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Use of Denaturing High-Performance Liquid Chromatography (DHPLC) to Characterize the Bacterial and Fungal Airway Microbiota of Cystic Fibrosis Patients[§]

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The aim of this study was to evaluate the use of denaturing high-performance liquid chromatography (DHPLC) to characterize cystic fibrosis (CF) airway microbiota including both bacteria and fungi. DHPLC conditions were first optimized using a mixture of V6, V7 and V8 region 16S rRNA gene PCR amplicons from 18 bacterial species commonly found in CF patients. Then, the microbial diversity of 4 sputum samples from 4 CF patients was analyzed using cultural methods, cloning/sequencing (for bacteria only) and DHPLC peak fraction collection/sequencing. DHPLC analysis allowed identifying more bacterial and fungal species than the classical culture methods, including well-recognized pathogens such as *Pseudomonas aeruginosa*. Even if a lower number of bacterial Operational Taxonomic Units (OTUs) was identified by DHPLC, it allowed to find OTUs unidentified by cloning/sequencing. The combination of both techniques permitted to correlate the majority of DHPLC peaks to defined OTUs. Finally, although *Aspergillus fumigatus* detection using DHPLC can still be improved, this technique clearly allowed to identify a higher number of fungal species versus classical culture-based methods. To conclude, DHPLC provided meaningful additional data concerning pathogenic bacteria and fungi as well as fastidious microorganisms present within the CF respiratory tract. DHPLC can be considered as a complementary technique to culture-dependent analyses in routine microbiological laboratories.

Keywords: denaturing high-performance liquid chromatography, airway microbiota, cystic fibrosis, cloning/sequencing, bacteria, fungi

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Introduction

Cystic Fibrosis (CF) is a human autosomal recessive disease that is common in Caucasian populations (Southern *et al.*, 2007). One of its many clinical manifestations is the irreversible decline in lung function, which is the main cause of mortality and morbidity in CF patients (Nixon *et al.*, 2001). Airway colonization and infection with opportunistic pathogens greatly contributes to lung destruction. For these reasons, accurate microbial detection and identification techniques are needed. In routine microbiology laboratories, microbial detection and identification (for both bacteria and fungi) traditionally rely on culture-dependent methods. The coexistence of microbial species, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus fumigatus*, has been well known for years in CF infections. Over the past decade, new methodologies such as fingerprinting techniques, cloning and sequencing or more recently high-throughput sequencing have led to a new paradigm. Indeed, CF airway microbiota is depicted as a complex microbial community and pulmonary infection in CF patients is no longer considered to be caused by a single species but rather polymicrobial (Canton and del Campo, 2010). Today, our understanding of the impact of such polymicrobial infections on the host is still limited. Nevertheless, it is currently accepted that microbiome composition is linked to pathogenic processes. Even harmless commensal bacteria may actually play a role, either in synergizing well-known pathogens such as *P. aeruginosa* (Parkins *et al.*, 2008), or in promoting patient stability as demonstrated with streptococci (Filkins *et al.*, 2012).

Denaturing high-performance liquid chromatography (DHPLC) is a novel community fingerprinting technique based on separation of multiple fragments of double-stranded DNA in denaturing mode. This technique has been successfully used to investigate bacterial and fungal diversity of environmental, clinical and food sources (Nazaret *et al.*, 2009; Wagner *et al.*, 2009).

The aim of this study was to use DHPLC to explore broncho-pulmonary microbiota of CF patients. First, we evaluated and optimized the resolution of DHPLC to discriminate 18 bacterial species commonly encountered in CF patients. Then, the bacterial microbiota of four sputa samples from four CF patients were characterized using collecting/sequencing approach of DHPLC peak fractions. Fungal diversity was also analyzed using a previously published DHPLC method (Delavenne *et al.*, 2011). The molecular data were compared with those obtained using a culture-

dependent technique.

Materials and Methods

Bacterial isolates

Twenty-one isolates belonging to 18 species were used in this study and included *P. aeruginosa* (one reference strain ATCC 27853 and two clinical isolates that we designated LAU and MAT), *P. putida*, *P. stutzeri*, *Burkholderia cepacia*, *B. multivorans*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, *Elizabethkingia meningoseptica*, *Haemophilus influenzae*, *Escherichia coli*, *S. aureus* CIP 65.10, *Streptococcus pneumoniae*, *S. mutans*, *Moraxella* (*Branhamella*) *catarrhalis*, *Mycobacterium avium*, *M. intracellulare, M. abscessus*, and *M. bollettii*. All strains were isolated from CF patient sputa except for strains ATCC 27853 (*P. aeruginosa*) and CIP 65.10 (*S. aureus*). Isolate identifications were previously conducted based on phenotypical and morphological criteria as well as and biochemical tests. All Gram-negative non-fermenting bacilli isolates were further identified by 16S rRNA gene sequencing as previously described (Moissenet *et al.*, 2005). The non-tuberculous mycobacteria were identified by a commercially available DNA strip assay (GenoType Mycobacterium CM/AS; Hain Lifescience, Germany). Moreover, a multilocus sequence analysis was also performed for both *M. abscessus* and *M. bollettii* isolates (Macheras *et al.*, 2010).

Sputum samples

Four sputum samples were collected from four CF patients: a 20 year-old woman (CF4), a 7 year-old boy (CF7), a 24 year-old man (CF46), and a 10 year-old girl (CF59). Sputum samples were fluidized for 15 min using a 1X dithiothreitol solution (Digest-EUR®, Eurobio) (v/v) prior to microbiological analysis. For each sputum sample, microbiological analyses were performed according to French guidelines (Anonyme, 2010) and based on recommendations by Koenig (1995) for bacteria and fungi, respectively. After microbiological analysis, samples were stored at -20°C until DNA extraction.

DNA extraction from bacterial isolates

Biomass scraped with a 10 µl-loop from 3 day BHI Agar cultures was suspended in 600 µl TE 10:1 (10 mM Tris, 1 mM EDTA, pH 8) in a microfuge tube containing 100 mg of silica beads (Sigma-Aldrich, Germany). Then, 40 µl of proteinase K (15 mg/ml in TE 10:1) and 100 µl of 20% SDS were added and incubated for 2 h at 55°C. Cells were disrupted 3 times using a Fastprep instrument (Bio101, Vista, USA) for 40 sec at a speed setting of 4 with 1-min incubation on ice prior to each treatment. Subsequently, DNA was purified as previously described (Gevers *et al.*, 2001) and quantified using a Nanodrop machine (Thermo, USA). DNA samples were diluted to 100 ng/µl prior to PCR amplification.

Total DNA extraction from sputum samples

After 5 min sonication using a bath sonicator (Elamsonic S10, Germany), total DNA was extracted from sputum samples using the QiaAmp DNA Mini Kit (QIAGEN GmbH, Germany) following manufacturer instructions ("Tissue protocol").

PCR-amplification of partial 16S rRNA gene fragments from pure bacterial DNA cultures and sputum samples

Primers 984GCf (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTA-3′) and 1378r (5′-CGGTGTGTACAAGGCCCGGGAAC-3′) were used to amplify the V6, V7, and V8 regions of the 16S rRNA gene from bacterial cultures and DNA extracts from sputum samples as previously described (Wagner *et al.*, 2009).

DHPLC analysis of 16S rRNA gene amplicons

PCR products obtained with primers 984GCf and 1378r were analyzed by DHPLC using the WAVE microbial analysis system (Transgenomic, USA) using a fluorescence detector after staining with the WAVE optimized HS staining solution (Transgenomic). This solution corresponds to a DNA-intercalating dye that increases the detection sensitivity.

Separation conditions in terms of flow rate and eluent gradients were carried out according to Wagner *et al.* (2009) with some modifications (see Supplementary data Table S1). Separation temperature and eluent gradient were optimized by mixing 1 µl of all 16S rRNA gene amplicons from bacterial species associated with CF to select for DHPLC conditions allowing for maximum peak separation. Then, each individual 16S rRNA gene amplicon was also separately injected to precisely identify the peaks obtained with the PCR amplicon mixture.

For DHPLC analyses of sputa samples, peak fractions were obtained as previously described (Delavenne *et al.*, 2011) by manually collecting two to three drops representing the top of the peak. One microliter of each fraction was directly used as template for re-amplification using the same primer pair and conditions described above, except that PCR amplification was achieved in a 35 µl reaction mix and only 20 cycles were performed. The resulting PCR product was re-injected into the DHPLC system (see Supplementary data Table S1 for conditions) and fraction collection and amplification were performed again.

PCR-amplification of the ITS1 region for fungal sputum DNA samples and DHPLC analysis

Semi-nested PCR assay was used to amplify the ITS1 region of fungal sputum DNA samples as previously described (Delavenne *et al.*, 2011). PCR products were separated by DHPLC on the WAVE microbial analysis system (Transgenomic) and detected using a fluorescence detector after staining with the WAVE optimized HS staining solution (Transgenomic). DHPLC analysis of ITS1 amplicons (see Supplementary data Table S2), peak fraction collection and re-amplification were performed as previously described (Delavenne *et al.*, 2011).

Partial 16S rRNA gene cloning and sequencing

PCR products obtained using primers 984GCf and 1378r from DNA extracted from sputum samples was ligated into

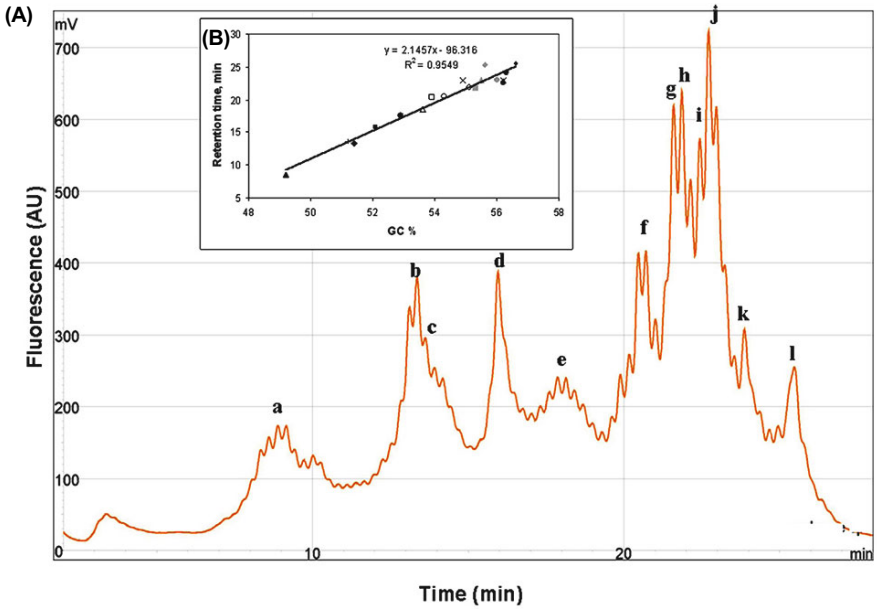

Fig. 1. DHPLC peak profile of a mixture of 16S rRNA gene amplicons from 18 bacterial species using primers 984GCf/1378r (A) and retention time of the PCR product of each species as a function of its GC% (B). a, *Staphylococcus aureus* (Rt=8.51 min, ▲); b, *Haemophilus influenzae* (Rt=13.32 min, +); c, *Elizabethkingia meningoseptica* (Rt=13.67 min, ◆); d, *Moraxella catarrhalis* (Rt=15.853 min, ■); e, *Streptococcus mutans* (Rt=17.53 min, ●), *Streptococcus pneumoniae* (Rt=18.55, △); f, *Escherichia coli* (Rt=20.34 min, □), *Pseudomonas stutzeri* (Rt=20.47 min, ○); g, *Stenotrophomonas maltophilia* (Rt=21.69 min, ■); h, *Pseudomonas aeruginosa* ATCC 27853, LAU and MAT (Rt=21.97 min, ◇); i, *Mycobacterium bolletti* (Rt=22.53 min, ■); j, *Mycobacterium abscessus* (Rt=22.91 min, ∗), *Pseudomonas putida* (Rt=22.94 min, ×), *Burkholderia multivorans* (Rt=22.98 min, ▲), *Burkhoderia cepacia* (Rt=22.99 min, ●); k, *Mycobacterium avium* (Rt=24.08 min, ●); l, *Achromobacter xylosoxidans* (Rt=25.36 min, ◆), *Mycobacterium intracellulare* (Rt=25.51 min, ◆).

the pCR4-TOPO vector (Invitrogen, USA). Recombinant pCR4-TOPO plasmids were used to transform *E. coli* TOP10 One Shot chemically competent cells according to manufacturer's instructions (Invitrogen). Fifty transformant clones were randomly picked, plasmid purification was performed according to Bigot and Charbit (2009) and inserts were amplified using primers 984GCf and 1378r before sequencing.

Sequencing of peak fractions and clones

Amplicons were sequenced at the Biogenouest sequencing platform at the "Station Biologique de Roscoff" (http://www.sb-roscoff.fr/SG/) using primer ITS1-F for fungi and 1378r for bacteria. ITS1 sequences were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST, October 2012) to determine the closest known relatives. 16S rDNA sequences were compared with sequences obtained from the Ribosomal Database Project (RDP), version 10.27 (Cole *et al.*, 2009), by using the SEQMATCH program to obtain S_{ab} values with database sequences. After sequence analyses, each distinct PCR product obtained from DHPLC and cloning/sequencing were injected into the DHPLC system to determine their respective retention times and to identify their corresponding peaks in the sputum DHPLC fingerprints.

Ethics

The Comité de Protection des Personnes VI-Ouest approved the protocol; all patients and their relatives gave written informed consent. The collection of archival specimens was registered with the French Ministry of Research and the Agence Régionale de l'Hospitalisation, No. DC-2008-214.

Results and Discussion

Optimization of DHPLC running conditions to analyze bacterial diversity in CF patients

DHPLC conditions in terms of flow rate and temperature were optimized on a mixture of PCR amplicons from bacterial species commonly found in CF patients. PCR amplicons were eluted between 8.5 and 25.5 min (Fig. 1A) and 16 out of 18 bacterial species had different retention times. The only exceptions were *B. multivorans* and *B. cepacia*, which had similar retention times (22.98 and 22.99 min, respectively). In addition, after injection of a mixture of 16S rRNA amplicons from all 18 species, 8 species were clearly resolved (Fig. 1A) including important pathogens such as *S. aureus*, *H. influenzae*, and *P. aeruginosa*. As previously observed (Wagner *et al.*, 2009), PCR product retention times were significantly correlated (r^2=0.95; p<0.001) with PCR amplicon GC% contents (Fig. 1B). As a result, while good PCR amplicon resolution was obtained for samples with different GC%, PCR product resolutions were more problematic when samples had very similar GC% contents. This was particularly noted for PCR products with GC% comprised between 53% and 56.6%. It is noteworthy that PCR product resolution may be further improved for species with high GC content by optimizing DHPLC running conditions. However, one objective in this study was to develop a DHPLC methodology to determine the molecular fingerprint of CF bacterial microbiota in a single run.

Bacterial diversity in CF patients

Cloning/sequencing and DHPLC peak fraction collection/sequencing techniques were performed to evaluate bacterial diversity of CF patients. Although the reference database covered 50% of bacterial genera usually found in CF sputa, DHPLC collecting/sequencing of peak fractions and cloning/sequencing analyses of CF sputa (Table 1 and Fig. 2) pro-

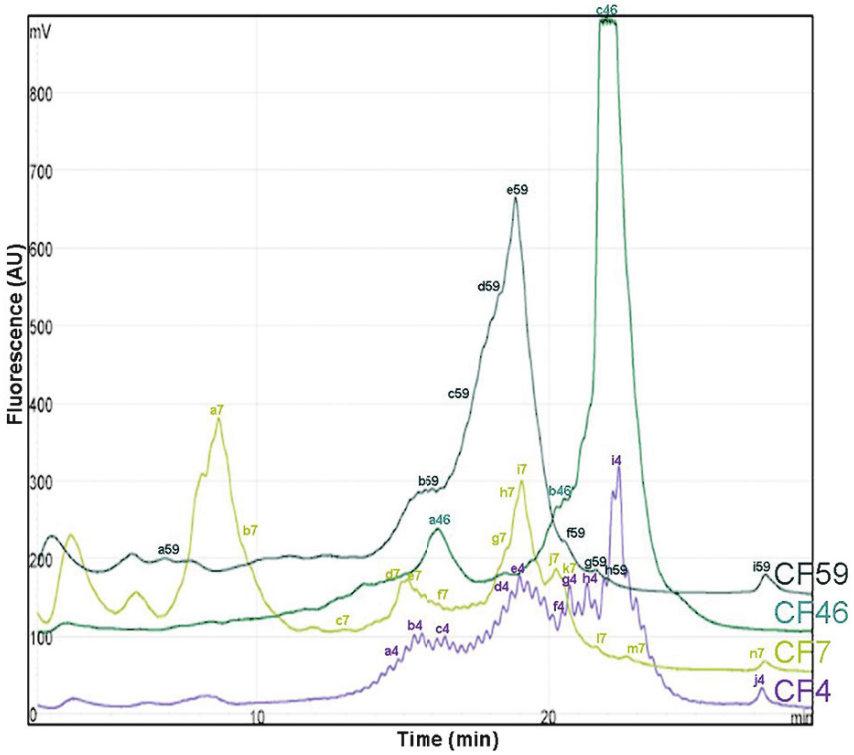

Fig. 2. DHPLC analysis of the bacterial diversity in the sputum of four cystic fibrosis patients (CF4, CF7, CF46, and CF59). See Table 2 for assignment of DHPLC peaks.

vided evidence that the bacterial reference database used to optimize DHPLC conditions was not exhaustive enough to identify all bacterial species present in CF sputa. Moreover, PCR products from different species co-eluted and the complexity of CF bacterial microbiota did not provide a base-line separation of amplicons. This drawback is well established in molecular fingerprinting techniques such as DGGE or TTGE (Marzorati *et al.*, 2008). However, DHPLC offers several advantages over the latter fingerprinting techniques such as short running time (<30 min), high reproducibility and less fastidious collection of peak fractions for sequencing.

In each CF sputum sample, the cloning/sequencing technique allowed to detect a higher number of Operational Taxonomic Units (OTUs) in comparison to DHPLC (Table 1). Five, 9, 1, and 3 OTUs were identified in the 4 CF sputa after collecting of peak fractions/sequencing using DHPLC, while 10, 11, 3, and 8 OTUs were found by cloning/sequencing. Similar OTUs were identified using both techniques such as those related to well-known CF patient pathogens like *P. aeruginosa* and *S. aureus* or oral bacteria like *Streptococcus* spp. Other OTUs were only recovered using DHPLC (e.g. *Actinomyces*, *Haemophilus* spp.). Indeed, we were able to collect peak template DNAs with low relative areas that were successfully sequenced. It is likely that such OTUs could have been identified by cloning/sequencing provided that an increased number of clones were analyzed. In contrast, several OTUs were only obtained using cloning/sequencing (e.g. *Gemella*, *Granulicatella*, *Scardovia*, *Rothia*, *Parvimonas* spp.). This result may be explained by the fact that their corresponding PCR products had retention times close to those of dominant species i.e., *Streptococcus* spp. and that PCR products of dominant species were much more abundant. Therefore, after reamplification and purification using DHPLC, PCR products related to the most dominant species were obtained. Nevertheless, by combining both techniques, it was possible to link the majority of DHPLC peaks to defined OTUs (Fig. 2).

Overall, 15 genera from the Firmicutes (*Gemella*, *Granulicatella*, *Parvimonas*, *Staphylococcus*, *Streptococcus*, *Veillonella*), Proteobacteria (*Escherichia*, *Haemophilus*, *Neisseria*, *Pseudomonas*) and Actinobacteria (*Actinomyces*, *Granulicatella*, *Micrococcus*, *Rothia*, *Scardovia*) phyla were detected (Table 1). The highest diversity was found in sputum CF7 followed by sputum CF4, CF59, and CF46 (Table 1). This result was in good agreement with the complexity of the molecular fingerprints obtained using DHPLC (Fig. 2).

Culture-based analysis of important pathogens found in CF revealed the presence of *S. aureus* at counts of 2×10^7, 10^8 and 10^5 CFU/ml in sputa CF4, CF7, and CF59, respectively, while *P. aeruginosa* counts were 10^2 and 10^8 CFU/ml in sputa CF7 and CF46, respectively. *E. coli* (10^3 CFU/ml) and presence of *M. abscessus* were also detected in sputum CF4 while *S. pneumoniae* (10^8 CFU/ml) was found in sputum CF59. The culture-independent techniques used in this study failed to detect *S. aureus* in CF4 and CF59 while OTUs related to *P. aeruginosa* were found in sputa CF4, CF46, and CF59. OTUs related to *S. pneumoniae* were also found in sputum CF59 but could not be differentiated from other *Streptococcus* spp. that shared the same S_{ab} score.

In agreement with recent studies (Nixon *et al.*, 2001; Rogers *et al.*, 2004; Harris *et al.*, 2007; Sibley *et al.*, 2008; Cox *et al.*, 2010; Klepac-Ceraj *et al.*, 2010; Guss *et al.*, 2011; van der Gast *et al.*, 2011), the culture-independent techniques showed that the bacterial microbiota of CF patients' airway tract is

Table 1. Bacterial diversity in sputum of 4 cystic fibrosis patients (CF4, CF7, CF46 and CF59) obtained after collecting/sequencing of DHPLC peak fractions (FP) and cloning/sequencing (CS)

Sample ID	DHPLC peak	Closest phylogenetic affiliations in the RDP database ($S_{a/b}$ score)
CF4		
CS38	a4	*Streptococcus salivarius** (1.0) *S. vestibularis** (1.0)
FP10	a4	*S. parauberis** (0.609) *S. salivarius** (0.602)
CS13	b4	*Veillonella. parvula* (0.931)
CS27	c4	*V. dispar* (0.981)
CS12	d4	*Rothia mucilaginosa* (0.961)
CS9	e4	*S. sanguinis** (1.0)
CS15	f4	*Escherichia coli** (0.983) *Salmonella enterica* (0.983) *Enterobacter cloacae* (0.983)
CS30	g4	*R. dentocariosa* (1.0)
CS2	h4	*S. suis** (0.803) *S. oralis** (0.803) *S. sanguinis** (0.803)
FP9	h4	*S. sanguinis** (0.517) *S. pyogenes** (0.509)
FP15	h4	*S. mitis** (0.652) *S. sanguinis** (0.652) *S. pneumoniae** (0.652)
CS14	i4	*Pseudomonas* sp.* (0.981) *P. aeruginosa** (0.964)
FP39	i4	*Pseudomonas* sp.* (1.0) *P. aeruginosa** (0.984)
FP66	j4	*Actinomyces naeslundii* (1.0) *A. oris* (1.0)
CS31	**	*Scardovia wiggsiae* (1.0)
CF46		
CS3	a46	*V. dispar* (0.978)
CS36	b46	*E. coli** (1.0) *H. alvei* (1.0) *Sh. boydii* (1.0)
FP3-1	c46	*P. aeruginosa** (1.0)
CS1	c46	*P. aeruginosa** (1.0)
CF7		
CS17	a7	*Staphylococcus aureus* subsp. *aureus** (1.0) *S. haemolyticus** (1.0)
FP8	a7	*S. aureus* subsp. *aureus** (1.0) *S. haemolyticus** (1.0)
CS9	b7	*N. flava* (0.983) *N. sicca* (0.983) *N. pharyngis* (0.983)
CS22	c7	*Haemophilus segnis** (0.677)
FP14	c7	*H. parainfluenzae** (0.882)
CS11	d7	*Parvimonas micra* (0.77)
CS16	e7	*V. rogosae* (1.0) *V. parvula* (1.0)
CS14	f7	*Granulicatella elegans* (0.904) *G. adiacens* (0.904) *G. para-adiacens* (0.904)
CS18	g7	*S. oralis** (0.964) *S. parasanguinis**(0.964) *O. gastrococcus* (0.964)
FP11	g7	*S. parasanguinis* (0.938) *S. oralis** (0.938) *Okadaella gastrococcus* (0.938)
CS5	h7	*S. mitis** (0.981) *S. pseudopneumoniae** (0.981) *S. pneumoniae** (0.981)
FP23	h7	*S. mitis** (0.72)
CS12	i7	*R. mucilaginosa* (1.0)
CS15	j7	*Neisseria mucosa* (0.946)
FP61	j7	*N. mucosa* (0.98)
CS19	k7	*E. coli** (1.0) *Hafnia alvei* (1.0) *Shigella boydii* (1.0)
FP56	l7	*S. dentirousetti** (0.591)
FP36	l7	*S. iniae** (0.684)
FP31	m7	*Micrococcus luteus* (0.724)
FP70	n7	*Actinomyces naeslundii* (0.986) *A. oris* (0.986) *A. viscosus* (0.986)
CF59		
CS24	a59	*Gemella haemolysans* (0.986)
CS6	b59	*V. caviae* (0.958)
CS33	c59	*V. parvula* (0.975)
CS14	d59	*R. mucilaginosa* (0.948)
FP10-3	e59	*S. oralis** (0.98) *S. mitis** (0.98) *S. pneumoniae** (0.98) *S. pseudopneumoniae** (0.98)
CS12	e59	*S. oralis** (0.938) *S. mitis** (0.938) *S. pneumoniae** (0.98) *S. sanguinis** (0.938)
CS13	e59	*V. atypica* (0.801)
CS46	f59	*E. coli** (1.0) *H. alvei* (1.0) *Sh. boydii* (1.0)
CS45	g59	*S. pyogenes** (0.733)
FP9-5	h59	*P. aeruginosa** (1.0)
FP14	i59	*A. naeslundii* (1.0) *A. oris* (1.0) *A. viscosus* (1.0)

*bacterial species affiliated to the 11 genera of the DHPLC reference database
***elution in washing peak

Table 2. Fungal diversity in sputum of 4 cystic fibrosis patients (CF4, CF7, CF46, and CF59) obtained after collecting/sequencing of DHPLC peak fractions

DHPLC peak[a]	Closest phylogenetic affiliation in the GenBank / EMBL /DDBJ / PDB databases (Accession number)	% similarity
CF4		
c	*Fusarium verticillioides* (JF499684)	99
e	*Pseudallescheria boydii* (HQ185349)	100
f	*Exophiala dermatitidis* (HE590083)	100
CF7		
b	*Candida albicans* (JN169123)	100
h	*Malassezia globosa* (GU291271)	94
CF46		
b	*Candida albicans* (JN169123)	100
d	*Acremonium strictum* (JF912337)	100
h	*Malassezia globosa* (AY387134)	98
CF59		
a	*Galactomyces geotrichum* (EU789402)	92
b	*Candida albicans* (JN606258)	100
g	*Malassezia restricta* (GU291270)	100
h	*Malassezia globosa* (GU291271)	92

[a] See legends from Fig. 3 for species assignment of DHPLC peak fractions

more diverse and complex than culture-based techniques. In the latter studies, well-recognized pathogens as well as emerging pathogens were observed. For two CF patients (CF4 and CF59), *P. aeruginosa* was detected by DHPLC (confirmed by a *P. aeruginosa* specific *opr*L qPCR method; data not shown) but not using classical culture methods. In a previous study mainly focused on *P. aeruginosa* detection, several DHPLC positive/culture negative cases were already reported (Nazaret *et al.*, 2009). In this study, CF patients became *P. aeruginosa* positive after a few months; this reinforces the lack of reliability observed when using cultural methods to detect such bacteria. For the four CF patients, six different species of anaerobic bacteria (i.e. *Veillonella*, *Scardovia*, *Parvimonas*, and *Actinomyces* spp.) were only detected by the culture-independent techniques. Since the study by Tunney *et al.* (2008) that clearly demonstrated the importance of anaerobes in CF sputa, numerous studies on CF airway tract microbiota, including the present study, have described anaerobic bacteria, especially belonging to the genus *Veillonella* (Duan *et al.*, 2003; Bittar *et al.*, 2008; Sibley *et al.*, 2008; Tunney *et al.*, 2008, 2011; Zhao *et al.*, 2012). Several studies have also highlighted an interaction between *P. aeruginosa* colonization and anaerobes with, in consequence, enhanced pathogenesis observed (Duan *et al.*, 2003; Zhao *et al.*, 2012). For example, co-aggregation between *P. aeruginosa* and *Actinomyces naeslundii* has been shown suggesting enhanced *P. aeruginosa* colonization and thereby higher susceptibility of the host to infection (Komiyama *et al.*, 1987). On one hand, the presence of anaerobic bacteria in sputum samples as well as species such *Neisseria mucosa* or alpha-hemolytic streptococci may be the sign of oral contamination of the sputum sample by oropharyngeal bacteria. In this context, a recent study suggested that oropharyngeal contamination could limit the accuracy of DNA-based techniques on upper-airway specimens (Goddard *et al.*, 2012). On the other hand, bacteria usually described as oral contaminants have also sought to potentially interplay with classical CF pathogens such as *P. aeruginosa* (Duan *et al.*, 2003; Parkins *et al.*, 2008) or to act in CF patient clinical status by promoting stability (Filkins *et al.*, 2012). Moreover, several studies have demonstrated that CF lungs can be colonized by indigenous oral bacteria. These bacteria were shown to have different T-RFLP profiles with CF patients lung microbiota and mouth microbiota and the presence of anaerobic bacteria was also shown in transtracheal aspiration (Brook and Fink, 1983; Rogers *et al.*, 2006).

Fungal diversity in CF patients

Fungal diversity in CF patients was assessed using DHPLC and a culture-based technique. CF sample fungal microbiota was found to be more diverse using DHPLC than the culture-dependent method. Using DHPLC, relatively low diversity was observed as compared to bacterial diversity with only 2, 3, 3, and 4 fungal species in sputa CF4, CF7, CF46, and CF59, respectively (Table 2 and Fig. 3). *C. albicans* and *Malassezia* spp. were detected in 3 out of 4 sputa while other species such as *Acremonium strictum*, *Exophiala dermatitidis*, *Fusarium verticilloides*, *Geotrichum candidum*, and *Pseudallescheria boydii* were only found in a single sputum sample (Table 2 and Fig. 3). Using the culture-based technique, *C. albicans* and *C. glabrata* was identified in sputa CF4 while *C. albicans* and *A. fumigatus* were identified in CF7 and CF59 samples.

Several hypotheses may be considered to explain the discrepant results between culture-dependent and independent

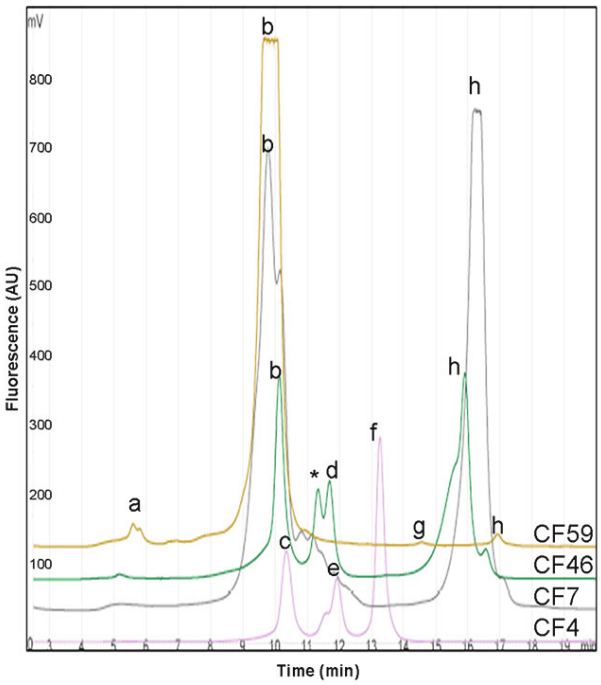

Fig. 3. DHPLC analysis of the fungal diversity in the sputum of four cystic fibrosis patients (CF4, CF7, CF46, and CF59). a, *Galactomyces geotrichum*; b, *Candida albicans*; c, *Fusarium verticillioides*; d, *Acremonium strictum*; e, *Pseudalescheria boydii*; f, *Exophiala dermatidis*; g, *Malassezia restricta*; h, *Malassezia globosa*; * non-assigned peak)

techniques. First, DHPLC analysis permitted a larger view of fungal diversity including fungal species that are quite fastidious to cultivate. For example, *Malassezia* spp., detected in patients CF7, CF46, and CF59, do not grow on conventional agar media (Gueho *et al.*, 1996) while recovery of *E. dermatitidis* may require up to 4 weeks incubation (Lipuma, 2010). Secondly, in contrast to culture-based techniques, semi-nested-PCR used for DNA amplification combined with DHPLC analysis can be used to detect subdominant and dominant fungal species as previously observed for yeast and filamentous fungi in raw milk samples (Delavenne *et al.*, 2011). Indeed, *C. albicans* and *A. strictum* found in patient CF46 and *Galactomyces geotrichum* found in patient CF59, may represent minor fungal populations not isolated on agar media. The antifungal treatment given to patient CF46 may have also inhibited *C. albicans* and *A. strictum* growth. Moreover, important discrepancies in terms of dominant and sub-dominant populations may have hindered their isolation on agar media.

Despite the fact that universal fungal primers were used for PCR amplification, we failed to detect *A. fumigatus* using DHPLC. Yet, this species was isolated from sputa of 3 CF patients (CF4, CF7, and CF59). A possible explanation may be that primer ITS1F, used in semi-nested PCR reactions, presented two mismatches in the 5′ end of *A. fumigatus* target DNA template (data not shown). These primer-template mismatches may have disturbed *A. fumigatus* DNA amplification thereby preferentially amplifying DNA from the other fungal species. Pulmonary colonization with *A. fumigatus* arises late during the cystic fibrosis progression and acts as a major aggravating factor of pulmonary dysfunction (Moss, 2010). The failure to detect *A. fumigatus* represents a weakness of the DHPLC technique used in this study. Nevertheless, DHPLC analysis identified other fungal species in sputum specimens, which frequencies are certainly underestimated although potentially pathogen. *P. boydii* detected in patient CF4, is assumed to be the second most frequent mould after *A. fumigatus* in CF patients presenting fungal pulmonary colonization (Cimon *et al.*, 2001). Interestingly, this species was isolated in patient CF4 several months after initial detection using DHPLC. *E. dermatitidis* seems to be a frequent pulmonary colonizer, specifically in CF patients presenting pancreatic insufficiency (Kondori *et al.*, 2011). Nonetheless, the pathogenic role of *E. dermatitidis* remains unclear (Kondori *et al.*, 2011), as is the case for *Fusarium* spp. and *Acremonium* spp. (Nagano *et al.*, 2009). These results provide additional data on the presence of these fungi in the respiratory tract of CF patients as recently reported by Nagano *et al.* (2009).

To conclude, DHPLC analysis combined or not with cloning/sequencing offered a more complete view of CF airway microbiota in contrast to traditional culture-based techniques, even if the latter still remain essential. DHPLC allowed to detect fastidious fungal and bacterial pathogens and well-recognized pathogens such as *P. aeruginosa* within the complex microbial community found in CF airways. Use of DHPLC in routine diagnosis would be interesting in microbiological laboratories specialized in CF sputum sample management. Indeed, this technique offers an interesting way (in terms of cost per analysis) to monitor the dynamics of bacterial and fungal microbiota of CF patients. For example, if a major change in molecular fingerprint is observed, collection/sequencing of a given fraction would be useful to identify which microorganisms caused the changes in microbiota composition. In addition, as CF airway fungal communities are much less complex that bacterial communities, DHPLC could clearly be used to describe such communities.

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