REVIEW 10.1111/j.1469-0691.2010.03236.x

Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients

F. Bittar and J.-M. Rolain

Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), CNRS-IRD, UMR 6236, Faculté de Médecine et de Pharmacie, Université de la Méditerranée, Marseille Cedex 05, France

Abstract

Respiratory infections remain a major threat to cystic fibrosis (CF) patients. The detection and correct identification of the bacteria implicated in these infections is critical for the therapeutic management of patients. The traditional methods of culture and phenotypic identification of bacteria lack both sensitivity and specificity because many bacteria can be missed and/or misidentified. Molecular analyses have recently emerged as useful means to resolve these problems, including molecular methods for accurate identification or detection of bacteria and molecular methods for evaluation of microbial diversity. These recent molecular technologies have increased the list of new and/or emerging pathogens and epidemic strains associated with CF patients. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of intact cells has also emerged recently as a powerful and rapid method for the routine identification of bacteria in clinical microbiology laboratories and will certainly represent the method of choice also for the routine identification of bacteria in the context of CF. Finally, recent data derived from molecular culture-independent analyses indicate the presence of a previously underestimated, complex microbial community in sputa from CF patients. Interestingly, full genome sequencing of some bacteria frequently recovered from CF patients has highlighted the fact that the lungs of CF patients are hotspots for lateral gene transfer and the adaptation of these ecosystems to a specific chronic condition.

Keywords: Bacterial diversity, cystic fibrosis, emerging bacteria, genome analysis, molecular identification, review

Clin Microbiol Infect 2010; **16:** 809–820

Corresponding author and reprint requests: J.-M. Rolain, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), CNRS-IRD, UMR 6236, Faculté de Médecine et de Pharmacie, Université de la Méditerranée, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France
E-mail: jean-marc.rolain@univmed.fr

Introduction

Cystic fibrosis (CF), the most common hereditary disease in Caucasian populations, results from mutations in the CF transmembrane conductance regulator (CFTR) gene and affects the function of almost all of the body's exocrine glands [1]. It is characterized by the production of abnormally viscous mucus by the affected glands, resulting mainly in impaired respiratory and pancreatic function. Respiratory infections, which start at an early age in the majority of people with CF, and airway inflammation represent the most serious threats during the disease, leading to pulmonary deterioration and respiratory failure. Finally, the high morbidity and mortality described in these subjects are the consequence of recurrent respiratory infections [2]. Therefore, the isolation and proper identification of CF pathogens are critical steps that have significant impact on antimicrobial treatment, patient management, cross-infection prevention and control in CF care units, as well as on the quality of life in these patients. The mechanisms by which the early acquisition of infection in CF patients occurs, especially in the case of *Pseudomonas aeruginosa* infection, were recently reviewed and include several hypotheses: low airway surface liquid leading to impaired mucociliary clearance, increased availability of cell surface receptors, reduced ingestion of bacteria by epithelial cells and/or low levels of molecules such as nitric oxide and antioxidant glutathione [3]. Although a few typical bacteria are traditionally involved in CF lung infections, including *Staphylococcus aureus*, *P. aeruginosa*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, patients with CF are susceptible to infection by other opportunistic bacterial species that are not usually pathogenic for healthy individuals [4], such as members of the *Burkholderia cepacia* complex,

Stenotrophomonas maltophilia, *Achromobacter xylosoxidans*, *Pandoraea* spp., *Ralstonia* spp., *Inquilinus limosus*, nontuberculous mycobacteria and other species [2,5–7]. The majority of these microbes are Gram-negative rods, environmental, multidrug resistant bacteria, and they persist, in spite of aggressive and prolonged courses of antibiotic treatment, in the patient's lung after biofilm formation. Thus, the infections become chronic with several exacerbation episodes per year. Accurate identification of the bacteria involved is an important step for understanding both the epidemiology and the clinical implications of emerging pathogens in the CF population. Recently, new molecular tools have led to an increase in the number of bacteria identified in the CF lung [8–10], indicating that the microbiome ecology in these patients is more complex than previously thought, which lead to the concept that CF is a polymicrobial infectious disease [11]. This review focuses on the different technologies used to identify and to detect bacteria in CF sputum samples (Fig. 1), including the current phenotypic methods and the recent molecular tools that have enabled: (i) the correct identification of misidentified bacteria; (ii) the discovery of new pathogens; (iii) the better characterization of the main pathogens; and finally (iv) an assessment of the complex microbial diversity in CF respiratory tracts using novel culture-independent approaches.

Conventional and Phenotypic Identification

The classical identification of bacteria isolated from respiratory samples recovered from CF patients is based mainly on different morphologic and metabolic characters, including Gram, growth at different temperature, enzyme activity (e.g. catalase, oxidase, lipase, phosphatase, etc.), carbon and nitrogen assimilation with different sugars and amino acid substrates, and antibiotic susceptibility profiles. Many of these characteristics can be tested simultaneously with commercial kits [e.g. the API (bioMérieux, Marcy l'Etoile, France) and RapID (Remel, Lenexa, KA, USA) systems] and automated apparatuses such as VITEK (bioMérieux), MicroScan-WalkAway (Dade Behring Inc.,. West Sacramento, CA, USA) and Phoenix (Becton-Dickinson, Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) (Fig. 1). The final identification, which takes between 6 and 48 h (depending on bacterial species) after bacterial growth, is given using automated algorithm analysis. This type of identification can be performed only on pure, isolated bacteria (Fig. 1). The agar plates used are adapted to isolate the main CF pathogens and usually include five agar plates, for Gram-positive bacteria, Gram-negative bacteria, fastidious bacteria and *Mycobacterium* spp., and *B. cepacia* complex-selective media [8]. Culture methods are useful for isolation of bacteria and are the first step for identification and antibiotic susceptibility testing. However, many problems can arise from this procedure (Table 1). First, the oropharyngeal flora and fungi can be cultured, aside from the pathogens, on the same media, making the isolation and/or detection of pathogenic bacteria in polymicrobial sputum more difficult [2]. In addition, in selective media (e.g. *B. cepacia* complex agar plates), other emerging bacteria, including *S. maltophilia*, *A. xylosoxidans*, *I. limosus*, and *Herbaspirillum* spp., can grow and lead to misidentification [12–15]. Conversely, many unculturable pathogens and anaerobes

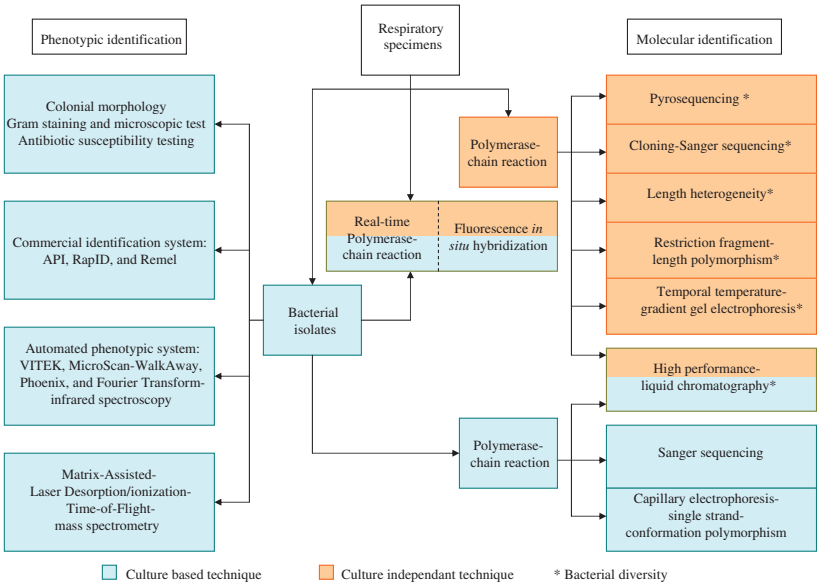

FIG. 1. The different technologies used to identify bacteria in cystic fibrosis sputum samples.

TABLE 1. Advantages and drawbacks of different techniques used for identification and/or detection of bacteria in cystic fibrosis patients

Tools	Advantages	Drawbacks
Culture and classical phenotypic identification	Enable bacterial isolation and antibiotic susceptibility testing	Missing bacteria (unculturable and/or bacteria missed in polymicrobial flora) Misidentification.
Molecular means for bacterial identification (isolates) and/or bacterial detection (isolates and sputa)	Correct identification of some bacterial genera and/or species	Missing bacteria. Misidentification in some cases due to lack of discriminatory power. No antibiotic susceptibility testing
Molecular means evaluating the biodiversity (sputa)	More exhaustive to detect and to evaluate the complex flora present	No isolated bacteria. No antibiotic susceptibility results

could be missed on these plates and thus not be detected, leading to a permanent requirement to develop new media. It is also known that mucoid *P. aeruginosa* can invade the agar plates, obscuring the presence of other bacteria in the same plate, or it can simply produce substances that inhibit their growth [16]. Moreover, misidentification of bacteria using the standard phenotypic procedures is increasingly reported in isolates recovered from CF patients.

Unusual [17] and misidentified [18–24] bacteria are mainly documented in the context of CF as a result of: (i) appropriate ecology in the CF lung that supports the growth of a wide variety of bacteria rarely seen in humans [17]; (ii) long-term airway colonization by some bacteria leading to phenotypic modifications as described, for example, in hypermutable [25,26] and mucoid variants of *P. aeruginosa* [27] and also in small-colony variants of several pathogens (e.g. *S. aureus* [28], *S. maltophilia* [29] *and P. aeruginosa* [30]); (iii) taxonomic changes concerning some bacteria and the description of new genera and/or species that are not included in the database of automated phenotypic identification systems [31]; (iv) the recent development of molecular identification methods with superior performance compared to that of commercial devices and biochemical testing [32,33]; and (v) some species that are closely related and have similar phenotypes that make accurate laboratory identification challenging [4].

Misidentification of the bacteria in sputa from CF patients remains problematic, especially of nonfermenting Gram-negative bacilli. Currently, differentiation of *Pandoraea* spp., *Ralstonia* spp. and *Burkholderia* spp. from each other using phenotypic tests can be difficult and misleading [2]. Similarly, confusion of *P. aeruginosa* with *A. xylosoxidans* or *S. maltophilia* has been recently described [23,34]. Indeed, several highly transmissible bacteria, such as *B. cepacia* and *P. aeruginosa*, may be misidentified, and this could delay the therapeutic management and infection control recommendations in CF centres [33,35]. Many studies amply illustrate this problem with high rates of isolated bacteria with incorrect or impossible identification [36]. Indeed, this problem is described in the literature for *P. aeruginosa* and *B. cepacia* [2,14,18,23] and was also encountered in our previous study where we demonstrated that such misidentifications were also made of other common bacteria, including *S. aureus* and *S. pneumoniae* [8]. It has been reported, for example, that only 57% of non-mucoid strains and 40% of mucoid strains of *P. aeruginosa* are correctly identified with the MicroScan Autoscan automated system, and that extending the incubation to 48 h improves identification [20]. As with mucoid *P. aeruginosa* strains, the identification of *B. cepacia* is difficult, and errors of identification are numerous (e.g. of phenotypically homogeneous bacteria of the genera *Burkholderia*, *Alcaligenes*, *Ralstonia*, *Stenotrophomonas* and *Pandoraea* [2,14,18]). Conversely, bacteria of the genus *Alcaligenes*, *Ralstonia pickettii* or *S. maltophilia* can be inaccurately identified as *B. cepacia* [14]. Moreover, *Segniliparus rugosus* can be confused with rapidly growing members of the genus *Mycobacterium* because of its rapid growth on culture media designed for mycobacteria [37]. Interestingly, in a recent multicentre quality assurance trial of identification of CF isolates conducted in 18 European countries, common and emerging CF pathogens were also misclassified by many participant laboratories or were not detected [38].

Finally, for certain bacteria, correct phenotypic identification is impossible because they are newly-discovered bacteria whose phenotypic profiles do not exist in the commercial identification system databases (API, Vitek 2). Over the last 10 years, recent taxonomic studies (benefitting from molecular approaches) have resulted in the description of an increasing number of new genera and/or species [31]. This is the case for *I. limosus*, which is isolated from sputum and mainly reported in CF patients [17,39–42]. The majority of the isolates were mucoid, grew on selective *B. cepacia* agar, were identified with API 20 NE as either *Sphingomonas paucimobilis* or *Agrobacterium radiobacter*, and were suscepti-

ble only to imipenem, ciprofloxacin and rifampicin [13,17, 39,40], suggesting that this bacterium is a multiresistant emerging bacterium in CF patients.

It is interesting to note that many other promising phenotypic strategies for the identification of bacteria have been recently applied to resolve the problem of misidentification, including the use of Fourier transform infrared spectroscopy [43] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [44–46] (Fig. 1).

MALDI-TOF MS for CF Bacterial Identification

This low-cost technique, which is based on protein fingerprint profiling of intact bacterial cells, has already been applied to identify bacteria recovered from CF patients. It allows rapid identification with a high degree of reliability and strong potential for correct identification, as well as the possibility of continuous system updating by enlarging the database with information for a wider range of new bacterial species and isolates. MALDI-TOF MS has been used successfully to characterize nonfermenting isolates of Gram-negative bacilli from CF patients after engineering the database with a set of reference strains [44]. Moreover, two other studies have also demonstrated that *B. cepacia* complex species, the more frequently misidentified CF pathogens, can be identified accurately using this methodology [45,46], which is essential for the timely management of CF patients. More recently, Seng *et al.* have described the successful use of MS in the routine clinical microbiology laboratory [47]. This successful advance in the application of MALDI-TOF MS for bacterial identification makes it possible, in the near future, for it to be used also for the routine identification of CF pathogens.

Molecular Means for the Correct Identification of Misidentified Bacteria

Although conventional phenotypic tests fail to produce accurate results [32,33], molecular methods such as PCR followed by sequencing (Fig. 1) offer an established, powerful and reliable option for the correct identification of bacteria (Table 1), especially nonfermenting Gram-negative bacilli [13,36,48–56].

Accurate identification of bacteria is important for both the epidemiology and the clinical implications of emerging pathogens in CF patients. For example, correct identification of genomovars within the *B. cepacia* complex is crucial because the presence of certain genomovars in CF patients may be hazardous. Indeed, *B. multivorans* (genomovar II) and *B. cenocepacia* (genomovar III) are the most virulent and transmissible species [57] associated with a severe decline in lung function and increased mortality rates. Moreover, lung transplantation is often avoided in the case of genomovar II- or III-infected CF patients with the aim of controlling patient-to-patient transmission [57].

Moreover, the introduction of molecular techniques, especially 16S rRNA gene amplification and sequencing, for the identification of misidentified or unidentified bacteria by traditional methods has revolutionized our current knowledge with the description of many emerging and multidrug-resistant bacteria that can be found in CF patients, as summarized in Table 2 [15,17,23,36,37,54,56,58–72].

Other novel molecular approaches have also been developed recently for rapid and accurate identification of bacteria in CF patients (Fig. 1). Some of these assays target a specific bacterial species using real-time PCR (RT-PCR) for identification of isolates, e.g. of the *B. cepacia* complex by multiplex *recA* and 16S rRNA gene RT-PCR [73], of *I. limosus* by 16S rRNA gene RT-PCR [13] and of *P. aeruginosa* by duplex *ecfX* and the *gyrB* gene RT-PCR [34]. Fluorescence *in situ* hybridization has also been used for *A. xylosoxidans*, *Alcaligenes faecalis* [74] and *B. cepacia* complex identification [75]. These different assays are both practical and appropriate for a modern clinical microbiology laboratory. Additionally, more recent and sophisticated approaches have been developed for the identification of bacteria (i.e. nonfermenting Gram-negative rods) including capillary electrophoresis-single-strand conformation polymorphism analysis [76] and ribosomal intergenic spacer analysis-high performance liquid chromatography [77].

Although 16S rRNA gene amplification and sequencing remains the universal reference standard technique for bacterial identification, its discriminatory ability for the identification of bacteria at the species level is, in some cases, limited and species-dependent (Table 1). In this situation, other genes should be analyzed for bacterial species or subspecies identification. This is the case for *B. cepacia* complex, *Burkholderia gladioli*, *Ralstonia* spp., *Pandoraea* spp. and *Mycobacterium* spp., for which other genes such as *recA*, 23S rRNA, *gyrB* and *rpo*B are more variable in sequence and have a higher discriminatory power for identification [78–81]. Moreover, for the *B. cepacia* complex, sequencing of several genes by using the multilocus sequence typing method and/or the polyphasic taxonomic approach are sometimes needed to resolve the difficulty of identification of strains at the subspecies level or for genotyping [57,78,82]. Typing of bacterial strains is also required to understand the epidemiology, routes or sources of infections, aiming to optimize healthcare

TABLE 2. Bacterial species described in the context of cystic fibrosis

Bacterial species	References	Bacterial species	References
Abiotrophia defectiva	[8,10,89]	*Moraxella catarrhalis*	[8]
Acetobacter indonesiensis	[58]	*Moraxella osloensis*	[17]
Achromobacter ruhlandi	[10]	*Mycobacterium abscessus*	[7,10]
Achromobacter xylosoxidans	[8,10]	*Mycobacterium avium*	[7]
Acinetobacter baumannii	[4]	*Mycobacterium simiae*	[7]
Acinetobacter sp.	[17]	*Neisseria cinerea*	[10]
Actinomyces graeventzii	[10]	*Neisseria* sp.	[8,87]
Actinomyces naeslundii	[70]	*Neisseria subflava*	[10]
Actinomyces odontolyticus	[10,70]	*Neissieria flava*	[70]
Actinomyces sp.	[8,87]	*Neissieria mucosa*	[70]
Actinomyces viscosus	[70]	*Nocardia asiatica*	[62]
Advenella incenata	[59]	*Nocardia asteroides*	[63]
Agrobacterium radiobacter	[36]	*Nocardia elegans*	[62]
Alcaligenes faecalis	[74]	*Nocardia farcinica*	[64]
Atopobium parvulum	[70]	*Nocardia transvalensis*	[62]
Atopostipes suicloacalis	[10]	*Novosphingobium* sp.	[10]
Bacillus licheniformus	[70]	*Ochrobactrum anthropi*	[36,56]
Bacillus pumilis	[70]	*Paenibacillus cineris*	[69]
Bacteroides fragilis	[89]	*Pandoraea apista*	[4]
Bdellovibrio sp.	[10]	*Pandoraea norimbergensis*	[4]
Bergeyella sp.	[8,87]	*Pandoraea pnomenusa*	[4]
Bifidobacterium longum	[70]	*Pandoraea pulmonicola*	[4]
Bifidobacterium sp.	[87]	*Pandoraea sputorum*	[4]
Bordetella avium	[60]	*Pantoea agglomerans*	[23]
Bordetella bronchiseptica/parapertussis	[61]	*Peptostreptococcus micros*	[70]
Bordetella hinzii	[17]	*Peptostreptococcus prevotii*	[70]
Bordetella petrii	[60]	*Peptostreptococcus* sp.	[8,87]
Bordetella sp.	[10]	*Porphyromonas* sp.	[8,10]
Brevundimonas diminuta	[32,56]	*Prevotella corporis*	[70]
Bulleidia moorei	[70]	*Prevotella denticola*	[8,10]
Burkholderia ambifaria	[2,57]	*Prevotella disiens*	[70]
Burkholderia anthina	[2,57]	*Prevotella melaninogenica*	[8,10,70]
Burkholderia arboris	[78]	*Prevotella oris*	[8,10,89]
Burkholderia cenocepacia	[2,4,57,78]	*Prevotella pallens*	[10,70]
Burkholderia cepacia	[2,57]	*Prevotella salivae*	[8,70]
Burkholderia contaminans	[78,82]	*Prevotella* sp.	[8,10,87]
Burkholderia dolosa	[4,57]	*Propionibacterium acnes*	[10,70]
Burkholderia fungorum	[17]	*Pseudomanas fluorescens*	[71]
Burkholderia gladioli	[79]	*Pseudomonas aeruginosa*	[8,10,87,89]
Burkholderia lata	[82]	*Pseudomonas alcaligenes*	[36]
Burkholderia multivorans	[2,4,8,57]	*Pseudomonas brassicacearum*	[36]
Burkholderia pseudomallei	[72]	*Pseudomonas mendocina*	[23]
Burkholderia pyrrocinia	[2,57]	*Pseudomonas putida*	[71]
Burkholderia stabilis	[2,57]	*Pseudomonas sacchoraphila*	[10]
Burkholderia vietnamiensis	[2,4,57]	*Pseudomonas* sp.	[88]
Campylobacter concisus	[10]	*Pseudomonas stutzeri*	[71]
Campylobacter sp.	[87]	*Pseudomonas synxantha*	[36]
Capnocytophaga infantium	[10]	*Ralstonia mannitolilytica*	[51]
Capnocytophaga sp.	[8]	*Ralstonia pickettii*	[51]
Carnobacterium sp.	[8]	*Ralstonia basilensis*	[51]
Caulobacter sp.	[10]	*Ralstonia gilardii*	[4,51]
Chryseobacterium gleum	[65]	*Ralstonia insidiosa*	[4,51]
Chryseobacterium indologenes	[65]	*Ralstonia metallidurans*	[51]
Chryseobacterium meningosepticum	[65]	*Ralstonia paucula*	[4,51]
Chryseobacterium sp.	[17,36]	*Ralstonia respiraculi*	[4,51]
Citrobacter murliniae	[89]	*Ralstonia taiwanensis*	[4,51]
Clostridium bartlettii	[10]	*Rhizobium radiobacter*	[17,23]
Clostridium hastiforme	[70]	*Rhizobium* sp.	[10]
Comamonas testosteroni	[10,17]	*Rickettsiales* sp.	[10]
Cupriavidus respiraculi	[23]	*Rothia dentocariosa*	[70,87]
Dialister pneumosintes	[8]	*Rothia mucilaginosa*	[8,70]
Dolosigranulum pigrum	[8,9]	*Sarcina ventriculi*	[89]
Eikenella corrodens	[8]	*Segniliparus rugosus*	[37]
Escherichia coli	[8,10,70,87]	*Selenomonas infelix*	[8]
Fusobacterium gonidiaformans	[89]	*Selenomonas noxia*	[8]
Fusobacterium nucleatum	[10,70]	*Selenomonas* sp.	[8]
Fusobacterium sp.	[10,87]	*Serratia marcescens*	[8]
Gemella bergeri	[10]	*Sphingomonas paucimobilis*	[36]
Gemella haemolysans	[8]	*Staphylococcus aureus*	[8,10,87]
Gemella morbillorum	[8]	*Staphylococcus epidermidis*	[70]
Gemella sanguinis	[8,10,70]	*Staphylococcus hominis*	[70]
Granulicatella adiacens	[8,10]	*Staphylococcus pasteuri*	[70]
Granulicatella elegans	[10]	*Staphylococcus saccharolyticus*	[70]
Granulicatella paradiacens	[8]	*Stenotrophomonas maltophilia*	[8,10,89]
Haemophilus influenzae	[8,10,87]	*Stomatococcus mucilaginosus*	[87]
Herbaspirillum frisingense	[15]	*Streptococcus agalactiae*	[66]
Herbaspirillum huttiense	[15]	*Streptococcus anginosus*	[8,70,95]
Herbaspirillum putei	[15]	*Streptococcus constellatus*	[8,70,88,95]
Herbaspirillum seropedicae	[15]	*Streptococcus cristatus*	[8,10,70]

TABLE 2. (Continued)

Bacterial species	References	Bacterial species	References
Herbaspirillum sp.	[17]	*Streptococcus genomosp.*	[8]
Inquilinus limosus	[10,13]	*Streptococcus gordonii*	[8,10]
Johnsonella sp.	[10]	*Streptococcus iniae*	[8]
Kingella denitrificans	[8]	*Streptococcus intermedius*	[70,95]
Kingella oralis	[8]	*Streptococcus milleri*	[10,67]
Klebsiella pneumoniae	[10]	*Streptococcus mitis*	[8,10,70]
Lachnospiraceae genomosp.	[8]	*Streptococcus oralis*	[70]
Lactobacillus casei	[10,70]	*Streptococcus parasanguis*	[8,10,70]
Lactobacillus delbruekii	[8]	*Streptococcus peroris*	[10]
Lactobacillus fermentum	[10]	*Streptococcus pneumoniae*	[8]
Lactobacillus salivarius	[70]	*Streptococcus salivarius*	[8,70]
Lactobacillus sp.	[87,88]	*Streptococcus sanguinis*	[8,10,70]
Lautropia mirabilis	[68]	*Streptococcus* sp.	[8,87]
Leptotrichia sp.	[89]	*Streptococcus thermophilus*	[70]
Leptotrichia wadeii	[10]	*Tannerella forsythensis*	[8]
Lysobacter enzymogenes	[10]	*Veillonella atypica*	[8,10,70]
Methylobacterium asaccharovorans	[10]	*Veillonella dispar*	[10,70]
Methylobacterium sp.	[10]	*Veillonella ratti*	[10]
Micrococcus luteus	[70]	*Veillonella* sp.	[8,87]
Mogibacterium timidum	[10]	*Xanthomonas* sp.	[17]

The references were chosen according to the first description of the bacterial species using either culture-dependant or culture-independent molecular methods, except for some classical pathogens where a review has been selected.

services and to prevent transmission between patients. It can also provide an idea of bacterial population dynamics. Epidemic emerging strains in CF patients have been comprehensively reported, especially for the principal pathogens, including *B. cepacia* complex [57], *P. aeruginosa* [83] and methicillin-resistant *S. aureus* [84,85]. The various genotyping methods that can be used have been reviewed recently [86]. However, although these techniques have been used for the correct identification of bacteria, they do not allow evaluation of the actual bacterial diversity, and so many bacterial species can be missed (Table 1).

Molecular Techniques for the Evaluation of Bacterial Diversity

Although routine culture methods yield limited microbiological information for CF sputa [16], current knowledge indicates that CF respiratory infections must be considered as polymicrobial infections [11]. Indeed, culture-independent studies using molecular tools (including 16S rRNA gene clonal library sequencing [8,10], 16S rRNA gene pyrosequencing [9], temporal temperature gradient gel electrophoresis 16S rRNA gene PCR [87] and 16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) profiling [88–91]) have allowed a more precise evaluation of the microbial diversity in the lungs of CF patients (Table 1). The strength of these different analyses lies in the fact that they can be performed directly from clinical samples without any need for culturing (Fig. 1). The results obtained demonstrate the existence of a large known and unknown cadre of bacterial species residing in CF lungs with a high prevalence of anaerobic bacteria and oropharyngeal flora (Table 2). Different approaches applying these culture-independent methods have been extensively used to study the microbial communities, including Sanger clone sequencing [8,10] and pyrosequencing [9]. In our 16S rRNA gene amplification and cloning study, 760 clones were obtained from 25 CF patients (children and adults), which resulted in the identification of 53 different bacterial species (Table 2) [8].

The mean number of bacterial species per sputum was 7.2 ± 3.9 (range 1–14). Interestingly, the mean number of detected bacterial species increased when ribosomal genes from more clones were sequenced, indicating the complexity of microbial communities in the samples from these patients [8]. Of the 53 bacterial species detected, 16 (30%) were anaerobic bacteria of different genera, such as *Prevotella*, *Veillonella*, *Porphyromonas* and *Selenomonas* (Table 2) [8]. Finally, many new or emerging bacterial species were also detected, including *Dolosigranulum pigrum*, *Dialister pneumosintes*, *Granulicatella adiacens* and *Rothia mucilaginosa* (Table 2) [8]. In a similar study, more clones (>6000) were screened from 28 CF children to identify 65 bacterial species (Table 2) [10]. The mean number of bacterial species per sputum was 5.3 ± 4.9 (range 1–21), and the percentage of anaerobes ranged from 27% to 93% of the clones examined.

Moreover, candidate pathogens such as *Prevotella denticola*, *Lysobacter enzymogenes* and members of the *Rickettsiales* were detected [10]. More recently, Armougom *et al.* described the use of pyrosequencing after 16S rRNA gene amplification to analyze the sputum sample of a CF child [9]. Although the

shortness of pyrosequencing reads combined with the weak nucleotide sequence variability of the 16S rDNA limited the assembly of full-length 16S rDNA, this technology provides a more reliable estimate of the relative abundance and species richness among the bacteria present [9].

Using reverse transcription T-RFLP, as compared to T-RFLP, Rogers *et al.* indicated that the majority of bacterial species detected in CF sputa are metabolically active and thus may be clinically significant and likely to participate in CF lung infection [88,92]. Moreover, Rogers *et al.* [93] demonstrated, by a comparison of T-RFLP profiles of isolates from sputum samples and mouthwash samples collected from the same adult CF patients, that these reported mixed florae cannot be a result of contamination of sputum by bacteria present in the oral cavity. The microflora may play an indirect role in the outcome of the disease by modulating gene expression via interspecies communication [94]. Moreover, these complex florae can enhance or regulate the expression of the virulence factors and the pathogenicity of other organisms (e.g. *P. aeruginosa*), as demonstrated using a rat lung infection model and genome-wide transcriptional analysis [94] and, more recently, using a *Drosophila* model [95]. In addition, the response of the host's innate immune system towards these infections is complex, highlighting the potential complexity of polymicrobial infections [95]. In another study, it was shown that bacteria of the *Streptococcus milleri* group are detected in many cases, and that these comprise the numerically dominant pathogens in 39% of acute CF pulmonary exacerbations and can establish chronic infections [91].

Anaerobes, not currently sought using conventional culture methods, were also detected frequently via these culture-independent assays [8,10]. However, it is not surprising to find anaerobic bacteria in CF lungs, especially after the establishment of chronic infection where many bacteria, including *P. aeruginosa* and *S. aureus*, reside in a biofilm under hypoxic conditions. This concept is in agreement with recent studies that revealed high numbers of anaerobic bacteria in CF sputum samples from which facultative anaerobic species such as *P. aeruginosa* and *S. aureus* were also isolated [70,96]. In these same studies, anaerobic bacteria were detected in much lower numbers in sputa from healthy individuals and from patients with pulmonary diseases other than CF [70,96].

It is also possible that these anaerobes are more frequently recovered from CF patients because they have been selected for after recurrent antimicrobial treatments as a result of being naturally resistant to certain antibiotics such as aminoglycosides [8]. Thus, the potential contribution of anaerobes in CF pathology requires more investigation in the future.

Finally, it is interesting to note that denaturing gradient gel electrophoresis, a technique used mainly for studying and screening CFTR mutations [97], has not been specifically used to screen bacterial communities in CF samples.

In conclusion, molecular culture-independent methods have provided an interesting opportunity to discover a large number of known and unknown bacteria, including anaerobes and new emerging pathogens, living in microbial communities in the lower respiratory tracts of CF patients. In the future, the list of bacteria associated with CF patients will continue to increase via the use of molecular tools. Further metagenomic analysis of CF sputa will also rapidly expand this list, leading to a more precise knowledge about these complex niches.

Molecular Characterization of Principal CF-Associated Bacteria and Whole-genome Sequencing

Full bacterial genome sequencing represents the most complete and powerful tool to understand the physiopathology of bacterial infection because it gives global information about the existing virulence factors, mechanisms of antibiotic resistance, and the genetic materials of a given bacterium. Although rapid advances in massive DNA sequencing and increased numbers of whole-genome sequences are available, only a few genomes of CF-associated strains have been sequenced to date. Indeed, only three epidemic strains, of *S. aureus* (CF-Marseille) [84], *P. aeruginosa* (LES) [98] and *B. cenocepacia* (J2315) [99], have been sequenced and published recently.

Genomic analyses of these isolates revealed the presence of numerous virulence and drug resistance functions [84,98,99] and mutated genes [100], reflecting the adaptive way of life of these pathogens in a specific niche, when exposed to aggressive and multiple antimicrobial therapies, and during established chronic infection. The main link found between these genomes was a high level of lateral gene transfer compared to non-CF isolates. For example, the J2315 genome contains 14 additional genomic islands (21% of the genome) compared to non-CF *B. cenocepacia* strains [99]. The functions of these islands appear to lie in the promotion of survival and pathogenesis in the CF lung [99]. Prophages that play an important role in genomic evolution of bacteria and genome diversity [101,102] via horizontal gene transfer were also found in these sequenced strains. Interestingly, recent studies demonstrate that some antibiotics, especially those frequently used in CF patients (e.g. ciprofloxacin, tobramycin, cotrimoxazole and imipenem), can

enhance phage mobility [84,103], leading to the frequent exchange of genetic material and spread of virulence and/or antibiotic resistance genes among bacterial species. More recently, metagenomic analysis of DNA virus communities in the respiratory tract of CF individuals has demonstrated a higher abundance of phage communities in these patients, especially *Staphylococcus* phages, that are associated with airway pathology [104]. Moreover, prophages were recently found as critical genomic determinants for competitiveness of the epidemic strain of *P. aeruginosa* in a chronic infection model [98]. According to these data, it is likely that phages participate in the physiopathology of the disease by rapid adaptation via lateral gene transfer. More studies are needed to illustrate the role of bacteriophages and other viruses in these infections using metagenomic analysis.

Microarray assays were also carried out aiming to characterize CF pathogens, especially *P. aeruginosa* and *S. aureus* isolates. This tool was used to study the regulation of gene expression concerning virulence factors, antimicrobial resistance and persistence factors during different stress conditions in *P. aeruginosa* [26,105–112], *B. cenocepacia* [113] and *S. aureus* [84,114], as well as to assess variation in the genomic repertoire of *P. aeruginosa* strains [115,116]. The results obtained again indicate an adaptive and defensive response of these opportunistic bacteria that may contribute to the morbidity and mortality in CF. More recently, a model for niche-transcriptional response derived by analyzing gene expression in different environmental niches via high-throughput cDNA sequencing, was performed in two *B. cenocepacia* isolates (one isolated from a CF patient and another from agricultural soil) and was informative for understanding the bacterial response to its ecology [117].

Although these two strains share a 99.8% average nucleotide identity in their conserved genes, many regulatory and potential virulence factors were over-expressed under conditions mimicking the CF lung compared to those of soil [117]. This may represent specific adaptations to the niches from which each strain was isolated [117]. Finally, interactions between CF-associated bacterial species were also studied using microarrays [94,118]. Interestingly, one of these analyses demonstrated that microflora can modulate gene expression of other pathogens, such as *P. aeruginosa* [94], again emphasizing that very little is known about the complex microbial infections in CF patients. Both genome sequencing of other bacterial species and assessment of their transcriptional responses in the CF niche can contribute effectively in the future to better define our current understanding of respiratory infections in CF patients.

Conclusions and Perspectives

Although bacterial infections in CF patients were considered, in the past, to be a result of only a few pathogens, recent advances in molecular methods have led to the recognition of an increasing number of bacteria associated with these patients. This trend is reliant on a better identification of bacteria or a more exhaustive analysis of sputum samples.

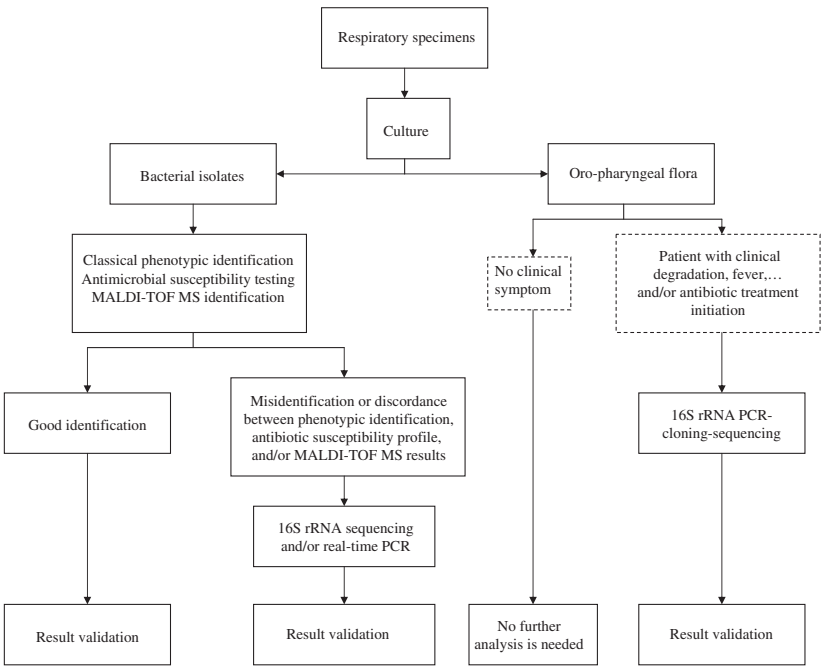

FIG. 2. Proposed strategy for the management and analysis of sputa from cystic fibrosis patients in clinical microbiology laboratories. MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

For these reasons, we are of the opinion that a new strategy for the correct identification of bacterial isolates recovered from sputum samples (as proposed in Fig. 2) should be implemented in modern clinical microbiology laboratories.

We consider that MALDI-TOF MS for the routine identification of bacteria should be the gold standard in the future [47]. If an isolated bacterium is not identified correctly or if there is discordance between the phenotypic identification and antibiotic susceptibility profile or MALDI-TOF MS results, the identification of the strain by partial 16S rRNA gene sequencing is warranted, especially for bacteria isolated on CEPACIA agar. Amplification by PCR followed by cloning must be reserved for specific cases, especially when it is impossible to isolate and identify a known pathogen from sputa among polymicrobial oral flora in a patient with clinical degradation associated with fever, an inflammatory syndrome, decreased respiratory function, and/or following lung transplantation. By contrast, it would be interesting to develop specific real-time PCR methods, using probes for the identification of several pathogens (including *P. aeruginosa* [119], *B. cepacia* [73] and *I. limosus* [13]), in specific situations.

Metagenomic studies using high-throughput sequencing analysis are ongoing in many clinical microbiology areas [120]. In the future, metagenomic approaches for profiling the CF lung microbiome will help to expand the known diversity and allow a better understanding of the physiopathology of the complex respiratory infections in the context of this disease. This was recently demonstrated by an analysis targeting the respiratory DNA virus communities in CF patients, indicating that the disease state, as a result of the specific environment in the CF airway, is defined by metabolism and not by taxonomy [104]. Moreover, monitoring microbial changes or, more specifically, gene expression using oligonucleotide microarrays, can provide a more comprehensive view of the biodynamics of micro-organisms through chronic infections, especially during antimicrobial therapy [121,122]. Other interesting approaches via DNA microarrays will be to develop multiple probe arrays able to detect quickly, accurately and simultaneously, and without culturing, the best known microbiota in the respiratory tract of CF patients. In the future, such an assay may take an important place in the modern clinical microbiology and diagnosis laboratories for the timely management of infection in CF patients.

Transparency Declaration

The authors have no conflicts of interest to declare.

References

1. Ratjen F, Doring G. Cystic fibrosis. *Lancet* 2003; 361: 681–689.
2. Miller MB, Gilligan PH. Laboratory aspects of management of chronic pulmonary infections in patients with cystic fibrosis. *J Clin Microbiol* 2003; 41: 4009–4015.
3. Davies JC. *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev* 2002; 3: 128–134.
4. Davies JC, Rubin BK. Emerging and unusual gram-negative infections in cystic fibrosis. *Semin Respir Crit Care Med* 2007; 28: 312–321.
5. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002; 15: 194–222.
6. Hutchison ML, Govan JR. Pathogenicity of microbes associated with cystic fibrosis. *Microbes Infect* 1999; 1: 1005–1014.
7. Levy I, Grisaru-Soen G, Lerner-Geva L *et al.* Multicenter cross-sectional study of nontuberculous mycobacterial infections among cystic fibrosis patients, Israel. *Emerg Infect Dis* 2008; 14: 378–384.
8. Bittar F, Richet H, Dubus JC *et al.* Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS ONE* 2008; 3: e2908.
9. Armougom F, Bittar F, Stremler N *et al.* Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. *Eur J Clin Microbiol Infect Dis* 2009; 28: 1151–1154.
10. Harris JK, De Groote MA, Sagel SD *et al.* Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proc Natl Acad Sci USA* 2007; 104: 20529–20533.
11. Sibley CD, Rabin H, Surette MG. Cystic fibrosis: a polymicrobial infectious disease. *Future Microbiol* 2006; 1: 53–61.
12. Henry DA, Campbell ME, Lipuma JJ, Speert DP. Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium. *J Clin Microbiol* 1997; 35: 614–619.
13. Bittar F, Leydier A, Bosdure E *et al. Inquilinus limosus* and cystic fibrosis. *Emerg Infect Dis* 2008; 14: 993–995.
14. Henry D, Campbell M, McGimpsey C *et al.* Comparison of isolation media for recovery of *Burkholderia cepacia* complex from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol* 1999; 37: 1004–1007.
15. Spilker T, Uluer AZ, Marty FM *et al.* Recovery of *Herbaspirillum* species from persons with cystic fibrosis. *J Clin Microbiol* 2008; 46: 2774–2777.
16. van BA, Renders NH, Smith S, Overbeek SE, Verbrugh HA. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. *FEMS Immunol Med Microbiol* 2000; 27: 51–57.
17. Coenye T, Goris J, Spilker T, Vandamme P, Lipuma JJ. Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of *Inquilinus limosus* gen. nov., sp. nov. *J Clin Microbiol* 2002; 40: 2062–2069.
18. McMenamin JD, Zaccone TM, Coenye T, Vandamme P, Lipuma JJ. Misidentification of *Burkholderia cepacia* in US cystic fibrosis treatment centers: an analysis of 1,051 recent sputum isolates. *Chest* 2000; 117: 1661–1665.
19. Atkinson RM, Lipuma JJ, Rosenbluth DB, Dunne WM Jr. Chronic colonization with *Pandoraea apista* in cystic fibrosis patients determined by repetitive-element-sequence PCR. *J Clin Microbiol* 2006; 44: 833–836.
20. Saiman L, Burns JL, Larone D, Chen Y, Garber E, Whittier S. Evaluation of MicroScan Autoscan for identification of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *J Clin Microbiol* 2003; 41: 492–494.
21. Pimentel JD, MacLeod C. Misidentification of *Pandoraea sputorum* isolated from sputum of a patient with cystic fibrosis and review of

Pandoraea species infections in transplant patients. *J Clin Microbiol* 2008; 46: 3165–3168.
22. Saiman L, Chen Y, Tabibi S *et al.* Identification and antimicrobial susceptibility of *Alcaligenes xylosoxidans* isolated from patients with cystic fibrosis. *J Clin Microbiol* 2001; 39: 3942–3945.
23. Kidd TJ, Ramsay KA, Hu H *et al.* Low rates of *Pseudomonas aeruginosa* misidentification in isolates from cystic fibrosis patients. *J Clin Microbiol* 2009; 47: 1503–1509.
24. Burdge DR, Noble MA, Campbell ME, Krell VL, Speert DP. *Xanthomonas maltophilia* misidentified as *Pseudomonas cepacia* in cultures of sputum from patients with cystic fibrosis: a diagnostic pitfall with major clinical implications. *Clin Infect Dis* 1995; 20: 445–448.
25. Hoboth C, Hoffmann R, Eichner A *et al.* Dynamics of adaptive microevolution of hypermutable *Pseudomonas aeruginosa* during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis* 2009; 200: 118–130.
26. Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I. Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother* 2008; 61: 1053–1056.
27. Murray TS, Egan M, Kazmierczak BI. *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patients. *Curr Opin Pediatr* 2007; 19: 83–88.
28. Kahl B, Herrmann M, Everding AS *et al.* Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J Infect Dis* 1998; 177: 1023–1029.
29. Anderson SW, Stapp JR, Burns JL, Qin X. Characterization of small-colony-variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *J Clin Microbiol* 2007; 45: 529–535.
30. Oliver A, Canton R, Campo P, Baquero F, Blazquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 2000; 288: 1251–1254.
31. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 2007; 45: 2761–2764.
32. Kiska DL, Kerr A, Jones MC *et al.* Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J Clin Microbiol* 1996; 34: 886–891.
33. Shelly DB, Spilker T, Gracely EJ, Coenye T, Vandamme P, Lipuma JJ. Utility of commercial systems for identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture. *J Clin Microbiol* 2000; 38: 3112–3115.
34. Anuj SN, Whiley DM, Kidd TJ *et al.* Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the *ecfX* and the *gyrB* genes. *Diagn Microbiol Infect Dis* 2009; 63: 127–131.
35. Saiman L, Siegel J. Infection control in cystic fibrosis. *Clin Microbiol Rev* 2004; 17: 57–71.
36. Wellinghausen N, Kothe J, Wirths B, Sigge A, Poppert S. Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. *J Clin Microbiol* 2005; 43: 4070–4075.
37. Butler WR, Sheils CA, Brown-Elliott BA *et al.* First isolations of *Segniliparus rugosus* from patients with cystic fibrosis. *J Clin Microbiol* 2007; 45: 3449–3452.
38. Hogardt M, Ulrich J, Riehn-Kopp H, Tummler B. EuroCareCF quality assessment of diagnostic microbiology of cystic fibrosis isolates. *J Clin Microbiol* 2009; 47: 3435–3438.
39. Pitulle C, Citron DM, Bochner B, Barbers R, Appleman MD. Novel bacterium isolated from a lung transplant patient with cystic fibrosis. *J Clin Microbiol* 1999; 37: 3851–3855.
40. Wellinghausen N, Essig A, Sommerburg O. *Inquilinus limosus* in patients with cystic fibrosis, Germany. *Emerg Infect Dis* 2005; 11: 457–459.
41. Chiron R, Marchandin H, Counil F *et al.* Clinical and microbiological features of *Inquilinus* sp. isolates from five patients with cystic fibrosis. *J Clin Microbiol* 2005; 43: 3938–3943.
42. Schmoldt S, Latzin P, Heesemann J, Griese M, Imhof A, Hogardt M. Clonal analysis of *Inquilinus limosus* isolates from six cystic fibrosis patients and specific serum antibody response. *J Med Microbiol* 2006; 55: 1425–1433.
43. Bosch A, Minan A, Vescina C *et al.* Fourier transform infrared spectroscopy for rapid identification of nonfermenting gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. *J Clin Microbiol* 2008; 46: 2535–2546.
44. Degand N, Carbonnelle E, Dauphin B *et al.* Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of nonfermenting gram-negative bacilli isolated from cystic fibrosis patients. *J Clin Microbiol* 2008; 46: 3361–3367.
45. Vanlaere E, Sergeant K, Dawyndt P *et al.* Matrix-assisted laser desorption ionisation-time-of of-flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex. *J Microbiol Methods* 2008; 75: 279–286.
46. Minan A, Bosch A, Lasch P *et al.* Rapid identification of *Burkholderia cepacia* complex species including strains of the novel Taxon K, recovered from cystic fibrosis patients by intact cell MALDI-ToF mass spectrometry. *Analyst* 2009; 134: 1138–1148.
47. Seng P, Drancourt M, Gouriet F *et al.* Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis* 2009; 49: 543–551.
48. Ferroni A, Sermet-Gaudelus I, Abachin E *et al.* [Phenotypic and genotypic characteristics of non fermenting atypical strains recovered from cystic fibrosis patients]. *Pathol Biol (Paris)* 2003; 51: 405–411.
49. Ferroni A, Sermet-Gaudelus I, Abachin E *et al.* Use of 16S rRNA gene sequencing for identification of nonfermenting gram-negative bacilli recovered from patients attending a single cystic fibrosis center. *J Clin Microbiol* 2002; 40: 3793–3797.
50. Moissenet D, Bingen E, Arlet G, Vu-Thien H. [Use of 16S rRNA gene sequencing for identification of '*Pseudomonas*-like' isolates from sputum of patients with cystic fibrosis]. *Pathol Biol (Paris)* 2005; 53: 500–502.
51. Coenye T, Spilker T, Reik R, Vandamme P, Lipuma JJ. Use of PCR analyses to define the distribution of *Ralstonia* species recovered from patients with cystic fibrosis. *J Clin Microbiol* 2005; 43: 3463–3466.
52. Coenye T, Vandamme P, Lipuma JJ. Infection by *Ralstonia* species in cystic fibrosis patients: identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction. *Emerg Infect Dis* 2002; 8: 692–696.
53. Liu L, Coenye T, Burns JL, Whitby PW, Stull TL, Lipuma JJ. Ribosomal DNA-directed PCR for identification of *Achromobacter* (*Alcaligenes*) *xylosoxidans* recovered from sputum samples from cystic fibrosis patients. *J Clin Microbiol* 2002; 40: 1210–1213.
54. Coenye T, Liu L, Vandamme P, Lipuma JJ. Identification of *Pandoraea* species by 16S ribosomal DNA-based PCR assays. *J Clin Microbiol* 2001; 39: 4452–4455.
55. Spilker T, Coenye T, Vandamme P, Lipuma JJ. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *J Clin Microbiol* 2004; 42: 2074–2079.
56. Menuet M, Bittar F, Stremler N *et al.* First isolation of two colistin-resistant emerging pathogens, *Brevundimonas diminuta* and *Ochrobactrum anthropi*, in a woman with cystic fibrosis: a case report. *J Med Case Reports* 2008; 2: 373.
57. Mahenthiralingam E, Baldwin A, Dowson CG. *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. *J Appl Microbiol* 2008; 104: 1539–1551.

58. Bittar F, Reynaud-Gaubert M, Thomas P, Boniface S, Raoult D, Rolain JM. *Acetobacter indonesiensis* pneumonia after lung transplant. *Emerg Infect Dis* 2008; 14: 997–998.
59. Coenye T, Vanlaere E, Samyn E, Falsen E, Larsson P, Vandamme P. *Advenella incenata* gen. nov., sp. nov., a novel member of the *Alcaligenaceae*, isolated from various clinical samples. *Int J Syst Evol Microbiol* 2005; 55: 251–256.
60. Spilker T, Liwienski AA, Lipuma JJ. Identification of *Bordetella* spp. in respiratory specimens from individuals with cystic fibrosis. *Clin Microbiol Infect* 2008; 14: 504–506.
61. Wallet F, Perez T, Armand S, Wallaert B, Courcol RJ. Pneumonia due to *Bordetella bronchiseptica* in a cystic fibrosis patient: 16S rRNA sequencing for diagnosis confirmation. *J Clin Microbiol* 2002; 40: 2300–2301.
62. Barrio MI, Martinez MC, Prados C, Giron RM, Maiz L, Martinez MT. Isolation of *Nocardia* species patients with cystic fibrosis. *Arch Bronconeumol* 2008; 44: 109–112.
63. Lumb R, Greville H, Martin J, Sangster N, Holmes M. *Nocardia asteroides* isolated from three patients with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* 2002; 21: 230–233.
64. Petersen BE, Jenkins SG, Yuan S, Lamm C, Szporn AH. *Nocardia farcinica* isolated from bronchoalveolar lavage fluid of a child with cystic fibrosis. *Pediatr Infect Dis J* 2007; 26: 858–859.
65. Lambiase A, Del PM, Raia V, Sepe A, Ferri P, Rossano F. *Chryseobacterium* respiratory tract infections in patients with cystic fibrosis. *J Infect* 2007; 55: 518–523.
66. Eickel V, Kahl B, Reinisch B *et al.* Emergence of respiratory *Streptococcus agalactiae* isolates in cystic fibrosis patients. *PLoS ONE* 2009; 4: e4650.
67. Parkins MD, Sibley CD, Surette MG, Rabin HR. The *Streptococcus milleri* group – an unrecognized cause of disease in cystic fibrosis: a case series and literature review. *Pediatr Pulmonol* 2008; 43: 490–497.
68. Ben Dekhil SM, Peel MM, Lennox VA, Stackebrandt E, Sly LI. Isolation of *Lautropia mirabilis* from sputa of a cystic fibrosis patient. *J Clin Microbiol* 1997; 35: 1024–1026.
69. Leao RS, Pereira RH, Ferreira AG, Lima AN, Albano RM, Marques EA. First report of *Paenibacillus cineris* from a patient with cystic fibrosis. *Diagn Microbiol Infect Dis* 2010; 66: 101–103.
70. Tunney MM, Field TR, Moriarty TF *et al.* Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 2008; 177: 995–1001.
71. Clarke L, Moore JE, Millar BC *et al.* Development of a diagnostic PCR assay that targets a heat-shock protein gene (groES) for detection of *Pseudomonas* spp. in cystic fibrosis patients. *J Med Microbiol* 2003; 52: 759–763.
72. O'Carroll MR, Kidd TJ, Coulter C *et al. Burkholderia pseudomallei:* another emerging pathogen in cystic fibrosis. *Thorax* 2003; 58: 1087–1091.
73. Pimentel JD, Dubedat SM, EL ND, Benn RA. Identification of isolates within the *Burkholderia cepacia* complex by a multiplex *recA* and 16S rRNA gene real-time PCR assay. *J Clin Microbiol* 2007; 45: 3853–3854.
74. Wellinghausen N, Wirths B, Poppert S. Fluorescence in situ hybridization for rapid identification of *Achromobacter xylosoxidans* and *Alcaligenes faecalis* recovered from cystic fibrosis patients. *J Clin Microbiol* 2006; 44: 3415–3417.
75. Brown AR, Govan JR. Assessment of fluorescent in situ hybridization and PCR-based methods for rapid identification of *Burkholderia cepacia* complex organisms directly from sputum samples. *J Clin Microbiol* 2007; 45: 1920–1926.
76. Ghozzi R, Morand P, Ferroni A *et al.* Capillary electrophoresis-single-strand conformation polymorphism analysis for rapid identification of *Pseudomonas aeruginosa* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J Clin Microbiol* 1999; 37: 3374–3379.
77. Nazaret S, Assade F, Brothier E, Freydiere AM, Bellon G, Cournoyer B. RISA-HPLC analysis of lung bacterial colonizers of cystic fibrosis children. *J Microbiol Methods* 2009; 76: 58–69.
78. Cesarini S, Bevivino A, Tabacchioni S, Chiarini L, Dalmastri C. *RecA* gene sequence and multilocus sequence typing for species-level resolution of *Burkholderia cepacia* complex isolates. *Lett Appl Microbiol* 2009; 49: 580–588.
79. Whitby PW, Pope LC, Carter KB, Lipuma JJ, Stull TL. Species-specific PCR as a tool for the identification of *Burkholderia gladioli*. *J Clin Microbiol* 2000; 38: 282–285.
80. Coenye T, Lipuma JJ. Use of the *gyrB* gene for the identification of *Pandoraea* species. *FEMS Microbiol Lett* 2002; 208: 15–19.
81. Kim BJ, Lee SH, Lyu MA *et al.* Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpo*B). *J Clin Microbiol* 1999; 37: 1714–1720.
82. Vanlaere E, Baldwin A, Gevers D *et al.* Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int J Syst Evol Microbiol* 2009; 59: 102–111.
83. Cheng K, Smyth RL, Govan JR *et al.* Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 1996; 348: 639–642.
84. Rolain JM, Francois P, Hernandez D *et al.* Genomic analysis of an emerging multiresistant *Staphylococcus aureus* strain rapidly spreading in cystic fibrosis patients revealed the presence of an antibiotic inducible bacteriophage. *Biol Direct* 2009; 4: 1.
85. Glikman D, Siegel JD, David MZ *et al.* Complex molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolates from children with cystic fibrosis in the era of epidemic community-associated methicillin-resistant *S. aureus*. *Chest* 2008; 133: 1381–1387.
86. Li W, Raoult D, Fournier PE. Bacterial strain typing in the genomic era. *FEMS Microbiol Rev* 2009; 33: 892–916.
87. Kolak M, Karpati F, Monstein HJ, Jonasson J. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. *Int J Med Microbiol* 2003; 293: 309–317.
88. Rogers GB, Daniels TW, Tuck A *et al.* Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC Pulm Med* 2009; 9: 14.
89. Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 2003; 41: 3548–3558.
90. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 2004; 42: 5176–5183.
91. 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* 2008; 105: 15070–15075.
92. Rogers GB, Carroll MP, Serisier DJ *et al.* Bacterial activity in cystic fibrosis lung infections. *Respir Res* 2005; 6: 49.
93. Rogers GB, Carroll MP, Serisier DJ *et al.* Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. *J Clin Microbiol* 2006; 44: 2601–2604.
94. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol* 2003; 50: 1477–1491.

95. Sibley CD, Duan K, Fischer C *et al.* Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog* 2008; 4: e1000184.
96. Worlitzsch D, Rintelen C, Bohm K *et al.* Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clin Microbiol Infect* 2009; 15: 454–460.
97. Picci L, Cameran M, Marangon O *et al.* A 10-year large-scale cystic fibrosis carrier screening in the Italian population. *J Cyst Fibros* 2010; 9: 29–35.
98. Winstanley C, Langille MG, Fothergill JL *et al.* Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool epidemic strain of *Pseudomonas aeruginosa*. *Genome Res* 2009; 19: 12–23.
99. Holden MT, Seth-Smith HM, Crossman LC *et al.* The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J Bacteriol* 2009; 191: 261–277.
100. Smith EE, Buckley DG, Wu Z *et al.* Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* 2006; 103: 8487–8492.
101. Finnan S, Morrissey JP, O'Gara F, Boyd EF. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. *J Clin Microbiol* 2004; 42: 5783–5792.
102. Brockhurst MA, Buckling A, Rainey PB. The effect of a bacteriophage on diversification of the opportunistic bacterial pathogen, *Pseudomonas aeruginosa*. *Proc Biol Sci* 2005; 272: 1385–1391.
103. Goerke C, Koller J, Wolz C. Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; 50: 171–177.
104. Willner D, Furlan M, Haynes M *et al.* Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS ONE* 2009; 4: e7370.
105. Firoved AM, Deretic V. Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* 2003; 185: 1071–1081.
106. Wolfgang MC, Jyot J, Goodman AL, Ramphal R, Lory S. *Pseudomonas aeruginosa* regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. *Proc Natl Acad Sci USA* 2004; 101: 6664–6668.
107. Firoved AM, Ornatowski W, Deretic V. Microarray analysis reveals induction of lipoprotein genes in mucoid *Pseudomonas aeruginosa*: implications for inflammation in cystic fibrosis. *Infect Immun* 2004; 72: 5012–5018.
108. Bragonzi A, Worlitzsch D, Pier GB *et al.* Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. *J Infect Dis* 2005; 192: 410–419.
109. Chang W, Small DA, Toghrol F, Bentley WE. Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide. *BMC Genomics* 2005; 6: 115.
110. Son MS, Matthews WJ Jr, Kang Y, Nguyen DT, Hoang TT. In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* 2007; 75: 5313–5324.
111. Manos J, Arthur J, Rose B *et al.* Transcriptome analyses and biofilm-forming characteristics of a clonal *Pseudomonas aeruginosa* from the cystic fibrosis lung. *J Med Microbiol* 2008; 57: 1454–1465.
112. Platt MD, Schurr MJ, Sauer K *et al.* Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. *J Bacteriol* 2008; 190: 2739–2758.
113. Drevinek P, Holden MT, Ge Z *et al.* Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum. *BMC Infect Dis* 2008; 8: 121.
114. Moisan H, Brouillette E, Jacob CL, Langlois-Begin P, Michaud S, Malouin F. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J Bacteriol* 2006; 188: 64–76.
115. Wolfgang MC, Kulasekara BR, Liang X *et al.* Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2003; 100: 8484–8489.
116. Ernst RK, D'Argenio DA, Ichikawa JK *et al.* Genome mosaicism is conserved but not unique in *Pseudomonas aeruginosa* isolates from the airways of young children with cystic fibrosis. *Environ Microbiol* 2003; 5: 1341–1349.
117. Yoder-Himes DR, Chain PS, Zhu Y *et al.* Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. *Proc Natl Acad Sci USA* 2009; 106: 3976–3981.
118. Mashburn LM, Jett AM, Akins DR, Whiteley M. *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during in vivo coculture. *J Bacteriol* 2005; 187: 554–566.
119. Qin X, Emerson J, Stapp J, Stapp L, Abe P, Burns JL. Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. *J Clin Microbiol* 2003; 41: 4312–4317.
120. Petrosino JF, Highlander S, Luna RA, Gibbs RA, Versalovic J. Metagenomic pyrosequencing and microbial identification. *Clin Chem* 2009; 55: 856–866.
121. Anderson GG, Moreau-Marquis S, Stanton BA, O'Toole GA. In vitro analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun* 2008; 76: 1423–1433.
122. Bagge N, Schuster M, Hentzer M *et al. Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother* 2004; 48: 1175–1187.