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Atypical Bacteria in the CF Airways: Diversity, Clinical Consequences, Emergence and Adaptation

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1. Introduction

The inventory of atypical[1] bacteria that may be found in the airways of cystic fibrosis (CF) patients besides well-known typical CF pathogens like *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex has greatly evolved over the past decades. Progressively, species initially considered as atypical in the CF airways, mainly gram-negative bacilli including *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and non-tuberculous mycobacteria have received more and more attention. They are now considered as usual CF-associated bacteria although their role in the disease progression is not fully elucidated (Beringer *et al*., 2000; Foweraker, 2009; Hauser *et al*., 2011). At the same time, other species occasionally identified from the respiratory secretions of CF patients were in turn regarded as atypical species in CF (for a recent review, see Hauser *et al*., 2011). This resulted from both the increasing number of metagenomic studies conducted on CF airways microbiota and a more systematic use of 16S ribosomal RNA (rRNA) gene sequencing to identify bacteria cultured from CF respiratory tract (CFRT) samples (CFRTS).

Recent findings of "new" atypical bacterial species in CF airways by both cultivation-based and cultivation-independent studies gave novel insights in microbiology of the CFRT. However, pathogenesis and clinical significance of these bacteria remain unclear, i.e. adaptation to the CF airways niche, positive or negative interaction between organisms, impact on the respiratory status of the patients. Similarly, antimicrobial susceptibility pattern is more often not investigated for atypical bacteria.

In this chapter, we propose a review of both published studies and personal findings about atypical bacterial species found in CF airways by both cultivation-based and cultivation-independent studies. Personal data came from our expertise of 16S rRNA gene-based identification of atypical bacteria cultured from CFRTS in patients attending the CF center of

[1]The term "atypical" will be used herein to design bacterial species rarely reported in the literature dealing with the microbiology of respiratory tract in CF patients.

the Montpellier University Hospital[2]. Most of these atypical bacteria are either environmental bacteria or belong to the human respiratory tract microbiota. In this context, examples will be mainly taken from a selected panel of bacteria in our area of expertise, including human anaerobic bacteria and environmental alphaproteobacteria: *Agrobacterium*, acetic acid bacteria, *Ochrobactrum* and *Inquilinus*.

2. CF airways, a niche for environmental opportunistic pathogens

CF airways represent a particular niche in which recent cultivation-based and cultivation-independent studies revealed several atypical microbes, which were recently reviewed by Hauser *et al.* (Hauser *et al*., 2011). Most of these bacteria are from environmental origin and act as opportunistic pathogens. Some isolates corresponded to unknown taxa when cultivated from CFRTS and supported the description of novel species such as *Inquilinus limosus* (Coenye et al., 2002). Other isolates corresponded to species isolated for the first time in man such as the acetic acid bacteria of the genus *Gluconobacter* previously recovered from environmental and food sources only (Alauzet *et al*., 2010). We previously reported eight other species detected in CF airways samples but so far described in food or environmental samples only: *Acetobacter fabarum*, *Advenella kashmirensis*, *Aquamicrobium lusatiense*, *Chryseobacterium bovis*, *Phyllobacterium myrsinacearum*, *Pseudomonas brenneri*, *Shinella yambaruensis* and *Sphingomonas pseudosanguinis* (Jumas-Bilak *et al*., 2011). This diversity suggested that the microbiota of the CF airways niche was far to be fully described. Finally, other isolates were identified as environmental bacteria also known to cause opportunistic infections in immunocompromised patients, for example members of the genera *Agrobacterium* or *Ochrobactrum* (Menuet *et al*., 2008).

2.1 Atypical bacteria identified by molecular means in our center

The following paragraphs and Table 1 present a summary about the atypical bacterial species cultivated from the respiratory tract of CF patients and identified by 16S rRNA gene sequencing in our center.

Methods performed for bacterial DNA extraction, 16S rRNA gene amplification and sequencing and sequence analysis were described elsewhere; particularly a threshold of 98.7% was considered for species identification (Stackebrandt & Ebers, 2006; Teyssier *et al*., 2003). A total of 23 atypical taxa were identified in 30 CFRTS from 25 patients. Three patients were colonized by 2 to 3 of these atypical species recovered either in a sample or in two distantly sampled specimens. Fourteen species had never been reported in man before being identified in CF patients, 6 were previously isolated in human clinical samples but were not previously reported in CF patients and 3 species were only isolated in CF patients. Among the taxa not previously isolated in man, four have been found in cultivation-independent studies of human biological samples, *Cupriavidus metallidurans* in skin microbiota (Grice *et al*., 2009), *Cupriavidus respiraculi* in small intestine microbiota (Franck *et al*., 2007), *P. myrsinacearum* in vaginal microbiota (Hyman *et al*., 2005) and *S. pseudosanguinis* in diabetic wound microbiota (Grice *et al*., 2010). Species subjected to detailed paragraphs were not included in Table 1. They were chosen to complete available recent reviews and/or to give information from personal data.

[2]Caring for more than 200 children and adults each year - 95 adults and 110 children in 2009, the CF center of the Montpellier University Hospital is a large regional French CF center.

Bacterial species (Patient designation)	**Non-human isolation**	**Isolation in non-CF patients**	**Isolation from CF RTS**	**Selected reference**
Advenella kashmirensis (1)	Temperate orchard soil	NPR	NPR	Ghosh *et al.*, 2005
Aquamicrobium lusatiense (2)	Activated sludge	NPR	NPR	Fritsche *et al.*, 1999
Chromobacterium aquaticum (9)	Spring-water	NPR	NPR	Young *et al.*, 2008
Chryseobacterium bovis (**3**)	Cow's milk	NPR	NPR	Hantsis-Zacharov *et al.*, 2008
Comamonas koreensis (**4**)	Forest sediment, wetland	NPR	NPR	Chang *et al.*, 2002
Phyllobacterium myrsinacearum (5)	Leaf nodules of tropical plants	NPR	NPR	Mergaert *et al.*, 2002
Pseudomonas brenneri (**6**)	Natural mineral waters	NPR	NPR	Baïda *et al.*, 2001
Pseudomonas nitroreducens (10)	Rhizospheric soil	NPR	NPR	Korade *et al.*, 2009
Shinella yambaruensis (7)	Soil	NPR	NPR	Matsui *et al.*, 2009
Sphingomonas pseudosanguinis (8)	Water reservoir of air humidifier	NPR	NPR	Kämpfer *et al.*, 2007a
Chryseobacterium indologenes (**3**)	Water, soil, hospital environment	Various	NPR	Lin *et al.*, 2010
Delftia tsuruhatensis (16-18)	Agricultural soil, bioreactor, activated sludge, rhizoplane	Catheter	NPR	Preiswerk *et al.*, 2011
Microbacterium sp.[a] (**3**)	Rhizosphere, mosquito, medical wastes	Various	NPR	Gneiding *et al.*, 2008
Nocardia cyriacigeorgica[b] (12-15)	Soil, animals (bovin, cat, dog)	Various	NPR	Schlaberg *et al.*, 2008
Tsukamurella sp.[c] (**4**)	Activated sludge	Blood, RTS, brain, cornea	NPR	Sheng *et al.*, 2009
Wautersiella falsenii (11)	Poultry	Various	NPR	Kämpfer *et al.*, 2006
Cupriavidus metallidurans (**19**)	Industrial biotopes	NPR	2 isolates	Coenye *et al.*, 2005
Cupriavidus respiraculi (20, 21)	NPR	NPR	23 isolates	Coenye *et al.*, 2005
Pandoraea apista (**22**)	NPR	NPR	22 isolates	Atkinson *et al.*, 2006
Pandoraea pulmonicola (23)	NPR	NPR	2 isolates	Coenye *et al.*, 2000
Bordetella petrii (24)	Polluted soil, river sediment, marine sponges, grass root	Bone, RTS	5 isolates	Spilker *et al.*, 2008
Brevundimonas diminuta (25)	Water, marine soil, petroleum oil, food	Various	1 isolate	Menuet *et al.*, 2008
Nocardia farcinica[b] (**22**)	Activated sludge, animals	Various	3 isolates	Bittar *et al.*, 2010

[a] 16S rRNA gene sequencing did not allow discrimination between *Microbacterium oxydans* and *Microbacterium paraoxydans*.
[b] Species identification was achieved by the Observatoire National des Nocardioses laboratory, Lyon, France, due to lack of discrimination between several nocardial species using 16S rRNA gene sequencing.
[c] No discrimination between *Tsukamurella tyrosinosolvens* and *Tsukamurella pulmonis*.
NPR, not previously reported; RTS, respiratory tract sample
Patients' designation in bold type indicated patients with other samples positive for atypical species listed either in Table 1 or in Table 3.

Table 1. Atypical bacterial species identified by 16S rDNA sequencing in CFRTS from patients attending the center of the University Hospital of Montpellier and general data on isolation in non-human specimens, in non-CF and CF patients.

2.2 *Inquilinus*

I. limosus is a gram-negative bacilli that grew slowly with non-pigmented and extremely mucoid colonies (Figures 1C and 1E) (Coenye *et al*., 2002). This alphaproteobacteria belongs to the order *Rhodospirillales* and to the family *Rhodospirillaceae* that groups environmental non-sulfur purple bacteria (Table 2). Members of this family were never isolated in man except for *I. limosus* that appeared human-associated. Since its characterization from CFRTS in 2002, *I. limosus* was regularly reported, mainly from CFRT (Bittar *et al*., 2008a; Chiron *et al*., 2005; Coenye *et al*., 2002). Most patients with *Inquilinus* were chronically colonized by *P. aeruginosa* and *Inquilinus* chronic colonization appeared usual in CF patients (Chiron *et al*., 2005; Hayes *et al*., 2009; Schmoldt *et al*., 2006). Typing *Inquilinus* strains by random amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) revealed no cross-transmission within centers and a diversity of contamination sources (Chiron *et al*., 2005; Schmoldt *et al*., 2006).

In our center, a 21-year-old patient is chronically colonized by *Inquilinus* sp. since the age of 12 years (Chiron *et al*., 2005). The patient has chronic colonization by a methicillin-susceptible *S. aureus* and was transiently colonized by *P. aeruginosa* (3 *P. aeruginosa* strains isolated since *Inquilinus* recovery and no *P. aeruginosa* isolated since 4 years). *Inquilinus* sp. bacterial load ranged from 10^4 to up to 10^8 CFU/ml depending on the sample, representing the dominant or one of the major species in the sputum samples. Despite environmental investigation, the source for infection remained unknown for this patient. Environmental sources for *Inquilinus* contamination are highly suspected but they were never traced. More generally, no environmental niche for *I. limosus* is detected when screening sequences deposited for environmental clones in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

2.3 Acetic acid bacteria

Members of the genus *Acetobacter*, *Gluconobacter* and *Asaia* were recently isolated from the respiratory tract of CF patients. These gram-negative bacilli belong to the family *Acetobacteraceae*, the second family forming the order *Rhodospirillales* in the alphaproteobacteria together with *Rhodospirillaceae*, the family of *Inquilinus* (Table 2). *Acetobacter*, *Gluconobacter* and *Asaia* are Acetic Acid Bacteria (AAB) characterized by their ability to oxidize alcohols or sugars, leading to the production of acetic acid. AAB are commonly found in soil or are associated with plants. They have been used in industrial food processing throughout human history, especially to convert wine to vinegar and to produce tropical fermented products (Yamada & Yukphan, 2008). The first report of human infection involving AAB dates from 2004, i.e. a case of peritonitis associated with *Asaia bogorensis* (Snyder *et al*., 2004). Since then, AAB have increasingly been reported as organisms potentially infecting humans and were firstly recognized in a CF patient in 2008 (Alauzet *et al*., 2010; Bittar *et al*., 2008a).

We reported four additional AAB isolates in 3 CF patients as follows: (i) successive isolation of an *Asaia* sp. and of two unrelated *Gluconobacter* sp. in a 2-year-old CF patient, (ii) a *Gluconobacter* sp. isolate unrelated to the strains from previous patient and recovered in a 3-year-old CF patient followed at the same CF center, (iii) an *A. fabarum* isolate in a 19.5-year-old CF patient (Alauzet *et al*., 2010; Jumas-Bilak *et al*., 2011). In these cases, AAB were

recovered at low bacterial load in the sputum samples (≤8 x 10^3 CFU/ml). AAB strains usually grew in 24 to 72 h at 30°C on various agar media selective for gram-negative bacteria except MacConkey agar whereas culture on the same media at 37°C yielded very tiny colonies (Figure 1D) (Alauzet *et al*., 2010; Bittar *et al*., 2008a). More generally, reports of *A. fabarum*, an AAB species recently characterized from fermented Ghanaian cocoa beans, and of *Gluconobacter* sp. increased the list of AAB, recently recognized as emerging opportunistic human pathogens, recovered from human samples (Alauzet *et al*., 2010; Cleenwerck *et al*., 2008).

2.4 *Agrobacterium*

Members of the order *Rhizobiales* and of the family *Rhizobiaceae*, *Agrobacterium* spp. are gram-negative, non-fastidious, non-fermentative short rods that form mucoid or non-mucoid colonies on agar media (Dunne *et al*., 1993). *Agrobacterium* are recovered from soil rhizosphere and are well-known plant-associated bacteria that may be phytopathogens. Modified strains of *Agrobacterium tumefaciens* are widely used in plant engineering. In the past two decades, *Agrobacterium radiobacter* has been recognized as an opportunistic human pathogen responsible for nosocomial infections, mainly bacteremia, peritonitis, and urinary tract infections despite virulence considered to be low (Chen *et al*., 2008; Edmond *et al*., 1993). In 2002, four *A. radiobacter* strains have been isolated from the respiratory tract of CF patients and it has been suggested that *A. radiobacter* may have the potential to spread from patient to patient (Coenye *et al*., 2002).

In 2010, we reported 19 additional isolates of *Agrobacterium* sp. from 17 CF patients; strains were analyzed by multilocus sequence typing (MLST) showing 11 different Sequence Types (STs), 17 of the 19 strains belonging to the genovar A7, a genovar that contained only clinical strains and probably adapted to human beings. Diversity in a single ST was demonstrated by PFGE showing that cross-contamination between patients did not occur in our center (Aujoulat *et al*., 2010). *A. radiobacter* was mainly recognized during transient colonization, no other isolate being recovered in the follow-up of most patients (19 patients out of the 22 currently colonized in our center, 86.4%). Patients' age at *A. radiobacter* isolation ranged from 6 months to 29 years (mean age, 9 years). Successive episodes of colonization occurred in 3 patients, from 2 months to 1.5 year apart. One of these cases was investigated by typing methods showing 2 unrelated isolates recovered 1.5 year apart. In three patients, two *A. radiobacter* isolates with different cultural characteristics were observed in a sample. One of these cases was further investigated showing the patient to be colonized by two genetically different and genomically unrelated strains. Bacterial load in samples was relatively low for most cases comprised between 10^2 and 10^3 CFU/ml except for three samples where the load was higher (from 10^4 to 2 x 10^6 CFU/ml) but *A. radiobacter* was not the dominant species. These samples were taken during scheduled consultation for two patients and during exacerbation attributed to *H. influenzae* for the third patient. Bacterial species mainly co-isolated are the usual pathogens *S. aureus* (9 patients) and *H. influenzae* (5 patients) while co-isolation of *P. aeruginosa* was noted in one patient only. Of note, *A. radiobacter* is usually recovered in highly diversified polymicrobial cultures associated with other rarely isolated organisms like *Acinetobacter* spp., *B. diminuta*, *Comamonas acidovorans*, *C. indologenes*, *C. respiraculi*, *D. tsuruhatensis*, *Enterobacteriaceae* (9 different species), *Ochrobactrum anthropi*, *Tsukamurella* sp. or *Roseomonas* sp. (16 out of 22 patients).

2.5 *Ochrobactrum*

Another member of the order *Rhizobiales* in alphaproteobacteria, the genus *Ochrobactrum* groups bacteria increasingly reported in CF (Menuet *et al*., 2008; Yagüe-Muñoz *et al*., 2010). First considered as an emerging pathogen by Menuet *et al*., *O. anthropi* was responsible for a bacteremia in CF. *Ochrobactrum* spp. are gram-negative non-fermentative oxidase-positive short rods recovered from a wide variety of environmental sources (water, soil, rhizosphere) as well as from plants, animals and human. Five species, *O. anthropi*, *O. intermedium*, *O. pseudintermedium*, *O. haematophilum* and *O. pseudogrignonense* were recovered from human samples; the first two species being increasingly reported as opportunistic pathogens mainly during nosocomial infections, particularly bacteremia and endocarditis (Kämpfer *et al*., 2007b; Teyssier *et al*. 2005, 2007). *Ochrobactrum* spp. do not present exigent cultural requirements and colony morphology depends on the species (Teyssier & Jumas-Bilak, 2011).

In our center, 14 patients are colonized by *Ochrobactrum* spp. strains (mean age, 3 years [10 months-18 years]) and 35 isolates were recovered (one to 10 isolates per patient). Serial isolates were isolated in seven patients. *O. anthropi* was the major species recovered in CF patients, all the isolates except three being identified as *O. anthropi*. Moreover, *O. intermedium* (n=1) and *O. pseudogrignonense* (n=2) were isolated from patients also colonized by *O. anthropi*. The relative importance of species observed in CF did not reflect the distribution of species in the general population where *O. intermedium* was more frequently represented (Teyssier *et al*., 2003). In a collection of 66 *Ochrobactrum* spp. from the non-CF population attending the University Hospital of Montpellier, species identified by molecular means were distributed as follows: *O. anthropi* (n=37, 56.1%), *O. intermedium* (n=25, 37.9%), *O. pseudogrignonense* (n=1, 1.5%), and *O. pseudintermedium* (n=3, 4.5%) (unpublished data). Two strains of *O. pseudogrignonense* recovered from human clinical samples (blood and ear) supported the description of the species in 2007, then this recently described species was recovered from CFRTS, two patients attending our center being colonized by unrelated *O. pseudogrignonense* strains. Main associated bacteria were *S. maltophilia* (8 patients), *Enterobacteriaceae* (7 patients), and *S. aureus* and *H. influenzae* (6 patients each). Concomitant isolation of *P. aeruginosa* was observed for 6 samples from 4 patients while co-isolation of atypical species like *A. radiobacter*, *Acinetobacter* spp., *Alcaligenes* spp., *A. kashmirensis*, *C. acidovorans*, *C. indologenes*, *D. tsuruhatensis* and *S. paucimobilis* was frequently observed. Bacterial load was comprised between 10^2 and 4 x 10^4 CFU of *Ochrobactrum* spp./ml and in most samples *Ochrobactrum* spp. were not the dominant species. Molecular typing based on PFGE and MLST revealed a high level of diversity among isolates showing that no epidemic strains spread occurred in our center (Romano *et al*., 2009). The same typing methods showed that serial isolates recovered from a patient could correspond to successive colonization by unrelated strains. Such successive episodes of colonization were observed in 5 patients. By contrast, chronic colonization was noted over a 10-month period (4 serial isolates) for one patient with intercurrent isolation of unrelated *O. anthropi* strains and for a 3-year period in a second patient (2 isolates). Complex route of colonization by *Ochrobactrum* spp. in CF is revealed here and warrants further investigation to search for the diversity of sources.

2.6 How unusual are atypical CF-associated bacteria?

2.6.1 Incidence of atypical species in CF

When looking at the isolation frequency of the atypical bacteria described by Hauser *et al*. in our CF center, we found that most of the taxa cited were recovered from sputum samples of the patients thereby underlining that these species were not exceptionally isolated from CFRTS (Hauser *et al*., 2011).

Strains whose identification was confirmed by 16S rRNA gene sequencing were listed in Table 1. Regarding other taxa detailed herein, AAB have been recovered from the respiratory tract of about 1% of the patients attending our CF center and are still to be considered as an unusual isolation in CF. *Agrobacterium* and *O. anthropi* isolates were found in about 10% and 7% of the patients, respectively and should not be considered anymore as unusual species in CFRT. Other species of *Ochrobactrum* are still to be considered as very unusually isolated in CF. A unique patient was chronically colonized by *Inquilinus* sp. in our CF center (0.5% of the patients) while higher incidence was reported in other CF centers. Notably, a higher incidence is reported in a neighbor region in South of France because *Inquilinus* was reported in 2.8% out of 145 CF patients, incidence varying according to age from 1.2% in children to 4.9% in adult patients (Bittar *et al*., 2008a). *Inquilinus* sp. was not found in the respiratory tract of non-CF patients (Bittar *et al*., 2008a). Interestingly, we observed that patients colonized by one of the previous taxa are often simultaneously or successively colonized by other of these species. For example, among the 22 patients with at least one isolate of *Agrobacterium* sp., 9 had at least one episode of colonization by another environmental alphaproteobacteria. The two patients with AAB were also colonized by *A. radiobacter* alone or associated with *O. anthropi* colonization. Other species or genera cited by Hauser *et al*. that were isolated in our center but not detailed here are *Acinetobacter* spp., *Chryseobacterium* spp. and members of the family *Enterobacteriaceae*.

2.6.2 Atypical or underestimated species?

Modification in cultivation and identification methods applied for CFRTS processing may explain an increasing rate of recovery of some species during CF. For example, AAB are increasingly recognized as emerging human opportunistic pathogens and their frequency may probably be underestimated because of their growth characteristics, particularly their faint growth at 37°C, a default temperature setting in routine medical microbiology, and because of the difficulty with identifying these microorganisms. For instance, the recovery of AAB in CF was related to the use of *Burkholderia cepacia* complex selective agar that is incubated for a prolonged incubation time (5 days) at 30°C. Therefore, the recovery of *Asaia* and *Gluconobacter* is enhanced because they resisted to antibiotics included in the medium and they grew in such cultivation conditions while no growth is observed on MacConkey agar plates incubated for 3 days at 30°C or 37°C (Alauzet *et al*., 2010) (Figure 1D).

Growth of atypical bacteria on different media and at different incubation temperatures is shown in Figure 1.

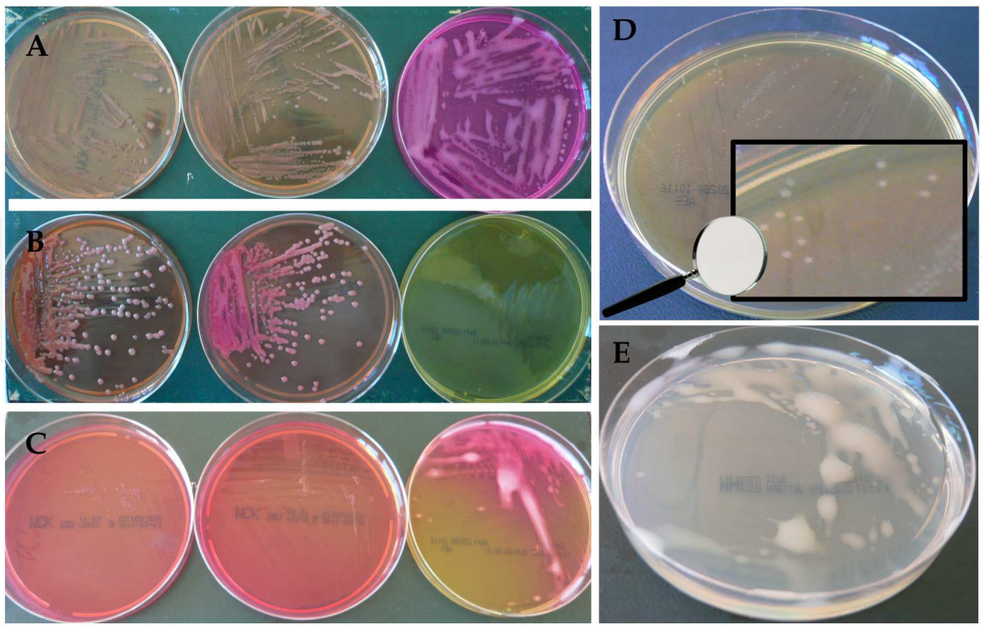

Fig. 1. Growth of *O. anthropi* (A), *A. radiobacter* (B) and *Inquilinus* sp. (C) on MacConkey agar plates (bioMérieux) incubated at 37°C (left) or 30°C (middle) and on the *Burkholderia cepacia*-selective medium Cepacia agar (AES) incubated at 30°C (right); growth of *Gluconobacter* sp. on *B. cepacia*-selective medium Cepacia agar (D), and growth of *Inquilinus* sp. on Mueller-Hinton agar (bioMérieux) (E) (incubation time was 3 days in all cases).

In addition to limitation related to cultivation conditions, recovery of atypical species could also be impaired by the routine practice of CFRT sampling. Indeed, the unique sample usually submitted to bacteriological analysis was shown to be insufficient for recognition of all bacteria that may colonize the patient, including CF pathogens (Rogers *et al*., 2010).

Regarding identification, molecular-based methods revealed up to 25% of isolates without correct phenotypic identification (Bittar *et al*., 2008b). *A. radiobacter* strains are accurately identified with API 20NE strip or VITEK2 GN card (bioMérieux) but *A. radiobacter* is named *Rhizobium radiobacter* in API and VITEK2 databases due to confusing taxonomy in these genera (Aujoulat *et al*., 2010; Otto-Karg *et al*., 2009; Teyssier *et al*., 2009). Identification could be more difficult for other taxa. *O. anthropi* is the sole species of the genus included in API and VITEK2 databases. Both systems permit genus-level identification and sequencing of either 16S rRNA gene or another housekeeping gene should be performed for species identification. Although *Inquilinus* sp. showed several notable characteristics that will be discussed below, i.e. mucoid phenotype, characteristic multiresistant pattern to antibiotics and ability to persist in the CF airways, its identification remains difficult. This emerging pathogen was either not detected or misclassified by laboratories (Bittar *et al*., 2008b; Hogard *et al*., 2009). Since 2011, a second species has been described in the genus, *Inquilinus ginsengisoli*, isolated from soil (Jung *et al*., 2011). This species could be differentiated from *I. limosus* by a careful 16S rRNA gene analysis. Similarly, both genera of AAB recovered in CF patients, i.e. *Asaia* and *Gluconobacter* required molecular methods for their identification. However, some closely related species belonging to these genera might remain unidentified despite sequencing housekeeping genes in addition to 16S rRNA gene (Alauzet *et al*., 2010).

Recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) was used for identification of non-fermentative gram-negative bacilli isolated from CF patients (Degand *et al*., 2008). A few atypical species covered by this chapter were included, i.e. 1 *B. hinzii*, 1 *I. limosus*, 1 *C. respiraculi*. The three isolates were correctly identified by the system. Another study evaluating the system for identification of environmental members of the family *Rhizobiaceae* including *Agrobacterium* (= *Rhizobium*) *radiobacter* showed comparable performances to housekeeping gene sequence analysis suggesting that this species might be correctly identified by the system if included in the "CF" database (Ferreira *et al*., 2011). Additional studies on a larger panel of isolates are needed to precisely evaluate the performance of the system for identifying all the diversity of atypical bacteria that may be encountered in CF samples.

2.7 Taxonomic diversity of atypical bacteria in CF

Atypical taxa isolated from CFRTS in our center and identified by 16S rRNA gene sequencing are distributed among 3 phyla, the phylum *Proteobacteria* being the most represented. The Table 2 gives the taxonomic repartition of the atypical taxa in the 3 major phyla. In the *Proteobacteria*, gram-negative bacilli of the class *Alphaproteobacteria* account for the majority of atypical taxa identified (Table 2). This may suggest that patients are more frequently in contact with the environmental niches of these species and/or that these taxa have an increased potential to colonize the CFRT.

Phylum	Class	Order	Family	Taxon
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Actinomyces graevenitzii*
			Microbacteriaceae	*Microbacterium* sp.
			Nocardiaceae	*Nocardia* spp.
			Tsukamurellaceae	*Tsukamurella* sp.
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium* spp.
				Wautersiella falsenii
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas diminuta*
		Rhizobiales	*Brucellaceae*	*Ochrobactrum* spp.
			Phyllobacteriaceae	*Aquamicrobium lusatiense*
				Phyllobacterium myrsinacearum
			Rhizobiaceae	*Agrobacterium* sp.
				Shinella yambaruensis
		Rhodospirillales	*Acetobacteraceae*	*Acetobacter fabarum*
				Asaia sp.
				Gluconobacter sp.
			Rhodospirillaceae	*Inquilinus limosus*
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas pseudosanguinis*
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Advenella kashmirensis*
				Bordetella spp.
			Burkholderiaceae	*Cupriavidus* spp.
				Pandoraea spp.
			Comamonadaceae	*Comamonas koreensis*
				Delftia tsuruhatensis
		Neisseriales	*Neisseriaceae*	*Chromobacterium aquaticum*
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas brenneri*
				Pseudomonas nitroreducens

Table 2. Taxonomic lineages for atypical bacteria identified from CFRTS in our center using 16S rRNA gene sequencing.

2.8 Unknown cultivated bacterial taxa in CF

Diversity of the cultivable part of the CFRT microbiota remains underestimated. In the past decade, 12 novel species were characterized based on isolates recovered in CFRTS, i.e. 7 *Burkholderia* species, 3 *Ralstonia* species (of which 2 are yet reclassified in the genus *Cupriavidus*), *Advenella incenata* and *I. limosus* (PubMed search on August the 1st, 2011 with key words "sp. nov." and "cystic fibrosis"). In our center, identification based on 16S rDNA revealed 5 potential novel taxa cultivated from CFRTS (Table 3). Of note, 2 patients had other samples positive for atypical species listed in Table 1. Altogether these data illustrate the diversity not fully explored of bacteria that could be cultivated from CFRTS.

Isolate reference	Patient no.	16S rDNA sequence similarity with the closest known species (%, name of the species)	Taxonomic interpretation*
29 dec. 2009, 2320	26	96%, *Corynebacterium durum*	Novel species in the genus *Corynebacterium*
15 jun. 2007, 5197	**19**	96.5%, *Cupriavidus metallidurans*	Novel species in the genus *Cupriavidus*
21 jul. 2009, 2477	27	97.6%, *Roseomonas cervicalis*	Probable novel species in the genus *Roseomonas*
20 nov. 2009, 5248	28	98.2%, *Cupriavidus respiraculi*	Probable novel species in the genus *Cupriavidus*
15 apr. 2005, 5138	**6**	98.5%, *Cupriavidus respiraculi*	Probable novel species in the genus *Cupriavidus*

* Isolates showing less than 97% of sequence similarity with the closest known species were considered as novel taxa (Stackebrandt & Goebel, 1994); isolates showing between 97% and 98.7% of sequence similarity with the closest known species were considered as probable novel taxa (Stackebrandt & Ebers, 2006). Bold type indicated patients with other samples positive for atypical species as listed in Table 1.

Table 3. Unknown taxa in the CFRTS from patients attending the CF center of the University Hospital of Montpellier.

3. Atypical pathologic communities in CF

3.1 Microbial diversity in CFRT assessed by cultivation-independent studies

Over the last decade, our knowledge of the bacterial diversity in CFRT microbiota has evolved due to cultivation-independent methods. Terminal Restriction Fragment Length Polymorphism Profiling, Temporal Temperature Gradient gel Electrophoresis or sequencing were employed to characterize 16S ribosomal DNA in CFRT community and revealed both a higher biodiversity than previously presumed and several atypical organisms (Bittar *et al*., 2008b; Guss *et al*., 2011; Kolak *et al*., 2003; Rogers *et al*., 2004).

Comparison between cultivation-based methods and cultivation independent methods revealed the overlooked part of bacteria present in CFRTS including species recovered from the oral microbiota during health and diseases, atypical species of unknown pathogenicity and well-known bacterial species pathogenic for CF patients (Bittar *et al*., 2008b; Rogers *et al*., 2009; van Belkum *et al*., 2000). For example, Bittar *et al*., studying 25 CF sputum samples

showed that 53 species were found using the PCR-cloning-sequencing approach while only 13 were cultivated (Bittar *et al*., 2008b). Sixteen species only found by the molecular method corresponded to anaerobes not covered by the cultivation conditions used in the study. Nevertheless, several aerobic and anaerobic species detected by the genomic method corresponded to species unusually or firstly detected in CF (Bittar *et al*., 2008b).

Additional unexpected bacteria were described in other cultivation-independent studies. For example, Harris *et al*. reported in a 14-year-old CF patient the first occurrence in man of sequences corresponding to the alphaproteobacteria *Chelatococcus asaccharovorans* (Harris *et al*., 2007). These sequences represented 5% of the total sequences found in the corresponding specimen. Some of the other unexpected bacteria overlooked by cultivation-based methods and revealed by cultivation-independent methods may be clinically relevant in CF. Indeed, CF candidate pathogens were identified by Harris *et al*. comparing microbiota from CF and non-CF patients as follows: *Prevotella denticola*, *Lysobacter* sp., and *Rickettsiales* sp. (Harris *et al*., 2007). Interestingly, members of the genus *Lysobacter* are gram-negative bacilli showing similar environmental lifestyle as *Agrobacterium* and *Ochrobactrum*, i.e. isolated from soil, rhizosphere and plant-associated samples. *Lysobacter* spp. were also found as dominant species on the human tongue dorsum and recovered from the surface of prosthetic hip joints (Dempsey *et al*., 2007; Riggio *et al*., 2008). Unless additional arguments are given, *Lysobacter* sp. should be considered with caution as a CF potential pathogen. Finally, cultivation-independent studies revealed the occurrence of several unknown taxa in the CFRTS like novel members of the order *Rickettsiales* and of the family *Coxiellaceae* (the latter representing 11% of the sequences found in association with those of *Chelatococcus asaccharovorans*) (Harris *et al*., 2007). Regarding bacterial taxa developed in this chapter, i.e. *Inquilinus*, acetic acid bacteria, *Agrobacterium* and *Ochrobactrum*, sequences of *Inquilinus* sp. were recovered in some cultivation-independent studies while the other taxa were not found (Bittar *et al*., 2008b). In addition to patients' sampling methods, these taxa may be overlooked in molecular-based approaches due to their minority in the CFRT microbiota, a hypothesis congruent with the low bacterial load observed in culture for these bacteria.

3.2 Dysbiosis in the CF polymicrobial disease and example of anaerobes

Comparison of microbiota diversity in CF patients and in control groups, as well as cultivation-independent studies suggested that CF should be considered as a polymicrobial disease (Klepac-Ceraj *et al*., 2010; Sibley *et al*., 2006). A major recent finding revealed that lung function is significantly and positively correlated with the bacterial species richness of the global microbiota (van der Gast *et al*., 2011). Considering the inter-individual variations in the microbiota composition, van der Gast *et al*. studying microbiota composition of sputum samples from 14 adult CF patients further showed that both core and satellite taxa are significantly correlated with lung function (van der Gast *et al*., 2011). Moreover, any disequilibrium in the consortium of microorganisms found in the CFRT may have clinical consequences on the clinical status as previously observed in other dysbiosis-associated diseases like bacterial vaginosis (Oakley *et al*., 2008). Although the total number of bacterial species observed in a population of CF patients was shown significantly more diverse than that observed in a control group including bronchiectasis patients (Bittar *et al*., 2008b), at the individual level, libraries of lower complexity were observed in CF compared to a control group (Harris *et al*., 2007). Such a low diversity observed in CF patients may reflect the enrichment of a pathogenic species

and/or the consequence of dysbiosis and so possibly signify bacterial involvement in disease (Harris *et al*., 2007). For example, among the 28 CF patients included in the study, more than the half (53.6%) had less than 5 organisms detected and one of these microorganisms dominated the poorly diversified microbiota (from 63 to 98% of the sequences). For 8 patients, all sequences corresponded to a unique bacterial taxon (*S. aureus*, *Lysobacter* sp., *S. maltophilia*, *P. aeruginosa* and *Mycobacterium abscessus*). Atypical bacteria proposed as CF candidate pathogens and cited above, i.e. *P. denticola*, *Lysobacter* sp., and *Rickettsiales* sp. were all encountered in dysbiotic environment. Indeed, these sequences were each recovered in a CF patient as a major or as the unique sequence among sequences of the patient's microbiota, representing 56%, 100% and 36% of the total sequences, respectively (Harris *et al*., 2007). Based on the example of *P. denticola*, we will discuss further on the role of anaerobes, particularly those belonging to the genus *Prevotella,* in the dysbiosis that may occur in CF (for a review on anaerobic bacteria infection in cystic fibrosis airway disease, see Lambiase *et al*., 2010).

In both cultivation-based and cultivation-independent studies, particular attention was recently paid to anaerobic microflora, which had received little attention before (Bittar *et al*., 2008b; Harris *et al*., 2007; Tunney *et al*., 2008). Indeed, anaerobic cultures are not performed in the routine practice of sputum samples and thus anaerobic bacteria are totally ignored except when specifically studied (Jewes & Spencer, 1990). Tunney *et al*. assessing anaerobic bacteria in CF children by means of culture of bronchoalveolar lavage fluid samples demonstrated that anaerobic bacteria are: (i) frequently present in the airway specimens, (ii) in higher numbers than in healthy volunteers, and (iii) generally different species compared with those detected in the non-CF control group (Tunney *et al*., 2008). Identification of the anaerobes isolated revealed 14 different genera with the genus *Prevotella*, being the most frequently isolated before *Veillonella*, *Propionibacterium*, and *Actinomyces*. *Prevotella* spp. were present in 22% to > 80% of the CF patients depending on the study while found in 10% of healthy patients (Field *et al*., 2010; Tunney *et al*., 2008). Based on cultivation-independent methods, Bittar *et al*. showed the anaerobes to represent 30.2% of the detected species in CF sputum specimens (16/53 species) with *Prevotella* sequences being dominant (48.7%) among sequences corresponding to anaerobes (Bittar *et al*., 2008b). *Prevotella melaninogenica* is usually the most common species identified in these studies. Strict anaerobes are well-known oral species. However, they were not regarded to be simply contaminants because of their diversity and abundance within the CF airways compared to non-CF population (Jones, 2011). Among them, *Prevotella* spp., already known as contributing to the consortia of microorganisms involved in several human pathologies attributed to dysbiosis, may contribute to CF airway disease (Alauzet *et al*., 2010; Field *et al*., 2010).

What remains also unknown is how the quantitative and/or qualitative modification in the composition of the microbiota would affect interactions between organisms. These interactions were previously demonstrated in complex microbiota like modulation of *P. aeruginosa* gene expression by host microflora through interspecies communication (Duan *et al*., 2003; Sibley *et al*., 2008b). It was hypothesized that *Prevotella* spp. may also modulate *P. aeruginosa* virulence gene expression as well as growth and virulence of the potential CF pathogen of the *Streptococcus milleri* group (Field et al., 2010; Shinzato *et al*., 1994; Sibley *et al*., 2008a). Unfortunately, *Prevotella* spp. were not included by Sibley *et al*. in the 40 oropharyngeal species tested for both microbe–microbe and polymicrobe–host interactions in *Drosophila melanogaster* infection model (Sibley *et al*., 2008b).

4. Pathogenesis and clinical consequences of colonization by atypical bacteria

Harris *et al*. previously hypothesized that atypical bacteria may explain inflammation in the absence of documented pathogens as well as failure to standard treatment in CF (Harris *et al*., 2007). The clinical significance in CF of bacteria detailed in this chapter remains unclear, as it is still the case for more frequently isolated species like *A. xylosoxidans* (Hauser *et al*., 2011).

Regarding anaerobes, Worlitzsch *et al*. showed that patients with and without obligate anaerobes in sputum specimens did not differ in lung function (Worlitzsch *et al*., 2009). AAB, *Agrobacterium*, *Inquilinus* and *Ochrobactrum* members are considered as opportunistic human pathogens, being involved in systemic or severe infections in immunocompromised patients or patients with underlying diseases/conditions (Alauzet *et al*., 2010; Chen *et al*., 2008; Cieslak *et al*., 1996; Kiratisin *et al*., 2006).

Case reports documented potential virulence in CF for some of these atypical bacteria. For AAB, the first case report in CF documented *Acetobacter indonesiensis* isolation during pneumonia occurring after lung transplant. The bacterium was considered to be the primary cause of the infection because of clinical improvement after adapted antimicrobial therapy (Bittar *et al*., 2008a). By contrast, *Gluconobacter* and *Asaia* sp. could not be incriminated in the evolution of the disease because of a favorable clinical evolution without any specific treatment (Alauzet *et al*., 2010). Case reports also witnessed for a potential pathogenic power of *Ochrobactrum* sp. in CF patients because *O. anthropi* was previously involved: (i) in association with *B. diminuta* in a case of acute pneumonia in a 17-year-old CF patient showing clinical improvement after adapted antimicrobial therapy including imipenem and tobramycin (Menuet *et al*., 2008), (ii) in a case of bacteremia in children (Yagüe-Muñoz *et al*., 2010). *Inquilinus* isolation was associated either with acute pulmonary exacerbation, respiratory decline without signs of acute exacerbation or stable respiratory status (Chiron *et al*., 2005; Schmoldt *et al*., 2006). To date, the patient chronically colonized by *Inquilinus* sp. in our center has stable respiratory status. *Inquilinus* sp. was also responsible for prosthetic valve endocarditis in a tetralogy of Fallot patient (Kiratisin *et al*., 2006). Additional arguments in favor of pathogenic potential of *Inquilinus* sp. are: (i) specific serum antibody response found in patients with *Inquilinus* sp. (Schmoldt *et al*., 2006), (ii) mucoid characteristic of *Inquilinus* sp. that could be related to exopolysaccharides, recognised as important virulence factors in lung infections, showing novel structures with usual components and similarity with *P. aeruginosa* exopolysaccharides (Herasimenka *et al*., 2007). Finally, no clinical data associated with *A. radiobacter* isolation in CF are currently available. From our personal data, *A. radiobacter* was mainly a transient colonizer of the CFRT while *Ochrobactrum* spp. displayed more complex relationships with CF host. These species were usually recovered at low bacterial load and in mixed cultures from the respiratory secretions sampled in patients during scheduled consultations. Their recovery was mainly associated with stable respiratory status but in some cases, respiratory decline with or without signs of acute exacerbation were noted. In these cases, multiple species were simultaneously isolated from the CFRTS, making it difficult to attribute signs and symptoms to a specific bacterium.

Treatment against these species was usually not started except in one case of *A. radiobacter* isolation that was recovered at low bacterial load but in pure culture in a context of respiratory decline in a 4-year-old patient. Trimethoprim/sulfamethoxazole treatment for 15 days led to eradication of the species from the airways and clinical improvement in this patient. In another patient with deteriorated respiratory status, antimicrobial treatment associating ciprofloxacin and trimethoprim/sulfamethoxazole was established. According to the antibiograms, this treatment was effective against all bacteria cultured from the sputum specimen, i.e. *A. radiobacter* but also *D. tsuruhatensis*, two enterobacteria and *S. aureus*, the major species in the sample and showed efficacy on the clinical status of the patient.

Of note, *Agrobacterium* and/or *Ochrobactrum* spp. were relatively frequently isolated after antimicrobial treatment against *P. aeruginosa* or *S. aureus* due to their resistance to amoxicillin/clavulanic acid, ceftazidime, tobramycin and/or colistin. Indeed, as previously described for other mild opportunistic pathogens of environmental origin like *S. maltophilia*; acetic acid bacteria, *Inquilinus* and *Ochrobactrum* spp. displayed a high level of resistance to antibacterial compounds (Alauzet *et al*., 2010; Berg *et al*., 2005; Bittar *et* al, 2008a; Thoma *et al*., 2009) (Figure 2).

Multiresistance-encoding genetic determinant has only been characterized for *O. anthropi* as a chromosomal class C beta-lactamase named OCH-1 while remaining unknown for *Agrobacterium*, AAB, *Inquilinus* sp. and other *Ochrobactrum* species (Nadjar *et al*., 2001). Moreover, majority of these species displayed intrinsic resistance to colistin. *Agrobacterium* sp. resisted to several drugs used in CF patients like ceftazidime and tobramycin but displayed resistance to less drugs than AAB, *Inquilinus* and *Ochrobactrum*.

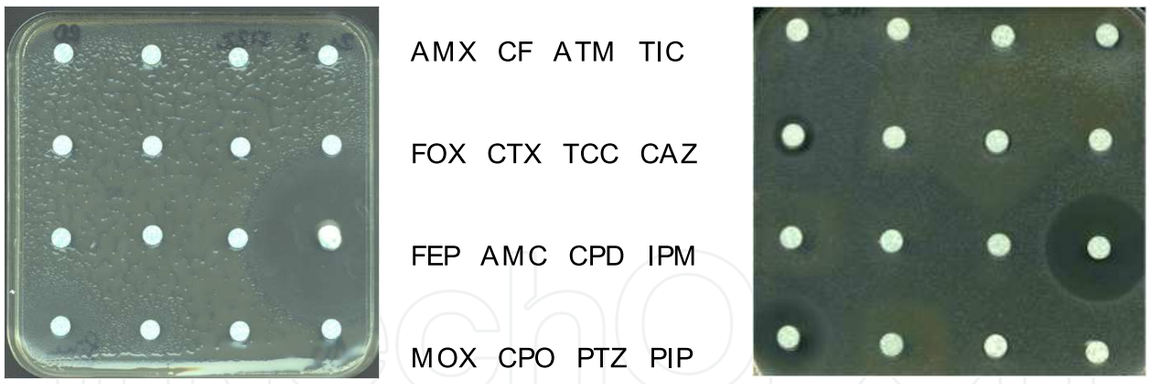

Fig. 2. Multiresistance pattern to β-lactam agents observed for *Inquilinus* sp. (left) and *O. intermedium* (right) by disk diffusion assay (antibiotic disk position is indicated by corresponding drug abbreviation in the middle).

Abbreviations and concentrations for antibiotics indicated according to disk position are: AMX, amoxicillin (25 µg); CF, cephalotin (30 µg); ATM, aztreonam (30 µg); TIC, ticarcillin (75 µg); FOX, cefoxitin (30 µg); CTX, cefotaxime (30 µg); CAZ, ceftazidime (30µg); TCC, ticarcillin/clavulanic acid (75 µg /10 µg); FEP, cefepime (30 µg); AMC, amoxicillin/clavulanic acid (20 µg /10 µg); CPD, cefpodoxime (30 µg); IPM, imipenem (10 µg); MOX, latamoxef (30 µg); CPO, cefpirome (30 µg); PTZ, piperacillin/tazobactam (75 µg /10 µg); PIP, piperacillin (75 µg).

There are too few isolates reported in the literature and even fewer CF case reports involving these atypical bacteria to drawn conclusion on their clinical relevance in CF. Moreover, interactions between these atypical species and other organisms within the CFRT microbiota is unknown. Knowledge on the virulence of these atypical bacteria required rigorous description and follow-up of cases involving such bacteria. Moreover, case control studies will also be needed to determine their clinical implication in CF patients as well as risk factors for acquisition. From our experience, it could be hypothesized that these species may be selected by antimicrobial therapy against pathogens due to their resistance or multidrug resistance. This has been previously suggested for *S. maltophilia,* which has a predilection to infect CF patients with more advanced disease and consequently more frequently exposed to broad-spectrum antibiotics (Hauser *et al*., 2011). Similarly, the increased use of nebulized colistin in CF patients may select specific colistin-resistant bacteria as previously suggested for *B. diminuta* (Menuet *et al*., 2008).

Besides intrinsic resistance, several bacteria present in the CFRT microbiota may acquire additional resistance mechanisms. Development of multidrug resistance is a frequent finding in CF and is usually mediated by combination of several resistance mechanisms (Poole, 2011). Acquired multidrug resistance may also be encoded by extended spectrum β-lactamases (ESBLs) and carbapenemases, which are increasingly reported in pathogens commonly found in CF (*P. aeruginosa*, *S. maltophilia*). *Enterobacteriaceae* can harbor ESBL-encoding genes localized on mobile genetic elements that may be transferable between members of the community. Although such observations remain rare (Cantón *et al*., 1997; 2 unpublished isolates in our centre), microbiologists have to be aware of multidrug resistant enterobacteria in CF due to pandemic dissemination of some enzymes like CTX-M ESBLs in the global population (Cantón *et al*., 2006). In this context, atypical bacteria from environmental origin, even transiently colonizing CFRT may constitute a reservoir of resistance determinants that can be mobilized into the microbial community, thereby contributing to the global increase of the microbiota resistance (Wright, 2010). Moreover, antibiotic degrading diffusible enzymes that may be secreted by atypical bacteria are a matter of concern. Indeed, antimicrobial treatment against pathogens associated with these atypical bacteria may become ineffective due to antibiotic hydrolysis by these enzymes. Altogether, bacteria showing multidrug resistance whether this resistance is innate or acquired contribute to the increase of the global resistome of the CFRT microbiota.

5. Adaptation of atypical bacteria to the CF airways niche

5.1 Adaptation to CFRT conditions

Airways of CF patients represent an ecological niche recognized as a model system for studies on bacterial adaptation. Indeed, in this specific niche, the bacteria incoming from the outer environment are submitted to complex selective forces from microbiological, immunological, physiological and biochemical environment of the CF airways that may drive the evolution of the corresponding microorganism (Yang *et al*., 2011a, 2011b). Several microbial species appear to be well adapted to survival within the CF airways (Hauser *et al*., 2011). Some species may adapt by forming colony variants, i.e. small-colony variants for *S. aureus* and *P. aeruginosa* or mucoid colony variants for *P. aeruginosa*, favoring resistance to

antibiotics, evasion to immune system and then long-term persistence in the CF airways. *P. aeruginosa*, the most studied pathogen, may adapt by a wide range of mechanisms that were recently reviewed (Hauser *et al*., 2011).

For atypical bacteria considered herein, little is known about the mechanisms of adaptation to host environment i.e. to CFRT. Extrapolating from *P. aeruginosa*, mucoid phenotype (*Inquilinus*, *Ochrobactrum*), antibiotic resistance (*Inquilinus*, *Ochrobactrum*, *Agrobacterium*), modifications in lipopolysaccharide (gram-negative genera) or existence of subsets of host-adapted strains (*Ochrobactrum, Agrobacterium*) are traits that may favor adaptation to CFRT. Although not yet further investigated, we observed diversification in both colonial morphology and antibiotype (susceptibility/resistance to fluoroquinolones) after a 6-year period of *Inquilinus* sp. colonization. Moreover, population genetics revealed lineages of *Agrobacterium* and *O. anthropi* adapted to man but not specifically CF-adapted in contrast to *P. aeruginosa* for which two genotypes were shown as specifically-associated with CF (Aujoulat *et al*., 2010; Romano *et al*., 2009; van Mansfeld *et al*., 2010). In addition, some other mechanisms of adaptation may be suspected for bacteria covered by this chapter. As far as AAB are concerned, it was previously hypothesized that they might specifically colonize the CFRT in relation to their ability to grow in acidic conditions. Indeed, this particular metabolic trait may confer a selective advantage to these bacteria in the acidified CF airways (Alauzet *et al*., 2010; Poschet *et al*., 2002).

5.2 Adaptative evolution in the CFRT

CFRT is a compartimentalized niche, which is spatially and temporally heterogeneous according to the anatomic site and to the period of disease evolution. A variable but relatively closed niche could drive diversification and adaptative evolution of the microbiota. In addition, mortality agents such as host immunity, antibiotics and lysogenic phages could lead to the diversification of a bacterial population that contribute to the persistence of the infection, as previously described for *P. aeruginosa* in the CF lung (Brockhurst *et al*., 2005).

The ability to switch to hypermutable phenotypes by rapid acquisition of mutations at an unusually high rate lead to phenotypic diversification as observed for *H. influenzae*, *S. aureus* and *P. aeruginosa* (Hauser *et al*., 2011). Besides hypermutation, genome plasticity is a mechanism for a bacterium to diversify its population and to adapt in various environments. Genomic rearrangements have been described in *P. aeruginosa* to switch from a saprophytic to a pathogenic lifestyle. Large chromosomal inversions are associated with insertion sequences duplication in *P. aeruginosa* strains isolated in CF. These events, by disrupting genes, have been shown to be involved in phenotypic adaptation of the strains to their particular environment (Coyne *et al*., 2010).

Genomic macrorestriction followed by PFGE is an efficient tool to follow genomic rearrangements, particularly in bacterial species poorly investigated at the genetic level, as atypical species isolated from CFRT. Genomic evolution was previously demonstrated in serial isolates recovered from the respiratory tract of a non-CF patient chronically colonized over a 1.5-year period by *O. intermedium* (Teyssier *et al*., 2003). The clone evolved *in vivo* by a deletion of a 150 kb-genomic fragment, which included one copy of ribosomal operon. It was suggested that: (i) the new genomic organization gave a selective advantage to the

strain *in vivo*, (ii) the genomic reduction may be an adaptative phenomenon of this free-living bacterium to the narrow ecological niche represented by the human respiratory tract. Indeed, the relation between host-restricted lifestyle and a small genome size is patent in bacteria, particularly in alphaproteobacteria (Moreno, 1998). We recently hypothesize that a phenomenon of genomic rearrangement might also be observed in *Inquilinus* sp. strains. Investigation was conducted on 21 serial isolates recovered during the 9-year follow-up of a CF patient chronically colonized by *Inquilinus* sp. PFGE analysis revealed the genomic stability of the strains while showing the existence of two closely related variants. Genomic reduction is suspected in one of the two co-existing variants suggesting that an adaptation process to human host is ongoing in these isolates but this should be further investigated (Teyssier *et al*., 2011). This hypothesis was supported by comparison of sequential *Inquilinus* isolates recovered in one German patient revealing identical RAPD profiles but slightly different protein patterns. Expression of two antigens disappeared between successively isolated strains and was considered as suggestive of an adaptation of the *Inquilinus* clone during the course of infection (Schmoldt *et al*., 2006). As observed by PFGE, the two variants co-existed in the respiratory tract.

6. Hypothesis: CFRT is a hotspot for emergence of human pathogens

The emergence of human pathogens from environment involves a dramatic jump in the lifestyle of bacteria. CFRT and atypical bacteria provide examples for different types of lifestyle switches. Bacteria like *Ochrobactrum* spp. have a very versatile behavior with no obvious pathogenicity except in man but with close association with diverse organisms, association that can be as close as symbiosis (Teyssier *et al*., 2004). In the respiratory tract, *O. intermedium* can evolve by genomic reduction until displaying a genome structure similar to *Brucella*, an intracellular strict pathogen phylogenetically related to *Ochrobactrum* spp. (Teyssier *et al*., 2003). In some cases, pathogenic microorganisms are capable to cause disease in a variety of organisms that may belong to different biological kingdoms of life. Such cross-kingdom pathogenesis could be illustrated by *A. radiobacter/tumefaciens* that causes both plant and human diseases. The virulence factors involved in phytopathogenicity are not found in clinical strains (unpublished data). However, multi-locus phylogeny showed that the clinical strains belonged to an epidemic clone among the *Agrobacterium* spp. population. This epidemic clone so-called genovar A7 could correspond to a new species in the genus and presented some phenotypic characters such as growth at 40 °C, which is a basic trait for human pathogenicity (Aujoulat *et al*., 2011). The bacterial lifestyle needs basic requirements such as temperature, water, nutrients and pH optima. In human-associated *Agrobacterium*, growth at high temperature is an emergent character. In other cases, basic requirements may be similar in both pathogenic and harmless members of neighbor clades, hence considered to be pre-existing adaptations. Different members of the *Acetobacteraceae* could infect human suggesting that they shared common traits, such as ability to grow at acidic pH, that permits their opportunistic growth, particularly in CFRT.

One primary condition for colonization and pathogenicity is the probability to meet the bacteria that implies living in close proximity. AAB could be considered as "domestic" for human since they were used for a long time in food processing. This situation differed from that observed for *Inquilinus*, which is the sole genus to include human-adapted bacteria in

Rhodospirillaceae. I. limosus emerged from the family *Rhodospirillaceae*, a group found in soil and plant but totally unrelated to human beings. Association with coral, sponge and cuttlefish is also described, particularly in the neighborhood of the genus *Inquilinus*. It is noteworthy that, when associated with animals, *Rhodospirillaceae* are found in mucous or gelatinous environment, such as the egg capsule in sepia. 16S rDNA sequences of *I. ginsengisoli* described from the soil of ginseng fields differed slightly from that of *I. limosus* type strain (Jung *et al*., 2011). 16S rDNA-based phylogeny showed a short robust branch (bootstrap value at 85) that groups all the clinical isolates and only one environmental isolate from roots of a perennial grass of Thar Desert in India (Figure 3). In a rooted tree, this branch appeared as supporting the most recently emerging species in the *Inquilinus* genus (data not shown). Finally, no relationship with human or other mammals was found for bacteria in the phylogenetic neighborhood of *Inquilinus*. Therefore, we could hypothetize that the association of *I. limosus* with CFRT resulted from a recent emergence by a speciation process of a human-adapted species from a group that experimented life in mucous environment.

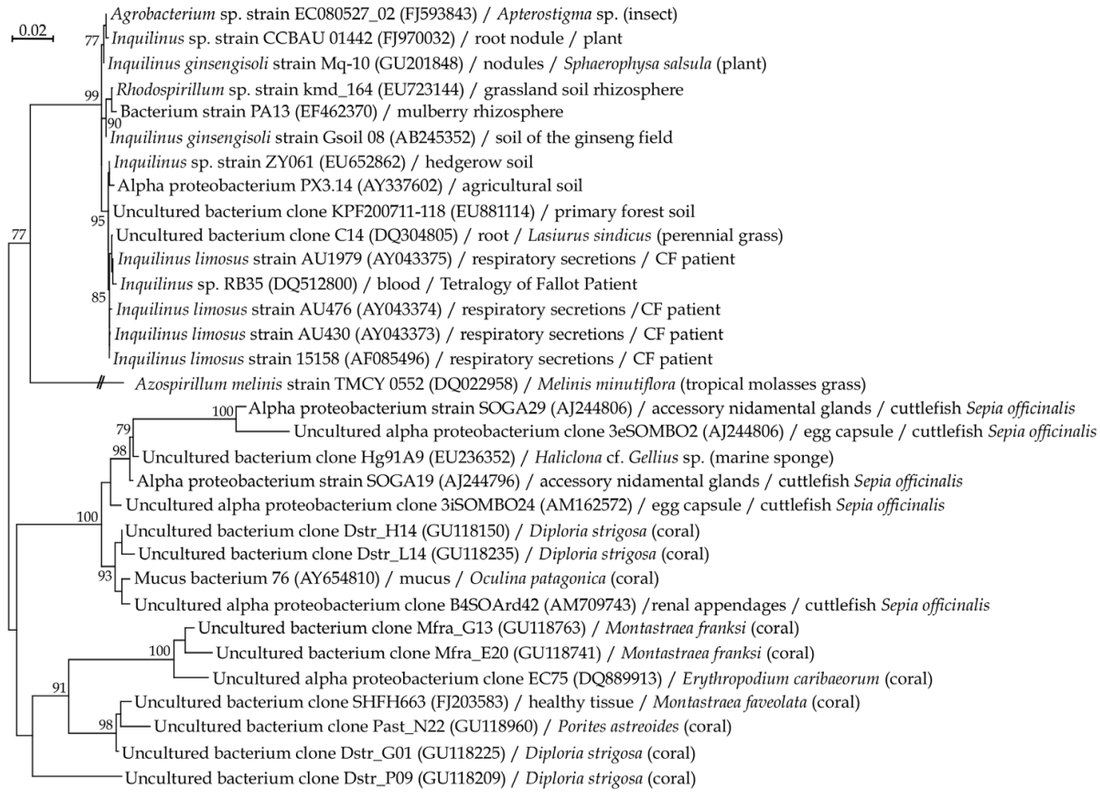

Fig. 3. Maximum-likelihood phylogenetic tree based on partial 16S rRNA gene sequences showing relationship among members of the genus *Inquilinus* and between *Inquilinus* spp. and selected close relatives from the family *Rhodospirillaceae*. Genbank accession numbers, isolation source and host follow sequence names. Numbers at nodes indicate percentage bootstrap support, based on analysis of 100 replicates. They are indicated for major nodes when >70. Bar, 0.02 substitutions per site.

We showed that the manner that CFRT niche drive the speciation and the adaptative evolution of bacteria is not univocal. However, the CFRT formed an abnormal human niche with basic conditions that allow the installation of environmental bacteria generally unrelated to human beings. Bacteria associated with CFRT, occupy two types of anatomical regions: i) the lower regions of the respiratory tract that are normally free of bacteria ii) the upper regions of the respiratory tract that are normally colonized by a resident microbiota. In CFRT, both dysbiosis and colonization by atypical environmental bacteria lead to a modified ecosystem where bacterial interactions may be unbalanced. The CFRT could be considered as an ecosystem with an emerging community where many bacteria belonging to different phyla interact and exchange genes at an increased rate. Lateral gene exchange is recognized as main innovation source for bacteria. They can acquire new genomic repertoires from which clonal specialists could emerge. Hotspots of interaction and exchange such as amoeba and rhizosphere have been previously described (Berg *et al*., 2005; Saisongkorh *et al*., 2010). The hypothesis that CFRT could play this role is reinforced by the number of atypical bacteria observed in this niche and by the probable emergence of specific sub-populations or species. Thus, emergence of opportunistic human pathogens from environmental origin may be first recognized from the CF airways model, a niche for bacterial adaptation and emergence (Yang *et al*., 2011b).

7. Conclusion

Atypical bacteria are increasingly recognized in CF. These bacteria may be considered as emerging from both biological and methodological points of view in CF. Recognition of these atypical bacteria should be encouraged in the perspective of a more complete description of their prevalence, relative importance of encountered species, antimicrobial susceptibility patterns and clinical relevance.

Despite some candidate pathogens were proposed, the role of these atypical bacteria in the disease evolution is unknown. More than considering individual pathogenic species, the diversity of the microbiota is more and more considered as an important marker in the evolution of the disease. Klepac-Ceraj *et al*. recently suggested that community composition might be a better predictor of disease progression than the presence of *P. aeruginosa* alone (Klepac-Ceraj *et al*., 2010) and van der Gast *et al*. showed that taxa richness decreased with a reduction in lung function (van der Gast *et al*., 2011). From this point of view, each taxon contributing to increase the global diversity of the microbiota appeared important whatever its identification.

Finally, whether these species may interact with other members of the CFRT microbiota and with more common pathogens to influence the onset and/or the evolution of colonization/infection by typical pathogens like *P. aeruginosa* are great questions for future advances in CF-associated infectious diseases.

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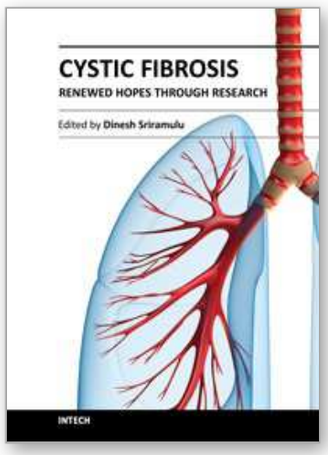

Cystic Fibrosis - Renewed Hopes Through Research

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Living healthy is all one wants, but the genetics behind creation of every human is different. As a curse or human agony, some are born with congenital defects in their menu of the genome. Just one has to live with that! The complexity of cystic fibrosis condition, which is rather a slow-killer, affects various organ systems of the human body complicating further with secondary infections. That's what makes the disease so puzzling for which scientists around the world are trying to understand better and to find a cure. Though they narrowed down to a single target gene, the tentacles of the disease reach many unknown corners of the human body. Decades of scientific research in the field of chronic illnesses like this one surely increased the level of life expectancy. This book is the compilation of interesting chapters contributed by eminent interdisciplinary scientists around the world trying to make the life of cystic fibrosis patients better.

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