Review

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Studying bacterial infections through culture-independent approaches

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The ability to characterize accurately the cause of infection is fundamental to effective treatment. The impact of any antimicrobial agents used to treat infection will, however, always be constrained by both the appropriateness of their use and our ability to determine their effectiveness. Traditional culture-based diagnostic microbiology is, in many cases, unable to provide this information. Molecular microbiological approaches that assess the content of clinical samples in a culture-independent manner promise to change dramatically the types of data that are obtained routinely from clinical samples. We argue that, in addition to the technical advance that these methodologies offer, a conceptual advance in the way that we reflect on the information generated is also required. Through the development of both of these advances, our understanding of infection, as well as the ways in which infections can be treated, may be improved. In the analysis of the microbiological content of certain clinical samples, such as blood, cerebrospinal fluid, brain and bone biopsy, culture-independent approaches have been well documented. Herein, we discuss how extensions to such studies can shape our understanding of infection at the many sites of the human body where a mixed flora, or in more ecological terms, a community of microbes, is present. To do this, we consider the underlying principles that underpin diagnostic systems, describe the ways in which these systems can be applied to community characterization, and discuss the significance of the data generated. We propose that at all locations within the human body where infection is routinely initiated within the context of a community of microbes, the same principles will apply. To consider this further, we take insights from areas such as the gut, oral cavity and skin. The main focus here is understanding respiratory tract infection, and specifically the infections of the cystic fibrosis lung. The impact that the use of culture-independent, molecular analyses will have on the way we approach the treatment of infections is also considered.

Introduction

The need to establish congruence between a pathological condition and a single causative agent, as required by Koch's postulates, has shaped the development of diagnostic microbiology. As such, this has been of great importance in characterizing many aetiological agents. Moreover, this approach defined the underlying philosophy of how microbes from many different environments have been studied. A process that isolates a single organism in axenic culture can be appropriate as an assay for certain types of infection. For instance, where a disease state results from the entry of a single causative agent into the bloodstream, as is the case with members of the genus *Borrelia* and Lyme disease (Coulter *et al.*, 2005). However, there is a growing need to consider infection within the context of a complex microbial milieu. This is reflected in the fact that bacteria infecting human tissues often

comprise mixed communities, particularly when mucosal barriers have been compromised (Brogden *et al.*, 2005). Furthermore, the expression of social behaviour by bacteria, such as the organization of structured bacterial communities in biofilms (Xavier & Foster, 2007; Nadell *et al.*, 2009) has significant, and typically adverse, consequences for therapy (Socransky & Haffajee, 2002; Costerton, 2005; Chavez de Paz, 2007; Christensen *et al.*, 2007; Tre-Hardy *et al.*, 2009). These findings alone already challenge the appropriateness of the study of bacterial infections based on single strains isolated through *in vitro* cultivation.

This may be further emphasized through the physiological states, such as viable but non-culturable, in which bacterial cells can exist (Colwell, 2000; Anderson *et al.*, 2004). Moreover, from many body sites, the number of cells that grow is typically less than those detected. This

phenomenon is illustrated by work carried out by Socransky *et al.* (1963), where it was estimated that approximately 50% of the cells in the debris from the human gingival crevice remained uncultured despite the use of chemically diverse growth media, and both aerobic and anaerobic conditions. The impact of physiological status, together with the impact of immune response and antibiotic therapy, on bacterial cells will be considered later in this work. However, to fully understand the process of an infection developing in areas that contain bacterial communities, such as the gut, the respiratory tract and skin, it must be considered within the complex microbial context.

An encounter with an organism capable of causing disease will not necessarily result in frank infection. The expression of a particular type of activity by the pathogen, the host or both may be necessary for infection to occur, with a wide range of factors, not least the composition and behaviour of the microbial community, potentially important. The complexities are illustrated by a number of phenomena, such as the ability of pathogenic bacteria to exist in a carrier state, where the host does not develop disease, but harbours an infective organism that may cause disease in those to whom it is transmitted. For example, *Neisseria meningitidis* or *Vibrio cholerae*, cause disease in some individuals but not others (Yazdankhah & Caugant, 2004; Vanden Broeck *et al.*, 2007). A further example is the ability of pathogenic bacteria to turn on or off disease-producing genes depending on circumstance, such as the production of exotoxins by *Clostridium difficile* (Voth & Ballard, 2005). Interactions between bacteria and host surfaces, therefore, represent a continuum, from transient contact, through colonization to pathogenic activity and ultimately infection.

Such considerations of course bring a far greater complexity to the examination of causative agents and their role in pathogenesis. Despite this, the possibility that a more extensive range of factors influence the development or the exacerbation of a disease cannot be ignored. Identifying these factors and understanding their impact may in itself provide an opportunity to predict, and ultimately modify, infection. For these reasons, molecular microbiological approaches that allow the elucidation of the mechanisms involved will result in a fundamental shift in the way in which infection is studied and understood. Before considering examples of infective processes, it is important to describe the molecular microbiological approaches that allow a culture-free means of analysis of clinical samples.

Development of bacterial community profiling

We know that natural environments contain a diverse array and number of microbes (Whitman *et al.*, 1998). Speculation over the association of microbes with disease also goes back thousands of years, with early reference to this made by Marcus Terentius Varro in 36 BC with a warning over the danger of locating homesteads near

swamps due to the presence of organisms that could cause disease, but which were too small to be seen (Cato & Varro, 1935). In 1546, Girolamo Fracastoro speculated about the existence of contagious particles (Fracastoro, 1930). However, it was not until the advances made in microscopy and microbiology by scientists such as Van Leeuwenhoek, Pasteur and ultimately Koch that identification of specific micro-organisms could be performed. These advances led to the idea that particular aetiological agents were associated with certain diseases. Furthermore, in setting out his postulates, Koch produced guidelines for establishing this relationship between a micro-organism and the causation of an infectious disease (Koch, 1884).

The principle that to isolate a microbe it must be separated from the bacterial milieu by enrichment culture – a process of selection using conditions favourable to its growth, whilst excluding other species – was further developed by Martinus Beijerinck (Brock *et al.*, 1994). This principle forms the basis of traditional, culture-based diagnostic microbiology, and persists to the present. Despite the effectiveness of this, even at the beginning of the 20th century there was a recognition that certain infections were in fact polymicrobial in nature (Loux & Coplin, 1902; White, 1902).

However, without a theoretical basis on which to construct models of polymicrobial activity, the identification of specific bacterial species in infections is limited in its predictive ability, and provides microbiologists with few insights (Prosser *et al.*, 2007). The process of developing such a basis is further hindered by the absence of the tools and disciplines of ecological theory from the contemporary mindset in microbiology (Prosser *et al.*, 2007). However, the potential importance of the polymicrobial nature of infections led to an investigation of these interactions, with parallels drawn from many areas of microbial ecology (Smith, 1982; Rotstein *et al.*, 1985; Brogden *et al.*, 2005; Brown & Buckling, 2008).

Culture-based diagnostic microbiology relies on our detection of aetiological agents through our ability to provide the conditions they require to grow *in vitro*. In many cases, even where the involvement of a specific pathogen is suspected, it can be extremely difficult to cultivate it in artificial media. This was illustrated by the difficulty encountered in identifying a causative agent for Whipple's disease. Here, despite Whipple reporting in 1907 the presence of rod-shaped micro-organisms in the vacuole of macrophages after silver-staining (Whipple, 1907), the agent remained obscure until 1999. Although *Tropheryma whipplei* was propagated in human fibroblast cells in that year (Raoult *et al.*, 2000), it was only through genomics-based design of a cell-free culture medium that *T. whipplei* was finally grown in axenic culture (Renesto *et al.*, 2003).

Being able to characterize accurately which bacterial species are involved in an infection becomes more difficult still when many different species are present. In such circumstances, the size of the pool of unknown species

cannot easily be determined and the ability to associate particular species with particular conditions is challenging. In gut microbial ecosystems, culture-independent surveys of 16S rRNA gene diversity have indicated that more than 75 % of the phylotypes detected in the human large intestine do not correspond closely to known cultured species (Suau *et al.*, 1999; Eckburg *et al.*, 2005; Flint *et al.*, 2007), an even greater proportion than reported by Socransky *et al.* (1963) using culture-based approaches.

The question of whether unculturable species exist is the subject of debate. Gest (2008) has pointed out that with sufficient effort and at least some cells in the correct physiological state, there is no fundamental boundary to *in vitro* cultivation. Indeed, as our understanding of niches such as the cystic fibrosis (CF) lung increases, so does our ability to replicate it as a growth environment *in vitro* (Palmer *et al.*, 2007a). However, it must also be recognized that where the effort required is prohibitive, some species become effectively unculturable in routine diagnostic microbiology. More fundamentally, where species cannot be cultured using standard media, under standard conditions, there may be no indication of their presence in a sample. In contrast to traditional culture-based diagnostics, molecular genetic approaches avoid the need for *in vitro* cultivation. In particular, the advent of PCR amplification provided a ready basis for the development of assays to exploit differences in DNA sequences. The importance of PCR to clinical microbiology is increasingly clear. Searching the PubMed database, the numbers of 'hits' for the annual number of publications linking those terms more than doubled in the decade since 1998. The range of applications of PCR (reviewed by Whelen & Persing, 1996) and real-time PCR (reviewed by Espy *et al.*, 2006) in clinical microbiology is equally increasing.

One of the greatest advantages conferred by PCR is that it allows detection of bacterial species to be based on nucleic acids extracted directly from clinical samples. The process typically involves the extraction of total nucleic acids from a clinical sample, followed by the amplification of the region of interest using specific oligonucleotide primers. In this way, the need for culture prior to detection is removed. Species-specific, culture-independent, PCR assays have been developed for the detection of a wide range of bacterial pathogens – *Clostridium difficile*, group B *Streptococcus*, *Bacillus anthracis* (Bergseng *et al.*, 2007; Kane *et al.*, 2008; Sloan *et al.*, 2008). By basing species identification on DNA sequence, the accuracy of identification is increased. Spilker *et al.* (2008) illustrated this in the context of CF lung infections by showing the high frequency of misidentification of *Bordetella* spp. by diagnostic microbiology laboratories based on culture analysis – a situation that has previously disguised the relatively high prevalence of these species in CF airway infections. Furthermore, these species-specific PCR assays confer a greater level of sensitivity of detection compared to conventional culture-based diagnostics (Chia *et al.*, 2004; Azzari *et al.*, 2008; Chiba *et al.*, 2009). This increased

sensitivity can be important, for example, when attempting to detect fastidious bacteria (Fenollar & Raoult, 2004). Equally, where the available specimen has been collected after antibiotic therapy, or where transportation conditions have been poor, this increased sensitivity may be crucial (Davies *et al.*, 2006; Rosey *et al.*, 2007). The more recent development of real-time PCR has not only provided a means to determine the bacterial load in a sample with a high degree of speed and accuracy, but also further increased the sensitivity of these assays (Rosey *et al.*, 2007).

Although these culture-independent approaches provide significant improvements in accuracy, the use of species-specific PCR techniques is equivalent to the use of selective media in culture-dependent approaches. They require a prediction to be made as to which agent is likely to be associated with a particular sample, and have a practical limit to the number of species-specific assays that can be performed. In partial response to this, multiplex PCR systems that can detect multiple species of interest have been developed for certain contexts. For example, Benson *et al.* (2008) developed an assay that detects gene-specific DNA sequences of six respiratory bacterial species (*Streptococcus pneumoniae*, *N. meningitidis*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae*). Systems such as this can be useful in rapidly detecting the presence of a number of known pathogens using a single assay. However, as with single-target PCR assays, multiplex PCR systems require predetermination of the bacterial species likely to be present in a given infection. Furthermore, these tests are used often with the assumption that detecting the presence of an agent is sufficient for pathology to be inferred. This theme will be reconsidered later; however, it is worth stressing again here that bacteria rarely exist in monocultures and that species–species interactions can profoundly affect the behaviour of individual species (Wuertz *et al.*, 2004; Dubey *et al.*, 2006; Andersson *et al.*, 2008; Van der Heijden *et al.*, 2008).

The identification of regions of particular phylogenetically informative genes that are conserved across large sections of the domain Bacteria offers an alternative approach. These conserved regions can be used to amplify sequences from any bacterial content of a sample, rather than just a single species. This represents a fundamentally different process to either culture-based assays, species-specific PCR or multiplex PCR. Also key to this process is that between the conserved regions there is sufficient sequence variation to allow species discrimination, allowing detection of bacterial species without the need to predict which pathogen(s) may be present. Whilst a number of genes have been used for such 'broad-range PCR', the most important of these phylogenetically informative regions is the 16S rRNA gene, which contains both the highly conserved and highly variable regions required (Clarridge, 2004). This can be performed either on strains of bacteria already isolated, or as will form the focus later, directly on clinical samples. This process has become

increasingly important to clinical microbiology – since their introduction, ribosomal gene sequencing studies have allowed the identification of novel bacterial species from human samples (Woo *et al.*, 2008). Arguably, one of the main reasons to use PCR though has been in relation to the analysis of samples regarded as culture negative. Here, studies have shown the importance of broad-range PCR in securing a diagnosis where traditional culture has not been successful (Harris & Hartley, 2003).

Working with samples containing many different species of course means that a mix of PCR products will be generated. To resolve information from these products, one or more strategies can be applied. Of these, the most commonly used include single strand conformation polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, 16S rRNA gene sequencing and terminal RFLP (T-RFLP) profiling (Nocker *et al.*, 2007; Juste *et al.*, 2008; Malik *et al.*, 2008). Each of these techniques exploit the variable internal regions directly or indirectly to resolve the different PCR products into separate species or groups of species. The selection of the technique to be used and the sequence to be targeted depend on both the characteristics of the community to be analysed and the type of data that is required (Nocker *et al.*, 2007; Juste *et al.*, 2008). Such broad-range PCR-based assays have been widely used to detect bacteria in samples as clinically diverse as heart-valve material, cerebrospinal fluid and synovial fluid (Jalava *et al.*, 2001; Rothman *et al.*, 2002; Gauduchon *et al.*, 2003; Podglajen *et al.*, 2003; Saravolatz *et al.*, 2003; Schuurman *et al.*, 2004), and have been shown to provide an increased level of sensitivity compared to conventional culture-based diagnostics (Rantakokko-Jalava *et al.*, 2000). The coupling of universal PCR with community profiling techniques therefore allows the direct characterization of the bacterial community in a clinical sample, and provides the theoretical ability to identify all bacterial species present, including those refractory to cultivation.

The ability to characterize accurately bacterial communities may be crucial if pathogenesis is related to changes in community composition. Such scenarios may arise in a number of contexts. For example, the development of vaginosis is believed to involve the modification of a normal bacterial flora to one that is associated with pathogenesis. This process involves the depletion of *Lactobacillus* populations, which are usually dominant in the vagina of healthy women, and an increase in a mixture of other bacterial species, often including *Gardnerella vaginalis*, Gram-positive anaerobic cocci such as *Peptostreptococcus* species, Gram-negative anaerobic rods such as *Prevotella* species, *Mycoplasma hominis* and *Ureaplasma urealyticum*, and sometimes *Mobiluncus* species (Hill, 1993; Pybus & Onderdonk, 1999; Persson *et al.*, 2009). Another example is in the development of human dental caries. Here, the expression of certain virulence factors by *Streptococcus mutans*, an important aetiological agent, has been shown to be inhibited by other species of

oral bacteria through interference with their cell–cell signalling mechanism (Wang & Kuramitsu, 2005). Furthermore, reports of the role of interactions associated with the development of disease in the oral cavity are numerous (Grenier & Mayrand, 1986; Liljemark & Bloomquist, 1996). Moreover, multispecies bacterial infections can occur in regions normally free from bacteria, such as in the development of chronic respiratory infections that occur in CF or chronic obstructive pulmonary disease (COPD) airways (Rogers *et al.*, 2003, 2004; Katznelson, 2006; Sibley *et al.*, 2006; Veeramachaneni & Sethi, 2006; Harris *et al.*, 2007). Not all ‘invasions’ are detrimental to the host though, as, for example, probiotic bacteria can be exploited to prevent the pathogenic effects of bacteria (Cremonini *et al.*, 2001; Hamilton-Miller, 2003; Lutgendorff *et al.*, 2008).

Combined, this means that an incomplete, and possibly distorted, picture of infection is obtained when only selective assays are employed. Knowing that alone, however, raises the question of what types of data are most clinically informative. To be able to understand this, we need first to understand more about the bacterial community through intensive study of a number of model systems such as the CF lung.

Why study the bacterial communities associated with the CF lung?

The lower respiratory tract of CF patients represents an ideal habitat for investigating the processes that are involved in the development and dynamics of polymicrobial infections (Table 1). In CF, the basic defect that results in abnormal functioning of the CF transmembrane conductance regulator protein principally relates to abnormal ion transfer across epithelial cell surfaces, resulting in impaired mucociliary clearance (Clunes & Boucher, 2007; Coakley & Boucher, 2007). The lungs of patients with CF are normal *in utero* and, before the onset of infection and inflammation, represent as favourable an anatomical niche for bacterial colonization as those without CF. After birth the lower airways are exposed to a diverse range of bacteria, amongst other material, through their constant ventilation and close proximity to the communities of the upper respiratory tract and oral cavity. In healthy individuals the mucociliary escalator helps to remove bacteria from the lower airways. However, when these systems fail to function efficiently, as is the case in CF (Matsui *et al.*, 1998; Boucher, 2004), bacteria that gain access to these regions may begin the process of colonization (Accurso, 1997). Although poorly understood at present, this process may begin shortly after birth, with a typical progression towards chronic infection and inflammation.

Once colonization has occurred, lung infections in CF patients are characterized by periods of relative stability, punctuated by infective exacerbations (Goss & Burns, 2007). These exacerbations have a significantly negative

Table 1. Rationale for studying chronic lower respiratory infections (LRIs)

	Comment/reference
Clinical importance	LRIs are thought to be the third most important cause of mortality globally accounting for more than 4 million deaths annually (Murray & Lopez, 1997). In 2004, the World Health Organization estimated that LRIs were responsible for 6.8 % of deaths worldwide (WHO, 2004).
Economic burden	The treatment of LRIs represents a significant proportion of total healthcare expenditure (Monte <i>et al.</i> , 2008).
Complex bacterial communities	Chronic LRIs are polymicrobial (Sibley <i>et al.</i> , 2006; Sethi & Murphy, 2008), and can involve higher bacterial interactions both within species, for instance, in the formation of biofilms (Kobayashi, 2005), and between species, resulting in changes to virulence levels (Sibley <i>et al.</i> , 2008a).
Diverse array of niches	The lower respiratory tract provides a range of environmental niches that can be colonized by bacteria, including, in some disease states, anaerobic environments (Worlitzsch <i>et al.</i> , 2002; Yoon <i>et al.</i> , 2002).
Lack of understanding of the role of infective bacteria	The number of bacterial species associated with chronic respiratory infections is increasing, with the role of the majority as yet unknown. However, it must be assumed that each has the potential to adversely affect patient health.
Lack of effective treatment	Despite repeated high dose courses of intravenous antibiotics, the impact on colonizing flora is modest, and, at best, results in a management of infective exacerbations.
Transfer of knowledge	LRIs, such as CF, can be used as models to investigate bacterial behaviour in infection, generating knowledge that can be transferred to other polymicrobial infections.

effect on both quality of life (Britto *et al.*, 2002) and survival (Liou *et al.*, 2001), with a median predicted survival in the UK of 35.2 years (CFT, 2007). During exacerbations, antibiotics are administered to reduce sputum bacterial load (Ramsey, 1996) with an expectation of an improvement of pulmonary symptoms (Regelmann *et al.*, 1990).

For many years, culture-based diagnostic microbiology has been employed to characterize the microbial content of CF sputum in an attempt to gain a better understanding of the relationship between infection and disease. Based on this process, a relatively small number of bacterial species have been considered as having an important role in CF lung disease (Razvi & Saiman, 2007), most notably *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Staphylococcus aureus*, *H. influenzae* and *Stenotrophomonas maltophilia* (Gilligan, 1991; Heijerman, 2005). Several studies have documented additional species in the CF lung, albeit less frequently, and include among others *Achromobacter* spp., *Pandoraea* spp., *Ralstonia* and non-tuberculous mycobacteria (Gilligan, 1991; Coenye *et al.*, 2002; Lambiase *et al.*, 2006).

Our original working hypothesis was that, in common with the findings of these culture-based studies, a range of species wider than those traditionally associated with the CF lung would be detected using culture-independent approaches. Given the importance of maintaining lung function in these patients, we also hypothesized that any bacterial species might be significant in the progression of lung disease. An ability to manage more effectively chronic bacterial infections in the CF lung could dramatically improve both the longevity of CF patients and their quality of life. Some of the first information on this came in 2003 through the culture-independent approach of T-RFLP profiling, which involves electrophoretic resolution of PCR

products based on the relative position of restriction sites. This technique was used to analyse the bacterial content of 92 sputum samples from adult CF patients attending a single CF clinic in the UK. On average, more than 14 separate terminal restriction fragment (T-RF) bands, representing one or more bacterial species, were detected in each of the patients. Nearly 80 % of the five most common species detected were not any of the five key CF species (*P. aeruginosa*, *Burkholderia cepacia* complex, *Staphylococcus aureus*, *H. influenzae* and *Stenotrophomonas maltophilia*). Furthermore, extensive 16S rRNA gene clone sequence analysis showed that these additional species came from a wide range of different phylogenetic branches of the domain Bacteria (Heijerman, 2005).

It is difficult to deal succinctly with such a wide diversity of species. To give some insight though, the 'key' CF pathogens fall into two divisions of bacteria – Proteobacteria (*H. influenzae*, *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Alcaligenes xylosoxidans*) and Firmicutes (*Staphylococcus aureus*). Culture-independent studies have shown that these bacterial divisions are also represented by species of the genera *Veillonella*, *Streptococcus*, *Abiotrophia* and *Gemella* (Firmicutes), and *Neisseria* and *Acinetobacter* (Proteobacteria). However, culture-independent studies have further shown that species belonging to three other bacterial divisions are also commonly resolved: Actinobacteria (including *Actinomyces* spp., *Rothia* spp.), Bacteroides/Chlorobi (including *Prevotella* spp., *Porphyromonas* spp., *Capnocytophaga* spp., *Treponema* spp.) and Fusobacteria (*Fusobacterium* spp.). With the ever increasing capacity to sequence in depth the species in any clinical sample, the diversity observed will only rise further.

An unexpected finding of this first study was that genera such as the anaerobes *Veillonella* and *Prevotella* formed a

particularly well-represented group. However, this may be less surprising once the nature of the CF respiratory tract is considered in more detail. Once colonized, the CF airways are both chemically and physically diverse, containing complex nutrients and carbon sources. Importantly, they also contain regions with a range of oxygen potentials allowing both aerobic and anaerobic growth (Yoon *et al.*, 2002).

The process of determining the identities of all the species detected in studies such as these is ongoing, with the degree to which the bacteria in different individuals are similar yet to emerge. The need to determine the significance to CF lung disease of individual species is, however, clear. Initial studies on a limited number of adult CF patients indicated that the same set of species could be detected over the course of a year in each patient, despite the occurrence of periods of infective exacerbation and the extensive use of intravenous antibiotic treatment (F. A. Stressmann, A. Walker, G. B. Rogers, T. V. W. Daniels, A. Lilley, M. P. Carroll, C. J. van der Gast & K. D. Bruce, unpublished results). As such, this emphasizes the importance of determining the individual roles that these species play.

We also asked more directly how culture-based microbiology would compare to culture independent T-RFLP profiling of the bacterial communities present in the CF lung. For the purposes of this experiment, each band visualized represents a species. Examples of two such comparative analyses are shown in the profiles in Fig. 1.

Fig. 1. Two sets of T-RFLP profiles that have each been generated from both nucleic acids extracted directly from sputum samples and from cultures derived from those samples. Culture-based profiles involved DNA extraction from pooled cultures generated on blood agar, cystine-lactose-electrolyte-deficient agar, Columbia CNA agar, chocolate agar, *Pseudomonas*-selective agar and *Burkholderia cepacia* complex-selective media in aerobic culture at 37 °C. Arrows indicate notable differences in the T-RF banding patterns.

Here, two CF sputum samples have been divided, with one aliquot being used to inoculate the range of media routinely used for CF diagnostic microbiology, the other being subjected to culture-independent analysis. All the material cultured after 40 h was subjected to nucleic acid extraction. DNA was also extracted, without cultivation, from the second aliquot. Once the ribosomal genes were amplified and analysed by T-RFLP profiling, some marked and fundamental differences were seen. Discrepancies in the ability to detect bands derived from different species using the two approaches, or differences in the band intensities obtained, could be due to a number of factors. Firstly, it must be recognized that like all techniques, culture-independent approaches themselves can introduce bias, e.g. in terms of primer selectivity, threshold over background and differential cell lysis (Schutte *et al.*, 2008). This topic has been reviewed extensively (Von Wintzingerode *et al.*, 1997). Whilst this issue needs to be further addressed in relation to clinical microbiology, other factors may in fact be more significant. Clearly, certain bacterial species grow particularly well in the culture conditions used, for example *Staphylococcus aureus*. Moreover, species that require particular growth conditions that are not part of routine CF sputa diagnostic microbiology were detected in the culture-independent approach, for example those requiring anaerobic conditions most notably here again within the genus *Prevotella*. Many species that fall into this second group are present at levels comparable to, or often greater than, those of species considered previously to be the key CF pathogens. Currently, however, the presence of these species is not taken into account, with treatment strategies designed around the small number believed to be of clinical significance. Considering this further, conventional culture-based diagnostics has in certain studies shown little correlation with clinical parameters (Gibson *et al.*, 2003) or with culture-independent studies (Rogers *et al.*, 2009). For this reason, many of the current microbiological data generated are often viewed as being of little value by clinicians when selecting therapeutic interventions. Furthermore, diagnostic analysis, as currently employed, is unlikely to identify pathogens that are emerging, such as *Burkholderia pseudomallei* (Holland *et al.*, 2002; O'Carroll *et al.*, 2003) and the '*Streptococcus milleri*' group (Parkins *et al.*, 2008), let alone those not previously described. This last point has been highlighted through the wide range of previously unreported potential pathogens identified through the use of broad-range molecular analyses (Rogers *et al.*, 2004; Bittar *et al.*, 2008; Sibley *et al.*, 2008b).

So far, this review has discussed some of the disadvantages of the use of conventional culture-based approaches in the characterization of polymicrobial infections. Furthermore, the development of the culture-independent techniques, which have the potential to circumvent many of those flaws, has been described. However, it is now important to consider how the application of these approaches can best be used to develop our understanding of the processes

involved in infection, again, using CF lung infections as a model system.

Healthy and diseased airway flora – sampling and diversity

In healthy, non-CF individuals, bacteria are cleared from the airways, and are, therefore, denied the opportunity to colonize them. Despite this, induced sputum samples taken from healthy individuals were found to contain far higher numbers of bacterial species, albeit at much lower loads, than had been obtained for expectorated CF sputa (Rogers *et al.*, 2005b). Setting aside the difference in the sample collection method, the apparent difference in diversity between the two sample sets is likely to be due to the presence in the CF airways of some species in very high numbers. This will have the effect of masking the presence of other less prevalent, non-colonizing species by pushing them effectively below a threshold of detection.

Furthermore, the exposure of the airways to inhaled bacteria, as well as the existence of bacterial flora in the upper airways and oral cavity, may affect the composition of CF clinical samples. As described above, this seems to be the case with certain oral anaerobes being detected in the CF lung. Whilst, in comparison to established populations in the lower airways, inhaled bacteria are likely to be present in very low numbers, the potential for contamination of CF respiratory samples with upper airway flora was a concern. Sputum passes through the upper airways and oral cavity during expectoration, and a significant proportion of the bacteria isolated by conventional microbiology are classified as 'oral flora' in diagnostic reports. The presence of such species as a clinically significant factor would require a fundamental change in the way routine microbiological data are interpreted. For this reason, early reports of anaerobic species, known to colonize the oral cavity, in CF sputum were somewhat controversial. However, as more evidence of a substantive role of anaerobic species in CF lung infections is generated (Sibley *et al.*, 2008b; Tunney *et al.*, 2008) the need to consider the implications of their presence in CF sputa increases. For these reasons it is essential to establish whether anaerobic species are genuinely colonizing the lower airways. There are a number of strategies by which this can be achieved.

One way to address the problem of contamination is to analyse samples with minimized exposure to upper airway bacteria. These include bronchoalveolar lavage (BAL) samples, where material can be collected from the lower airways using a protective brush to prevent contamination during the introduction and removal of the bronchoscope. A limited study of BAL samples has indicated the presence of 'oral flora', including *Prevotella* spp. and *Veillonella* spp., in the lower airways of CF infants (G. B. Rogers, M. S. Payne, J. P. Legg, G. J. Connett, F. A. Stressmann, A. Walker, T. V. W. Daniels, M. P. Carroll & K. D. Bruce, unpublished results). Another approach is by the analysis

of samples that have not passed through the upper airways and oral cavity, and are, therefore, less exposed to contamination, such as samples obtained as trans-tracheal aspirates (TTA). TTA analysis is, however, rarely performed due to the highly invasive nature of the sample collection procedure. Despite this, aerobic and anaerobic culture of TTA samples from paediatric patients has revealed the presence of anaerobes, including *Veillonella* species, in the majority of cases (Brook & Fink, 1983). Furthermore, comparison of sputum and mouthwash samples from CF patients using molecular profiling techniques showed sputum samples not to be subject to profound contamination by oral cavity bacteria, and provided further evidence of colonization of the CF lung by oral bacterial species (Rogers *et al.*, 2006). Despite studies of this type, further work is required to establish the size and nature of these populations of anaerobic bacteria in the lower airways. More generally, however, we are still left with communities of CF lung bacteria that are present but not as mere contaminants of sampling methodology.

How are the bacterial communities in the CF lung established?

The difficulty of treating established bacterial communities in CF patients has led to great importance being attached to determining the mechanisms that drive community development in paediatric patients. A better understanding of the mechanisms key to this process would offer an opportunity to disrupt it. Attempts to elucidate these mechanisms are, however, hampered by the fact that infants rarely produce sputum, and material collected from higher up the respiratory tract may not predict lower respiratory pathogens (Ramsey *et al.*, 1991; Armstrong *et al.*, 1995).

Whilst limited in the depth of data that they can provide, and remaining focused on species of known clinical significance, culture-dependent analyses have given some insights into the mechanisms that may be involved in airway colonization. For example, data derived using this approach indicate that *Staphylococcus aureus* and non-encapsulated *H. influenzae* are isolated by culture-based microbiology early in life, whereas nearly all CF patients become infected with *P. aeruginosa* over time (Renders *et al.*, 2001). Furthermore, as patients acquire *P. aeruginosa*, *Staphylococcus aureus* tends to be detected less frequently (Machan *et al.*, 1992; CFF, 2007), although both species are commonly co-isolated (Hoffman *et al.*, 2006). A mechanism for this relationship has been suggested involving the production by *P. aeruginosa* of an anti-staphylococcal 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) when co-infecting CF airways with *Staphylococcus aureus* (Machan *et al.*, 1992). However, this compound also protects *Staphylococcus aureus* during co-culture from commonly used aminoglycoside antibiotics such as tobramycin (Hoffman *et al.*, 2006). Furthermore, it has been shown that prolonged growth of *Staphylococcus aureus* with *P.*

aeruginosa selects for typical *Staphylococcus aureus* small-colony variants, which have stable aminoglycoside resistance (Miller *et al.*, 1980) and are persistent in chronic infections, including those found in CF (Proctor *et al.*, 2006). It has, therefore, been suggested that *Staphylococcus aureus* density within CF airways reflects a balance between the suppressive effects of antibiotics and HQNO, and HQNO-mediated protection from aminoglycosides (Hoffman *et al.*, 2006).

Many of the processes likely to be important in the bacterial colonization of CF airways, either within or between species, are mediated by both concentration-dependent auto-inducer-mediated signalling systems (quorum sensing) (Waters & Bassler, 2005) and concentration-independent signalling systems (Lee *et al.*, 2007). Quorum-sensing systems, in particular, have been implicated in a number of activities that have a significant clinical impact in chronic respiratory infections, for example, the formation of biofilms (Parsek & Singh, 2003; Kobayashi, 2005), the production of virulence factors such as pyocyanin and elastase (Winstanley & Fothergill, 2009), and the production of bacteriocins (Fontaine *et al.*, 2007).

In addition to determining the behaviour of particular species in CF (Molina *et al.*, 2008; Moreau-Marquis *et al.*, 2008), there is also evidence from some culture-based studies that quorum sensing is implicated in the polymicrobial nature of these infections. Cross-talk between the quorum-sensing systems, involving the recognition of the signal produced by a different species, has been shown to be capable of affecting the behaviour of CF pathogens. Unidirectional cross-talk occurs between *P. aeruginosa* and *Burkholderia cepacia* complex, involving the recognition by *Burkholderia cepacia* of the C4 3-oxo-C12 homoserine lactones produced by *P. aeruginosa* at low concentrations to activate its *cep* quorum-sensing system (Eberl & Tümmler, 2004). Since *P. aeruginosa* colonization often precedes *Burkholderia cepacia* in CF infections, a non-specific quorum sensing could be one mechanism by which the latter is able to develop as a multispecies community with *P. aeruginosa*, and thereby colonize the lower airways (Jayaraman & Wood, 2008). Furthermore, quorum-sensing systems are known to be employed by a wide range of bacterial species (Jayaraman & Wood, 2008). This is suggestive not only of a potential mechanism by which CF bacterial communities might be structured, but also of a possible means by which the development of infections could be disrupted (Janssens *et al.*, 2008; Kiran *et al.*, 2008).

In addition to direct interaction through quorum-sensing systems, diverse bacterial communities also provide an opportunity for horizontal gene transfer. This is a process that allows for rapid transfer of genes under strong selection, such as genes that encode antibiotic resistance (Salysers *et al.*, 2004). The facilitation of horizontal gene transfer has clear implications for bacterial communities in infections, particularly those that have high exposure to antibiotics, as in the CF lung.

Succession, the orderly process of community development moving towards a state of equilibrium (Odum, 1969, Cotgreave & Forseth, 2002) may also play a role. There is evidence for the involvement of succession in the establishment of bacterial communities in non-CF clinical contexts. For example, the colonization of the neonatal gut (Favier *et al.*, 2002; Palmer *et al.*, 2007b), the establishment of bacterial flora in the oral cavity (Nyvad & Kilian, 1990; Li *et al.*, 2004; Jenkinson & Lamont, 2005; Kolenbrander *et al.*, 2006) and the development of infections in the root canal system (Fabricius *et al.*, 1982; Tani-Ishii *et al.*, 1994; Siqueira, 2002).

It is clear that these are dynamic processes. The presence of non-invasive micro-organisms in the airways can lead to damage to the respiratory mucosa due to the production of exotoxins, leading to inhibition of ciliary function and damage to the bronchial epithelium (Denny, 1974; Wilson & Cole, 1988; Kanthakumar *et al.*, 1993), inhibition of mucociliary transport (Munro *et al.*, 1989; Read *et al.*, 1992), alteration of respiratory epithelial ion transport (Stutts *et al.*, 1986; Graham *et al.*, 1993), and stimulation of mucus secretion (Somerville *et al.*, 1992). These processes have been extensively reviewed by Cole (1997).

A model of the process by which infection is established in skin wounds has been developed (Edwards & Harding, 2004) and parallels can be drawn between skin wounds and the CF airways. Both of these contexts represent a hitherto sterile niche, but one that is proximal to an existing bacterial flora present on the surface of the skin, or the upper airways in the case of CF. As such, many of the key components of the model developed for colonization of skin wounds may be reflected in chronic lower airway infections.

In this model, the path to the establishment of infections involves a number of separate stages: contamination – the presence of non-replicating organisms; colonization – the presence of replicating micro-organisms in the absence of tissue damage; and finally infection – the presence of replicating organisms with subsequent host injury (Dow *et al.*, 1999). The transition to infection is influenced by a number of factors, particularly the number of bacteria (g tissue^{-1}), the virulence and pathogenicity of the organism, and the ability of the host to mount an effective immune response (Wysocki, 2002). The infective dose required for infection varies from species to species, and is influenced by the organism's interactions with surrounding microflora (Bowler, 2003). In an analogous CF model, factors such as the ability to clear bacteria from the airways, and the impairment of the immune response, may influence the point at which a threshold bacterial load is reached, thus, making colonization possible.

The timescale over which a bacterial community develops in the CF airways appears to be short. Studies of the microbial content of BAL samples have indicated that, by 3 months of age, nearly 40% of infants identified by a neonatal CF screening program had a lower respiratory

tract infection (Davis, 1999). Furthermore, culture-independent T-RFLP analysis of BAL samples from sputum-producing paediatric patients showed that, even at an early age, there were no significant differences between the bacterial communities detected and those found in samples from CF adults in terms of diversity (Rogers *et al.*, 2005a). These findings are of critical importance given the fact that current treatment practice for these patients is aimed at preventing initial colonization and eradication of populations of particular species believed to be of key clinical significance (Balfour-Lynn & Elborn, 2007).

Whilst there is evidence that the diversity of bacterial communities in the airways of CF patients increases during early life, the division of patients into 'paediatric' and 'adult' is not necessarily relevant when considering airway infection. The age at which the bacterial communities in these infections reach a given level of complexity could be influenced by a range of factors and may vary considerably between individuals. It is not yet clear whether a 'climax community' exists, or whether the community is in a continued state of flux coupled with a decline in respiratory function. However, it is possible that particular species might be representative of particular stages in the process of development, and as such, may be clinically informative.

Can a community be regarded as pathogenic?

In a thoughtful paper, Jenkinson and Lamont raised the possibility of a microbial community being pathogenic (Jenkinson & Lamont, 2005). This would be the most potentially alarming consequence for the human host – that the community together is more damaging than any individual component species alone. The evidence is increasing that this may be more than just a hypothetical construct.

The ability of members of the bacterial flora to influence the virulence of *P. aeruginosa* in CF respiratory infections has been investigated using a *Drosophila melanogaster* infection model. Sibley *et al.* (2008a) demonstrated that in this model environment oropharyngeal species could be classed into three distinct groups – in addition to species that were either virulent, or avirulent regardless of the presence or absence of *P. aeruginosa*, there was a further group that were not pathogenic alone, but in combination with *P. aeruginosa* dramatically reduced the survival rates of the host organism. These data clearly suggest that the dismissal of oropharyngeal flora as clinically insignificant when identified in sputum samples could be short sighted. Therefore, the scope for such interactions to influence the virulence of key pathogens, such as *P. aeruginosa*, is greatly increased in a bacterial community containing many different species. This ability of 'non-pathogens' to influence the behaviour of pathogens again underlines the inappropriateness of considering, in isolation, individual members of polymicrobial infections.

A further level of complexity is provided by the fact that, with time, the process of interaction with the host may lead to changes in the genomes of infecting organisms. For example, it has been shown that *P. aeruginosa* strains present in advanced CF infections differ systematically from those of 'wild-type' *P. aeruginosa* (Smith *et al.*, 2006; Yang *et al.*, 2008). This process involved the mutation of many genes that code for virulence factors important in the process of establishment of infection, including genes functioning in O-antigen biosynthesis, type III secretion, twitching motility, exotoxin A regulation, multidrug efflux, osmotic balance, phenazine biosynthesis, quorum sensing and iron acquisition (Smith *et al.*, 2006). The reasons for this are not fully clear. However, strong conflict and selection pressures can arise among multiple species and strains in biofilms, and spontaneous mutation can generate conflict even within biofilms initiated by genetically identical cells (Diggle *et al.*, 2007; Nadell *et al.*, 2009). In this way, the same bacterial species may result in very different clinical outcomes at different stages of infection.

Again, further insights into the processes involved in CF infections can be gained from examination of polymicrobial infections in other contexts. For example, important parallels can be drawn for the study of chronic wounds. Wounds, such as those associated with venous leg ulceration, are often colonized by bacterial communities comprising more than 20 separate species (Bowler & Davies, 1999; Davies *et al.*, 2004; Gjodsbol *et al.*, 2006; Dowd *et al.*, 2008). Significantly, the diversity of the bacterial communities in these wounds influences their size and healing time (Robson *et al.*, 1999; Bowler *et al.*, 2001; Ryan, 2007). Crucially, it is the number of different bacterial species present that relates to wound healing, rather than certain individual bacterial species. Specifically, it has been shown that the presence of four or more bacterial species in such wounds correlates positively with non-healing (Hill *et al.*, 2003). This significance of polymicrobial nature of the communities suggests key roles for synergistic processes (Bowler, 2003).

Therefore, through greater characterization of the bacterial communities present in CF airway infections, it may be possible to establish links between particular community traits, relating to either their composition or their activity, and poor clinical prognosis. Furthermore, these traits may originate not from the presence of particular species in the bacterial community, but from the interactions between different community members, and between the community and the host. As such, it may be helpful to consider such communities as being, in themselves, pathogenic. Extending this, of course, is the realization that community pathogenicity is not 'fixed', and may be differentially realized at different times due to host, microbial, or other, factors. This suggests, therefore, that greater efforts will be required to understand pathogenic community dynamics. We consider this to be a particularly pressing issue given its potential clinical significance.

Tailoring molecular profiling to the study of dynamic systems – a second generation of community profiling

Generating snapshots of bacterial communities may help to develop a clearer picture of what constitutes healthy or disease community characteristics, or to identify species whose presence may be clinically significant. However, such information is of relatively little interest if our aim is to be able to predict and prevent infections, or ameliorate exacerbations. For this, we need to apply culture-independent profiling longitudinally.

When studying dynamic systems, it is essential that what is measured changes as rapidly as the community that it represents. Bacterial community profiling has typically involved using total DNA extracted directly from a sample as a template for PCR amplification. However, this has disadvantages when used to characterize short-term changes in community structure. Firstly, measurements based on DNA respond only slowly to changes in bacterial populations. DNA-based signals generated from bacteria that are highly metabolically active, senescent or dead can be of equal intensity. This is a serious concern. For example, following antibiotic treatment for patients with infective endocarditis, the process of clearing bacterial DNA can be slow (Rover *et al.*, 2005). Rover *et al.* (2005) also showed DNA from different species differing in the length of persistence, with for example, streptococcal DNA detected for longer than other bacterial species. Furthermore, due to the stability of extracellular DNA in the CF airways, there is potential for signals to be generated from DNA released from lysed bacterial cells long after they have ceased to be viable. In the same way, the transition between a senescent population and a rapidly growing one can only be detected once the population has expanded. For these reasons, attempts to detect the impact of therapies using DNA-based systems are severely hampered, and the diagnostic value of techniques of this kind are reduced.

However, the contribution of both extracellular DNA and DNA that is derived from bacterial cells that are no longer viable can be minimized. Propidium monoazide (PMA) added to the clinical sample prior to nucleic extraction, is able to enter cells whose structural integrity has been lost and intercalate into their DNA. Exposure to a bright light source causes covalent cross-links to form, rendering the DNA unable to act as a PCR template. In contrast, PMA is unable to enter cells whose cell wall is intact. Treatment of samples in this way results in bacterial community profiles being generated solely from DNA contained within viable bacteria (Fig. 2).

Application of this approach to CF sputum samples suggests that the presence of dead bacterial cells can significantly bias profiles from samples that are untreated with PMA (Rogers *et al.*, 2008). This is of clear importance in scenarios such as the CF lung where the impact on

Fig. 2. Two sets of T-RFLP profiles that have each been generated from untreated and PMA-treated CF sputum sample aliquots. Arrows indicate notable differences in the T-RF band intensities.

bacteria of both the host immune response and antibiotics is high.

The use of procedures such as these to base community profiling on only those bacteria that have the potential to have a clinical impact offers many new opportunities. For example, they could reveal the degree to which antibiotic therapies are bactericidal towards different populations within the overall community. However, what they cannot do is indicate the degree to which therapies have a bacteriostatic impact. Furthermore, they will not differentiate between a population that is limited in some way, and therefore has a low metabolic rate, and one that is highly metabolically active. This is an issue that can be addressed, however, by switching of the basis of the bacterial community profiling from rRNA genes to rRNA. In this way, it is possible to limit bacterial community profiles to only those cells that are metabolically active.

By reverse transcribing 16S rRNA extracted directly from samples, a pool of complementary DNA (cDNA) is generated that can then be used as template for PCR. Whilst the number of rRNA gene copies per cell is fixed for a given species, the number of rRNA copies varies with metabolic activity – the higher the metabolic rate the more ribosomes required for transcription. Therefore, by performing DNA- and RNA-based profiling on a sample in parallel, it is possible to determine both the relative

abundance of a bacterial species, and its relative metabolic activity. This strategy has been successfully applied to CF sputum samples and has revealed that, within individual samples, different populations of bacteria range from senescent to highly active (Fig. 3) (Rogers *et al.*, 2005b). Such an approach could be particularly valuable in identifying early positive or negative responses to antimicrobial treatments.

By providing an indication of the relative metabolic rates of different community members, comparative profiling of ribosomal genes and their RNA transcripts offers the potential to be informative regarding a number of key processes. Firstly, this approach can indicate where populations are senescent, and as such represent organisms that, whilst possibly being of reduced clinical significance at the time of profiling, have the potential to rapidly become highly significant over a short period of time were circumstances to change. Secondly, where highly metabolically active populations are identified, the likely expansion of a population can be predicted. In this way, it may be possible to intervene early and thereby reduce the clinical impact of an exacerbation. Finally, it is known that metabolic activity levels can influence the impact of antimicrobial therapies (Pamp *et al.*, 2008); therefore, determining the levels of metabolic activity of populations of bacteria within the community could inform of the likely degree of success that different therapeutic interventions would have. Furthermore, such data concerning the dynamics of polymicrobial infections would greatly advantage attempts at their treatment in a wide range of clinical contexts, and the need to include such approaches in the more general study of infection is clear.

Fig. 3. Regions of two T-RFLP profiles generated from a single sample. The profile on the left was generated from rDNA, whereas the profile on the right was generated from reverse transcribed rRNA. The relative intensities of T-RF bands produced by a given bacterial population indicate that there is a range of levels of metabolic activity in the community, ranging from inactive to highly active.

Comparative profiling approaches are not without their problems. The most significant of these is ensuring that comparisons are made like with like. Whilst rRNA levels are determined by metabolic activity levels, rRNA gene levels are typically constant for a given species; however, they are not constant between species. The number of ribosomal gene copies can range from 1 to 15 (Bercovier *et al.*, 1986; Andersson *et al.*, 1995; Rainey *et al.*, 1996); therefore, having a significant effect on the size of the template available for amplification. Furthermore, whilst rRNA is relatively unstable and is broken down rapidly, DNA is able to persist in a sample long after the cell that produced it has died. This is likely to result in some bacterial populations appearing to be present but inactive, when in fact no viable population is present.

However, by combining some of the advances that have been made recently in community profiling, such as basing analysis on cDNA, and the use of PMA treatment to prevent the contribution of DNA from non-viable cells, it may be possible to overcome these issues and generate a bacterial community profile that accurately characterizes species prevalence, viability and metabolic activity. The development of such a strategy would greatly improve our ability to understand the underlying processes that govern the dynamics of polymicrobial infections. The issue of variation in DNA signals due to variation in ribosomal operon number is significant. However, it can be argued that it would be sufficient to base profiling on rRNA alone. Since levels of rRNA are proportional to activity of a given population, they can be equated with the clinical impact of that population.

Characterization of CF airway infections

For the potential of molecular microbiological analysis to be realized, it is important that it is applied to the study of chronic CF lung infections in a focused way. To be of greatest use in the treatment of patients, such investigations should be targeted towards answering three key sets of questions.

- (1) The treatment of established chronic bacterial infections in the CF lung is difficult to manage effectively. Ultimately, the prevention of such infections from becoming established should be a fundamental research goal. Drawing from this, we need to understand how bacterial communities develop. In other systems this occurs through a process of succession, to a 'climax community', in which a state of equilibrium is reached where the community changes little unless the environment is disrupted (Cotgreave & Forseth, 2002). Such climax communities have been reported in the human body, e.g. in gut microbiology (Falk *et al.*, 1998) and root canal infections (Siqueira *et al.*, 2002). Analogously, are we able to characterize an ordered progression towards a climax community in CF infections starting from the

initial bacterial colonization in infants? If so, can points be identified at which this process might be disrupted?

(2) If such climax communities exist in CF infections, what are their characteristics? Is there more than one type, and if so, what are their implications for disease severity and the treatment of infection? Currently, the presence of certain species is used as an indicator of both prognosis and response to certain interventions, for example, the presence of *Burkholderia cenocepacia* is associated with poor survival rates following lung transplantation (Boussaud *et al.*, 2008; Murray *et al.*, 2008). By extension, in CF infections, what characteristics relate to particular clinical markers and outcomes? This may be particularly important in relation to infective exacerbations.

(3) Can these data inform clinicians about the impact made by a course of treatment? Currently, the efficacy of the use of antibiotics to treat bacterial infections in the CF airways can only be assessed indirectly, primarily through measures such as lung function and general well-being. The degree to which interventions are successful in interfering with bacterial growth – their primary role – is not addressed and so cannot be used to shape treatment.

The success of the application of molecular microbiological profiling to CF lung infections will be determined by the degree to which it can address these questions.

Future considerations

The application of the molecular approaches outlined here to the study of chronic bacterial respiratory infections promises to greatly increase our understanding of the mechanisms of disease and provide a basis for improving therapeutic interventions. However, there are three key areas that should be addressed to ensure the advantages that they confer are fully exploited.

Firstly, in parallel to the use of molecular profiling to further our understanding of the basic processes of infection, it is important that they are also developed to provide frontline clinical diagnostics. For this to happen, a translational strategy is required to identify features of infection that are of high clinical significance and develop diagnostic protocols that can provide clinicians with data that are useful in the design of therapeutic regimes.

Secondly, a realization of the implications for treatment of the polymicrobial nature of CF airway disease must occur. Rather than being based on the clinical significance of species studied in isolation, a model of disease where the bacterial community as a whole is considered as the 'pathogen' would be helpful. As recognized in polymicrobial infections in other areas of the body, it is not necessarily possible to achieve eradication of infection, and instead the aim should be to achieve a host manageable bioburden (Bowler, 2003). As such, the suitability of treatment

practices such as the administration of 14 day courses of intravenous antibiotics in response to infective exacerbations should be reviewed. Originally designed to achieve eradication of bacteria in infections, such approaches may serve to increase antibiotic resistance in the bacterial community and drug-related side-effects in the patient, but do not necessarily provide substantial advantages over shorter antibiotic courses. Furthermore, the use of antibiotic prophylaxis, in an attempt to manage populations of bacteria between exacerbations, may be ineffective, and possibly counter-productive.

Finally, the development and refinement of molecular profiling technologies is constant. For example, more effective means of resolving mixed bacterial PCR products generated from multispecies templates are being devised. These include MS-based approaches, such as the Ibis T5000 system (Ecker *et al.*, 2006, 2008), that are able to derive species identities based on base composition signatures of the sequences amplified (Ecker *et al.*, 2006, 2008). In addition, ultra-high throughput sequencing (Braslavsky *et al.*, 2003; Margulies *et al.*, 2005; Shendure *et al.*, 2005; Bentley *et al.*, 2008) now offers the opportunity to generate highly detailed bacterial community composition data. It is vital that advances such as these are fully exploited in the characterization of CF lung infections.

Meta-community analysis has already been employed to determine the resistance reservoir in soil communities (Schmitt *et al.*, 2006; Allen *et al.*, 2008), and a similar approach could allow the determination of the total pool of resistance markers present in chronic respiratory infection or group of infections. This is particularly important given the mobile nature of many of the sequences that confer antibiotic resistance (Butaye *et al.*, 2003; Bennett, 2008) and the reduction in treatment efficacy that can result from their spread. Whilst this review has focused primarily on bacterial infection, clinical situations will often be further complicated by the presence of viruses or fungal species. The truly polymicrobial nature of many infections also needs to be recognized. Ways of studying this are increasing. For example, the development of culture-independent strategies for the characterization of fungal diversity (Bouchara *et al.*, 2009), as well as the increasing recognition of the value of molecular diagnostics in the detection of respiratory viruses (Mahony, 2008), offers an opportunity to examine more closely the relationships between these groups of pathogens. Whilst adding further complexity to our understanding of community pathogenicity, this process will, however, be necessary for the most appropriate means of treatment to be given. Other areas of interest include changing the level of phylogenetic resolution of total community profiling approaches, by target sequence selection, the profiling of strains within species of interest would be possible, or using the ability to determine changes in levels of expression of specific virulence genes *in vivo* using quantitative PCR to characterize responses to changes in patient health or treatment efficacy.

Conclusion

Data derived from molecular microbiological investigations of CF respiratory infections have led to the realization that they involve bacterial communities, with multiple diverse bacterial species identified in individual samples (Rogers *et al.*, 2004; Sibley *et al.*, 2008b). Clearly if some or all of the species new to CF lung microbiology are of relevance to the progression of lung disease then they need to be factored into clinical consideration. However, based on the evidence from both CF and wider contexts, it may be necessary to view the bacterial community as a pathogenic entity in its own right that can express different degrees of virulence at different times. There is an increasing recognition of the need to introduce novel systems to deal with many of the problems inherent to culture-dependent systems. A conceptual shift – towards an understanding of more complex diagnostic scenarios – is needed just as much as the technical means by which to deliver this on a routine basis. Whilst the work discussed here is aimed at benefiting CF patients, it is clear that achieving a deeper understanding of the processes involved in bacterial pathogenesis in this context will provide insight into the aetiology of other polymicrobial infections.

It is now 5 years since Raoult *et al.* (2004) published their thought-provoking article on the future of clinical microbiology. There are clear trends in the direction of much of what they envisaged. Along with these technical and structural advances, the way that we view infection will also continue to change.

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