Distribution of innate efflux-mediated aminoglycoside resistance among different *Achromobacter* species

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Abstract

Achromobacter spp. are emerging respiratory pathogens in cystic fibrosis patients. Since 2013 the genus *Achromobacter* includes 15 species for which innate antibiotic resistance is unknown. Previously the AxyXY-OprZ efflux system has been described to confer aminoglycoside (AG) resistance in *A. xylosoxidans*. Nevertheless, some *Achromobacter* spp. strains are susceptible to AG. This study including 49 *Achromobacter* isolates reveals that AG resistance is correlated with different *Achromobacter* spp. It is noteworthy that the *axyXY-oprZ* operon is detected only in AG-resistant species, including the most frequently encountered in cystic fibrosis patients: *A. xylosoxidans, A. ruhlandii, A. dolens* and *A. insuavis*.

Keywords: *Achromobacter*, aminoglycoside resistance, AxyXY-OprZ, cystic fibrosis, *nrdA*, selection pressure
Original Submission: 12 October 2015; **Revised Submission:** 27 November 2015; **Accepted:** 27 November 2015
Article published online: 12 December 2015

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Achromobacter spp. are nonfermenting Gram-negative bacilli considered as emerging pathogens in cystic fibrosis (CF) patients [1,2]. Since the description of the type species, *A. xylosoxidans* [3], 14 other species have been ranked into the genus *Achromobacter: A. piechaudii* and *A. ruhlandii* [4], *A. denitrificans* [5], *A. spanius* and *A. insolitus* [6], *A. marplatensis* [7], *A. animicus, A. mucicolens, A. pulmonis* and *A. spiritinus* [8], *A. insuavis, A. aegrifaciens, A. anxifer* and *A. dolens* [9], and 6 other genogroups. These 21 species and genogroups can be distinguished by the multilocus sequence typing (MLST) scheme proposed by Spilker *et al.* [10]. The study demonstrated that sequencing a 765 bp internal fragment of the only *nrdA* gene is sufficient for correct identification [11]. Because of the actual difficulty in performing accurate species identification, most isolates are still referred by default as *A. xylosoxidans,* preventing the evaluation of the real epidemiology and clinical impact of each species. Moreover, the data about the mechanisms of innate antibiotic resistance are scarce [12,13]. The AxyXY-OprZ RND efflux system confers resistance to aminoglycosides (AG) in *A. xylosoxidans* AXX-A since reclassified as *A. insuavis* (accession number AFRQ01000000). Nevertheless, AG, which take an important part in CF antimicrobial therapy, remain active against some isolates of *Achromobacter* spp. [14,15].

We sought to describe the distribution of AG-resistant isolates among the different species of the genus *Achromobacter* and to search for the *axyXY-oprZ* efflux operon in AG-resistant and -susceptible isolates to assess if AG resistance is correlated with the presence of the operon.

Forty-nine *Achromobacter* isolates harbouring various AG resistance patterns were included in this study: 21 from CF patients' sputum, 20 from non-CF clinical samples and eight from environmental samples (Table 1). Most of them (n = 35) were collected in our laboratory; nine collection strains were purchased from the Institut Pasteur, France, including six type strains, and five were kindly provided by J. J. LiPuma (Department of Pediatrics and Communicable Diseases, University of Michigan Medical School). Isolates were identified at the genus level either by using the conventional biochemical method API 20NE (bioMérieux) or by sequencing the 16S rRNA gene. The identification to the species level was performed by sequencing the 765 bp internal *nrdA* fragment followed by *Achromobacter* PubMLST database query (http://pubmlst.org/achromobacter/). Minimal inhibitory concentrations (MICs) of tobramycin, amikacin, gentamicin and netilmicin were measured by the Etest method (bioMérieux). Mueller-Hinton agar plates were inoculated by swabbing from a 0.5 McFarland turbidity bacterial suspension, and MICs were recorded after overnight incubation at 37°C by two persons independently. The phenotype "AG-susceptible" (AG-S) was attributed to isolates susceptible to all

http://dx.doi.org/10.1016/j.nmni.2015.11.013

TABLE 1. *Achromobacter* isolates and main results

nrdA identification	Isolate	Origin	MIC (mg/L)				AG S/R	PCR *axyY*	PCR *oprZ*
			TOB	AMK	GEN	NET			
A. aegrifaciens	ACH-CF-D59	CF sputum[a]	>256	>256	>256	>256	R	+	+
A. aegrifaciens	ACH-CF-802	CF sputum[a]	64	48	16	32	R	+	+
A. aegrifaciens	ACH-ENV-2	Hospital hand-washing sink[a]	12	8	3	8	R	+	+
A. aegrifaciens	ACH-CF-766	CF sputum[a]	192	48	12	64	R	+	+
A. animicus	ACH-CF-864	CF sputum[a]	1.5	4	1	1	S	−	−
A. animicus	ACH-NCF-33	Catheter[a]	1.5	6	2	2	S	−	−
A. animicus	ACH-CF-D63	CF sputum[a]	1.5	4	1.5	1	S	−	−
A. animicus	ACH-CF-D64	CF sputum[a]	2	8	2	1.5	S	−	−
A. animicus	ACH-CF-D65	CF sputum[a]	1	3	1.5	1.5	S	−	−
A. animicus	ACH-CF-711	CF sputum[a]	2	4	1.5	1.5	S	−	−
A. denitrificans	CIP-77.15T	Soil	32	256	64	64	R	+	+
A. dolens	AU18822	CF sputum	>256	64	>256	12	R	+	+
A. dolens	AU20310	CF sputum	>256	>256	>256	128	R	+	+
A. genogroup 12	ACH-ENV-3	Dialysis water[a]	3	32	8	8	R	+	+
Novel species	ACH-CF-583	CF sputum[a]	24	>256	96	>256	R	+	+
A. insolitus	CIP-108202T	Leg wound	256	32	48	>256	R	+	+
A. insuavis	ACH-CF-476	CF sputum[a]	>256	>256	>256	>256	R	+	+
A. insuavis	ACH-CF-777	CF sputum[a]	96	>256	>256	>256	R	+	+
A. insuavis	AXX-A	Ear swab[a]	16	256	24	64	R	+	+
A. insuavis	CIP-102062	Blood	12	256	16	24	R	+	+
A. marplatensis	ACH-ENV-4	Lake[a]	12	256	24	32	R	+	+
A. mucicolens	ACH-NCF-34	Tracheal aspirate[a]	1.5	6	1.5	1.5	S	−	−
A. mucicolens	ACH-NCF-35	Tracheal aspirate[a]	2	8	2	1.5	S	−	−
A. mucicolens	ACH-NCF-36	Blood[a]	2	6	2	2	S	−	−
A. mucicolens	ACH-CF-510	CF sputum[a]	2	8	2	2	S	−	−
A. piechaudii	CIP-60.75T	Pharynx	1.5	6	3	3	S	−	−
A. ruhlandii	CIP-77.26T	Soil	8	24	12	16	R	+	+
A. ruhlandii	AU19877	CF sputum	16	>256	48	64	R	+	+
A. ruhlandii	AU19911	CF sputum	3	48	6	12	R	+	+
A. ruhlandii	AU19929	CF sputum	>256	>256	>256	>256	R	+	+
A. spanius	CIP-108199T	Blood	1.5	6	4	4	S	−	−
A. spanius	ACH-NCF-37	Foot wound[a]	1	4	1	1.5	S	−	−
A. spanius	ACH-CF-746	CF sputum[a]	2	6	2	2	S	−	−
A. xylosoxidans	ACH-CF-809	CF sputum[a]	128	>256	256	>256	R	+	+
A. xylosoxidans	ACH-NCF-39	Insertion-site skin swab[a]	24	>256	64	128	R	+	+
A. xylosoxidans	ACH-NCF-18	Tracheal aspirate[a]	64	>256	128	256	R	+	+
A. xylosoxidans	CIP-71.32T	Ear discharge	192	>256	>256	>256	R	+	+
A. xylosoxidans	CIP-101902	Pleural fluid	>256	>256	>256	>256	R	+	+
A. xylosoxidans	CIP-102236	Sputum	48	>256	256	>256	R	+	+
A. xylosoxidans	ACH-NCF-41	Sputum[a]	32	>256	64	128	R	+	+
A. xylosoxidans	ACH-NCF-42	Tracheal aspirate[a]	8	256	24	64	R	+	+
A. xylosoxidans	ACH-ENV-1	Dental instrument[a]	192	>256	>256	>256	R	+	+
A. xylosoxidans	ACH-NCF-13	Bronchial aspirate[a]	64	>256	128	>256	R	+	+
A. xylosoxidans	ACH-CF-805	CF sputum[a]	24	>256	48	192	R	+	+
A. xylosoxidans	ACH-NCF-11	Sputum[a]	16	256	32	48	R	+	+
A. xylosoxidans	ACH-CF-842	CF sputum[a]	8	192	24	32	R	+	+
A. xylosoxidans	ACH-NCF-40	Blood[a]	32	>256	96	192	R	+	+
A. xylosoxidans	ACH-ENV-5	River[a]	16	>256	24	64	R	+	+
A. xylosoxidans	ACH-ENV-6	Domestic hand-washing sink[a]	32	>256	48	128	R	+	+

AG, aminoglycoside; AMK, amikacin; CF, cystic fibrosis; GEN, gentamicin; MIC, minimum inhibitory concentration; NET, netilmicin; R, resistant.; S, susceptible; TOB, tobramycin.
[a]Isolates collected in our laboratory.

AG and the phenotype "AG-resistant" (AG-R) to the other by using the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints for *Pseudomonas* spp. (http://www.eucast.org/clinical_breakpoints/; version 5.0). Detection of the *axyXY-oprZ* operon was performed by 2 PCRs targeting the genes (a) *axyY*, encoding the RND transporter, and (b) *oprZ*, encoding the outer membrane factor. PCRs were carried out in reaction mixtures containing dNTP (0.2 mM), forward and reverse primers (0.25 μM each), Taq polymerase (Fermentas) (2.5 U) with the supplied buffer, $MgCl_2$ (1.5 mM), dimethyl sulfoxide (5% volume) and template DNA (1 μL), adjusted with water to a final volume of 50 μL. The cycling parameters were 94°C for 10 minutes, 30 cycles of 94°C for 90 seconds, annealing primers temperature for 90 sections, 72°C for 60 seconds, and 72°C for 10 minutes. The results are summarized in Table 1.

The *nrdA* sequences analysis allowed identification of 48 of the 49 isolates. *nrdA* sequence of ACH-CF-583 harboured 39 nucleotide differences, with its closest match in database (genogroup 19) (Fig. 1) indicating that this isolate belonged to a novel genogroup or a novel species. Fourteen of the 49 studied isolates were categorized as AG-S. Interestingly, all isolates belonging to a same species harboured the same AG resistance pattern. In the resistant species, the level of resistance was sometimes variable among the isolates. Nevertheless, none of these isolates had been categorized as susceptible for all four AG molecules. A variable expression level of the efflux operon might account for these differences as already observed for

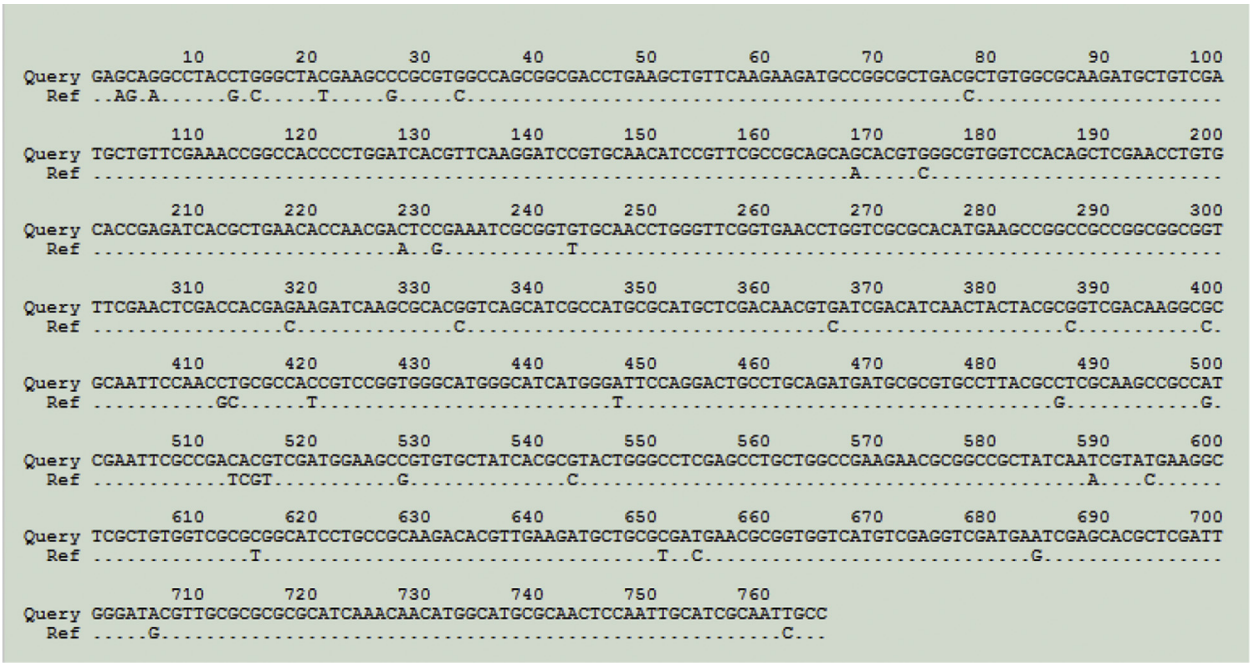

FIG. 1. ACH-CF-583 *nrdA* sequence alignment with its closest match (genogroup 19) in *Achromobacter* PubMLST database. Query: ACH-CF-583; Ref: closest match in database.

Pseudomonas aeruginosa [16]. Distribution of the AG-S and AG-R isolates according to species identification is represented in a dendrogram (Fig. 2) generated from the *nrdA* sequences using the neighbour-joining method with 1000 bootstrap replications (MEGA6). We also included in the dendrogram the *nrdA* sequences of the type strains (LMG) of the recently described novel species. AG-S isolates were divided up into two clusters of species including *A. animicus* and *A. mucicolens* (cluster 1), and *A. spanius* and *A. piechaudii* (cluster 2). These two clusters were supported by high bootstrap values. All other isolates were AG-R and did not belong to cluster 1 or 2. They included the 16 *A. xylosoxidans* isolates and all isolates from the species *A. aegrifaciens, A. denitrificans, A. dolens, A.* genogroup 12, *A. insolitus, A. insuavis, A. marplatensis* and *A. ruhlandii.* A similar tree topology was obtained by using the maximum likelihood and the maximum parsimony methods (data not shown). There was a perfect correlation between the AG resistance profile and the presence of the AxyXY-OprZ efflux system. Indeed, the operon was detected in all AG-R isolates and not in the AG-S ones. An additional PCR was performed in all AG-S isolates with primers designed in flanking sequences of the *axyXY-oprZ* operon and confirmed the absence of the whole operon. Because the GC content of the efflux operon is similar to that of whole genome, we hypothesized that a deletion occurred in the course of evolution. These findings indicate that susceptibility or resistance to AG might be a phenotypic trait correlated with *Achromobacter* species evolution.

To date, and to our knowledge, only four studies including an appropriate *Achromobacter* identification method report distribution of the different *Achromobacter* species in clinical samples. They indicate that *A. xylosoxidans* is the species the most frequently recovered from CF patients [11,17–19]. Other species are also widely prevalent: *A. ruhlandii, A. dolens* and *A. insuavis.* It is noteworthy that all isolates belonging to these four species have been categorized as AG-R in the present work. One can therefore wonder whether these AG-R species are more pathogenic than the AG-S ones, which might explain their high prevalence. One might also hypothesize that these species have emerged under the selection pressure of AG treatment frequently prescribed to those in the CF population.

In conclusion, AG resistance is the first phenotypic characteristic correlated with the different species of the genus *Achromobacter.* More studies including accurate species level identification are required in order to improve knowledge about the epidemiology and virulence of these pathogens. They might also help to elucidate whether the use of inhaled AG promotes selection of *Achromobacter* that belong to the species harbouring the AxyXY-OprZ efflux system.

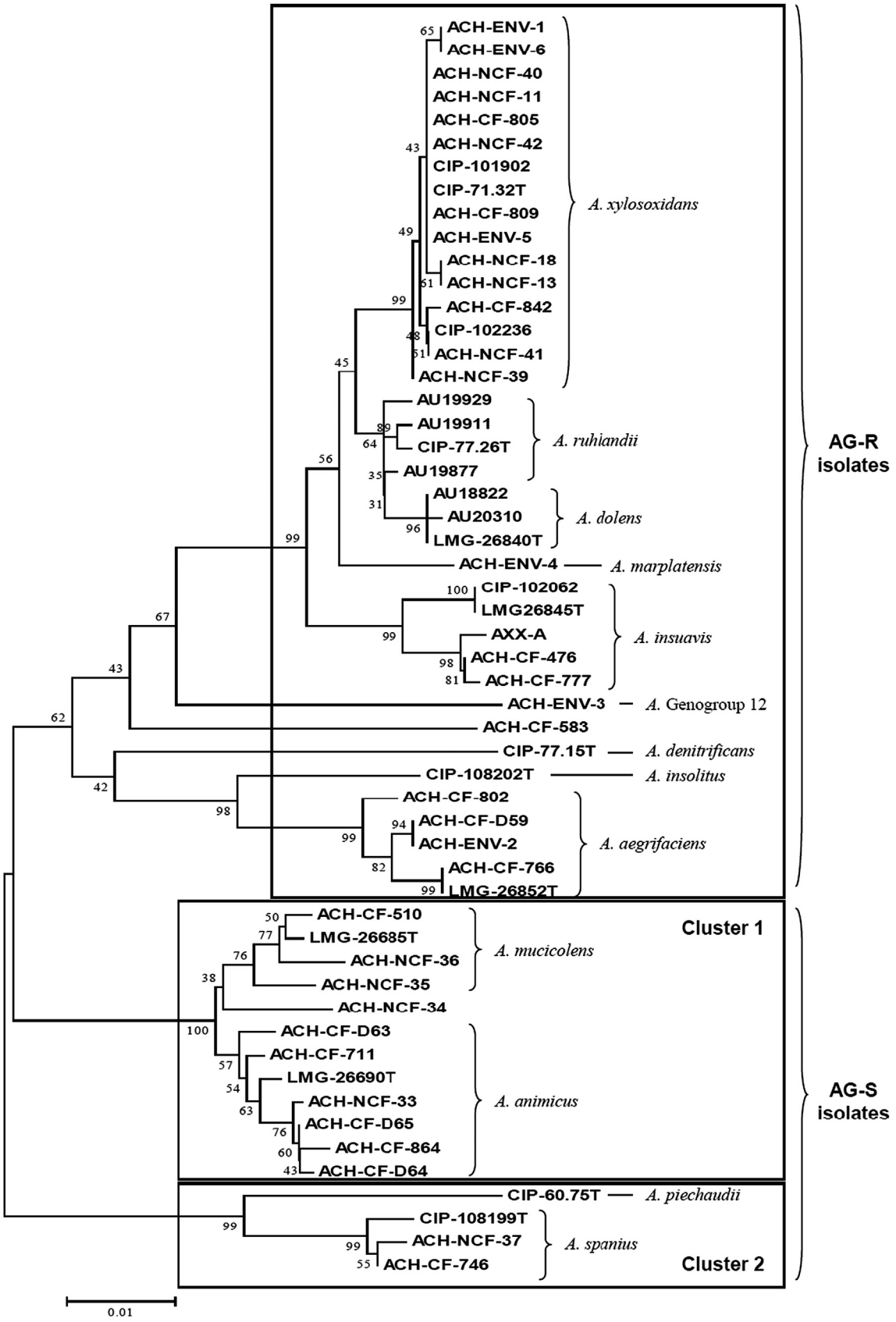

FIG. 2. Neighbour-joining dendrogram based on *nrdA* sequence. Numbers at nodes indicate bootstrap values. Scale bar indicates number of substitutions per site. AG-resistant (AG-R) isolates are in upper box; AG-susceptible (AG-S) isolates are in boxes 'cluster 1' and 'cluster 2.' AG resistance was not determined for the LMG strains.

Acknowledgements

We thank J. J. LiPuma for providing isolates and T. Spilker for his help in analysing *nrdA* sequences.

Conflict of interest

None declared.

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