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Fluorescence In Situ Hybridization for Rapid Identification of *Achromobacter xylosoxidans* and *Alcaligenes faecalis* Recovered from Cystic Fibrosis Patients

Nele Wellinghausen,* Beate Wirths, and Sven Poppert

Institute of Medical Microbiology and Hygiene, University Hospital of Ulm, Ulm, Germany

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Achromobacter xylosoxidans **is frequently isolated from the respiratory secretions of cystic fibrosis (CF) patients, but identification with biochemical tests is unreliable. We describe fluorescence in situ hybridization assays for the rapid identification of *Achromobacter xylosoxidans* and *Alcaligenes faecalis*. Both assays showed high sensitivities and high specificities with a collection of 155 nonfermenters from CF patients.**

Achromobacter xylosoxidans is a gram-negative, oxidase-positive, non-glucose-fermenting rod that is widely distributed in the environment. The species is found in two subspecies, called *A. xylosoxidans* subsp. *xylosoxidans* and *A. xylosoxidans* subsp. *denitrificans*. Due to the close relation to *Alcaligenes* spp., the species has intermittently been named *Alcaligenes* (28); but in 1998, use of the initial name, *Achromobacter xylosoxidans*, was again proposed (31).

Human infections caused by *A. xylosoxidans* mainly comprise nosocomial infections, like device-associated infections, bacteremia, meningitis, and peritonitis (1, 7, 9, 19, 21, 25, 26). In cystic fibrosis (CF) patients, *A. xylosoxidans* causes temporary and persistent infections of the respiratory tract (5, 11, 12, 18, 22, 24, 27). The rate of persistent infection among CF patients varies from 2.3% to 8.7% (5, 24), and the proportion of patients who are temporarily infected reaches up to 10% (14, 18, 24). Although our knowledge about the significance of this species in the deterioration of pulmonary function in CF patients is limited (24), accurate identification of *A. xylosoxidans* is desirable for the following reasons. First, it is the necessary prerequisite for assessment of the epidemiology and clinical significance of the species; second, *A. xylosoxidans* is frequently confused with other nonfermenters, like members of the *Burkholderia cepacia* complex, *Alcaligenes faecalis*, and *Pseudomonas* spp., which has a considerable impact on patient management (8, 17, 23, 29, 30); third, multidrug resistance is found in one-third of *A. xylosoxidans* isolates in CF patients (12, 14); and fourth, transmission of *A. xylosoxidans* strains between close contacts seem to occur more frequently than was previously assumed (13, 27).

Fluorescence in situ hybridization (FISH) has been proven to be a rapid, easy-to-perform, and comparably cheap method for the identification of bacterial pathogens (10, 20, 30). Therefore, we evaluated this technique for use for the identification of *A. xylosoxidans* and the closely related species *Alcaligenes faecalis* from CF patients. Both new FISH probes were evaluated with a panel of reference strains as well as 155 clinical isolates of gram-negative, oxidase-positive rods from CF patients.

Probes Ach-221 (5′-CGC TCY AAT AGT GCA AGG TC) and Alc-476 (5′-CTG CAG ATA CCG TCA GCA GT), which bind to 16S rRNA at positions 221 to 240 and 476 to 495, respectively, were designed by using the ARB program package (available at http://www.arb-home.de), synthesized, and 5′ labeled with the fluorescent dye Cy3 by Thermo Hybaid, Ulm, Germany. The probes were controlled by use of the ARB software as well as by a search of the sequences in the GenBank database with the BLAST program (2). Ten microliters of a bacterial suspension in 0.9% saline, prepared from an overnight culture on sheep blood agar or MacConkey agar, was applied to a glass slide, air dried, and fixed for 5 min in methanol. FISH was performed at 46°C, as described elsewhere (4, 16). Bacterial probe EUB-338 (5′-GCT GCC TCC CGT AGG AGT), which targets nearly all bacterial species (3), was used as a positive control on each slide. The optimal hybridization stringency was determined to be formamide concentrations of 50% for probe Ach-221 and 40% for probe Alc-476 by increasing the formamide concentrations in the hybridization buffer in increments of 10% and by lowering the salt concentration accordingly in the wash buffer (16).

The sensitivities and specificities of the FISH probes were evaluated in a blinded manner with a panel of reference strains, including closely related species as well as common CF pathogens (Table 1). Probe Ach-221 correctly identified both subspecies of *A. xylosoxidans*. It showed a faint cross-reaction with *Achromobacter ruhlandii* but not with other species (Table 1). Probe Alc-476 specifically identified both subspecies of *Alcaligenes faecalis*. Cross-reactions with other species were not detected (Table 1).

For clinical evaluation of the probes, all gram-negative, oxidase-positive, non-glucose-fermenting rods except morphologically typical *Pseudomonas aeruginosa* strains ($n = 155$) isolated from CF patients from 1 January 2003 to 5 April 2005 in our laboratory were used. This collection included 88 isolates that were already part of an earlier study (30). Species identification of clinical isolates was done by *P. aeruginosa*-specific FISH and by sequencing of the complete 16S rRNA gene, as described earlier (30). *A. xylosoxidans*-specific probe Ach-221 correctly identified all isolates of *A. xylosoxidans* ($n = 25$) and,

* Corresponding author. Mailing address: Institute of Medical Microbiology and Hygiene, University Hospital of Ulm, Robert-Koch Str. 8, D-89081 Ulm, Germany. Phone: 49-731-50024602. Fax: 49-731-50023473. E-mail: nele.wellinghausen@uniklinik-ulm.de.

TABLE 1. Evaluation of the probes on reference strains

Strain[a]	Source[b]	Result with probe[c]:	
		Ach-221	Alc-476
Achromobacter piechaudii	ATCC 43552	−	−
Achromobacter ruhlandii	ATCC 15749	(+)	−
Achromobacter xylosoxidans subsp. *denitrificans*	ATCC 15173	+	−
Achromobacter xylosoxidans subsp. *xylosoxidans*	ATCC 27061	+	−
Alcaligenes faecalis subsp. *faecalis*	ATCC 8750	−	+
Alcaligenes faecalis subsp. *faecalis*	ATCC 35655	−	+
Alcaligenes faecalis subsp. *parafaecalis*	DSM 13975	−	+
Bordetella avium	ATCC 35086	−	−
Bordetella bronchiseptica	ci (1)	−	−
Bordetella hinzii	ATCC 51783	−	−
Bordetella holmesii	ATCC 51541	−	−
Bordetella parapertussis	DSM 4922	−	−
Bordetella pertussis	ATCC 9797	−	−
Burkholderia cepacia genomovar I	ATCC 25416	−	−
Burkholderia cenocepacia	DSM 16553^T	−	−
Burkholderia cenocepacia	ci (1)	−	−
Burkholderia dolosa	DSM 16088^T	−	−
Burkholderia gladioli	ATCC 10248	−	−
Burkholderia multivorans	DSM 13243	−	−
Flavimonas oryzihabitans	ci (1)	−	−
Flavobacterium odoratimimus	QC 02/05	−	−
Pandoraea apista	DSM 16535^T	−	−
Pandoraea norimbergensis	DSM 11628^T	−	−
Pandoraea pnomenusa	DSM 16536^T	−	−
Pandoraea pulmonicola	DSM 16583^T	−	−
Pandoraea species	ci (2)	−	−
Pseudomonas aeruginosa	ATCC 27853	−	−
Pseudomonas luteola	QC 02/05	−	−
Pseudomonas putida	ci (1)	−	−
Pseudomonas stutzeri	ATCC 17588	−	−
Ralstonia gilardii	ATCC 700815	−	−
Ralstonia mannitolilytica	DSM 17512^T	−	−
Ralstonia pickettii	ATCC 27511	−	−
Stenotrophomonas maltophilia	ATCC 13637	−	−
Stenotrophomonas maltophilia	ci (2)	−	−
Tetrathiobacter kashmirensis	ci (1)	−	−

[a] Species identification was confirmed by complete 16S rRNA gene sequencing.
[b] QC, external quality control strain of the Institute for Standardization and Documentation in the Medical Laboratory. ci, clinical isolate (number of isolates).
[c] +, positive; (+), weakly positive; −, negative.

TABLE 2. Evaluation of the probes on oxidase-positive nonfermenters from CF patients

Species	No. of isolates	No. of isolates with positive result with probe:	
		Ach-221	Alc-476
Achromobacter xylosoxidans subsp. *xylosoxidans*	25	25	0
Agrobacterium radiobacter	2	0	0
Alcaligenes faecalis	1	0	1
Burkholderia multivorans	21	0	0
Chryseobacterium species	3	1	0
Inquilinus limosus	1	0	0
Ochrobactrum anthropi	1	0	0
Pseudomonas aeruginosa	93	0	0
Pseudomonas alcaligenes	1	0	0
Pseudomonas brassicacearum	1	0	0
Pseudomonas fluorescens	1	0	0
Pseudomonas putida	1	0	0
Pseudomonas stutzeri	2	0	0
Pseudomonas synxantha	1	0	0
Sphingomonas paucimobilis	1	0	0

thus, showed a sensitivity of 100%. It cross-reacted only with one isolate of a *Chryseobacterium* sp., resulting in a specificity of 99.2%. The single strain of *Alcaligenes faecalis* subsp. *faecalis* that was isolated during the study period was correctly identified with the *Alcaligenes faecalis*-specific probe (Table 2). The probe did not cross-react with any species, resulting in a specificity of 100%.

A. xylosoxidans became recognized as an "emerging" CF pathogen in the 1980s and has been studied with growing interest during the last decade. Ferroni et al. (8) reported that *A. xylosoxidans* was the second most frequent gram-negative nonfermenter, after *Pseudomonas aeruginosa*, recovered from among 1,093 isolates from 148 patients. Nevertheless, identification of *A. xylosoxidans* is still a challenge for the clinical microbiologist; and by using biochemical and commercial tests, it is frequently confused with other nonfermenters, especially members of the *Burkholderia cepacia* complex (8, 17, 23, 29, 30). Modern molecular methods, like sequencing of ribosomal genes, allow accurate identification of gram-negative rods, including *A. xylosoxidans*, from CF patients (6, 8, 17). Despite its exactness, DNA sequencing is, however, not preferable for routine identification of common CF pathogens like *A. xylosoxidans* due to its comparatively high costs and the requirement for specific technical equipment. In addition, public DNA databases that do not involve quality control measures also contain faulty or incorrectly assigned sequences, leading to the misidentification of isolates. By alignment of public sequences with sequences of American Type Culture Collection strains, we found, for example, that GenBank entry AJ509012, assigned as *Alcaligenes faecalis*, is in fact a 16S rRNA gene sequence of *A. xylosoxidans* subsp. *xylosoxidans*. Recently, Liu et al. (15) described a ribosomal DNA-directed PCR assay for the identification of both subspecies of *A. xylosoxidans* from CF patients. The PCR primer cross bound, however, to *A. ruhlandii* and *Achromobacter piechaudii* (15).

Our newly designed *A. xylosoxidans*-specific probe Ach-221 showed excellent sensitivity and specificity for the identification of *A. xylosoxidans*. Due to the close relationship, weak cross binding was seen with *A. ruhlandii*, but the probe did not cross bind to the pathogenic species *A. piechaudii*. The cross-reaction to *A. ruhlandii* is clinically negligible since this species has not yet been found in human specimens. The observed false-positive result of probe Ach-221 with a single isolate of a *Chryseobacterium* sp. is probably caused by unspecific binding of the probe to an unknown target since we did not find homologous sequences in the probe and the 16S rRNA gene of this *Chryseobacterium* strain. The *Alcaligenes faecalis*-specific probe also showed excellent sensitivity and specificity and is, therefore, a valuable adjunct to the *A. xylosoxidans*-specific probe. Nevertheless, due to the small number of *Alcaligenes* isolates included in this study and the continued emergence of new bacterial species, especially gram-negative rods, in CF patients, further evaluation and critical assessment of the probes are necessary.

In conclusion, the FISH probes described here are excellent

tools for the rapid and specific identification of *A. xylosoxidans* and *Alcaligenes faecalis* isolates from the respiratory secretions of CF patients. The FISH assay can be performed within 2.5 h and, thus, allows the correct identification of these species on the same day that positive cultures from a clinical specimen are obtained. Due to its low cost and simplicity, the FISH assay is also very useful for the large screening studies that are necessary for final evaluation of the clinical significance of *A. xylosoxidans* in CF patients.

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