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True Microbiota Involved in Chronic Lung Infection of Cystic Fibrosis Patients Found by Culturing and 16S rRNA Gene Analysis▽

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Patients suffering from cystic fibrosis (CF) develop chronic lung infection. In this study, we investigated the microorganisms present in transplanted CF lungs ($n = 5$) by standard culturing and 16S rRNA gene analysis. A correspondence between culturing and the molecular methods was observed. In conclusion, standard culturing seems reliable for the identification of the dominating pathogens.

Cystic fibrosis (CF) is the most common lethal autosomal recessively inherited disorder of Caucasians. Although several organs are affected, the most severe effect is observed in the lungs, which is the major cause of deaths of patients (5). Here, genetic alterations of the chloride channel in epithelial cells lead to dehydration of the airway mucus, increasing its viscosity. This means that the cilia are unable to transport the mucus in which inhaled material and, importantly, bacteria are entrapped, enabling microorganisms to colonize and establish infections within the mucus (9). In the early stages of CF, intermittent colonizations occur, which can be treated with antibiotics (10). Establishment of chronic infection occurs over time and is characterized by the formation and establishment of bacterial aggregates (the so-called biofilms) (1, 5). Formation of biofilm is problematic since not only does this afford protection against the different components of the host defense in the lungs but the bacteria also become extremely tolerant to antibiotics (1, 4, 5). Most pathogenic bacteria are easily diagnosed by standard culture-based techniques; however, many less well recognized bacteria can be difficult to culture due to their growth requirements or being very slow growing or not growing at all if the patient has been treated with antibiotics. In these cases, the standard culture techniques may fail to detect these bacteria and detect only the more readily culturable bacteria (14). In the CF centers in Denmark, an intensive antibiotic treatment strategy has been shown to prolong the life expectancy of the CF patients (10). In recent studies, the chronically infected lungs of CF patients have been observed to harbor multiple species (19, 21). However, the strict antibiotic strategy employed in Denmark has led to only a small variety of microorganisms being found in the lungs of CF patients, compared to what is found in other studies (8, 18, 23). In a previous study, we applied fluorescence *in situ* hybridization (FISH) using peptide nucleic acid (PNA) probes to investigate the spatial distribution of *Pseudomonas aeruginosa* in the lungs of end-stage Danish CF patients by using both general and specific probes and found *P. aeruginosa* to be present alone (1). The end stage is defined as the time when the lungs are destroyed and the lung function is reduced to an extent where lung transplantation is required for the patient to survive (3).

In the present study, we investigated the true microbiota of the end-stage CF lung by investigating fresh samples directly from explanted lungs of Danish CF patients undergoing double lung transplantations. This was to avoid possible contamination by the patient's oral and pharyngeal flora during expectoration of sputum, which is the typical type of sample investigated in CF studies.

We included 34 lung tissue and mucopurulent pus/sputum samples excised directly and sterilely from the lungs of five Danish end-stage CF patients undergoing double lung transplantation at Rigshospitalet (Copenhagen, Denmark). The lungs were collected with the consent of the patients and in accordance with the biomedical project protocol (KF-01278432) approved by the Danish Council of Ethics. To investigate the microorganisms of the true microbiota present within the lungs of the patients, both standard culturing and 16S rRNA gene analysis were performed. All culture experiments were performed at the Department of Clinical Microbiology, Rigshospitalet (Copenhagen University Hospital, Denmark), according to standard protocols (2). All samples were incubated both aerobically and anaerobically. Aerobic culturing was performed on blood agar, chocolate agar, and eosin-methylene blue (EMB) agar with an incubation time of up to 1 week. Anaerobic culturing was performed on blood agar and chocolate agar, using an atmosphere of 7% CO_2 and 7% H_2 in N_2 for up to 2 weeks.

Before extraction of DNA for 16S rRNA gene analysis, samples were lysed by proteinase K (40 μl) and ATL buffer (360 μl) from the DNeasy blood and tissue kit (Qiagen, Copenhagen, Denmark) for each 500 mg of tissue and incubated overnight at 56°C. The samples were then centrifuged at 13,000

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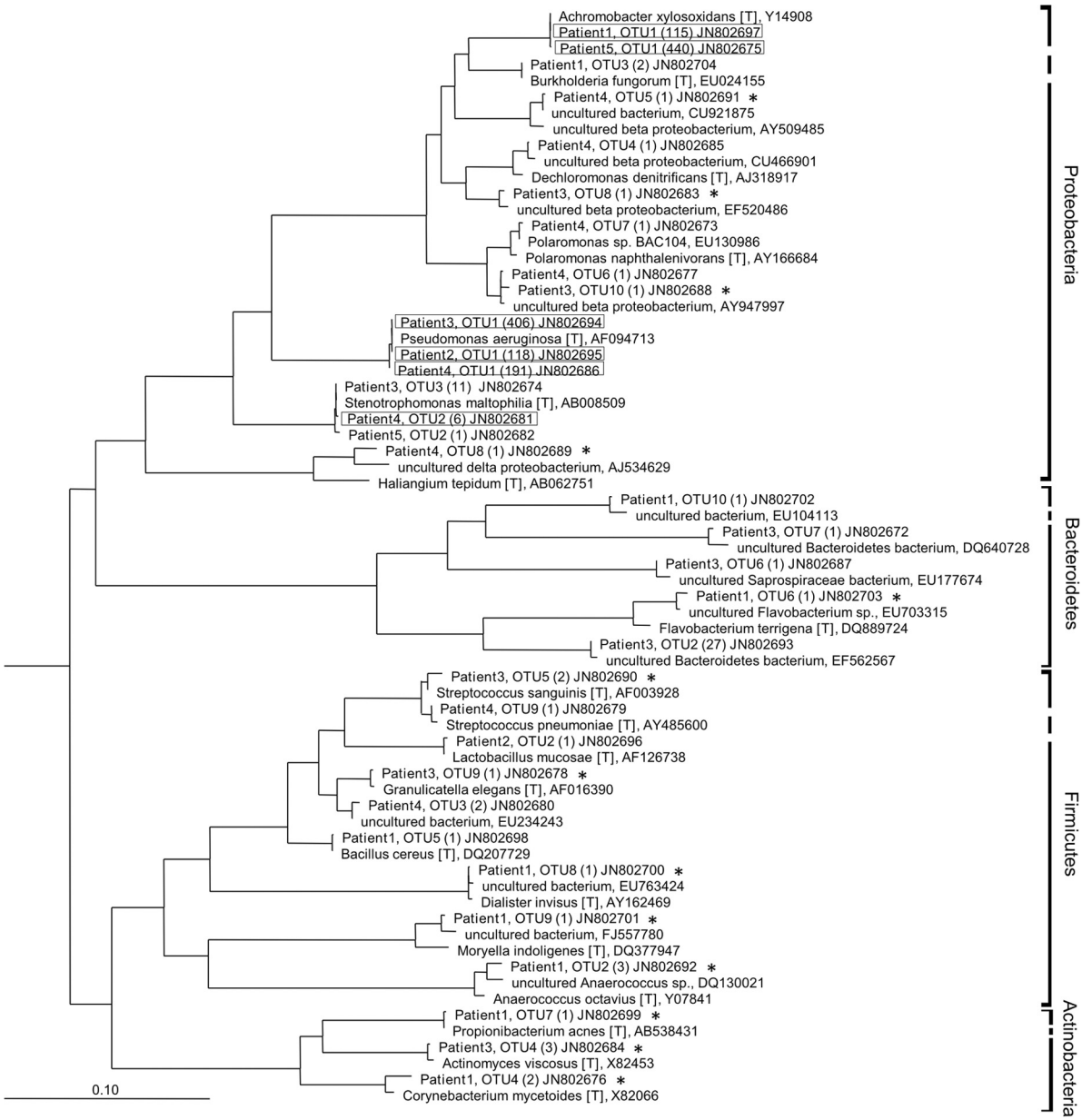

FIG. 1. Maximum likelihood tree of the sequences in the clone libraries with their closest relatives. The OTUs from the clone libraries from the five patients are given with the numbers of sequences in parentheses. The out-group (consisting of 24 sequences of the *Chloroflexi* phylum) was set as the root, not shown in the figure. The scale bar represents a 10% deviation of sequence. Asterisks indicate sequences where identification by BLAST search gave different results. The identities of microorganisms found by culturing are highlighted by a box; these are also the clones most often identified in the respective clone libraries.

rpm for 1 min, and DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's protocol (revision 6560-200-07DEC); starting from step 6, DNA was eluted with 60 μl diethyl pyrocarbonate (DEPC)-treated water. Nearly full-length 16S rRNA genes were amplified as described in the literature (22), using two different combinations of universal bacterial primers: 26F (5′-AGAGTTTGATCCTGGCTCAG-3′) with either 1390R (5′-GACGGGCGGTGTCTACAA-3′) or 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (15). The resulting 16S rRNA gene fragments were pooled and purified using Nucleospin Extract II columns (Macherey-Nagel, Düren, Germany). The PCR products were cloned into a pCR4-TOPO vector, transformed into One Shot Top 10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA), and incubated overnight at 37°C on LB agar plates containing 50 μg/ml kanamycin and 50 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Either plasmids were purified using the Illustra TempliPhi DNA amplification kit (GE Healthcare, Brøndby, Denmark) and sequenced commercially by Macrogen (South Korea), or plasmid purification was performed by Macrogen before sequencing. Sequences were obtained using

TABLE 1. Overview of bacteria found in the explanted lung samples by culturing and 16S rRNA gene analysis

Patient	Culturing	16S rRNA gene analysis		
		Species (BLAST)[a]	OTU	No.[b]
Patient 1	*Achromobacter xylosoxidans*	*Achromobacter xylosoxidans*	1	115
		Uncultured bacterium	2*[c]	3
		Burkholderia fungorum	3	2
		Uncultured bacterium	4*	2
		Bacillus cereus	5	1
		Uncultured *Flavobacterium*	6*	1
		Uncultured bacterium	7*	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9*	1
		Uncultured bacterium	10*	1
Patient 2	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	1	118
		Lactobacillus mucosae	2	1
Patient 3	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	1	406
		Uncultured *Bacteroidetes* bacterium	2	27
		Stenotrophomonas maltophilia	3	11
		Uncultured bacterium	4*	3
		Uncultured bacterium	5*	2
		Uncultured *Saprospiraceae* bacterium	6	1
		Uncultured *Bacteroidetes* bacterium	7	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9*	1
		Uncultured bacterium	10*	1
Patient 4	*Pseudomonas aeruginosa* *Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa*	1	191
		Stenotrophomonas maltophilia	2	6
		Uncultured bacterium	3	2
		Uncultured betaproteobacterium	4	1
		Uncultured betaproteobacterium	5*	1
		Uncultured betaproteobacterium	6	1
		Polaromonas sp.	7	1
		Uncultured bacterium	8*	1
		Uncultured bacterium	9	1
Patient 5	*Achromobacter xylosoxidans*	*Achromobacter xylosoxidans*	1	440
		Stenotrophomonas maltophilia	2	1

[a] The species found by 16S rRNA gene analysis is given by the closest relatives of the bacterial OTUs in clone libraries for the patients.
[b] The number of sequences that make up the OTUs.
[c] Asterisks indicate clones where the identification by BLAST differed from the identification made by phylogenetic analysis.

the M13F primer (5′-GTAAAACGACGGCCAGT-3′) and checked for chimeric sequences with the program Bellerophon (12), using the Huber-Hugenholtz correction and a window size of 300 nucleotides. The BlastN function in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for initial identification of closest relatives with standard parameter settings, except that the database was set to the nucleotide collection (nr/nt).

Alignment of the sequences was performed using the SILVA web aligner (17) with default settings and refined manually in ARB (16). The sequences from the 34 clone libraries were compiled into overall libraries for each of the 5 patients; within these, the sequences were grouped into operational taxonomy units (OTUs) if they had a sequence similarity of more than 97% (13). Representative clones for all OTUs were also sequenced using the M13R primer (5′-GCGGATAACAATTT CACACAGG-3′) in order to obtain consensus sequences covering the entire length of the fragments. Consensus sequences representing the different OTUs and their closest relatives in the nonredundant SSU Ref database from SILVA release 104 were used for calculation of trees by distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software but omitting hypervariable regions of the gene. Twenty-four out-group sequences from the phylum *Chloroflexi* were added to the tree calculations.

Culture analysis showed the presence of monospecies infection in the lungs of four patients, and two bacterial species were found for the last patient (patient 4). No growth of anaerobic bacteria was observed. The isolated bacteria were *P. aeruginosa*, *Stenotrophomonas maltophilia*, or *Achromobacter xylosoxidans* (Table 1), and the same result was found on all types of media used. The 5 patients expectorated sputum just prior to their lung transplantation. The culture analysis of this sputum revealed the exact same bacteria as those found by the culture analysis from the explanted lungs (not shown). The initial identification of clone library sequences (as determined by BLAST search) showed that the organisms found by culture analysis were present in high numbers in the clone libraries (Table 1). The phylogenetic trees (neighbor joining, maximum parsimony, and maximum likelihood) were constructed to vi-

sualize the phylogenetic relationship of the microorganisms and showed congruent topology (the maximum likelihood tree is shown in Fig. 1). The locations of the sequences in the tree confirmed the result of the BLAST search and in several cases gave identification of sequences that had been determined to be uncultured bacteria by BLAST search, as indicated by asterisks in Table 1 and Fig. 1. This is due to the fact that, unlike the BLAST tool at NCBI, only quality-checked sequences were used in the ARB database used. Another factor is that, in ARB, the secondary structure of the 16S rRNA gene was taken into account. Some of the bacteria identified in the clone libraries have previously been associated with cystic fibrosis, such as *Stenotrophomonas maltophilia* (6, 7, 20), *Burkholderia fungorum* (6, 19), and *Streptococcus* sp. (13), but the clinical relevance of these bacteria and others found in small amounts in the samples is unknown (2, 11). Compared to the results obtained by culture analysis, the 16S rRNA gene analysis showed a greater diversity of bacteria, with sequences distributed into 4 phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. As the bacteria found by culturing were also represented by the highest numbers of sequences in the clone libraries, it is very likely that these bacteria were dominant in the lung. We are currently investigating this thoroughly by FISH and quantitative PCR.

The results presented here correlate with results that we have previously published (1) that the end-stage CF lung harbors relatively few bacterial species that could be identified by culturing. However, this might not represent the other levels of chronic infection in the CF lungs. In fact, many of the non-end-stage CF patients at the Copenhagen CF Clinic harbor several species in their lungs, which should also be investigated further.

Nucleotide sequence accession numbers. The nonredundant, nearly full-length 16S rRNA gene sequences representing each OTU obtained in this study were deposited in GenBank under the accession numbers JN802672 to JN802704.

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