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Shared Genotypes of *Achromobacter xylosoxidans* Strains Isolated from Patients at a Cystic Fibrosis Rehabilitation Center

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During a study examining transmission of *Pseudomonas aeruginosa* among 76 cystic fibrosis patients in a rehabilitation center, where patients stay in close contact during prolonged periods, several clusters of patients carrying genotypically identical *P. aeruginosa*, as well as two clusters of 4 and 10 patients, respectively, colonized with genotypically identical *Achromobacter xylosoxidans* strains, were discovered.

Clonal spread of *Pseudomonas aeruginosa* strains has recently been reported in United Kingdom and Australian cystic fibrosis (CF) centers (1, 5, 11, 16). This seems to be an emerging infection control problem in CF centers, necessitating the segregation of *P. aeruginosa* colonized and noncolonized patients. A large longitudinal study in British Columbia, Canada, however, did not identify *P. aeruginosa* patient-to-patient transmission in that CF population (21).

Therefore we started a study to determine the prevalence and risk of transmission of *P. aeruginosa* among cystic fibrosis patients in a Belgian rehabilitation center (24). The *P. aeruginosa*-colonized patients lived there together as in a boarding school, with shared dining and living facilities but with separate bedrooms.

During this study we also identified other nonfermenting gram-negative bacilli present together with *P. aeruginosa*. Predominant among these were *Achromobacter xylosoxidans* isolates. Moreover, using randomly amplified polymorphic DNA analysis and amplified fragment length polymorphism (AFLP) typing, we found that several patients were carrying a common genotype of *A. xylosoxidans*.

The taxonomic position of *A. xylosoxidans* has been uncertain during the last decades, leading to name changes from *Achromobacter* to *Alcaligenes* and back to *Achromobacter*. The species was described as the type species of the genus *Achromobacter* (27). Later on, Kersters and De Ley (13) proposed to transfer the type species of the genus *Achromobacter* to the genus *Alcaligenes*. However, more recently, the results of phylogenetic analyses of 16S rRNA nucleotide sequences and a difference of more than 10% in GC content of DNA demonstrated that *Achromobacter xylosoxidans* and *Alcaligenes faecalis*, the type species of the genus *Alcaligenes*, belong to two distinct genera, respectively, *Achromobacter* and *Alcaligenes* (26).

Isolation and identification. Lactose-negative colonies on McConkey agar were isolated on Mueller-Hinton agar containing 5% sheep blood and subsequently tested for oxidase activity. Oxidase-positive isolates were further identified using tDNA-PCR in combination with fluorescent capillary electrophoresis (2). This approach enables us to distinguish between gram-negative nonfermenters such as *P. aeruginosa*, *Burkholderia* species, and *Achromobacter* species (unpublished data) by comparing the tDNA-PCR fingerprints of unknowns with those of reference strains in a library (available at http://allserv.ugent.be/~mvaneech/LBR.html). Identification of isolates as *Achromobacter xylosoxidans* was confirmed by using API20 NE (bioMérieux, Marcy l'Etoile, France). Some of the isolates, identified genotypically as *A. xylosoxidans*, were initially considered atypical *P. aeruginosa* in our routine laboratory. Due to the diversity of colonial morphologies and biochemical reactivity encountered, misdiagnosis of gram-negative nonfermenters cultured from CF sputum may occur. In one study, misidentification of 11% of *A. xylosoxidans* strains was reported (20).

Genotyping. For each patient, the *A. xylosoxidans* isolates were first genotyped by means of arbitrarily primed PCR, using alkaline cell lysis for DNA extraction and randomly amplified polymorphic DNA analysis with Ready-to-Go beads (Amersham Biosciences AB, Uppsala, Sweden) with primer ERIC2 (AAGTAAGTGACTGGGGTGAGCG) at an annealing temperature of 35°C, as described previously (9). For the purpose of selective restriction fragment amplification (AFLP), total bacterial DNA was isolated from fresh cultures on tryptic soy agar by using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). AFLP with one fluorescent primer (fAFLP) and with fragment length analysis by means of ABI310 (Applied Biosystems, Foster City, Calif.)-based capillary electrophoresis was carried out basically as described previously (22). Briefly, a combined restriction-ligation procedure was used in which 10 ng of total genomic DNA was incubated with 2 pmol of EcoRI adapter, 20 pmol of MseI adapter, 1 U of EcoRI (Amersham Biosciences), 1 U of MseI (New England Biolabs, Beverly, Mass.), 50 mM NaCl, 50 ng of bovine serum albumin per μl (Roche, Basel, Switzerland), and 4 U of T4 DNA ligase (Amersham Biosciences) in a total volume of 10 μl of 1× reaction buffer for 3 h at 37°C, after which the mixture was diluted 20 times with Tris buffer (Tris [10 mM]–EDTA [0.1 mM], pH 8.0).

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TABLE 1. Adapter and primer sequences used for fAFLP-based genotyping

Adapter or primer	Sequence
EcoI adapter 1	5′ CTCGTAGACTGCGTACC
EcoI adapter 2	5′ AATTGGTACGCAGTCTAC
MseI adapter 1	5′ GACGATGAGTCCTGAG
MseI adapter 2	5′ TACTCAGGACTCATC
EcoRI + 0 primer	5′ (tet)GACTGCGTACCAATTC
MseI + C primer	5′ GATGAGTCCTGAGTAAC

For the selective amplification of the restriction fragments, five microliters of the diluted restriction-ligation mixture was used for amplification in a volume of 10 μl under the following conditions: 0.4 μM TET-labeled EcoR + 0 primer, 1.2 μM *Mse* + C primer (Eurogentec, Seraing, Belgium) (E1), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1× reaction buffer, and 1 U of GoldStar DNA polymerase (Eurogentec). After 2 min of incubation at 72°C and at 94°C the cycling conditions were 36 cycles of 30 s at 94°C, 30 s at 65 to 56°C, and 60 s at 72°C. During the first 13 cycles, the annealing temperature was lowered by 0.7°C per cycle. After an additional 10 min of incubation at 72°C, the samples were cooled. An overview of PCR primers and adapter sequences is shown in Table 1. To one μl of PCR product were added 12 μl of deionized formamide and 0.3 μl of GS-400 High Density size standard and 0.2 μl of GS-500 size standard, which both contain ROX-labeled fragments in the range of 50 to 500 bp, and this mixture was electrophoresed on an ABI PRISM 310 system (Applied Biosystems, Foster City, Calif.).

A total of 102 *A. xylosoxidans* isolates were cultured from the sputa of a total of 13 patients out of a population of 76 patients studied (Table 2). The sputum cultures of the remaining 63 patients were negative for *A. xylosoxidans*. Only four genotypes were established on the basis of fAFLP genotyping. Two genotypes, designated S and V, were found in 4 and 10 patients, respectively, with 1 patient carrying both genotypes. Therefore, we designated these patients S1 to S3 (patients carrying only genotype S), B (the patient carrying both genotypes), and V1 to V9 (patients carrying only genotype V). Two patients, S3 and B, each had a separate *A. xylosoxidans* genotype in addition to their cluster strains. These genotypes were designated "S3 other" and "B other." Patients V6 and V7 were siblings, carrying not only the same *A. xylosoxidans* genotype V but also identical *P. aeruginosa* genotypes (J). For both siblings, the *P. aeruginosa* genotype (J) was already present at arrival, but the shared *A. xylosoxidans* was acquired after an interval of 6 months (Table 3 and Table 4). Another two patients (V4 and V5), with a common *A. xylosoxidans* genotype (V), also had a *P. aeruginosa* genotype (Y) in common. Only one of the two patients carried the Y genotype at arrival.

Patients B and V2 also shared the same *P. aeruginosa* genotype (U). Patient V2 already carried this genotype at arrival; B acquired it during the fourth month of their overlapping stay. In a previous report (24) we showed that the majority of shared

TABLE 3. Times during study period 1 in which patients stayed at the rehabilitation center

Patient	Patient stay occurred during[a]:							
	January		February		March		April	
	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4
V1								
V2	VU	VU	VU	VU	VU	VU	—	VU
V3								
V4								
V5	—	—	—	—	—	—	Y	
V6	J	J	J	J	J	J	J	JV
V7	J	J	J	J	J	J	J	J
V8								
V9							—	
B	—	—	—	—	—	—	—	U
S1								
S2						S	S	
S3	S	—	S	S	S	—	—	S

[a] Underlining represents the time periods during which patients stayed at the rehabilitation center. Capital letters represent shared genotypes of *A. xylosoxidans* (S, V) and *P. aeruginosa* (J, U, Y).

TABLE 2. Patients and the different genotypes of *P. aeruginosa* and *A. xylosoxidans* isolates

Patient	Total no. of isolates	Total no. of genotypes[a]	No. of isolates of indicated genotype											
			A. xylosoxidans			*P. aeruginosa*								
			S	V	Other	J	I	O	Q	R	T	U	Y	Other
S1	5	1/0	5											
S2	2	1/0	2											
S3	36	2/1	25		2[b]			9						
B	32	3/3	1	1	14[c]							2		1, 13
V1	30	1/1		4										26
V2	63	1/1		24								39		
V3	11	1/1		3							8			
V4	13	1/1		4									9	
V5	37	1/3		7						1			5	24
V6	61	1/1		2		59								
V7	28	1/1		1		27								
V8	16	1/1		6			10							
V9	15	1/3		1					8					2, 4

[a] Number of different *A. xylosoxidans*/*P. aeruginosa* genotypes for this patient.
[b] Designation of the additional *A. xylosoxidans* genotype of patient S3 is "S3 other."
[c] Designation of the additional *A. xylosoxidans* genotype of patient B is "B other."

TABLE 4. Times during study period 2 in which patients stayed at the rehabilitation center[a]

Patient	Patient stay occurred during[a]:																							
	2001								2002															
	September		October		November		December		January		February		March		April		May		June		July		August		October
	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4
V1									V	V														
V2	VU	U	U	U	U	U	U	U	U	U	U		VU					VU						—
V3		—									V						V							
V4						VY	VY	VY	Y	Y	Y	Y		—	—		—			—	—		V	
V5	Y	Y	V	—						V	V	—	—	—	—		—		—	—	—			VY
V6																								V
V7		J	J	JV	J	J	J	—											—	J	—	—	—	—
V8			—	V	V	V						—	V				—							—
V9			—	—											V					V	—			
B				—	—	—	—				SV	—	—	—	—					U	—			
S1					S	S																		
S2																								
S3	—	—	S	S	S	S	S	S	S	—	—	—	—	—	—	—	—	—	—	—				

[a] Underlining represents the time periods during which patients stayed at the rehabilitation center. Capital letters represent shared genotypes of *A. xylosoxidans* (S, V) and *P. aeruginosa* (J, U, Y). No new isolations were made during September and the first half of October 2002.

P. aeruginosa genotypes were already cultured at arrival, so patient-to-patient transmission seemed to have happened mostly in the past, before segregation was introduced (since 1992) and before infection control practices such as daily decontamination of the sinks and water closets by alternatively rinsing with vinegar and liquid bleach (since 1995).

The 10 V-cluster patients came from seven different CF centers. During the first study period (from 8 January 2001 until 30 April 2001) (Table 3), 7 of these patients had an overlapping stay: in only one patient (V2) the V genotype was already cultured at arrival and in one patient (V6) a "new infection" could be suspected, and the five other patients remained free of the V genotype during this period. In the second study period (from 1 September 2001 until 23 October 2002) (Table 4), three patients (V2, V4, and V8) already carried the V genotype at (re)admission. However, possibly new infections with the V genotype could be suspected for six patients (V1, V3, V5, V7, V9, and B), all occurring within a period of 6 months (between 1 October 2001 and 1 April 2002).

Three of the four S-genotype patients were followed at the same CF center (B, S1, and S2). The S genotype was already cultured at arrival in three patients (S1, S2, and S3). The fourth patient (B) only showed his S genotype at readmission, after an absence of 3 months. During the previous stays of this patient—lasting 4 and 2 months, respectively—cultures were always negative for the S genotype. It is therefore possible that patient-to-patient transmission took place at the CF center, where the patient was followed with two other S-cluster patients.

All patients colonized with *A. xylosoxidans*, except patients S1 and S2, also carried *P. aeruginosa* genotypes, with patient B having up to three different *P. aeruginosa* genotypes in addition to his/her three *A. xylosoxidans* genotypes. These data at first glance appear to point to a strong tendency of cocolonization with *P. aeruginosa* and *A. xylosoxidans* but may be biased, since the study included only patients assumed to be colonized with *P. aeruginosa*. No study was undertaken to establish in how many cases patients were carrying *A. xylosoxidans* without *P. aeruginosa*. Also, Tan et al. (23) reported that most patients carrying *A. xylosoxidans* were colonized by *P. aeruginosa*, and in another study, six of the eight patients with *A. xylosoxidans* were also colonized with *P. aeruginosa* (17).

We found, for the same population, 14 clusters of *P. aeruginosa*, comprising 2 to 10 patients per cluster (24). Thus, the largest cluster of *A. xylosoxidans* colonization (10 patients) was the same size as the largest *P. aeruginosa* cluster. Furthermore, when a patient was colonized by *A. xylosoxidans*, its isolates always belonged to one of two shared genotypes, with patient B even colonized by isolates of both genotypes, whereas in the same population 45/76 patients (59%) were found carrying a *P. aeruginosa* strain not related to any other strain and thus with a separate genotype. So it can be stated that there was much less genotypic diversity among the *A. xylosoxidans* strains observed compared with the *P. aeruginosa* strains for the same patient population.

A. xylosoxidans has been recognized as an emerging CF pathogen since one study published in 1985 (14) and later on in several others (3, 4, 6, 7, 8). Fabbri et al. (7) identified 12 of the 71 isolates (16.9%) from 24 patients as *A. xylosoxidans*. Ferroni

et al. (8) reported *A. xylosoxidans* to be the second most frequent gram-negative nonfermenter, after *P. aeruginosa*, among 1,093 isolates from 148 patients (10 isolates from 8 patients). Moissenet et al. (17) reported colonization with *A. xylosoxidans* in 6% of 120 CF children, with a mean age of 14.2 years for colonized children. Still, the prevalence may be higher, since in one study *A. xylosoxidans* was detected in CF patients only after usage of a selective medium (18). A phase III study of aerosolized tobramycin showed that a much higher number of the 595 patients were colonized with *Stenotrophomonas maltophilia*, *A. xylosoxidans*, *Aspergillus* species, and oxacillin-resistant *Staphylococcus aureus* than had been established by the CF foundation patient registry efforts (4). In this study, *A. xylosoxidans* (in 8.7% of the patients) was almost as frequently isolated as *S. maltophilia* (10.3%).

Although colonization of CF patients with *A. xylosoxidans* is well established, epidemiological studies thus far have been unable to establish evidence of much transmission of strains of this species. Dunne and Maisch (6) in 1995 had already reported persistent colonization of outpatients with *A. xylosoxidans* but did rule out patient-to-patient transmission by means of PCR-based genotyping. Vu-Thien et al. (25) found persistent colonization of patients with *Burkholderia cepacia*, *S. maltophilia*, and *A. xylosoxidans* but could show the presence of identical isolates in different patients only for *B. cepacia*. In a large study including 92 *A. xylosoxidans*-positive patients from 46 centers, Krzewinski et al. (15) found five pairs of patients, which included two pairs of siblings and one pair of friends, with genotypically identical *A. xylosoxidans* isolates. Furthermore, one additional instance of cross-colonization with *A. xylosoxidans* in two siblings (19) and the presence of the same strain in two of eight colonized children (17) were reported. Our study revealed two large clusters of patients colonized by the same *A. xylosoxidans* strains. These findings are supported by a recent publication of Kanellopoulou et al. (12) that showed that *A. xylosoxidans* isolates of five colonized CF patients were genetically related, suggesting a common-source outbreak.

Although for most of the nonfermenting gram-negative rods the disk diffusion antibiogram is not validated by the CLSI (formerly NCCLS), we carried out an antibiogram to evaluate its possible usefulness as a preliminary typing technique. Several interpretation problems were apparent, such as heterogeneous growth in the inhibition zone and unclear inhibition zone borders. In our hands this approach indicated that all isolates except one showed large inhibition zones for piperacillin (30 to 45 mm) and showed no inhibition zone for amikacin, gentamicin, ofloxacin, ampicillin, aztreonam, temocillin, cefuroxime, and cefotaxime. The activity of ceftazidime and meropenem was very variable, while that of cotrimoxazole and colimycin was difficult to interpret. Fabbri et al. (7) found this organism to be the least susceptible for antibiotics among gram-negative nonfermenting rods and concluded that ceftazidime was most active. We found very variable results for ceftazidime, while for only one strain (B other) no large inhibition zone for piperacillin was observed. Also, Saiman et al. (20) found piperacillin among the most active antibiotics in vitro. There were no consistent susceptibility characteristics which enabled one to differentiate between the four genotypes, but the susceptibility pattern, i.e., colimycin resistance and combined resistance to aminoglycosides and quinolones, was considered to be helpful in distinguishing this species from *P. aeruginosa*.

The pathogenic potential of these newly emerging CF "pathogens" has been ill studied. One study (10) addressed the endotoxic potential of eight species of gram-negative organisms, including *A. xylosoxidans*, and found that, with the exception of *S. maltophilia*, lipopolysaccharide extracted from all of the bacteria upregulated, by various degrees, expression of each of the proinflammatory cytokines assayed. Given the high antibiotic resistance observed in this and previous studies and taking into account that some strains may be transmissible, it may be advisable to pay attention to the presence of *A. xylosoxidans* in the lungs of cystic fibrosis patients. Tan et al. (23) showed in their retrospective case-controlled study of 557 CF patients that the 13 patients that were chronically infected with *A. xylosoxidans* did not deteriorate more in clinical or pulmonary function than patients colonized with *P. aeruginosa* only. More clinical data will be necessary in the future to resolve the issue regarding the pathogenicity of *A. xylosoxidans* in CF patients.

In summary, although several authors have indicated that transmissibility of *A. xylosoxidans* is low, we report the occurrence of genotypically identical strains of this species among two clusters of CF patients attending the same rehabilitation center.

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