* biofilms show that the mutation rates within these species can be increased up to 500-fold, and that *S. aureus* adopts a drastically different growth phenotype reminiscent of isolates recovered directly from the CF lung. To date, there is only one report in which a polymicrobial biofilm derived from CF sputum has been reconstituted *in vitro* [99]. That study focused on how antimicrobials affect the population, and growth conditions were not optimized to represent the physiology in the CF airway. By more closely recapitulating the CF-associated chemical environment, it may be possible to maintain polymicrobial biofilms more representative of those residing in the airways of CF patients.

One major limitation of *in vitro* models is the lack of spatial organization closely resembling that of a host, and which is provided to some degree in *in vivo* infection models. To overcome this deficiency of *in vitro* model systems, an *ex vivo* infection model has recently been developed [100] to provide a tractable and high-throughput system that can be easily used to mimic the airway architecture within a laboratory environment. Using porcine lungs, a waste by-product of the food industry, and ASM, this *ex vivo* model provides an effective and ethical solution to introducing spatial organization into longitudinal culture studies. Furthermore, different sections of lung tissue can

be used to mimic either upper or lower respiratory tract infections [101]. So far only PA isolates have been cultured within this model and these results demonstrate that *ex vivo* models are an effective experimental approach to study CF infection. It would be fascinating to introduce the polymicrobial community derived from CF sputum into an *ex vivo* model and assess how this shapes the trajectory of, for example, PA evolution.

Perhaps the largest barrier to the use of *in vitro* polymicrobial infection models is a lack of host cells that likely play a role in influencing the composition and behavior of the microbial consortium. *In vitro* models will prove crucial for the study of microbe-microbe interactions and will effectively reveal information about how species are able to interact with one another. *In vivo* models on the other hand are not as well suited to study interspecies interactions, nor are they as experimentally reproducible or tractable. They do (however) allow a more direct insight into how hosts respond to infection. A combination of both *in vitro* and *in vivo* polymicrobial models would allow for changes in microbial populations and in the chemical environment to be correlated with host responses.

Human cell–culture infection models provide an alternative approach to introduce elements of the host response into *in vitro* studies, and in the future, may allow more direct investigation of human–microbe interactions. Such *in vitro* cell culture infection models have already been successfully used to study a monolayer of human bronchiole epithelial cells homozygous for the ΔF508 CFTR mutation infected with a co-culture of *S. aureus* and PA [102]. It was found that PA decreases the viability of *S. aureus* and causes the species to adopt fermentative metabolic pathways. These results strongly correlate with changes reported to occur prior to the onset of APEs, and provide clear evidence that this of *in vitro* infection model may effectively represent the CF airway environment. Advances in cell culture procedures and gene editing tools mean that it may soon be feasible to routinely culture cell lines derived directly from individual CF patients [103], which could, in turn, be infected with polymicrobial populations gathered from the sputum of the same patient. The generation of such 'personalized infection models' would allow an unparalleled advantage over the use of 'generic' cell-lines and *in vitro* models to study microbial populations, allowing a more efficacious testing of antimicrobial action prior to treatment, bridging the 'bench-to-bedside' gap.

In silico polymicrobial models

The study of polymicrobial communities is not simply confined to 'the wet lab'. With our understanding of microbial metabolic pathways being continually refined, new, more accurate *in silico* modeling of polymicrobial communities is becoming readily available. Advancements in the semi-curation of complex microbial communities, using software such as ModelSeed and AGORA, have led to the metabolic modeling of CF-associated bacterial communities from published 16S rDNA data [104]. With better formulation for ensuring host-derived metabolite balance across the community (through use of SteadyCom software), sample-specific heterogenous communities can be generated that accurately predict which CF-pathogens come to dominate a polymicrobial community. *In silico* models are becoming a highly valued tool to derive metabolic inferences that may be difficult to obtain or observe experimentally and provide a theoretical framework for interpreting *in vitro* studies.

Computational predictive models are limited by the type and quality of data gathered from experimental studies. This means that they poorly predict the rare and hard to cultivate microbial species present within CF-associated microbial communities. Additionally, strain variants and evolutionary 'cheats' cannot yet be accounted for within *in silico* models, meaning that these models do not predict or incorporate phenotypic adaptation among the species present. Similarly, the striking spatial heterogeneity reported within the airways of CF patients is not easily captured in *in silico* models. Despite these limitations, *in silico* models do provide a useful a framework for further experimental analyses, informing on experimental design and interpretation.

Conclusion & future perspective

Culture-independent examination of sputum samples expectorated from CF patients have revealed that a diverse and varied microbial population (including bacteria and fungi) is associated with the airways of CF patients [14,15,18–22]. Increasing evidence suggests that interspecies interactions between members of this microbial consortium elicit mutual influence on the gene expression profile and metabolic pathways adopted by both pathogenic and typically nonpathogenic species. It is now clear that CF-associated infections must be considered as polymicrobial in nature, and that a better understanding of how microbial cross talk impacts upon the expression of virulence factors will be essential if we are to develop improved therapeutic interventions.

A lack of models enabling the stable, long-term cultivation of polymicrobial communities derived from CF patients, as well as some historical bias, has meant that until recently, most researchers focused their studies on the primary CF-associated pathogens, for example, PA and *S. aureus*, neglecting the impact of less abundant or more

fastidious species. The generation of such polymicrobial-infection models would be of enormous benefit to the research community, allowing the physiologically representative response(s) of the whole microbial community to common therapeutic interventions to be more effectively monitored. Furthermore, the phenotypic adaptation and evolutionary events leading to dominance of key pathogens, or the onset of a decline in lung function could be picked apart and better understood. Through understanding how interspecies interactions and changes within a microbial population lead to a worsened disease status, it may be possible to identify novel or improved therapeutic interventions to help alleviate disease symptoms and improve the quality of life for CF patients.

Animal models of CF are paramount for studying the pathophysiological progression of the disease within mammalian systems. However, as discussed earlier, the closer these models come to mimicking the human disease, the more complex and experimentally costly they become. Improvements in CF pigs and the development of CF sheep likely present the best chance for the development of an effective animal-based infection model, yet a lack of experimental evidence within the literature makes it difficult to conclude whether this will ever be realized. The introduction of PA to the existing microbiota of CF pigs would certainly help to reveal if an *in vivo* polymicrobial infection model could be attained in principle.

In vitro models provide a feasible approach for the recapitulation of sputum-derived polymicrobial communities within the laboratory, especially given the development of ASM and *ex vivo* porcine lung models. *In vitro* models are cost-effective, lack ethical concerns (i.e., are 3Rs-compliant) and hopefully will allow for high-throughput, chronic models of infection to be easily set up without the need for specialized animal handling facilities. A highly defined, experimentally tuneable chemical environment and lack of host-to-host variability means *in vitro* models would provide the perfect system to identify subtle, but key, changes occurring in the community.

Over the course of the next decade we predict that a viable and physiologically representative *in vitro* polymicrobial models will be developed and see widespread uptake. Such models will hopefully allow the economical recapitulation of an entire CF-associated polymicrobial community in the laboratory environment. Key areas to be examined using such an experimental model would include: understanding how the introduction of PA to an established polymicrobial population influences changes in community architecture and the chemical environment (secretome, nutrient availability etc), how the microbial population responds to external stressors such as antimicrobial agents, identification of the signaling pathways driving key phenotypic adaptations, and so on. Following their successful development and implementation, such *in vitro* models may allow novel therapeutic avenues to be explored, potentially leading to an improvement in quality of life for CF patients and possibly other chronic respiratory diseases involving polymicrobial infection.

Executive summary

Cystic fibrosis is a polymicrobial disease
- Culture-independent profiling has revealed a diverse and varied polymicrobial community is associated with the cystic fibrosis (CF) airways.
- Members of this community interact with one another, causing modulations in gene expression that potentially impact upon disease severity.
- No models currently exist that permit the recapitulation of a true CF-associated polymicrobial community in the laboratory.

Murine models of CF
- CF mice are incapable of developing spontaneous airway infections and microbial challenges are rapidly cleared.
- Only *Pseudomonas aeruginosa* isolates immobilized within beads that are instilled into the lung allow a semi-chronic infection scenario to be established.
- Although mice provide limited insights into the host-response to infection, murine models are not suited for the development of polymicrobial infection models.

Ferret & pig models of CF
- Both CF ferrets and CF pigs develop spontaneous airway infection with a range of microbial species soon after birth.
- Infections in CF ferrets induce fatal, hyperinflammatory immune responses that severely limit their use for development as polymicrobial models.
- The airway microbiota of CF pigs most closely resembles that of adolescent human patients, although infection with *P. aeruginosa* has not been examined.
- Costs and complexity hinder the uptake of CF pig models, and as a result, little research has been undertaken into airway infections using this model.

Plant & invertebrate models of CF
- Plant and invertebrate models provide high-throughput platforms to screen mutant libraries for a limited range of genes required for *in vivo* growth and virulence.
- Plant and invertebrate models support the growth of only a limited spectrum of CF-associated microbial species.

***In vitro* polymicrobial models**
- The development of *in vitro* polymicrobial models provides a cost-effective, tractable and robust system for studying subtle interspecies interactions.
- *In vitro* models potentially allow long-term, 'chronic infection' to be recapitulated.
- Artificial sputum media is a defined synthetic medium closely mimicking the nutritional composition of CF-derived sputum. artificial sputum media provides a physiologically relevant *in vitro* culture medium to permit the growth of CF-associated species.
- Using sections of porcine lungs, an *ex vivo* culture system has been developed that introduces a spatial structure similar to that of the CF-airway into *in vitro* models.

***In silico* polymicrobial models**
- *In silico* models can be used to accurately recapitulate polymicrobial communities from published experimental data inputs, and can yield information that may be difficult to obtain experimentally, but can help to guide experimental design in *in vitro* and *in vivo* models.
- Such models are limited by the quality and quantity of data gathered from experimental studies and so poorly represent hard-to-cultivate microbial species. However, as more (and better) input data is gathered, *in silico* models will inevitably improve.

References

Papers of special note have been highlighted as: ● of interest; ●● of considerable interest

1. Cystic Fibrosis Foundation. www.cff.org

2. Knowles MR, Durie PR. What is cystic fibrosis? *N. Engl. J. Med.* 347(6), 439–442 (2002).

3. Cystic fibrosis mutation database. http://www.genet.sickkids.on.20ca/cftr/

4. Boucher RC. An overview of the pathogenesis of cystic fibrosis lung disease. *Adv. Drug. Deliv. Rev.* 54(11), 1359–1371 (2002).

5. Grasemann H, Ioannidis I, Tomkiewicz RP, de Groot H, Rubin BK, Ratjen F. Nitric oxide metabolites in cystic fibrosis lung disease. *Arch. Dis. Child.* 78(1), 49–53 (1998).

6. Jones KL, Hegab AH, Hillman BC *et al.* Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. *Pediatr. Pulmonol.* 30(2), 79–85 (2000).

7. Tate S, MacGregor G, Davis M, Innes JA, Greening AP. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 57(11), 926–929 (2002).

---

8.   Worlitzsch D, Tarran R, Ulrich M *et al.* Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J. Clin. Invest.* 109(3), 317–325 (2002).

9.   Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J. Bacteriol.* 189(8), 8079–8087 (2007).

10.   Ghio AJ, Roggli VL, Soukup JM, Richards JH, Randell SH, Muhlebach MS. Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. *J. Cyst. Fibros.* 12(4), 390–398 (2012).

11.   Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15(2), 194–222 (2002).

12.   Rajan S, Saiman L. Pulmonary infections in patients with cystic fibrosis. *Semin. Respir. Infect.* 17(1), 47–56 (2002).

13.   Elborn JS. Cystic fibrosis. *Lancet* 388(10059), 2519–2531 (2016).

14.   Carmody LA, Zhao J, Schloss PD *et al.* Changes in cystic fibrosis airway microbiota at pulmonary exacerbation. *Ann. Am. Thorac. Soc.* 10(3), 179–187 (2013).

15.   Carmody LA, Zhao J, Kalikin LM *et al.* The daily dynamics of cystic fibrosis airway microbiota during clinical stability and at exacerbation. *Microbiome.* 3(12), doi:10.1186/s40168-015-0074-9 (2015).

16.   Govan J, Nelson J. Microbiology of cystic fibrosis lung infections: themes and issues. *J. R. Soc. Med.* 86(Suppl. 20), 11–18 (1993).

17.   Conrad D, Haynes M, Salamon P, Rainey PB, Youle M, Rohwer F. Cystic fibrosis therapy: a community ecology perspective. *Am. J. Respir. Cell. Mol. Biol.* 48(2), 105–156 (2012).

18.   Zhao J, Schloss PD, Kalikin LM *et al.* Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc. Natl Acad. Sci USA* 109(15), 5089–5814 (2012).

19.   Boutin S, Graeber SY, Weitnauer M *et al.* Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. *PLoS ONE* 10(1), doi:10.1371/journal.pone.0116029 (2015).

20.   Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc. Natl Acad. Sci. USA* 105(39), 15070–15075 (2008).

21.   Rogers GB, Hoffman LR, Whiteley M, Daniels TW, Carroll MP, Bruce KD. Revealing the dynamics of polymicrobial infections: implications for antibiotic therapy. *Trends Microbiol.* 18(8), 357–364 (2010).

•   **Reviews the utility of next-generation sequencing approaches for discerning the complexity of polymicrobial communities, using cystic fibrosis (CF) as a case study.**

22.   Short FL, Murdoch SL, Ryan RP. Polymicrobial human disease: the ills of social networking. *Trends Microbiol.* 22(9), 508–516 (2014).

23.   Jorth P, Ehsan Z, Rezayat A *et al.* Direct lung sampling indicates that established pathogens dominate early infections in children with cystic fibrosis. *Cell Press.* 27(4), 1190–1204 (2019).

••   **Provides direct counter evidence against the notion that the CF-airways contain a vastly diverse and dynamic array of microorganisms.**

24.   Ibberson CB, Stacy A, Fleming D *et al.* Co-infecting microorganisms dramatically alter pathogen gene essentiality during polymicrobial infection. *Nat. Microbiol.* 2, 17079 (2017).

••   **A paper crucial to demonstrating that a change in gene expression occurs when species are co-cultured together, here through coculturing *Pseudomonas aeruginosa* (PA) and *S. aureus* ~25% of the essential genome in *S. aureus* is converted to 'nonessential' when compared with a mono-culture.**

25.   Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8(1), 15–25 (2010).

26.   Elias S, Banin E. Multi-species biofilms: living with friendly neighbors. *FEMS. Microbiol. Rev.* 36(5), 990–1004 (2012).

27.   Rogers GB, Carroll MP, Bruce KD. Studying bacterial infections through culture-independent approaches. *J. Med. Microbiol.* 58(11), 1401–1418 (2009).

28.   Rogers GB, Stressmann FA, Walker AW, Carroll MP, Bruce KD. Lung infections in cystic fibrosis; deriving clinical insight from microbial complexity. *Exp. Rev. Mol. Diag.* 10(2), 187–196 (2010).

29.   Leekha S, Terrell CL, Edson RS. General principles of antimicrobial therapy. *Mayo Clin. Proc.* 86(2), 156–167 (2011).

30.   Quinn RA, Lim YW, Maughan H, Conrad D, Rohwer F, Whiteson KL. Biogeochemical forces shape the composition and physiology of polymicrobial communities in the cystic fibrosis lung. *MBio.* 5(2), e00956–13 (2014).

31.   Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection. *Proc. Natl Acad. Sci. USA* 110(3), 1059–1064 (2013).

•   **Demonstrates that PA is able to interact with other species present within the environment, in this case Gram-positive bacteria, this interaction then leads to alterations in the production of virulence factors thought to be active against both prokaryotic and eukaryotic organisms.**

32.   Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin. Microbiol. Rev.* 25(1), 193–213 (2012).

33. Snouwaert JN, Brigman KK, Latour AM *et al.* An animal model for cystic fibrosis made by gene targeting. *Science.* 257(5073), 1083–1088 (1992).

34. Fisher JT, Zhang Y, Engelhardt JF. Comparative biology of cystic fibrosis animal models. *Meth. Mol. Biol.* 742, 311–334 (2011).

35. Rosen BH, Chanson M, Gawenis LR *et al.* Animal and model systems for studying cystic fibrosis. *J. Cyst. Fibros.* 17(2S), S28–S34 (2017).

36. Semaniakou A, Croll RP, Chappe V. Animal models in the pathophysiology of cystic fibrosis. *Front. Pharm.* 9(1475), doi:10.3389/fphar.2018.01475 (2019).

37. Guilbault C, Saeed Z, Downey GP, Radzioch D. Cystic fibrosis mouse models. *Am. J. Respir. Cell. Mol. Biol.* 36(1), 1–7 (2007).

38. Zhou Z, Duerr J, Johannesson B *et al.* The ENaC-overexpressing mouse as a model of cystic fibrosis lung disease. *J. Cyst. Fibros.* 10(S2), S172–S182 (2011).

39. Durie PR, Kent G, Phillips MJ, Ackerley CA. Characteristic multiorgan pathology of cystic fibrosis in a long-living cystic fibrosis transmembrane regulator knockout murine model. *Am. J. Pathol.* 164(4), 1481–14938 (2004).

40. Bruscia EM, Bonfield TL. Cystic fibrosis lung immunity: the role of the macrophage. *J. Innate. Immun.* 8(6), 550–563 (2016).

41. Cash HA, Woods DE, McCullough B, Johanson WG Jr, Bass JA. A rat model of chronic respiratory infection with *Pseudomonas aeruginosa. Am. Rev. Respir. Dis.* 119(3), 453–9 (1979).

42. Van Heeckeren AM, Schluchter MD, Xue W, Davis PB. Response to acute lung infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Am. J. Respir. Crit. Care. Med.* 173(3), 288–96 (2006).

43. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol. Microbiol.* 50(5), 1477–1491 (2003).

44. Hoffman N, Rasmussen TB, Jensen PØ *et al.* Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. *Infect. Imm.* 73(4), 2504–2514 (2005).

45. Moser C, Van Gennip M, Bjarnsholt T *et al.* Novel experimental *Pseudomonas aeruginosa* lung infection model mimicking long-term host-pathogen interactions in cystic fibrosis. *APMIS.* 117(2), 95–107 (2009).

46. Chattoraj SS, Murthy R, Ganesan S *et al. Pseudomonas aeruginosa* alginate promotes *Burkholderia cenocepacia* persistence in cystic fibrosis transmembrane conductance regulator knockout mice. *Infect. Immun.* 78(3), 984–993 (2010).

47. Munder A, Wölbeling F, Kerber-Momot T *et al.* Acute intratracheal *Pseudomonas aeruginosa* infection in cystic fibrosis mice is age dependent. *Respir. Res.* 12(148), doi:10.1186/1465-9921-12-148 (2011).

48. Baldan R, Cigana C, Testa F *et al.* Adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways influences virulence of *Staphylococcus aureus In vitro* and murine models of co-infection. *PLoS ONE* 9(3), e89614 (2014).

49. Cigana C, Lorè NI, Riva C *et al.* Tracking the immunopathological response to *Pseudomonas aeruginosa* during respiratory infections. *Sci. Rep.* 6, 21465 (2015).

50. Bayes H, Ritchie N, Irvine S, Evans TJ. A murine model of early *Pseudomonas aeruginosa* lung disease with transition to chronic infection. *Sci. Rep.* 6, 35838 (2016).

51. Fothergill JL, Neill DR, Loman N, Winstanley C, Kadioglu A. *Pseudomonas aeruginosa* adaptation in the nasopharyngeal reservoir leads to migration and persistence in the lungs. *Nat Commun.* 5, 4780 (2014).

52. Teichgräber V, Ulrich M, Endlich N *et al.* Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat Med.* 14(4), 382–391 (2008).

53. Bragonzi A, Farulla I, Paroni M *et al.* Modelling co-infection of the cystic fibrosis lung by *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* reveals influences on biofilm formation and host response. *PLoS ONE* 7(12), e52330 (2012).

54. Sun X, Sui H, Fisher JT *et al.* Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. *J. Clin. Invest.* 120(9), 3149–3160 (2010).

55. Sun X, Olivier AK, Liang B *et al.* Lung phenotype of juvenile and adult cystic-fibrosis transmembrane conductance regualator-knockout ferrets. *Am. J. Respir. Cell. Mol. Biol.* 50(3), 502–512 (2014).

56. Keiser NW, Birket SE, Evans IA *et al.* Defective innate immunity and hyperinflammation in newborn cystic fibrosis transmembrane conductance regulator-knockout ferret lungs. *Am. J. Respir. Cell. Mol. Biol.* 52(6), 683–694 (2015).

57. Rogers CS, Stoltz DA, Meyerholz DK *et al.* Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science.* 321(5897), 1837–1841 (2008).

58. Welsh MJ, Rogers CS, Stoltz DA, Meyerholz DK, Prather RS. Development of a porcine model of cystic fibrosis. *Trans. Am. Clin. Climatol. Assoc.* 120, 149–162 (2009).

59. Stoltz DA, Meyerholz DK, Pezzulo AA *et al.* Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci. Transl. Med.* 2(29), 29ra31 (2010).

60. Wine JJ. The development of lung disease in cystic fibrosis pigs. *Sci. Transl. Med.* 2(29), 29s20 (2010).

61. Fan Z, Perisse IV, Cotton CU *et al.* A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. *JCI insight.* 3(19), e123529 (2018).

62.   Hoffman LR, Hajjar AM. Ferreting out the role of infection in cystic fibrosis lung disease. *Am. J. Respir. Crit. Care. Med.* 197(10), 1243–1244 (2018).

63.   Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. Common virulence factors for bacterial pathogenicity in plants and animals. *Science.* 268(5219), 1899–1902 (1995).

64.   Rahme LG, Tan MW, Le L *et al.* Use of plant hosts to identify *Pseudomonas aeruginosa virulence* factors. *Proc. Natl Acad. Sci. USA* 94(24), 13245–13250 (1997).

65.   Garge S, Azimi S, Diggle SP. A simple mung bean infection model for studying the virulence of *Pseudomonas aeruginosa. Microbiology.* 164(5), 765–768 (2018).

66.   Powell JR, Ausubel FM. Models of *Caenorhabditis elegans* infection by bacterial and fungal pathogens. *Meth. Mol. Biol.* 415, 403–427 (2008).

67.   Marsh EK, May RC. *Caernohabditis elegans* a model organism for investigating immunity. *Appl. Environ. Bio.* 78(7), 2075–2081 (2007).

68.   Vega NM, Gore J. Stochastic assembly produces heterogenous communities in the *Caenorhabditis elegans* intestine. *PLoS Biol.* 15(3), e2000633 (2017).

69.   Cezairliyan BO, Vinayavekhin N, Grenfell-Lee D, Yuen GJ, Saghatelian A, Ausubel FM. Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans. PLoS Pathog.* 9, e1003101 (2013).

70.   Tan MW, Mahajan-Miklos S, Ausubel FM. Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc. Natl Acad. Sci. USA* 96(2), 715–720 (1999).

71.   Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. *J. Bacteriol.* 182(13), 3843–3845 (2000).

72.   Hill L. Evaluation of *Galleria mellonella* larvae for measuring efficacy and pharmacokinetics of antibiotic therapies against *Pseudomonas aeruginosa* infection. *Int. J. Antimicrob. Agents.* 43(3), 254–261 (2014).

73.   Racey D, Inglis RF, Harrison F, Oliver A, Buckling A. The effect of elevated mutation rates on the evolution of cooperation and virulence of *Pseudomonas aeruginosa. Evolution* 64(2), 515–521 (2010).

74.   Sibley CD, Duan K, Fischer C *et al.* Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog.* 4(10), e1000184 (2008).

75.   Lemaitre B. The road to Toll. *Nat Revs Immunol.* 4(7), 521–527 (2004).

76.   Quinn RA, Whiteson K, Lim YW *et al.* A Winogradsky-based culture system shows an associated between microbial fermentation and cystic fibrosis exacerbation. *ISME J.* 9(4), 1024–1038 (2015).

●   **Provides experimental *in vitro* evidence that significant chemical changes may be occurring at the onset of acute pulmonary exacerbations and suggests that these events are driven by changes in the composition of the microbial population.**

77.   Lopes SP, Azevedo NF, Pereira MO. Developing a model for cystic fibrosis sociomicrobiology based on antibiotic and environmental stress. *J. Med. Micro.* 307(8), 460–470 (2017).

78.   Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Curr. Protoc. Mol. Biol.* Chapter 25(Unit 25B.10), doi:10.1002/0471142727.mb25b10s94 (2011).

79.   Grahl N, Dolben EL, Filkins LM *et al.* Profiling of bacterial and fungal microbial communities in cystic fibrosis sputum using RNA. *mSphere.* 3(4), e00292–18 (2018).

80.   Cheng Y, Yam JKH, Cai Z. Population dynamics and transcriptomic responses of *Pseudomonas aeruginosa* in a complex laboratory microbial community. *NPJ Biofilms. Microbiomes* 5(1), doi:10.1038/s41522-018-0076-z (2019).

81.   Sriramulu DD, Lünsdorf H, Lam JS, Römling U. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *J. Med. Microbiol.* 54(7), 667–676 (2005).

82.   Haley CL, Colmer-Hamood JA, Hamood AN. Characterisation of biofilm-like structures formed by *Pseudomonas aeruginosa* in a synthetic mucus medium. *BMC. Microbiol.* 12(181), doi:10.1186/1471-2180-12-181. (2012).

83.   Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. *Proc. Natl Acad. Sci. USA.* 12(13), 4110–4115 (2015).

●   **The composition of a highly defined artificial sputum media (ASM) recipe is described, comparing the essential transcriptome of PA cultured within human derived sputum compared with this ASM found little difference in the essential gene expression-highlighting that this ASM may be particularly useful for culturing CF associated communities.**

84.   Frapwell CJ, Howlin RP, Soren O *et al.* Increased rates of genomic mutation in a biofilm co-culture model of *P. aeruginosa* and *S. aureus. BioRxiv* doi:10.1101/387233 (2018).

85.   Sousa AM, Monteiro R, Pereira MO. Unveiling the early events of *Pseudomonas aeruginosa* adaptation in cystic fibrosis airway environment using a long-term *in vitro* maintenance. *J. Med. Microbio.* 308(8), 1053–1064 (2018).

86.   Cornforth DM, Dees JL, Ibberson CB *et al. Pseudomonas aeruginosa* transcriptome during human infection. *Proc. Natl Acad. Sci. USA* 115(22), E5125–E5134 (2018).

87.   Tata M, Wolfinger TM, Amman F *et al.* RNASeq based transcriptional profiling of *Pseudomonas aeruginosa* PA14 after short- and long-term anoxic cultivation in synthetic cystic fibrosis sputum medium. *PLoS ONE* 11(1), e0147811 (2016).

88.   Nguyen AT, Oglesby-Sherrouse AG. Interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during co-cultivations and polymicrobial infections. *Appl. Microbiol. Biotechnol.* 100(14), 6141–6148 (2016).

89.   Limoli DH, Yang J, Khansaheb MK *et al. Staphylococcus aureus* and *Pseudomonas aeruginosa* co-infection is associated with cystic fibrosis related diabetes and poor clinical outcomes. *Eur. J. Clin. Microbiol. Infect. Dis.* 35(6), 947–953 (2016).

90.   Barth AL, Pitt TL. Auxotrophic Variants of *Pseudomonas aeruginosa* are selected from prototrophic wild-type strains in respiratory infections in patients with cystic fibrosis. *J. Clin. Microbiol.* 33(1), 37–40 (1995).

91.   Weigand MR, Sundin GW. General and inducible hypermutation facilitate parallel adaptation in *Pseudomonas aeruginosa* despite divergent mutation spectra. *Proc. Natl Acad. Sci. USA* 109(34), 13680–13685 (2012).

92.   Defoirdt T, Brackman G, Coenye T. Quorum sensing inhibitors: how strong is the evidence? *Trends. Microbiol.* 21(12), 619–624 (2013).

93.   Bjarnsholt T, Jensen PØ, Fiandaca MJ *et al. Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr. Pulmonol.* 44(6), 547–558 (2009).

94.   Lopes SP, Ceri H, Azevedo NF, Pereira MO. Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. *Int. J. Antimicrob. Agents.* 40(3), 260–263 (2012).

95.   Magalhães AP, Lopes SP, Pereira MO. Insights into cystic fibrosis polymicrobial consortia: the role of species interactions in biofilm development, phenotype, and response to in-use antibiotics. *Front Microbiol.* 7(2146), doi:10.3389/fmicb.2016.02146 (2017).

96.   Makovcova J Babak V, Kulich P, Masek J, Slany M, Cincarova L. Dynamics of mono- and dual-species biofilm formation and interactions between Staphylococcus aureus and Gram-negative bacteria. *Microb. Biotechnol.* 10(4), 819–832 (2017).

97.   Malhotra S, Limoli DH, English AE, Parsek MR, Wozniak DJ. Mixed communities of mucoid and non-mucoid *Pseudomonas aeruginosa* exhibit enhanced resistance to host antimicrobials. *MBio* 9(2), e00275–18 (2018).

98.   Woods PW, Haynes ZM, Mina EG, Marques CNH. Maintenance of *S. aureus* in co-culture with *P. aeruginosa* while growing as biofilms. *Front. Microbiol.* 9(3291), doi:10.3389/fmicb.208.03291 (2019).

99.   Spasenovski T, Carroll MP, Lilley AK, Payne MS, Bruce KD. Modelling the bacterial community associated with cystic fibrosis lung infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 29(3), 319–328 (2010).

100. Harrison F, Muruli A, Higgins S, Diggle SP. Development of an ex vivo porcine lung model for studying growth, virulence, and signalling of *Pseudomonas aeruginosa*. *Infect. Immun.* 82(8), 3312–3323 (2014).

101. Harrison F, Diggle SP. An ex vivo lung model to study bronchioles infected with *Pseudomonas aeruginosa* biofilms. *Microbiology.* 162(10), 1755–1760 (2016).

102. Filkins LM, Graber JA, Olson DG *et al.* Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives *S. aureus* towards fermentative metabolism and reduced viability in a cystic fibrosis model. *J. Bacteriol.* 197(14), 2252–2264 (2015).

103. Mou H, Brazauskas K, Rajagopal J. Personalized medicine for cystic fibrosis: establishing human model systems. *Pediatr. Pulmonol.* 50(S40), S14–23 (2015).

104. Henson MA, Orazi G, Phalak P, O'Toole GA. Metabolic modeling of cystic fibrosis airway communities predicts mechanisms of pathogen dominance. *mSystems.* 4(2), e00026–e00019 (2019).