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

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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.

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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 *Pandoraea*, seven *Pseudomonas aeruginosa*, six *Burkholderia gladioli*, six *Stenotrophomonas maltophilia*, and three *Ralstonia picketii* 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* LMG 1873^T, *Achromobacter ruhlandii* LMG 1866 ^{T}, *Achromobacter denitrificans* LMG 1231^T, *Alcaligenes faecalis* LMG 1229^T, *Bordetella pertussis* LMG 14455^T, *Bordetella parapertussis* LMG 14449^T, *Bordetella bronchiseptica* LMG 1231^T, *Bordetella hinzii* LMG 13501^T, *Bordetella avium* LMG 1852^T, *Bordetella holmesii* LMG 1873^T, and *Bordetella trematum* LMG 13506^T.

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 AU0665, 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, 16SF1 (5′-GCCTTCGGGTTGTAAAGCAC-3′), 16SF2 (5′-CCTTACCTACCCTTGACA-3′), 16SB1 (5′-GCGCTCGTTGCGGG

TABLE 1. 16S rDNA derived PCR primer sequences

Primer	Sequence (5′-3′)	Nucleotide positions[a]	Product size (bp)
UFPL	AGTTTGATCCTGGCTCAG	9–26	1,490
URPL	GGTTACCTTGTTACGACTT	1482–1500	
AX-F1	GCAGGAAAGAAACGTCGCGGGT	427–448	163
AX-B1	ATTTCACATCTTTCTTTCCG	576–595	

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

ACT-3′), and 16SB2 (5′-GTATTACCGCGGCTGCTG-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*, *Pandoraea*, 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, Gaithersburg, Md.), 250 mM concentrations of each deoxynucleotide triphosphate (Gibco-BRL), 1× PCR buffer (Gibco-BRL), 1.5 mM $MgCl_2$ (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 rRNA nucleotide sequences for strains AU0665, AU1011, and ATCC 9220 are AF411019, AF411020, and AF411021, respectively.

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

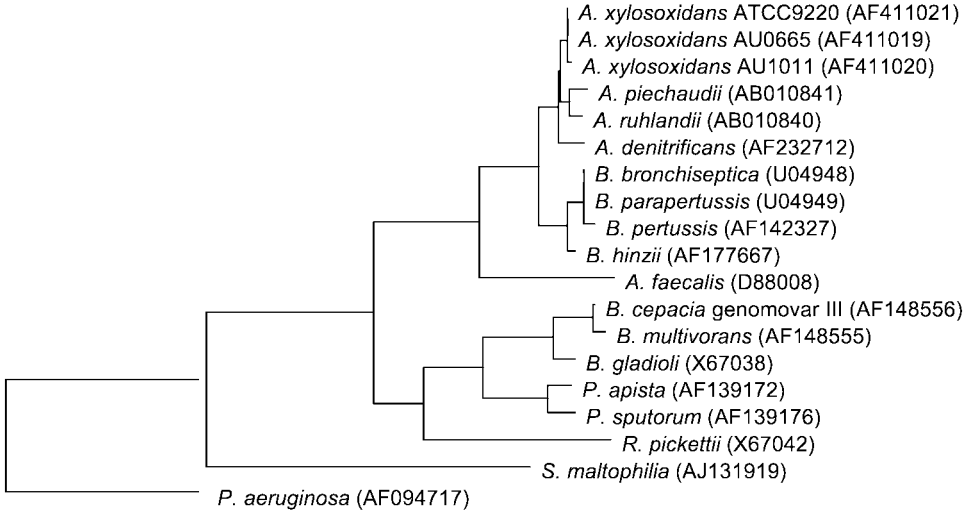

FIG. 1. Phylogenetic tree (based on neighbor-joining method analysis of 16S rDNA sequences) of the genus *Achromobacter* and related species. Numbers in parentheses are GenBank accession numbers for 16S rDNA nucleotide sequences of strains included. Bar, 10% sequence dissimilarity.

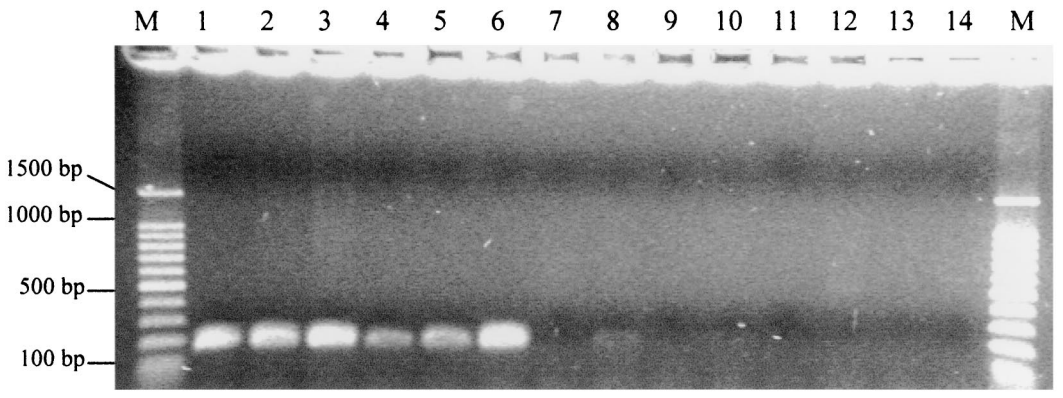

FIG. 2. PCR analysis of *A. xylosoxidans* and related species. Lanes: M, DNA marker; 1, *A. xylosoxidans* AU0665; 2, *A. xylosoxidans* AU1011; 3, *A. xylosoxidans* ATCC9220; 4, *A. piechaudii* LMG 1873^T; 5, *A. ruhlandii* LMG 1866^T; 6, *A. denitrificans* LMG 1231^T; 7, *Alcaligenes faecalis* LMG 1229^T; 8, *Bordetella hinzii* LMG 13501^T; 9, *Bordetella pertussis* LMG 14455^T; 10, *Bordetella parapertussis* LMG 14449^T; 11, *Bordetella bronchiseptica* LMG 1231^T; 12, *Burkholderia cepacia* genomovar III HI2147; 13, *Pandoraea apista* AU0003; 14, *Pseudomonas aeruginosa* AU0225.

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 *Pseudomonas aeruginosa*, but *A. xylosoxidans* and other nonfermenting species such as *Ralstonia* spp., *Stenotrophomonas maltophilia*, and species in the *Burkholderia cepacia* complex also infect the CF lung. Burns et al. found that 52 (8.7%) of 595 CF patients were infected with *A. xylosoxidans* (2). Although *A. xylosoxidans* is capable of chronic infection in CF (21, 22), its role in contributing to pulmonary decline is not clear.

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 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 *Pandoraea* 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 *Pandoraea* isolates tested, as well as *Bordetella gladioli*, *Stenotrophomonas maltophilia*, *Ralstonia picketii*, 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.

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