Ribosomal DNA-Directed PCR for Identification of Achromobacter (Alcaligenes) xylosoxidans Recovered from Sputum Samples from Cystic Fibrosis Patients

Lixia Liu, Tom Coenye, Jane L. Burns, Paul W. Whitby, Terrence L. Stuhl, and John J. LiPuma

Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, Michigan 48109; Department of Pediatrics, University of Washington, Seattle, Washington 98105; and Departments of Pediatrics and Microbiology/Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Received 25 September 2001/Returned for modification 18 December 2001/Accepted 20 January 2002

The opportunistic human pathogen Achromobacter (Alcaligenes) xylosoxidans has been recovered with increasing frequency from respiratory tract culture of persons with cystic fibrosis (CF). However, confusion of this species with other closely related respiratory pathogens has limited studies to better elucidate its epidemiology, natural history, and pathogenic role in CF. Misidentification of A. xylosoxidans as Burkholderia cepacia complex is especially problematic and presents a challenge to effective infection control in CF. To address the problem of accurate identification of A. xylosoxidans, we developed a PCR assay based on a 16S ribosomal DNA sequence. In an analysis of 149 isolates that included 47 A. xylosoxidans and several related glucose-nonfermenting species recovered from CF sputum, the sensitivity and specificity of this PCR assay were determined to be 100 and 97%, respectively. The availability of this assay will enhance identification of A. xylosoxidans, thereby facilitating study of the pathogenic role of this species and improving infection control efforts in CF.

The taxonomy of the genus Alcaligenes has undergone a number of changes during the last 20 years. The species Achromobacter (Alcaligenes) xylosoxidans has consecutively been named Achromobacter xylosoxidans, Alcaligenes denitrificans subsp. xylosoxidans, and Alcaligenes xylosoxidans subsp. xylosoxidans (26). More recently, the name Achromobacter xylosoxidans was again proposed (31).

A. xylosoxidans is an aerobic, oxidase- and catalase-positive, non-lactose-fermenting, gram-negative bacillus that is widely distributed in the natural environment. It is an opportunistic human pathogen capable of causing a variety of infections, including bacteremia, meningitis, pneumonia, and peritonitis (4, 5, 7, 14, 28). Nosocomial outbreaks attributed to disinfectant solutions, dialysis fluids, saline solution, and deionized water contaminated with this species have been described (8, 9, 19, 24, 25, 27).

A. xylosoxidans is also capable of persistent infection of the respiratory tract of persons with cystic fibrosis (CF) (2, 6, 22), although its precise role in contributing to pulmonary decline in this population is not clear. Nevertheless, this species is important in CF; it infects some 9% of CF patients (2) and is frequently confused with species within the Burkholderia cepacia complex (1, 20). Infection with these latter species is associated with significantly increased rates of morbidity and mortality in CF, and stringent infection control efforts are employed to prevent infection (16). Misidentification of A. xylosoxidans and related nonfermenting species seriously compromises infection control measures and confounds efforts to more clearly understand the epidemiology and natural history of infection in CF. To enable more accurate identification of A. xylosoxidans, we developed PCR assays based on 16S rRNA gene sequences.

MATERIALS AND METHODS

Bacterial strains. Bacterial isolates were obtained from the Burkholderia cepacia Research Laboratory and Repository (University of Michigan, Ann Arbor) or the Children’s Hospital and Regional Medical Center (Seattle, Wash.). Reference strains were obtained from the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium) or the American Type Culture Collection (Manassas, Va.). A total of 149 isolates were studied. Among these were 48 A. xylosoxidans isolates (47 recovered from CF sputum [2] and the reference isolate ATCC 9220) and 101 isolates representing phylogenetically related species and other species that may be encountered in CF sputum. Among these were 53 Burkholderia cepacia complex, 15 Pseudomonas aeruginosa, six Burkholderia gladioli, six Stenotrophomonas maltophilia, and three Ralstonia pickettii isolates, all of which were identified by using polyphasic analyses, including species-specific PCR assays previously described (3, 17, 29, 30). Also included were Achromobacter piechaudii strain LM1873T, A. xylosoxidans, Achromobacter denitrificans strain LMG 1231T, Alcaligenes faecalis strain LMG 1229T, Bordetella pertussis strain LMG 14455T, Bordetella parapertussis strain LMG 14449T, Bordetella bronchiseptica strain LMG 1231T, Bordetella hinzii strain LMG 13501T, Bordetella avium strain LMG 1852T, Bordetella holmesii strain LMG 1873T, and Bordetella trematum strain LMG 13506T.

DNA preparation. DNA was prepared by heating one or two colonies (picked from an overnight grown plate) at 95°C for 15 min in 20 μl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 M NaOH. After lysis, 180 μl of sterile distilled water was added to the lysis buffer, and the DNA solutions were stored at −20°C.

Amplification and sequence determination of 16S rRNA genes. The nearly complete sequence (corresponding to positions 9 to 1500 in the Escherichia coli numbering system) of the 16S rRNA gene of A. xylosoxidans strains AU0065, AU1011, and ATCC 9220 were amplified by PCR by using Pfu DNA polymerase (Stratagene, La Jolla, Calif.) with conserved primers UFPL and URPL as previously described (Table 1) (17). The resultant amplicons were purified by using the Promega Wizard PCR Prep DNA purification kit (Promega, Madison, Wis.) according to the manufacturer’s instructions. DNA sequence analysis was performed with an Applied Biosystems 377XL DNA sequencer and the protocols of the manufacturer (PE Applied Biosystems, Foster City, Calif.) by using the BigDye Terminator Cycle Sequencing Ready Reaction kit. The sequencing primers used were UFPL, URPL, 16S F1 (5′-GGCTTGGGGTGTGTAAAGCA-GC-3′), 16S F2 (5′-CTTACCTACCCCTGAC-3′), 16S B1 (5′-GGCTCGTTGCGG

* Corresponding author. Mailing address: Department of Pediatrics and Communicable Diseases, 8323 MSRB III, Box 0646, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0646. Phone: (734) 936-9767. Fax: (734) 764-4279. E-mail: jlipuma@umich.edu.
ACT-3'), and 16SB2 (5'-GTATACCCGCGGTGCTG-3'). Sequence assembly was performed by using EditSeq (DNAStar, Inc., Madison, Wis.).

Development of primers for species-specific PCR assays. The 16S ribosomal DNA (rDNA) sequences of AU0665, AU1011, and ATCC 9220 were aligned to rDNA sequences of all Achromobacter, Alcaligenes, Bordetella, Burkholderia, Pantoaea, and Ralstonia species available in the GenBank database by using the MegAlign software package (DNASTar). Putative species-specific signature sequences were detected, and primers targeting these sequences were developed. A phylogenetic tree based on the 16S rDNA alignments was constructed by using the MegAlign (DNASTar) software package to demonstrate the relationship of the species studied to A. xylosoxidans (Fig. 1).

PCR. PCR assays were performed in 25-μl reaction mixtures, containing 2 μl of template, 1 U of Taq polymerase (Gibco-BRL), 250 mM concentrations of each deoxynucleotide triphosphate (Gibco-BRL), 1× PCR buffer (Gibco-BRL), 1.5 mM MgCl2 (Gibco-BRL), and a 1 μM concentration of each oligonucleotide primer. Amplification was carried out by using a PTC-100 programmable thermal cycler (MJ Research, Watertown, Mass.). After initial denaturation for 3 min at 95°C, 30 amplification cycles were completed, each consisting of 1 min at 94°C, 1 min at 56°C, and 1 min 30 s at 72°C. A final extension of 10 min at 72°C was applied. Negative control PCRs with all reaction mixture components except template DNA were employed for every experiment. A. xylosoxidans AU0665 was used as a positive control.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rDNA nucleotide sequences for strains AU0665, AU1011, and ATCC 9220 are AF411019, AF411020, and AF411021, respectively.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Nucleotide positions</th>
<th>Product size (bp)</th>
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<tr>
<td>UFPL</td>
<td>AGTTTGATCCTGGCTCAG</td>
<td>9-26</td>
<td>1,490</td>
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<tr>
<td>URPL</td>
<td>GGTACCCTGGTACAGCTT</td>
<td>1482-1500</td>
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</tr>
<tr>
<td>AX-F1</td>
<td>GCCAGAAAGAGAAACGCGGGT</td>
<td>427-448</td>
<td>163</td>
</tr>
<tr>
<td>AX-B1</td>
<td>ATTTACATCTTTTTCGCGG</td>
<td>576-595</td>
<td></td>
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</tbody>
</table>

* Numbering corresponds to 16S rDNA sequences in GenBank whose accession numbers are provided in the text.

RESULTS

16S rRNA gene alignment and primer design. 16S rRNA gene sequences from A. xylosoxidans AU0665, AU1011, and ATCC 9220 were aligned to each other and to sequences of related bacteria retrieved from GenBank. The sequences of AU0665 and ATCC 9220 were 100% identical; the sequence of AU1011 differed by 1 bp. Multiple sequence alignments revealed 92 to 99% identities of these sequences to the 16S rRNA genes of other Achromobacter (or Alcaligenes) species. Species-level signature sequences were identified and primers AX-F1 and AX-B1 were designed to target these (Table 1).

Sensitivity and specificity of PCR assay. Figure 2 illustrates the results of PCR with primer pair AX-F1 and AX-B1. Products of the predicted sizes were detected in the three A. xylosoxidans strains used for 16S rRNA sequencing analyses. Products were also obtained for the reference strains of the closely related species A. piechaudii, A. ruhlandii, and A. denitrificans. Alcaligenes faecalis and all other species tested were negative; however, testing of Bordetella hinzii yielded an inconsistent and faintly positive reaction.

PCR analyses of each of the 149 test bacteria with primers AX-F1 and AX-B1 were carried out. With a test panel of 149 isolates, the results were as follows: for A. xylosoxidans, there were 48 positive isolates and no negative isolates; for all other spp., there were 3 positive isolates and 98 negative isolates. (The three positive results were obtained with reference strains of A. piechaudii, A. ruhlandii, and A. denitrificans.) The sensitivity and specificity of the PCR assays for A. xylosoxidans were 100 and 97%, respectively.

DISCUSSION

Although A. xylosoxidans is a relatively uncommon human pathogen, it is capable of causing invasive infection in both
immunocompromised and immunocompetent hosts (5). The species is widely distributed in the natural environment, especially in oligotrophic aquatic niches. It is nutritionally quite versatile; some strains can use aminopolycarboxylate-chelating agents (e.g., EDTA) as sole carbon sources, and others can degrade aromatic hydrocarbon compounds, including benzene and toluene (10, 23). Pseudoepidemics and true nosocomial outbreaks due to contamination of disinfectant solutions, including those containing quaternary ammonium compounds, have been reported (8, 9).

In CF, chronic infection of the respiratory tract leads to progressive pulmonary destruction and respiratory failure. The primary pathogen involved is progressive pulmonary destruction and respiratory failure. The species in the genus Stenotrophomonas, including those containing quaternary ammonium compounds, have been reported (8, 9).

The epidemiology of A. xylosoxidans in CF also requires further elucidation. A recent study demonstrated that the majority of 92 A. xylosoxidans culture-positive CF patients (from 46 U.S. cities) harbored unique strain types based on randomly amplified polymorphic DNA typing (13). Unlike the pattern seen with P. aeruginosa, these patients appeared to acquire a single strain of A. xylosoxidans, which was only rarely and transiently replaced with a second. Unfortunately, studies to better assess the epidemiology and natural history of infection in CF have been hampered by difficulties with accurate laboratory identification of this and related species.

The confusion of A. xylosoxidans with species of the Burkholderia cepacia complex is particularly troublesome (1, 20). The latter species also infect ca. 10% of adult CF patients but, in contrast to A. xylosoxidans, infection is more clearly associated with an adverse clinical outcome, and the spread of specific clones among CF patients is well documented (15). Because infection with Burkholderia cepacia complex species is generally refractory to antimicrobial therapy, prevention of acquisition is a mainstay of patient management. Stringent infection control measures are intended to segregate persons infected with Burkholderia cepacia complex from other CF patients (16). Obviously, accurate identification of nonfermenting species from CF sputum is critical to these efforts. To this end, several PCR assays based on 16S rRNA gene sequence have been developed recently for identification of Burkholderia cepacia complex species (17, 18), as well as other CF pathogens, including Burkholderia gladioli (30), Stenotrophomonas maltophilia (29), and Pandorea species (3).

To design a PCR assay for identification of A. xylosoxidans, we similarly sought species-specific signature sequences in 16S rRNA genes. The high degree of sequence identity among Achromobacter species offered limited opportunity to design species-specific primers. Although primer AX-F1 targets species-specific sequences in the 16S rRNA gene, primer AX-B1 is directed against a sequence shared by most Achromobacter species. A PCR assay employing these primers showed excellent sensitivity for A. xylosoxidans, detecting all isolates tested. However, three closely related Achromobacter species (A. ruhlandii, A. piechaudii, and A. denitrificans) (Fig. 1) also gave a positive reaction. These species are soil commensals represented by a very limited number of described strains that are not known to be pathogenic for humans (12) (although intravenous catheter related bacteremia due to A. piechaudii in an immunocompromised cancer patient has been reported recently [11]). These species are not known to infect CF patients; however, we were careful to also test several other species that are recovered from CF sputum and have been confused with A. xylosoxidans based on phenotypic analyses alone. Negative results were obtained with closely related respiratory pathogens in the genus Bordetella, including Bordetella bronchiseptica, Bordetella parapertussis, and Bordetella pertussis. All Burkholderia cepacia complex and Pandorea species tested, as well as Bordetella gladioli, Stenotrophomonas maltophilia, Ralstonia picketti, and Pseudomonas aeruginosa isolates, were also negative.

The availability of a PCR-based assay for identification of A. xylosoxidans will facilitate further studies of human infection
due to this pathogen. This will be particularly important in investigation of nosocomial outbreaks and in CF, wherein this species is being found with increasing frequency. Most importantly, a reliable PCR assay for A. xylosoxidans will allow better differentiation of this species from phenotypically similar species that also infect in CF. This will significantly enhance clinical management and infection control in this vulnerable patient population.

ACKNOWLEDGMENTS

This work was supported by grants (to J.J.L. and T.L.S.) from the Cystic Fibrosis Foundation. T.C. is supported with funding from the Carroll Haas Research Fund in Cystic Fibrosis. We thank Monica Bender and Alissa Martin for technical support.

REFERENCES