

Novel Bacterium Isolated from a Lung Transplant Patient with Cystic Fibrosis

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The major clinical problem for patients with cystic fibrosis (CF) is progressive loss of pulmonary function, usually due to chronic bacterial infections. A patient with CF and a lung transplant was severely infected with a previously unidentified gram-negative bacterium. We isolated this organism (strain DS15158) from the patient and characterized it by phylogenetic analysis of the small-subunit rRNA and biochemically by the BIOLOG GN MicroPlate assay, fatty acid analysis, and various standard laboratory tests. No close match to any other organism could be found. Isolate DS15158 represents a new genus-level divergence within the bacterial subdivision α -Proteobacteria on the basis of the 16S rRNA gene analysis.

Cystic fibrosis (CF) is a lethal genetic disease of childhood and the most commonly inherited fatal disease of Caucasians. It can cause disruption of the exocrine function of the pancreas, the intestinal glands, biliary tree, bronchial glands, and sweat glands. Exocrine glands secrete abnormally thick mucus, which obstructs the pancreas and which causes chronic infections of the lungs. CF arises from over 720 different mutations in the gene encoding a plasma membrane protein that is designated the CF transmembrane conductance regulator. Individuals with CF who lack the CF transmembrane conductance regulator are generally subject to chronic microbial inflammations, mostly caused by mucoid *Pseudomonas aeruginosa* (14). Other microorganisms that are associated with CF are *Burkholderia cepacia*, *Sientrophomonas maltophilia*, *Haemophilus influenzae*, *Staphylococcus aureus*, other uncharacterized bacterial species, and atypical mycobacteria (7, 16, 17). Fungi, such as *Aspergillus* spp. (14) and *Candida albicans* (9), have also been reported to colonize patients with CF (6). These infections coupled with a dysfunctional host immune system contribute to the progressive decline in pulmonary function among CF patients and for most of them can cause death before the age of 30. Treatments vary, but they may include antibiotics, DNase to thin the mucus, aerosols that relieve constriction of the airways, and physical therapy.

We isolated a previously unidentified, very mucoid gram-negative rod (VMGNR) from the respiratory track of a 22-year-old female with CF and end-stage lung disease who had received a bilateral living lobar transplant in February 1998 from two healthy relatives who did not have CF. The perioperative antimicrobial therapy included imipenem, tobramycin, ceftazidime, and aerosolized colistin sulfate. Postoperatively, the imipenem, ceftazidime, and aerosolized colistin sulfate were continued. The immunosuppressive regimen included tacrolimus, mycophenolate mofetil, and methylprednisolone sodium succinate (Solu-Medrol). Cultures of her explants grew *P. aeruginosa*, *Proteus mirabilis*, and the VMGNR. Postoperatively, chest X rays showed increasing infiltrates. The patient underwent bronchoscopy with bronchoalveolar lavage (BAL)

on the 8th postoperative day. The BAL specimen did not grow any organisms. A BAL specimen obtained on the 16th postoperative day grew *Enterococcus*, coagulase-negative *Staphylococcus* sp., and the VMGNR, which was initially identified as “mucoid *Pseudomonas* sp.” She continued to develop new infiltrates, and on the 28th postoperative day she underwent a bronchoscopy with BAL and transbronchial biopsy. The BAL specimen grew a few colonies of *Alcaligenes xylosoxidans* that were susceptible to ceftazidime, piperacillin, ciprofloxacin, and imipenem. Her condition slowly improved, and she was discharged from the hospital 6 weeks after the transplant. A follow-up outpatient surveillance bronchoscopy with BAL was performed 2 months posttransplantation, and the BAL specimen did not grow any organisms. The VMGNR has not been recovered from any other specimens during the past year, although during the past 6 months she has developed a slightly elevated leukocyte count with a persistent bandemia of up to 65%.

Conventional biochemical and microbiological tests including the BIOLOG GN MicroPlate assay and whole-cell cellular fatty acid (CFA) analysis showed no match to any species in their extensive databases. Commercially available phylogenetic analysis that was based on only part (500 nucleotides [nt]) of the small-subunit rRNA gene (16S rDNA) sequence derived from strain DS15158 also failed to match this isolate to a known organism. Therefore, to determine the correct relationship of DS15158 to other organisms in the 16S rRNA-based phylogenetic tree, we have used the full-length 16S rRNA for phylogenetic sequence analysis (13, 20). On the basis of this analysis we suggest that isolate DS15158 represents a new genus-level divergence within the bacterial subdivision α -Proteobacteria.

MATERIALS AND METHODS

Laboratory Growth and Susceptibility Tests. Isolate DS15158 was streaked onto Trypticase soy agar supplemented with 5% sheep blood (BA), MacConkey agar, colistin-nalidixic blood agar, chocolate agar, Mueller-Hinton agar, and colistin-nalidixic acid-phenylethyl alcohol (Rose) blood agar (Hardy Diagnostics, Santa Maria, Calif.). The plates were incubated at 35°C and were examined daily for 5 days. Additional BA plates were incubated at 25 and 42°C. Colonies from 48-h cultures were used to inoculate different identification kits commonly used to identify gram-negative bacteria, according to the directions of the manufacturers: the API 20E system (bioMérieux, St. Louis, Mo.), BBL Crystal Enteric/Nonfermenter ID kit (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and RapID NF Plus system (Remel, Lenexa, Kans.).

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TABLE 1. Biochemical characterization of bacterial isolate DS15158

Characteristic or test	Reaction ^a
Growth on:	
BA at:	
37°C	+
42°C	+
25°C	+ (slower)
MacConkey agar	Poor
CNA-PEA ^b agar	Poor
Triple sugar iron agar	Alkaline, no change
OF glucose	-
Oxidase	+
Catalase	+
Indole	-
Motility	-
Esculin hydrolysis	+
Gelatin liquefaction	-
Urease	+
Nitrate reduction	-
Nitrite reduction	-
H ₂ S production (Sims)	-
Methyl red	-
Voges-Proskauer	-
Utilization of citrate (Simmons)	+
MBM acetamide	-
Malonate	-
Aliphatic thiol	-
Growth in 6% NaCl broth	+
Arginine dihydrolase (Moellers)	-
Lysine decarboxylase (Moellers)	-
Ornithine decarboxylase (Moellers)	-
Phenylalanine deaminase	-
Enzyme production	
Phosphatase	+
β-Glucosidase	+
β-Galactosidase	+
N-Acetyl-glucosaminidase	+
Phosphorylcholinase	+
Proline aminopeptidase	+
Pyroglutaminyl aminopeptidase	+
γ-L-Glutamyl aminopeptidase	+
Tryptophan aminopeptidase	+
N-Benzyl-arginine aminopeptidase	+
Triglyceride hydrolase	+
β-Glucuronidase	-

^a +, positive; -, negative.

^b CNA, colistin nalidixic acid blood agar; PEA, phenylethyl alcohol blood agar.

The following tests and media were used as described elsewhere (12): tests for oxidase, indole, and catalase with 3% hydrogen peroxide, triple sugar iron agar, and tube tests for citrate, acetamide, methyl red, Voges-Proskauer, indole-nitrate broth, Sims H₂S-motility agar, phenylalanine deaminase, Moellers' arginine dihydrolase, and ornithine and lysine decarboxylases.

Initial testing for susceptibility to various antimicrobial agents (see Table 2) was performed by the disk diffusion method according to National Committee for Clinical Laboratory Standards guideline M2-A6 (12a). Subsequent testing was done by the broth microdilution method (National Committee for Clinical Laboratory Standards guideline M7-A4 [12b]) with two different inoculum concentrations corresponding to the no. 0.5 and no. 2 McFarland turbidity standards, respectively. Quantitative cultures were performed to determine the numbers of CFU per milliliter of both concentrations. Incubation was at 35°C for 24 and 40 h for the initial and final interpretations, respectively. The Etest (AB Biodisk, Solna, Sweden) was used to determine or confirm some MIC test results.

BIOLOG GN MicroPlate assay. The BIOLOG GN MicroPlate assay (BIOLOG Inc., Hayward, Calif.) classifies bacterial isolates on the basis of the ability of organisms to oxidize carbon sources (2, 5). The test provides 95 different carbon sources and was performed by the manufacturer with cultures that had been grown at 35°C for 72 h on BA.

CFA analysis. Whole-cell, long-chain fatty acids were determined with fatty acid methyl esters (FAME) by standard procedures (MIDI Laboratories, Newark, Del.) with cells harvested after incubation for 48 h at 35°C on BA.

TABLE 2. Susceptibility of bacterial isolate DS15158 to antimicrobial agents

Antimicrobial agent	MIC (μg/ml) ^a	Disk diffusion result	Etest MIC (μg/μl)
Levofloxacin	4		
Ofloxacin	8		
Ciprofloxacin	4		
Meropenem	2		
Imipenem	2		
Ceftriaxone	>32		
Cefotaxime	>32		
Cefipime	>32		
Ceftazidime	>32		
Ticarcillin-clavulanate	>128/2 ^b		
Piperacillin-tazobactam	>128/4 ^b		
Ampicillin-sulbactam	>32/16 ^b		
Vancomycin	>16		
Oxacillin	1	R ^c	>256
Doxycycline			16
Amikacin	32		
Tobramycin		R	
Gentamicin		R	
Polymyxin B		R	
Colistin		R	

^a Broth microdilution method, inoculum of 1.5×10^5 CFU/ml, 24 h of incubation.

^b Beta-lactam antibiotic-beta-lactamase inhibitor concentration.

^c R, growth occurred up to the disk.

Partial 16S rRNA gene analysis. Partial sequencing of the 16S rRNA gene (500 nts) was performed by MIDI Laboratories by standard procedures, and a phylogenetic analysis based on this partial 16S rDNA fragment was performed. The sequence data were compared to sequences in the MicroSeq database (PE Applied Biosystems), as well as to those in the databases for the Ribosomal Database Project (10) and GenBank (National Center for Biotechnology Information, Bethesda, Md.).

DNA isolation. DNA from ca. 100 μg of cells was isolated by bead beating as described earlier (8), except that the beads were not pretreated with acid. The lysate was extracted once each with phenol, phenol-chloroform (1:1; vol/vol), and phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol). DNA was precipitated by adding sodium acetate to a final concentration of 0.3 M and 2.5 volumes of ethanol. The pellet was washed with 70% ethanol, dried, resuspended in 10 mM Tris-HCl (pH 8 at 25°C)-1 mM EDTA, and analyzed on a 0.5% agarose gel.

PCR amplification of 16S rDNA. PCRs were performed with four different primer combinations: 8F (5'-AGAGTTTGATCCTGGCTCAG)-1391R (5'-GACGGGCGGTGWGTRCA; W = A or T, R = A or G), 8F-1492R (5'-GGTTACCTTGTTACGACTT), 515F (5'-GTGCCAGMGGCCGCGGTAA; M = A or C)-1391R, and 515F-1492R, respectively. Each 100-μl PCR mixture contained 8 U of *Taq* DNA polymerase (Promega), 200 ng of each primer, 300 ng of genomic DNA, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25°C), and 0.1% Triton X-100. All PCRs started with an initial denaturation step for 2 min at 94°C, followed by 25 amplification cycles (1 min at 92°C, 1 min at 45°C, and 1 min 30 s at 72°C) and a final extension step for 30 min at 72°C. PCR fragments were purified over Chromabond-100 TE spin columns (Clontech).

Sequencing of PCR products. Both DNA strands of the different PCR products (50 ng) were sequenced directly by cycle sequencing (24 cycles of 30 s at 96°C, 15 s at 45°C, and 4 min at 60°C) with AmpliTaq FS (PE Applied Biosystems) by using 20 ng of each PCR primer (see above).

Cloning of PCR products and sequencing of rDNA clones. Additionally, the PCR fragments were cloned into the T-Vector pCPT9 as described elsewhere (15). Transformation of *Escherichia coli* XL-1 Blue (Stratagene) was performed as described previously (11). Recombinants were selected by the blue or white color of the colonies, and plasmid DNA from several clones for every primer combination was isolated by using the QIAprep plasmid kit (QIAGEN). Both strands of recombinant plasmid DNA were sequenced by cycle sequencing as described for the PCR products (see above), except that 20 ng each of primer T7 (5'-GTAATACGACTCACTATAGGG) and primer SP6 (5'-ATTTAGGTGACACTATAG) and 200 ng of plasmid DNA were used. The sequences were analyzed on an ABI 373A automated DNA sequencer (PE Applied Biosystems) according to the manufacturer's protocol.

Phylogenetic data analysis. To determine the approximate phylogenetic affiliation of strain DS15158, the sequence data were initially compared to the sequence data available in databases by using BLAST (1). The sequence data were investigated for the presence of chimeric sequences by using CHECK_CHIMERA

(10), and the sequences were then aligned with the 16S rRNA sequences in the database of the program package ARB (18) that was manually expanded to 8,472 different 16S rRNA sequences. Data sets that were missing from ARB but that were determined by the BLAST search were retrieved from the GenBank database (5a) and were also aligned. 16S rRNA-based phylogenetic trees were constructed by distance matrix analysis of all available 16S rRNA sequences. A subset of *Proteobacteria* sequences that were at least 1,350 nts in length was then used to calculate a subtree by the neighbor-joining method of ARB. A subtree was also constructed by using the same data set and the parsimony tool of ARB. We have used a mask that was created manually and that included only regions with unambiguous alignments. The relative confidence of each phylogenetic analysis was estimated by bootstrap analysis, which included 100 replicates (3). Distance matrices were calculated by the neighbor-joining method.

Nucleotide sequence accession number. The sequence of strain DS15158 was deposited in GenBank (5a) under accession no. AF085496.

RESULTS

Strain DS15158 is a rod-shaped gram-negative bacterium that measures 1.5 to 2 μm in width by 3.5 μm in length. Examination by phase-contrast microscopy revealed a capsule surrounding the cell. Growth occurred at 35 and 42°C but was very slow at 25°C. This organism formed very slimy, nonpigmented colonies on nonselective media, including blood agar, chocolate agar, and Mueller-Hinton agar after 48 h. Growth on MacConkey agar was very slight after 3 days. Growth on Rose blood agar was very slight after 5 days.

Reactions by standard biochemical tests are summarized in Table 1. The API 20E kit system did not produce any positive reactions after 24 and 48 h of incubation. The RapID NF Plus system as well as the BBL Crystal system generated profiles that did not match those of any organism in the corresponding databases. A summary of the individual reactions is included in Table 1.

Susceptibilities to various antimicrobial agents are presented in Table 2. The suspension corresponding to the no. 0.5 McFarland standard had 4 × 10⁴ CFU/ml. The suspension corresponding to the no. 2 McFarland standard had 1.5 × 10⁵ CFU/ml. The MICs obtained with the inoculum of 1.5 × 10⁵ CFU/ml are given in Table 2. Incubation beyond 24 h did not significantly change the MICs.

The BIOLOG GN MicroPlate assay showed that the microorganism is capable of using 59 of the 95 carbon sources in the panel (Table 3). The closest match in the BIOLOG, release 3.70, database was to *Agrobacterium tumefaciens* subgroup A (also known as *Agrobacterium radiobacter*). The profile values were borderline for acceptability with a similarity value slightly greater than the minimum value of 0.5, and a carbon source distance value of five to seven carbon sources.

The CFA analysis with FAMES resulted in a profile that did not match that for any of the organisms in the MIDI Laboratories database. The major fatty acids found in isolate DS15158 are listed in Table 4.

Analysis of 500 nts of 16S rRNA by MIDI Laboratories resulted in a “no match” interpretation.

With 1,359 nts, the 16S rDNA sequence data for isolate DS15158 represent almost the full length of the organism’s 16S rRNA. All the sequences derived from the PCR products as well as from the recombinant plasmid DNA were identical. No chimeric sequences were detected. On the basis of 16S rRNA phylogenetic analysis, isolate DS15158 belongs to the bacterial subdivision α-*Proteobacteria* with no specific relative (Fig. 1 and 2). Both the neighbor-joining and the parsimony methods (data not shown) gave similar results with similar bootstrap support. On the basis of the sequence similarities of their 16S rRNAs (91.3%), the closest known relative of isolate DS15158 is *Azospirillum lipoferum* (Fig. 2).

TABLE 3. Carbon sources used by strain DS15158 in the BIOLOG GN MicroPlate assay

Carbon source	Carbon source
1. Dextrin	31. Methyl pyruvate
2. Glycogen	32. Monomethyl succinate
3. N-Acetyl-D-galactosamine	33. Acetic acid
4. N-Acetyl-D-glucosamine	34. cis-Aconitic acid
5. Adonitol	35. Citric acid
6. L-Arabinose	36. Formic acid
7. D-Arabitol	37. D-Galactonic acid lactone
8. Cellobiose	38. D-Gluconic acid
9. i-Erythritol	39. Beta-hydroxybutyric acid
10. D-Fructose	40. Alpha-ketoglutaric acid
11. L-Fucose	41. D,L-Lactic acid
12. D-Galactose	42. Malonic acid
13. Gentiobiose	43. Succinic acid
14. Alpha-D-glucose	44. Bromosuccinic acid
15. m-Inositol	45. Succinamic acid
16. Alpha-D-lactose	46. Alaninamide
17. Lactulose	47. D-alanine
18. Maltose	48. L-Alanine
19. D-Mannitol	49. L-alanyl-glycine
20. D-Mannose	50. L-asparagine
21. D-Melibiose	51. L-aspartic acid
22. Beta-methyl-D-glucoside	52. L-glutamic acid
23. D-Psicose	53. Glycyl-L-glutamic acid
24. D-Raffinose	54. L-Ornithine
25. L-Rhamnose	55. L-Proline
26. D-Sorbitol	56. L-Serine
27. Sucrose	57. D-L-Carnitine
28. D-Trehalose	58. Gamma-aminobutyric acid
29. Turanose	59. Inosine
30. Xylitol	

DISCUSSION

Several well-characterized bacteria and fungi are known to cause serious, chronic infections in patients with CF. We describe a bacterium (isolate DS15158) not previously reported to be associated with CF disease and not previously described.

As with other organisms isolated from patients with CF, this strain was resistant to most of the antimicrobial agents that we tested. By the broth microdilution test, this strain was found to be susceptible to oxacillin (MIC, 1 μg/ml). To confirm the

TABLE 4. FAME constituents of isolate DS15158

Fatty acid	% Total
13:1 ω 1c	0.90
Sum in feature 3 ^a	1.51
16:0	2.19
17:1 ω 6c	1.94
17:0	3.29
16:0 2 OH	1.86
16:0 3 OH	1.52
Sum in feature 6 ^b	1.91
Sum in feature 7 ^c	58.84
18:0	2.58
17:0 2 OH	2.03
17:0 3 OH	1.29
19:0 cyclo ω 8c	9.73
18:1 2 OH	4.93
18:0 2 OH	2.08
20:3 ω 6,9,12c	3.40

^a Summed feature 3, 12:0 aldehyde and/or 16:1 iso I and/or 14:0 3 OH.

^b Summed feature 6, 18:2 ω 6,9,12c and/or 18:0 anteiso.

^c Summed feature 7, 18:1 ω 7c and/or 18:1 ω 9c.

netic approach and as supported by the CFA analysis and the BIOLOG GN MicroPlate assay, we propose that DS15158 represents a new genus-level divergence within the α -*Proteobacteria*. The carbon source utilization profile of this organism is now contained in an updated BIOLOG database (release 4.0) under the provisional name "*Agrobacterium*-like—cystic fibrosis." This will allow laboratories to identify it in the future using a simple, commercially available test.

Although the use of rRNA sequences as a tool to study the natural relatedness of organisms is widely accepted, uncertainties in 16S rDNA sequences can arise because of sequencing errors, amplification errors, and the possibility of microheterogeneity (4). To minimize such errors, both strands of the 16S rDNA represented by PCR products and recombinant plasmid DNAs were sequenced. The fact that all sequences were identical was taken as additional evidence that DS15158 is derived from a pure culture.

DS15158 is a new organism isolated from a patient with CF. The next investigations needed include more detailed characterization and description of the new isolate, determination of its disease-causing potential, and determination of appropriate treatment.

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