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Role of Efflux in Antibiotic Resistance of *Achromobacter xylosoxidans* and *Achromobacter insuavis* Isolates From Patients With Cystic Fibrosis

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Achromobacter genus (including *Achromobacter xylosoxidans*, the most prevalent *Achromobacter* species in patients with cystic fibrosis) is poorly susceptible to most conventional antibiotics. Contribution of efflux by AxyABM, AxyXY-OprZ, and AxyEF-OprN and of target mutations were studied in clinical isolates of *A. xylosoxidans* and *Achromobacter insuavis.* Forty-one isolates longitudinally collected from 21 patients with CF were studied by whole-genome sequencing (WGS)-typing, determination of minimum inhibitory concentrations (MICs) of β-lactams, aminoglycosides, colistin, azithromycin, ciprofloxacin, chloramphenicol, and doxycycline, and expression (quantitative RT-PCR) and function (measure of the uptake of a fluorescent substrate) of efflux pumps. WGS-based typing resulted in 10 clusters comprising 2 or 3 isolates and 20 singletons. The efflux activity was high in strains with elevated MICs for amikacin or azithromycin. This work sheds a new light on the impact of efflux and target mutations in resistance of *Achromobacter* to several drugs.

Keywords: *Achromobacter*, efflux, target mutation, macrolide, fluoroquinolone, aminoglycoside

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INTRODUCTION

In adults with cystic fibrosis (CF), *Pseudomonas aeruginosa* is one of the main respiratory pathogens, but in recent years, other non-fermenting Gram-negative bacterial species, such as *Stenotrophomonas*, *Burkholderia*, or *Achromobacter* have been increasingly isolated (Cystic Fibrosis Foundation, 2020). This could be due, collectively, to a better eradication of *P. aeruginosa* by aggressive therapies, a lengthening of patients' life expectancy, and the development of new techniques for bacterial identification. Among these bacteria, *Achromobacter* spp. are ubiquitous environmental microorganisms, also part of the microbiota of the ear and the gastrointestinal tract (Steinberg and Del Rio, 2005). They may become opportunistic pathogens capable of causing a large variety of infections, including endophthalmitis, keratoconjunctivitis, catheter-associated bloodstream infection, endocarditis, pneumonia, meningitis, and peritonitis (Spilker et al., 2012). They are also isolated in patients with CF and cause serious respiratory tract infections (Swenson and Sadikot, 2015; Hoyle et al., 2018). *Achromobacter* can be found in up to 10% of the sputum samples collected from patients with CF, with *A. xylosoxidans* being the most prevalent

Achromobacter species, identified in 35–80% of the cases (Raidt et al., 2015; Amoureux et al., 2016; Gade et al., 2017; Isler et al., 2020). Its pathogenic role in CF remains unclear, but chronic colonization is associated with a decline in respiratory function (Edwards et al., 2017; Tetart et al., 2019; Marsac et al., 2021) and a higher risk of death or lung transplantation (Somayaji et al., 2017), suggesting the need for an active treatment. At this stage, however, there is no standard treatment protocols for *Achromobacter* infections in CF, and treatment options need to be selected on a case-by-case basis (Isler et al., 2020).

In a clinical perspective, ceftazidime, meropenem, ciprofloxacin, and colistin are representative of the classes for which EUCAST has published MIC distributions against *Achromobacter*.[1] Extended-spectrum β-lactams also often represent a first option for infections by *Achromobacter* in patients with CF (Swenson and Sadikot, 2015). Inhaled antibiotics (colistin, or tobramycin) proved useful complements to intravenous drugs (Wang et al., 2013). Tetracyclines and chloramphenicol are among the most active agents *in vitro* (Saiman et al., 2001). In addition, temocillin is indicated against *Burkholderia* (Zeiser et al., 2019), and azithromycin is widely used for its anti-virulence and immunomodulatory properties (Cramer et al., 2017), so that patients are also possibly exposed to these drugs even though they have no meaningful activity on *Achromobacter*.

Antibiotic selection remains a real challenge because *Achromobacter* displays not only innate but also frequent acquired multidrug resistance to a wide range of antibiotics commonly used for the management of infections by Gram-negative microorganisms (Traglia et al., 2012; Bador et al., 2013; Isler et al., 2020). Unfortunately, the knowledge of drug resistance mechanisms in this genus is limited. Genes located on mobile genetic elements, which encode β-lactamases or aminoglycoside-modifying enzymes or confer fluoroquinolone resistance, have been reported thus far (Hu et al., 2015; Isler et al., 2020) and contribute to acquired resistance. β-Lactamases can be highly diverse, including extended-spectrum (CTX-M, VEB-1) or AmpC-type (CMY-2, AmpC) β-lactamases hydrolyzing all beta-lactams except carbapenems, and plasmidic (IMP and VIM) carbapenemases (Isler et al., 2020). In addition, five predicted β-lactamase genes have been identified in the chromosome, encoding one class D ($bla_{OXA-114}$), one class C, two class B, and one class A enzymes (Doi et al., 2008).

Another potential resistance mechanism consists in active efflux through AxyABM, AxyXY-OprZ, or AxyEF-OprN pumps, which seem orthologs of MexAB-OprM, MexXY-OprA, and MexEF-OprN in *P. aeruginosa*, respectively (Bador et al., 2011, 2013; Nielsen et al., 2019; Magallon et al., 2021). The substrate specificity of these pumps and their impact on antibiotic activity is, however, different between these two species.

This study investigated resistance mechanisms in clinical isolates of *A. xylosoxidans* and the closely related species *Achromobacter insuavis*. To this effect, a collection of 41 isolates was assembled longitudinally from patients with CF, which allowed us to consider microevolution in specific genes.

[1]https://mic.eucast.org/

MATERIALS AND METHODS

Isolates, Identification, Whole-Genome Sequencing, and Relatedness

Forty-one successive isolates of *Achromobacter* spp. from sputum samples of 21 patients with CF and cultures remaining positive over prolonged time periods (0.3–11 years interval between the 2 successive samples; mean value, 4.4 years; **Table 1**) were collected at the CF centers of the University Hospital and Clemenshospital, Münster, Germany (2006–2017). *A. xylosoxidans* ATCC 27061 (Yabuuchi and Oyama, 1971) was used as a reference throughout this work, as being one of the few fully sequenced clinical isolates of *A. xylosoxidans* and for which the three efflux pumps of interest have been functionally characterized among the 6 identified (Hu et al., 2015). In addition, all sequences were compared to those of *A. insuavis* AXX-A, which shows a wild-type phenotype, in particular regarding its susceptibility to ciprofloxacin (Bador et al., 2011). *P. aeruginosa* ATCC 27853 (Fang et al., 2012) was also used as internal control for antimicrobial susceptibility testing. Two *A. insuavis* [Ai: parental clinical isolate, Ai ΔB/ΔY: Ai with deletions in *axyB* (Bador et al., 2011) or *axyY* (Bador et al., 2013)] were provided by Dr. Julien Bador, Department of Bacteriology, University Hospital of Dijon, Dijon, France. AX-08 and its deletion mutant in *axyE* were provided by Niels Norskov-Lauritsen, Aarhus University, Aahrus, Denmark (Nielsen et al., 2019).

Strain relatedness was analyzed and represented by a minimum spanning tree, after whole genome sequencing (WGS). Isolates were compared *via* WGS-based typing using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) (Dekker and Frank, 2016). After quality trimming, coding core genome regions were compared in a gene-by-gene approach (core genome multilocus sequence typing, cgMLST) using the SeqSphere + software version 6.0.2 (Ridom GmbH, Münster, Germany) and *A. xylosoxidans* ATCC 27061 within an *ad hoc* scheme as a reference sequence (GenBank accession number LN831029.1). SeqSphere + software was used to display the clonal relationship in a minimum-spanning tree. Threshold defining close genetic relation was set after plotting allelic changes over time of strains derived from each and the same patient. Species identification was performed using the *nrdA* gene data (Spilker et al., 2012). These were first extracted with the help of SeqSphere + from the WGS data *in silico* and thereafter subjected to *nrdA*_765 typing available by PubMLST. On the basis of all *nrdA* genes, a phylogenetic analysis was performed using software MEGA 11 (Tamura et al., 2021) in which the evolutionary history was inferred from a Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000) implying 1,000 bootstrap replications. The sequences of *rrl* encoding 23S rRNA, of *rpl4* and *rpl22* ribosomal genes and of *gyrA*, *gyrB*, *parE*, and *parC* were compared to the reference strains AXX-A and ATCC 27061 by pairwise sequence alignment.

Antimicrobial Susceptibility Testing

MICs were determined by broth microdilution according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2020)

TABLE 1 | Individual MICs for the reference strains and the 41 isolates.

Patient's number	Isolates[a]	Collection Date	Sampling interval (years)	MIC (mg/L)[b]																Gene expression level		
				CAZ	CAZ + AVI (32 mg/L)	MEM	PIP + TZB (4mg/L)	TIC	TIC + TZB (32 mg/L)	TIC + AVI (32 mg/L)	CST	AMK	AMK + BER (128 mg/L)	TOB	CIP	TMO	AZI	AZI + BER (128 mg/L)	CHL	DOX	*axyB*	*axyY*	*axyF*
–	ATCC 27061	2018		8	4	4	0.5	2	2	2	**4**	**1,024**	**64**	**256**	**4–8**	512	128	16	**64**	**16**	1	1	1
–	AXX-A			8	4	0.25	2	0.5	0.5	0.25	**2–4**	**128**	16	**128**	1–**2**	1,024–2,048	64	64	**16**	4	1.96	1.22	ND
–	AXX-A-Δ-*axyB*			4	2	0.125	16	512	0.25	0.125	**2–4**	**128**	16	**128**	1	256–512	64	64	4	2	3.08	1.80	ND
–	AXX-A-Δ-*axyY*			8	4	0.125	16	512	2	0.5	**2–4**	**32**	4	**32–64**	1	1,024–2,048	16	128	**16**	2	2.13	6.96	ND
–	AX08			2	2	0.25	1	2	2	2	1–2	**128**	16	**128**	2	256	32–64	8	**16**	**16**	4.57	3.96	3.40
–	AX08-Δ*axyE*			2	2	0.25	1	256	4	2	1–2	**128**	16	**128**	1	256	32–64	8	**16**	**8**	5.73	3.81	4.98
1	1.1	Dec 19, 2011	3.7	4	2	0.5	0.5	2	2	2	**8**	**512**	**64**	**64**	**8**	256	256	32	**32**	**16**	2.28	2.72	8.11
	1.7	Sep 2, 2015		**32**	**32**	**16**	**> 2,048**	**>2,048**	**> 2,048**	**>2,048**	**4**	**> 2,048**	**128**	**1,024**	**32**	> 2,048	256	16	**32**	**16**	3.16	4.15	0.57
2	2.1	Nov 30, 2016	0.8	4	nd	0.5	0.5	2	2	2	**8**	**512**	**32**	**128**	**4**	256	128	16	**32**	**16**	0.49	0.17	0.80
	2.3	Sep 20, 2017		4	nd	0.125	0.5	2	2	2	**16**	**512**	**32**	**128**	**4**	256	128	16	**16**	**8**	0.60	0.16	0.75
3	3.1[d]	Jan 5, 2012	–	4	nd	0.125	2	**128**	**128**	**128**	1	**128**	16	**128**	1	> 2,048	128	16	8	1	0.82	0.04	0.68
4	4.1	Feb 11, 2010	7.5	**64**	**16**	4	0.5	4	4	4	**1,024**	**> 2,048**	**256**	**512**	**4**	2,048	> 2,048	>2,048	8	**16**	2.43	1.72	2.23
	4.15	Aug 21, 2017		**64**	**32**	4	**32**	**32**	**32**	8	**> 2,048**	**>2,048**	**256**	**512**	**16**	2,048	> 2,048	>2,048	8	**16**	1.67	1.34	2.20
5	5.1	Aug 17, 2007	8.5	4	2	4	2	8	8	8	**16**	**2,048**	**32**	**256**	**16**	> 2,048	512	128	**32**	**8**	1.35	0.64	0.70
	5.12	Jan 12, 2016		**256**	**128**	**256**	**> 2,048**	**>2,048**	**> 2,048**	**>2,048**	**8**	**2,048**	**64**	**128**	**32**	> 2,048	>2,048	> 2,048	**32**	**16**	2.25	0.48	0.70
6	6.1[c]	Aug 17, 2006	11	**256**	**32**	**512**	**> 2,048**	**>2.048**	**> 2.048**	**64**	**1,024**	**> 2,048**	**64**	**256**	**16**	> 2,048	128	8	8	**16**	4.08	1.98	2.44
	6.14[c]	Sep 25, 2017		**256**	**32**	**256**	**> 2,048**	**>2,048**	**> 2,048**	**64**	1	16	16	4	**16**	> 2,048	8	8	**16**	4	4.19	0.11	1.73

(Continued)

TABLE 1 | (Continued)

Patient's number	Isolates[a]	Collection Date	Sampling interval (years)	MIC (mg/L)[b]																	Gene expression level		
				CAZ	CAZ + AVI (32 mg/L)	MEM	PIP + TZB (4mg/L)	TIC	TIC + TZB (32 mg/L)	TIC + AVI (32 mg/L)	CST	AMK	AMK + BER (128 mg/L)	TOB	CIP	TMO	AZI	AZI + BER (128 mg/L)	CHL	DOX	*axyB*	*axyY*	*axyF*
7	7.1	Aug 28, 2014	2.2	4	nd	0.25	1	2	2	2	**4**	**512**	**64**	**256**	**4**	512	128	16	**64**	**16**	0.96	0.47	1.47
	7.3	Nov 23, 2016		4	nd	4	0.25	2	2	2	**256**	**32**	8	**16**	**4**	256	128	16	**64**	**16**	1.65	0.65	2.07
8	8.1	Apr 21, 2010	4.7–5.5	**16**	8	2	8	4	4	4	**2,048**	**> 2,048**	**1,024**	**> 2,048**	**8**	>2,048	> 2,048	256	**16**	**8**	2.66	4.63	0.65
	8.6	Dec 3, 2014		**256**	**256**	**16**	**32**	**256**	**256**	**256**	**2,048**	**> 2,048**	**1,024**	**> 2,048**	**32**	>2,048	> 2,048	256	**16**	**8**	2.13	3.64	0.71
	8.7	Sep 3, 2015		**256**	**256**	**64**	**512**	**256**	**256**	**256**	**2,048**	**> 2,048**	**>2,048**	**> 2,048**	**32**	>2,048	> 2,048	256	**32**	4	2.03	3.53	0.71
9	9.1[c]	Jul 13, 2010	2–3.3	4	nd	1	1	4	4	4	**128**	**32**	8	**8**	**4**	512	64	8	**64**	**16**	1.03	0.26	6.53
	9.6[c]	Jun 19, 2012		4	nd	**64**	1	**1,024**	**1,024**	8	**256**	**128**	16	**16**	**8**	1,024	512	16	**512**	**32**	0.82	0.28	8.11
	9.8[c]	Nov 19, 2013		4	nd	4	0.5	2	2	1	**256**	**256**	**64**	**16**	**8**	256	> 2,048	32	**32**	**16**	5.17	4.87	5.82
10	10.1[c,d]	Feb 18, 2010	1.5	8	nd	**16**	**> 2,048**	**1,024**	**1,024**	**1,024**	**256**	16	8	4	**4**	> 2,048	32	32	**16**	**8**	1.31	0.08	0.73
	10.3[c,d]	Jul 26, 2011		8	nd	**128**	**> 2,048**	**1,024**	**1,024**	**128**	**4**	**64**	**32**	**32**	**128**	> 2,048	256	32	**512**	**32**	1.11	0.44	63.05
11	11.1	Dec 2, 2010	4.9	**256**	**256**	**128**	**> 2,048**	**2,048**	**2,048**	**2,048**	**> 2,048**	**>2,048**	**> 2,048**	**512**	**16**	> 2,048	>2,048	> 2,048	**32**	**8**	1.22	0.64	0.60
	11.10	Nov 10, 2015		**256**	**256**	**512**	**> 2,048**	**2,048**	**2,048**	**2,048**	**> 2,048**	**>2,048**	**> 2,048**	**1,024**	**16**	> 2,048	>2,048	> 2,048	**16**	**8**	1.86	3.02	0.43
12	12.1	Feb 1, 2008	8.5	4	nd	0.5	0.5	**512**	**512**	**512**	**16**	**256**	**32**	**256**	**4**	> 2,048	64	16	**32**	**16**	0.80	0.19	0.77
	12.9	Sep 12, 2016		4	nd	**32**	**> 2,048**	**512**	**512**	**512**	**32**	**256**	**32**	**128**	**4**	> 2,048	64	16	**32**	**16**	0.74	0.21	0.53
13	13.1	Aug 25, 2009	8.1	8	4	4	**> 2,048**	4	2	1	**16**	**512**	**32**	**256**	**16**	> 2,048	128	16	**32**	**16**	0.87	0.98	2.42
	13.15	Sep 20, 2017		**128**	**128**	**32**	**> 2,048**	**128**	**128**	**128**	**> 2,048**	512	32	**256**	**8**	> 2,048	128	8	**32**	**16**	1.69	0.75	1.75
14	14.2	Sep 30, 2014	1.8	**1,024**	**128**	**8**	**> 2,048**	**64**	**64**	**64**	**4**	256	64	**128**	**8**	> 2,048	>2,048	> 2,048	**16**	4	0.76	1.58	0.31

(Continued)

TABLE 1 | (Continued)

Patient's number	Isolates[a]	Collection Date	Sampling interval (years)	MIC (mg/L)[b]																Gene expression level		
				CAZ	CAZ + AVI (32 mg/L)	MEM	PIP + TZB (4mg/L)	TIC	TIC + TZB (32 mg/L)	TIC + AVI (32 mg/L)	CST	AMK	AMK + BER (128 mg/L)	TOB	CIP	TMO	AZI	AZI + BER (128 mg/L)	CHL	DOX	*axyB*	*axyY*	*axyF*
	14.4	Jul 6, 2016		**2,048**	**2,048**	**32**	**> 2,048**	**>2,048**	**> 2,048**	**>2,048**	2	1,024	64	**128**	**16**	> 2,048	>2,048	> 2,048	**16**	4	1.91	2.84	0.40
15	15.1[c,d]	Jul 1, 2009	4.1	4	nd	1	**> 2,048**	**512**	**512**	**256**	**4**	64	16	**16**	**4**	> 2,048	16	16	4	≤ 0.5	0.32	0.10	0.55
	15.4[c,d]	Aug 7, 2013		**2,048**	**2,048**	4	**> 2,048**	**>2,048**	**> 2,048**	**>2,048**	**4**	256	32	**128**	**4**	> 2,048	16	16	4	0.5	0.37	0.14	0.60
16	16.1	Nov 8, 2016	1.9	4	nd	1	0.5	4	4	2	**4**	16	4	**8**	**16**	512	16	16	**512**	1	0.96	0.23	201.60
	16.6	Sep 20, 2017		4	nd	0.5	0.5	4	4	2	2	16	4	**8**	**16**	512	16	16	**512**	1	0.96	0.24	119.89
17	17.1[c]	Dec 18, 2014	–	**1,024**	**64**	**128**	**64**	**> 2,048**	**>2,048**	**64**	**64**	1,024	32	**> 2,048**	**16**	>2,048	> 2,048	>2,048	**32**	**16**	1.09	1.11	0.90
18	18.1[c]	Aug 25, 2009	1.5	8	nd	2	1	**2,048**	**2,048**	**2,048**	2	128	16	**64**	**4**	2,048	64	16	**32**	**16**	0.65	0.11	0.98
	18.3[c]	Feb 15, 2011		8	nd	**64**	2	**2,048**	**2,048**	**2,048**	**4**	128	16	**64**	**8**	2,048	64	16	**32**	**16**	1.45	0.92	1.17
19	19.2[c]	Jan 5, 2010	0.35	4	nd	4	1	**1,024**	**1,024**	4	0.5	256	32	**32**	**32**	1,024	128	16	**512**	**16**	1.39	0.40	16.20
	19.5[c]	May 10, 2010		2	nd	4	0.5	**1,024**	**1,024**	4	1	8	8	4	**32**	1,024	64	8	**512**	**16**	1.27	0.14	8.89
26	26.3	Aug 16, 2017	–	4	nd	2	2	4	4	4	2	256	32	**64**	**4**	1,024	128	16	**64**	**8**	0.72	0.30	0.57
27	27.1[d]	Feb 18, 2010	3.75	2	nd	0.25	1	**> 2,048**	**>2,048**	**> 2,048**	2	64	16	**64**	**2**	512	64	16	**16**	0.5	1.21	0.11	1.06
	27.3[d]	Nov 12, 2013		2	nd	0.25	1	2	2	4	**4**	256	32	**64**	**4**	512	64	16	**32**	2	1.27	0.12	1.13

[a] *Strain numbering: first figure corresponds to the patient; second figure correspond to the isolate number in each patient (late isolate collected over the period of sampling).*
[b] *Values in bold are above the CLSI breakpoint (when available; see* ***Table 1*** *for values).*
[c] *Gray background highlights MICs of strains in which cephalosporinase activity was phenotypically detected.*
[d] *A. insuavis.*

for the following antibiotics (potency and origin): amikacin (Amukin 500 mg/2 ml for injection, S.A. Bristol-Myers Squibb, Belgium), azithromycin (100%, SMB, Brussels, Belgium), ceftazidime (2 g for IV injection, 72.5%, PAN Pharma, Luitré, France), chloramphenicol (98%, Sigma-Aldrich), ciprofloxacin (98%, Fluka, Sigma-Aldrich), colistin sulfate salt (79.6%, Sigma-Aldrich), doxycycline hyclate (86.6%, Sigma-Aldrich), meropenem (500 mg powder for solution for injection or infusion, 92%, Hospira UK Ltd., Hurley, United Kingdom), piperacillin (94.2%, Sigma-Aldrich, Maryland Heights, MO), temocillin (84%, Eumedica Pharmaceuticals, Manage, Belgium), ticarcillin disodium salt (85.2%, Sigma-Aldrich), and tobramycin (100%, Teva, Wilrijk, Belgium). Tazobactam sodium salt (92.4%, Cubist Pharmaceuticals, Lexington, MA) and avibactam (99.6%, AstraZeneca Pharmaceuticals, Waltham, MA) were used as inhibitors for β-lactamases. Berberine (chloride hydrate, 82.1%; Sigma-Aldrich), known to attenuate the MexXY-OprM/OprA-mediated aminoglycoside resistance in *P. aeruginosa* (Morita et al., 2016), was used to reduce AxyXY-OprZ activity.

Uptake of *N*-Phenyl-1-Naphthylamine

The uptake of the lipophilic probe *N*-phenyl-1-naphthylamine (NPN) was measured following the general methodology described previously (Lomovskaya et al., 2001). In brief, 10 ml of bacterial culture in exponential growth phase (OD_{620nm} 0.6) was harvested by centrifugation (3,000 *g*; 10 min) and resuspended in buffer (NaCl, 110 mM; KCl, 7 mM; NH_4Cl, 40 mM; NA_2HPO_4, 0.4 mM; Tris base, 52 mM; glucose, 0.2%; pH 7.5 adjusted with HCl). NPN was added at a final concentration of 10 μM, and cells were incubated 10 min at 37°C; then, 200-μl aliquots were dispensed in standard 96-well plates. The fluorescence signal (excitation/emission wavelengths, 340/410 nm) was measured on a Spectramax® multiplate reader (Molecular Devices, Sunnyvale, CA). The protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 97%, Sigma-Aldrich; final concentration, 100 μM) was used as a positive control; buffer solutions containing 10 μM NPN without cells or cells without NPN were used as blanks.

Phenotypic Screening of β-Lactamases

Cephalosporinase and carbapenemase activity was detected using the ESBL NDP/carba NP test (Nordmann et al., 2012a,b), adapted by using cefotaxime or imipenem (3 mg/ml) as substrates. *A. xylosoxidans* ATCC 27061 and *P. aeruginosa* ATCC 27853 were included as negative controls and clinical isolates of *P. aeruginosa* expressing IMP-13 or VIM-2 metallo-β-lactamases or *Klebsiella pneumoniae* expressing OXA-48, as positive controls.

Quantification of Efflux Gene Expression

The expression levels of a*xyB*, *axyY*, and *axyF* (encoding the inner membrane protein of AxyABM, AxyXY-OprZ, or AxyEF-OprN pumps, respectively) were quantified by real-time PCR, relative to those measured for the reference strain ATCC 27061. RNA was extracted (Invitrap Spin Cell RNA Mini Kit, 1061100300, STRATEC, Birkenfeld, Germany) from log-phase cultures ($OD_{620\ nm}$, around 0.7) and treated by DNase (TURBO DNA-free™ Kit, AM1907, Thermo Fisher Scientific, MA). The absence of DNA contamination was checked by performing a PCR on purified RNA, which did not amplify any residual material. cDNA was synthesized (Transcriptor First Strand cDNA Synthesis Kit, 04379012001, Roche, LifeSciences, Penzberg, Germany). A real-time PCR was performed on a CFX-96 machine (BIORAD, Hercules, CA) using SsoAdvanced™ Universal SYBR® Green Supermix, #1725271, the primers described in **Supplementary Table 1**, and 16S rRNA as housekeeping gene. Triplicates measurements were repeated in two independent experiments.

Statistical Analyses

Descriptive statistics and graphs were produced using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA) or JMP Pro v.14 (partition tree analysis; SAS Institute Inc., Cary, NC).

RESULTS

Genetic Relation of Strains

The clonal relationship of the 41 isolates (from 21 patients, including 18 pairs/triplets of early/late isolates) was analyzed by WGS typing. Species identification was confirmed *via* extracted *nrdA* gene data, resulting in *A. xylosoxidans* (isolates from patients 1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 17, 18, 19, and 26) or *A. insuavis* (isolates from patients 3, 10, 15, 16, and 27) according to the *nrdA*_765 typing scheme (**Supplementary Figure 1A**). As the isolates of patient 18 (18.1 and 18.3) are distantly related to all known species according to the phylogenetic analysis, these isolates may represent a new species within the genus *Achromobacter*.

After plotting allele changes in patient isolate pairs per year (range from 0.5 in pairs from patient 12 to 215.5 in pair from 11), the cutoff value for a very close genetic relation resulted in ≤ 20 alleles and for a close genetic relation in ≤ 57 alleles change per year. Results of WGS typing of the 41 *Achromobacter* isolates were displayed in two minimum spanning trees (MST) separated by species. Considering the allelic differences over time, MST of *A. xylosoxidans* strains resulted in 12 clusters (**Supplementary Figure 1B-A**, black borders) comprising 2, 3, or 5 very closely related or closely related isolates and 3 singletons *via* cgMLST algorithm of 5,778 target genes. Ten clusters harbored strains isolated from one patient and two clusters strains derived from two patients, illuminating the possibility of a patient-to-patient cross-transmission (patients 9 and 19; patients 7 and 26) of *A. xylosoxidans*. MST of *A. insuavis* strains resulted in four clusters (**Supplementary Figure 1B-B**, black borders) comprising two or three very closely related isolates. Three of these clusters harbored strains isolated from one patient, while one cluster included strains from two patients (patients 3 and 27), also giving hint for a patient-to-patient cross-transmission. Both MST reference genomes (GenBank accession numbers LN831029.1 and GCA_003096315.1) were not genetically related to any genotype from patient isolates of this study.

Minimum Inhibitory Concentrations, Resistance Profile

MICs were measured for antibiotics commonly used in CF and belonging to classes described as substrates for efflux in other Gram-negative bacteria (**Table 1** for individual values and **Table 2** for a summary).

Susceptibility was higher for β-lactams (37–61%) than for doxycycline (27%), colistin (22%), chloramphenicol (15%), aminoglycosides (7–12%), and ciprofloxacin (2%). High MIC_{50} were observed for temocillin and azithromycin (no susceptibility breakpoint set). Broad ranges of MIC values were observed for all drugs, and MIC_{50} were higher than the concentrations reachable in the serum of treated patients, except for ceftazidime, meropenem, and piperacillin/tazobactam. No systematic difference in MICs could be evidenced between strains identified as *A. xylosoxidans* or *A. insuavis*, but the number of strains in each species was too small to draw meaningful conclusions in this respect. When comparing longitudinally the MIC distributions in the 18 pairs/triplets of isolates, a significant increase was observed for ceftazidime, meropenem, and ciprofloxacin between early and late isolates (Wilcoxon matched-pairs signed-rank test: p: 0.03, 0.01, p: 0.01, respectively; see the changes in the median and geometric mean values for MICs of all drugs in early and late isolates in **Table 2** and the detailed analysis for drugs showing a significant loss in susceptibility between early and late isolates in **Figure 1**).

Phenotypic Screening of β-Lactamase Activity

The percentage of susceptibility to ticarcillin (37%; 15/41) was not modified in the presence of tazobactam, but increased to 46% (19/41) in the presence of avibactam (**Table 2**). Among the 16 isolates resistant to ceftazidime (MICs, 16–2,048 mg/L), MICs were decreased by 2.2 twofold dilutions on average by avibactam, with only one isolate (8.1; MIC, 16 mg/L) regaining susceptibility to ceftazidime (MIC with avibactam, 8 mg/L; **Table 1**). All isolates were also screened for β-lactamase activity using the NDP/carba NP phenotypic test. Fourteen isolates were displaying cephalosporinase activity (degradation of cefotaxime), among which 12 were resistant to ticarcillin, 7 to piperacillin-tazobactam, to ceftazidime, and 7 to meropenem, respectively (**Table 1** for an identification of these isolates). No carbapenemase activity was detected in the whole collection.

Efflux Pumps and Influence on Antibiotic Activity

We first showed a minor impact of deletion of each efflux pumps in reference strains on the MIC of the whole panel of antibiotics (**Table 1**). We therefore rather examined whether the expression levels of *axyB*, *axyY*, and *axyF* encoding the inner membrane protein of each of the three main RND efflux pumps were variable among isolates. The expression levels of these genes in early and late isolates are compared in **Supplementary Figure 2**. **Supplementary Figure 3** shows that there is a significant correlation between the level of expression of *axyB* and the MICs of amikacin, azithromycin, and meropenem, between the expression level of *axyY* and the MICs od amikacin (± berberine), tobramycin, azithromycin, and colistin, and between the expression level of *axyF* and the MICs of chloramphenicol (see **Supplementary Table 2** for statistical analyses).

The MIC of amikacin and azithromycin was then measured in the whole collection in the presence of the MexXY efflux attenuator berberine (Morita et al., 2016). At 1/4 MIC, berberine decreased these MICs, causing a reduction of the MIC_{50} of 2 and 3 doubling dilutions, respectively (**Tables 1**, **2**). Yet, no changes in the MIC were observed for a few strains with low or high MICs. Thus, amikacin activity remained unaffected by berberine in 3/9 isolates with MIC > 2,048 mg/L (isolates 8.7, 11.1, 11.10) and 1/1 and 1/4 isolates with MIC of 8 and 16 mg/L (isolates 19.5 and 6.14, respectively), respectively (**Supplementary Figure 4A**). Azithromycin activity was not improved by berberine in 8/12 isolates with MIC > 2,048 mg/L and 6/6 isolates with MIC ≤ 32 mg/L (**Supplementary Figure 4B**). In four pairs of isolates, the MICs of amikacin and azithromycin changed in parallel between the early and late isolates (late isolate more resistant for patients 9 and 10 or more susceptible for patients 6 and 19; see **Table 1**), giving us the opportunity to examine the relationship between this change in MIC and the expression levels of *axyB* or *axyY*. No systematic correlation was observed between MICs and the expression level of *axyB* (not shown) but well between MIC values and the expression levels of *axyY* (**Figure 2A**). We also evaluated the efflux activity in individual strains by measuring the fluorescence signal associated with the incorporation in bacterial membranes of *N*-phenyl-1-naphthylamine (NPN), a well-established substrate for efflux (Ocaktan et al., 1997). To validate this approach, we first showed that NPN accumulation was markedly increased in mutants of an *A. insuavis* reference strain deleted in *axyB* or *axyY* and in an *A. xylosoxidans* reference strain incubated with CCCP as compared to their wild-type counterparts (**Figure 2B**). NPN fluorescence was then measured in clinical isolates, with data stratified according to antibiotic MICs (**Figures 2C–E**). Isolates with low MICs for aminoglycosides or azithromycin showed significantly higher NPN accumulation (MIC threshold set at 256, 128, and 64 mg/L for amikacin, tobramycin, and azithromycin, respectively, by partition analysis).

Ribosomal Mutations and Decreased Azithromycin Activity

Fourteen isolates in this collection showed azithromycin MICs > 256 mg/L (2 isolates with a MIC of 512 mg/L and 12 isolates with a MIC > 2,048 mg/L, respectively; see **Table 1**). Ribosomal mutations in *rpl4*, *rpl22*, and *rrl* genes were therefore searched in these isolates and in 17 representative isolates with lower MICs (8–256 mg/L). The sequences of the isolates were first compared to those of *A. xylosoxidans* ATCC 27061. However, this reference strain does not show a wild-type phenotype (elevated MICs to some antibiotics; see **Table 1**). Since there is no fully sequenced wild-type *A. xylosoxidans*, we rather decided to compare all sequences to that of *A. insuavis* AXX-A which is more susceptible to meropenem, ticarcillin, aminoglycosides,

TABLE 2 | Antimicrobial susceptibility to different antibiotics among the 41 clinical isolates [including 18 pairs of successive isolates with 1–11 year's interval between the early (E) and late (L) sample] of *Achromobacter* spp.

Antibiotics[a]	CLSI susceptibility breakpoints (mg/L)[d] (S ≤)	Whole collection (*n* = 41)			Median of MICs (mg/L) from pairs of isolates (*n* = 36) (25–75% percentile)		Geom. Mean of MICs (mg/L) from pairs of isolates (*n* = 36) (with 95% CI)	
		% S	MIC_{50}	MIC range	E	L	E	L
CAZ*	8	61	4	2–2,048	4 (4–40)	20 (4–256)	12 (5–29)	32 (10–104)
MEM*	4	59	4	0.125–512	2 (0.5–6)	24 (4–96)	3 (1–7)	14 (4–47)
TZP	16	54	2	0.25–>2,048	1 (0.5–4,096)	2,304 (0.5–4,096)	15 (2–119)	91 (11–764)
TIC	16	37	256	2–>2,048	36 (4–1,536)	768 (3–4,096)	59 (13–274)	196 (41–927)
TIC + TZB[b]	16	37	256	2–>2,048	36 (4–1,536)	768 (3–4,096)	59 (13–274)	196 (41–927)
TIC + AVI[b]	16	46	64	1–>2,048	6 (2–768)	128 (3–2,048)	29 (7–118)	78 (18–343)
CST	2	22	8	0.5–>2,048	12 (4–640)	6 (3–1,152)	26 (7–102)	29 (6–131)
AMK	16	12	256	8–>2,048	384 (64–3,072)	256 (48–3,072)	335 (132–851)	299 (108–829)
AMK + BER[c]	16	32[e]	32	4–>2,048	32 (16–64)	32 (16–96)	45 (19–106)	53 (21–134)
TOB	4	7	64	4–>2,048	128 (24–256)	128 (16–384)	98 (41–233)	87 (33–228)
CIP*	1	2	8	1–128	6 (4–16)	16 (4–32)	7 (5–10)	13 (8–21)
TMO	NA[f]	NA	2,048	256–>2,048	3,072 (512–4,096)	4096 (512–4,096)	1625 (946–2,795)	1,756 (1,001–3,078)
AZI	NA	NA	128	8–>2,048	128 (64–2,304)	128 (64–4096)	196 (77–497)	256 (86–766)
AZI + BER[c]	NA	NA	16	8–>2,048	16 (16–192)	16 (16–2176)	53 (18–156)	62 (18–206)
CHL	8	15	32	4–512	32 (16–32)	32 (16–64)	31 (17–55)	38 (20–73)
DOX	4	27	16	≤0.5–32	16 (8–16)	16 (4–16)	8 (4–14)	8 (5–14)

[a] *CAZ, ceftazidime; MEM, meropenem; TZP, piperacillin/tazobactam; TIC, ticarcillin; CST, colistin; AMK, amikacin; TOB, tobramycin; CIP, ciprofloxacin; TMO, temocillin; AZI, azithromycin; DOX, doxycycline; CHL, chloramphenicol; TZB, tazobactam; AVI, avibactam; BER, berberine.*

[b] *Used at 32 mg/L.*

[c] *Used at 128 mg/L [1/4 MIC; pH of the medium remaining stable (7.3)].*

[d] *Breakpoints for "other non-Enterobacterales."*

[e] *Percentage calculated if considering AMK breakpoints.*

[f] *NA: not available (no breakpoints set for temocillin and azithromycin).*

**Significant differences in MIC distribution between E and L isolates (Wilcoxon matched-pairs signed-rank test) for CAZ (p: 0.03), MEM (p: 0.01), and CIP (p: 0.01).*

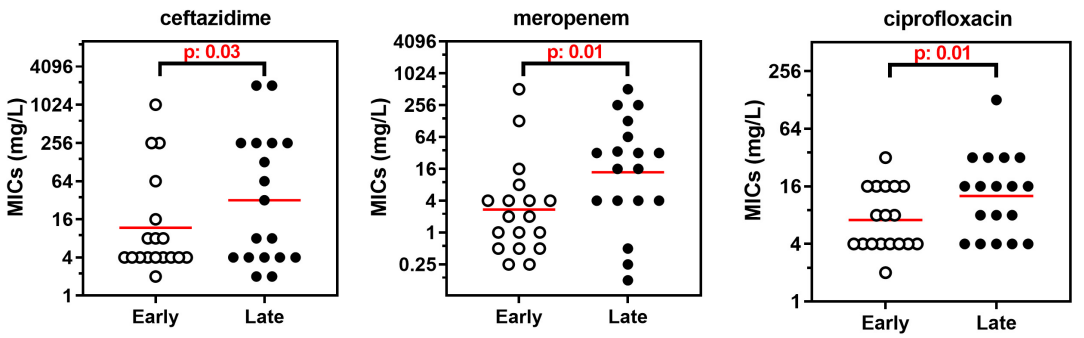

FIGURE 1 | MICs of 3 antibiotics in the 18 pairs of isolates collected successively in the same patients with an interval time ranging from 1 to 11 years. The graphs show the individual MIC for each isolate, with the geometric mean rep-resented by the red horizontal line. Only antibiotics for which a significant difference between the successive isolates (Early vs. late) was observed are illustrated (see **Table 1**). Statistical analysis: Wilcoxon matched-pairs signed-rank test.

ciprofloxacin, chloramphenicol, and doxycycline; **Table 1**). All the data are compiled in **Supplementary Table 3** together with azithromycin MICs (± berberine) and *axyB* or *axyX* gene expression levels. In this **Supplementary Table 3**, we specifically identify in blue color the mutations that distinguish *A. xysoloxidans* ATCC 27061 from *A. insuavis* AXX-A, and isolates are ordered to show close from one another those which share the same mutations, classified according to their MIC. Three silent mutations (T96C, T123C, and T411C) were commonly observed in *rpl4*, including in *A. xysoloxidans* ATCC 27061, while a few others were only seen in clinical isolates (C219G, C330T, C444T, and C414T). Two mutations leading to the replacement of an uncharged amino acid by a charged amino acid (Q65R and G69R) were detected in isolates with an

MIC of 512 mg/L (isolates 5.1 and 9.6). In *rpl22*, silent point mutations (T78C, T189C) were seen in *A. xysoloxidans* ATCC 27061 and in several clinical isolates. A missense conservative *rpl22* T134C (V45A) mutation was observed in the reference ATCC 27061 and in many isolates. It is most likely not related to resistance, as it was also found in isolates with an MIC of azithromycin of ≤ 64 mg/L (*viz.*, isolates 27.1, 27.3, or 10.1 and ATCC 27061). A series of mutations were commonly observed in *rrl*, independent of their azithromycin MIC in the presence of berberine (16 to > 2,048 mg/L), most of them being identified when comparing *A. xysoloxidans* ATCC 27061 and *A. insuavis* AXX-A. Among the 12 isolates with azithromycin MICs > 2,048 mg/L, 11 showed specific mutations in the *rrl* gene (A1284G, T1325C, A2043T, A2043G, A2044G, or C2596T). No specific mutation was identified in isolate 17.1. *axyY* expression was globally low in isolates that were more susceptible to azithromycin than the reference strain or in isolates presenting mutations in *rpl4* or *rpl22*. Conversely, *axyY* expression was variable among isolates harboring mutations in *rrl*, but at least one of the two efflux-associated genes was overexpressed in all isolates with MICs > 2,048 mg/L, except for isolates 17.1 and 11.1.

Mutations in Fluoroquinolone Targets and Resistance to Ciprofloxacin

Ciprofloxacin displayed marginal activity against this collection, with MIC ranging from 1 to 32 mg/L. As *A. xylosoxidans* shows an elevated MIC to ciprofloxacin (4 mg/L), its sequence was first compared with that of *A. insuavis* AXX-A. Several missense mutations (shown in green) in the sequence of *gyrA*, *gyrB*, *parC*, and *parE* were detected and an insertion of 3 amino acids at the end of the *gyrA* sequence, which were also found in other isolates with an MIC of 4 mg/L (**Supplementary Table 4**). Of note, the isolate 10.1, with an MIC of 4 mg/L and a basal expression of *axyF*, shows the same sequence for the four genes as *A. insuavis* AXX-A, which may suggest that the mutations seen in *A. xylosoxidans* ATCC 27061 are not necessarily explaining its elevated MIC. The isolate 3.1 (susceptible; MIC = 1 mg/L) showed only a few differences with the sequence of *A. insuavis* AXX-A, some of them being also found in *gyrB* or *parC* of *A. xylosoxidans* ATCC 27061. Other mutations were specifically identified in clinical isolates with ciprofloxacin MIC ≥ 4 mg/L, namely, T527S and M706V in *gyrA* (isolates from patients 18 and 8), N11T, G12S, N592S or N592G, S609A, A631T, A633S, and A634T in *gyrB* (isolates from patients 18, 15, and 5); V417L, E441Q, S482T, and K764R in *parC* (isolates from patients 9, 19, and 18); and V41I, A150T, and T593S in *parE* (isolate from patient 18). Lastly, other missense mutations, located exclusively in *gyrA* (Q83L, D87N, L454M, T881M; patients 4, 8, 16, 17) and *gyrB* (I683V; patient 5), were detected only in isolates with MICs ≥ 16 mg/L. Among all these mutations, only Q83L and D87N were located in the QRDR for *gyrA*. *axyF* expression was low in most of the isolates, except 10.3, 16.1, and 19.2 and associated with specific target mutations in 16.1 and 19.2. Of note, isolates 9.1 and 19.2 showed the same mutations in *parC* but different MICs depending on the level of expression of *axyF*. Noteworthy, the only isolate in the collection with a MIC of 128 mg/L (10.3, patient 10) did not show any specific QRDR mutation but rather a particularly high level of expression of *axyF* as compared to the previous isolate from the same patient (10.1). No other mutations were found in the isolates that are not shown in **Supplementary Table 4**.

DISCUSSION

This study highlights a major, and sometimes unexpected, role of efflux and target mutations in the resistance of *Achromobacter* isolated from patients with CF to specific antibiotic classes, thanks to the exploitation of a collection containing in majority longitudinal pairs of isolates from the same patients.

Whereas WGS-based typing has been successfully set up, a public cgMLST scheme for interpretation of genetic relatedness is lacking. Hence, an *ad hoc* scheme was established here [≤ 20 allele difference (de Been et al., 2015)]. Based on this strict criterion, only about half of the isolates kept a very high degree of relatedness over time, possibly indicating that genetic adaptations have taken place under selective pressure during antibiotic treatment. Although not powered as an epidemiological survey, our study shows that, among the tested drugs, the most active ones belong to the class of β-lactams, in accordance with previous reports from France (Amoureux et al., 2013; Dupont et al., 2017), Italy (Raso et al., 2008), United Kingdom (Okoliegbe et al., 2020), or United States (Duggan et al., 1996; Swenson and Sadikot, 2015). Nevertheless, remarkable changes in specific pairs allowed us to delineate important findings in terms of mechanisms of resistance, which was the main purpose of this work.

We first document a major role of efflux in resistance to several drugs, by evidencing quantitative correlations between the expression levels of the genes encoding efflux pumps and the increase in MICs of drug substrates, an aspect that was not examined in previous works (Bador et al., 2011, 2013; Magallon et al., 2021).

Regarding resistance to β-lactams, we did not observe any correlation between the expression of *axyB* and the MIC of ceftazidime, described as a substrate for this pump (Magallon et al., 2022), possibly because the concomitant contribution of cephalosporinase activity in resistance levels masks a potential contribution of efflux to this loss of susceptibility. This mechanism was phenotypically detected in one-third of the collection, but was not characterized at the molecular level, as it was out of the scope of this study. It is known for example that most *A. xylosoxidans* express a narrow-spectrum class D β-lactamase (Doi et al., 2008), but its role is considered marginal in resistance to cephalosporins or carbapenems (Doi et al., 2008; Amoureux et al., 2013; Papalia et al., 2020). Regarding meropenem specifically, no carbapenemase activity was detected in the collection, but we rather observed a correlation between MICs and *axyB* expression level. AxyABM has been previously shown to confer resistance to several cephalosporins and aztreonam (Bador et al., 2011) and, only very recently, to carbapenems as well (Magallon et al., 2022). In fact, previous work on carbapenems did not evidence this transport (Bador et al., 2011), probably because these authors used a strain with low meropenem MIC (0.094 mg/L), thus

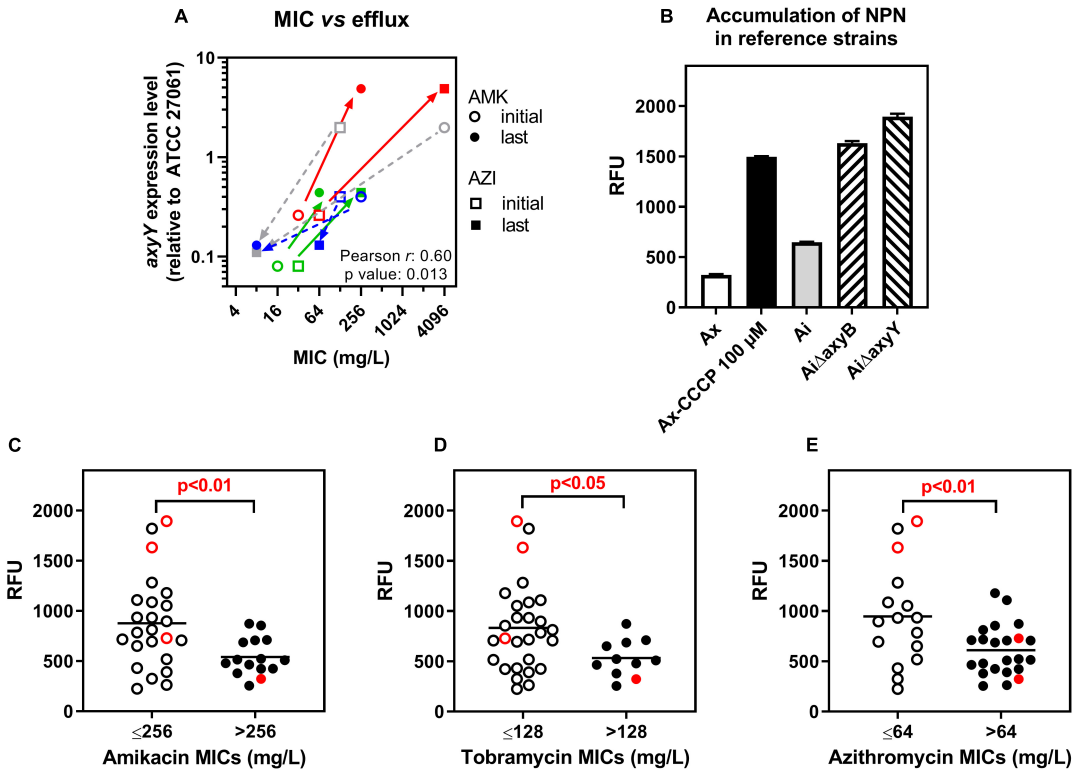

FIGURE 2 | Influence of active efflux on amikacin, tobramycin and azithromycin activity. **(A)** Correlation between the MIC of amikacin (AMK) or azithromycin (AZI) and the level of expression of *axyY* (relative to that in ATCC 27061) in 4 pairs of isolates, represented by different colors. Plain arrows joint data points of pairs for which the late isolate was more resistant [from patient 9 (red) and 10 (green)]; dotted arrows, those for which the late isolate was more susceptible [from patient 6 (gray) and 19 (dark blue)]. Pearson coefficient and *p*-values of the correlation are given. **(B)** Accumulation of the fluorophore NPN in reference strains (Ax: *A. xylosoxidans* ATCC 27061, Ai: *A. insuavis*), mutants (AiΔB: *A. insuavis* lacking *axyB* gene, AiΔY: *A. insuavis* lacking *axyY* gene), or in the presence of CCCP (100 μM) used as a positive control in Ax to reduce the activity of efflux systems, after 10 min of incubation at 37°C. Data are means ± SD of three independent determinations and are expressed in arbitrary fluorescence units (standardized initial inoculum for all strains). **(C–E)** Accumulation of NPN in the same conditions in reference and clinical isolates stratified as a function of their MICs for amikacin, tobramycin, or azithromycin. Red open-closed symbols: reference strains; black open-closed symbols: clinical isolates. Statistical analysis: partition tree to determine the MIC value splitting the distribution in 2 with the highest Logworth (-log *p*-value) value (*p*-value indicated on the graphs). The horizontal line corresponds to the mean value.

probably expressing the pump at a low level. This indicates the interest of also quantifying efflux pump expression level in order to better characterize their effects on susceptibility to drugs. The involvement of efflux in meropenem resistance seems to be a trait for CF isolates. We previously described that MexAB-OprM (homologous to AxyABM) plays a crucial role in meropenem resistance for *P. aeruginosa* isolates from CF (Chalhoub et al., 2016).

Aminoglycosides are considered as innately inactive against *A. xylosoxidans* (Bador et al., 2016) due to the constitutive expression of AxyXY-OprZ [expressed only in *Achromobacter* species resistant to aminoglycosides [*A. xylosoxidans*, *A. ruhlandii*, *A. dolens*, *A. insuavis*, *A. denitrificans*, *A. insolitus*, and *A. aegrifaciens* (Bador et al., 2016)]. We confirm the role of this efflux transporter in aminoglycoside resistance by demonstrating (a) the capacity of berberine to decrease aminoglycoside MIC and (b) a correlation between the expression level of *axyY* and the MICs of amikacin or tobramycin. Noteworthy, we found a few isolates that were susceptible to aminoglycosides, which could be ascribed to a particularly low level of expression of the pump. This impact of efflux on susceptibility to aminoglycosides is best seen for pairs 9, 10, 6, and 19 for which a commensurate change in MIC and in gene expression was noticed between early and late isolates. Likewise, we observed a correlation between *axyY* expression and colistin MICs, suggesting that it could be a substrate for AxyXY-OprZ. Of note, polymyxins susceptibility has been linked to MexXY-OprM/OprA expression in *P. aeruginosa* (Poole et al., 2015).

Macrolides act by inhibiting bacterial protein synthesis. A well-established resistance mechanism (in Gram-positive organisms) consists in mutations in 23S rRNA-encoding gene and in the ribosomal proteins L4 and L22 (Fyfe et al., 2016). We previously showed that both efflux and ribosomal mutations act in concert to confer high levels of resistance to macrolides in *P. aeruginosa* CF isolates (Mustafa et al., 2017). Here, we found a correlation between azithromycin MICs and the expression level of *axyB* and *axyY*, which is in line with the previously demonstrated role of efflux in macrolide resistance in *P. aeruginosa* (Morita et al., 2016). Surprisingly,

however, the MexXY efflux pump attenuator berberine was able to reduce azithromycin MICs only in a limited fraction of the collection, suggesting the presence of other resistance mechanisms. Genomic analysis revealed a series of ribosomal mutations, among which A2043T, A2043G, and A2044G in *rrl*, associated with a higher level of resistance than the mutation C2596T, as previously reported in CF *P. aeruginosa* (Mustafa et al., 2017; Colque et al., 2020). Other mutations (A1284G and T1325C) have not been described so far. In addition, in two isolates with an MIC of 512 mg/L, we found mutations in the ribosomal protein 4, namely, Q65R (never described) and G69R, previously reported in macrolide-resistant *Streptococcus pneumoniae* (Clark et al., 2007; Kosowska-Shick et al., 2008), linezolid-resistant *Staphylococcus epidermidis* (Mendes et al., 2012), and CF macrolide-resistant *Burkholderia multivorans* (G70R; corresponding position) (Silva et al., 2016).

Concerning fluoroquinolones, we could not evidence a clear role of efflux in resistance, since the level of expression of *axyF* was low in most of the isolates. Of note, however, two isolates from our collection with high level of expression in *axyF* (19.2, 10.3) also show high ciprofloxacin MICs, in the absence of target mutations (10.3) or in the presence of mutations similar to those observed in a more susceptible isolate with low *axyF* expression (19.2 vs. 9.1). This is coherent with the recent description of mutations in *axyT* (putative regulator of AxyEF-OprN) associated with overexpression of *axyF* in strains harboring high ciprofloxacin MIC even in the absence of QRDR mutations (Magallon et al., 2021). In the rest of the collection, we cannot exclude that efflux-mediated resistance could be masked by the impact of target mutations on MICs. Some mutations (Q83L and D87N in the QRDR of *gyrA*) have been previously reported as hot spots in *Escherichia coli* and *P. aeruginosa* (Bagel et al., 1999; Takenouchi et al., 1999), *Stenotrophomonas maltophilia* (Zhao et al., 2015), and environmental isolates of *Achromobacter* spp. (Furlan et al., 2018), while others have never been reported in *gyrA* (L454M and T881M) and *gyrB* (I683V), but are located outside of the QRDR regions. We note here that the mutation D87N was associated with an elevated ciprofloxacin MIC (32 mg/L) in the absence of overexpression of *axyF*, while D87G was associated with even higher MICs (64–128 mg/L) in isolates overexpressing *axyF* to high levels (Magallon et al., 2021). The mutations we reported in *parC/parE* were not previously described in *A. xylosoxidans* or other species, to the best of our knowledge, but are not located in the QRDR regions. It is established that mutations in the QRDR confer higher levels of resistance to fluoroquinolones than those in other regions (Yoshida et al., 1990; Belland et al., 1994). Conversely, most of the mutations reported by Magallon et al. (2021) and considered as not relevant for resistance were not seen here. It is also interesting to note that many mutations are evidenced in the isolates from patient 18, which we suspect to belong to a new species, and may thus simply represent variation in the gene sequence.

We did not study in details resistance mechanisms to tetracyclines and chloramphenicol, but it is remarkable that all isolates with elevated MICs to chloramphenicol were overexpressing *axyF*. We cannot exclude the concomitant presence of other resistance mechanisms, but only notice that chloramphenicol is also described as a good substrate for MexEF-OprN in *P. aeruginosa* (Maseda et al., 2000).

This study suffers from some limitations. First, the number of isolates remains limited, but this is due to the still relatively low proportion of patients colonized by this bacterial genus in the collecting centers. Second, we could not establish a link between resistance development and antibiotic use in each individual patient, which could be the topic of further investigations. Third, in close relationship with the two previous limitations, we could not study in details the evolution of resistance over time because the number of samples and the period of time during which they were collected was highly variable among patients, rendering difficult a statistically meaningful analysis. Fourth, we could not confirm all our hypotheses at the molecular level because this would require the construction of a large number of deletion mutants or of complemented strains, which would represent a work by itself. In particular, we noticed that the deletion of efflux pumps in reference strains is not sufficient to cause a significant phenotypic change in susceptibility, highlighting the interest of rather working with clinical isolates that show increased levels of expression for these transporters. Specifically, the comparison of successive isogenic isolates from the same patients allowed us to unambiguously evidence the role of resistance mechanisms that were expressed in one isolate from the pair and associated with a change in MIC, partially alleviating this limitation. Fifth, the only available ATCC reference strain does not show a wild-type profile of susceptibility for all antibiotics. It appeared nevertheless to us as an adequate control, being easily available to anyone, in the absence of fully sequenced isolate from human specimen harboring a wild-type phenotype. The AXX-A strain (considered as wild type) has been recently reclassified as *A. insuavis* (NCBI:txid1003200) but has nevertheless been used as a reference sequence in our genomic analyses, in order to prevent missing the identification of some mutations associated with resistance, especially for fluoroquinolones. Lastly, the definition and evaluation of a novel cgMLST scheme for WGS-based typing of *A. xylosoxidans* should be further investigated.

Nevertheless, the present work is among the first studies to shed some light on a number of different mechanisms that most likely contribute to explain the unusually high level of resistance to conventional antibiotics in *A. xylosoxidans* or the closely related species *A. insuavis.* Our data should therefore help to better apprehend bacterial response to antibiotic exposure and adapt antibiotherapy accordingly.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HC, BK, and FVB: conceptualization. HC and SK: methodology, formal analysis, and investigation. SK and BK: resources.

HC, SK, and FVB: writing—original draft preparation, funding acquisition. BK: writing—review and editing. BK and FVB: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.762307/full#supplementary-material

REFERENCES

Amoureux, L., Bador, J., Bounoua Zouak, F., Chapuis, A., de Curraize, C., and Neuwirth, C. (2016). Distribution of the species of Achromobacter in a French Cystic Fibrosis Centre and multilocus sequence typing analysis reveal the predominance of A. xylosoxidans and clonal relationships between some clinical and environmental isolates. *J. Cyst. Fibros.* 15, 486–494. doi: 10.1016/j.jcf.2015.12.009

Amoureux, L., Bador, J., Siebor, E., Taillefumier, N., Fanton, A., and Neuwirth, C. (2013). Epidemiology and resistance of Achromobacter xylosoxidans from cystic fibrosis patients in Dijon, Burgundy: first French data. *J. Cyst. Fibros.* 12, 170–176. doi: 10.1016/j.jcf.2012.08.005

Bador, J., Amoureux, L., Blanc, E., and Neuwirth, C. (2013). Innate aminoglycoside resistance of Achromobacter xylosoxidans is due to AxyXY-OprZ, an RND-type multidrug efflux pump. *Antimicrob. Agents Chemother.* 57, 603–605. doi: 10.1128/AAC.01243-12

Bador, J., Amoureux, L., Duez, J. M., Drabowicz, A., Siebor, E., Llanes, C., et al. (2011). First description of an RND-type multidrug efflux pump in Achromobacter xylosoxidans, AxyABM. *Antimicrob. Agents Chemother.* 55, 4912–4914. doi: 10.1128/AAC.00341-11

Bador, J., Neuwirth, C., Liszczynski, P., Mezier, M. C., Chretiennot, M., Grenot, E., et al. (2016). Distribution of innate efflux-mediated aminoglycoside resistance among different Achromobacter species. *New Microbes. New Infect.* 10, 1–5. doi: 10.1016/j.nmni.2015.11.013

Bagel, S., Hullen, V., Wiedemann, B., and Heisig, P. (1999). Impact of gyrA and parC mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. *Antimicrob. Agents Chemother.* 43, 868–875. doi: 10.1128/AAC.43.4.868

Belland, R. J., Morrison, S. G., Ison, C., and Huang, W. M. (1994). *Neisseria gonorrhoeae* acquires mutations in analogous regions of gyrA and parC in fluoroquinolone-resistant isolates. *Mol. Microbiol.* 14, 371–380. doi: 10.1111/j.1365-2958.1994.tb01297.x

Chalhoub, H., Saenz, Y., Rodriguez-Villalobos, H., Denis, O., Kahl, B. C., Tulkens, P. M., et al. (2016). High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations. *Int. J. Antimicrob. Agents* 48, 740–743. doi: 10.1016/j.ijantimicag.2016.09.012

Clark, C. L., Kosowska-Shick, K., Ednie, L. M., and Appelbaum, P. C. (2007). Capability of 11 antipneumococcal antibiotics to select for resistance by multistep and single-step methodologies. *Antimicrob. Agents Chemother.* 51, 4196–4201. doi: 10.1128/AAC.00827-07

Clinical and Laboratory Standards Institute (2020). *Performance Standards for Antimicrobial Susceptibility Testing; 30th Informational Supplement. CLSI document M100-S30*. Pennsylvania, PA: Clinical and Laboratory Standards Institute.

Colque, C. A., Albarracin Orio, A. G., Feliziani, S., Marvig, R. L., Tobares, A. R., Johansen, H. K., et al. (2020). Hypermutator *Pseudomonas aeruginosa* Exploits Multiple Genetic Pathways To Develop Multidrug Resistance during Long-Term Infections in the Airways of Cystic Fibrosis Patients. *Antimicrob. Agents Chemother.* 64, e2142–e2119. doi: 10.1128/AAC.02142-19

Cramer, C. L., Patterson, A., Alchakaki, A., and Soubani, A. O. (2017). Immunomodulatory indications of azithromycin in respiratory disease: a concise review for the clinician. *Postgrad. Med.* 129, 493–499. doi: 10.1080/00325481.2017.1285677

Cystic Fibrosis Foundation (2020). *2019 Patient Registry Annual Data Report*. Maryland, US: Cystic Fibrosis Foundation.

de Been, M., Pinholt, M., Top, J., Bletz, S., Mellmann, A., van Schaik, W., et al. (2015). Core Genome Multilocus Sequence Typing Scheme for High-Resolution Typing of Enterococcus faecium. *J. Clin. Microbiol.* 53, 3788–3797. doi: 10.1128/JCM.01946-15

Dekker, J. P., and Frank, K. M. (2016). Next-Generation Epidemiology: Using Real-Time Core Genome Multilocus Sequence Typing To Support Infection Control Policy. *J. Clin. Microbiol.* 54, 2850–2853. doi: 10.1128/JCM.01714-16

Doi, Y., Poirel, L., Paterson, D. L., and Nordmann, P. (2008). Characterization of a naturally occurring class D beta-lactamase from Achromobacter xylosoxidans. *Antimicrob. Agents Chemother.* 52, 1952–1956. doi: 10.1128/AAC.01463-07

Duggan, J. M., Goldstein, S. J., Chenoweth, C. E., Kauffman, C. A., and Bradley, S. F. (1996). Achromobacter xylosoxidans bacteremia: report of four cases and review of the literature. *Clin. Infect. Dis.* 23, 569–576. doi: 10.1093/clinids/23.3.569

Dupont, C., Jumas-Bilak, E., Michon, A. L., Chiron, R., and Marchandin, H. (2017). Impact of High Diversity of Achromobacter Populations within Cystic Fibrosis Sputum Samples on Antimicrobial Susceptibility Testing. *J. Clin. Microbiol.* 55, 206–215. doi: 10.1128/JCM.01843-16

Edwards, B. D., Greysson-Wong, J., Somayaji, R., Waddell, B., Whelan, F. J., Storey, D. G., et al. (2017). Prevalence and Outcomes of Achromobacter Species Infections in Adults with Cystic Fibrosis: a North American Cohort Study. *J. Clin. Microbiol.* 55, 2074–2085. doi: 10.1128/JCM.02556-16

Fang, X., Fang, Z., Zhao, J., Zou, Y., Li, T., Wang, J., et al. (2012). Draft genome sequence of *Pseudomonas aeruginosa* strain ATCC 27853. *J. Bacteriol.* 194, 3755. doi: 10.1128/jb.00690-12

Furlan, J. P. R., Sanchez, D. G., Gallo, I. F. L., and Stehling, E. G. (2018). Replicon typing of plasmids in environmental Achromobacter sp. producing quinolone-resistant determinants. *APMIS* 126, 864–869. doi: 10.1111/apm.12896

Fyfe, C., Grossman, T. H., Kerstein, K., and Sutcliffe, J. (2016). Resistance to Macrolide Antibiotics in Public Health Pathogens. *Cold Spring Harb. Perspect. Med.* 6:a025395. doi: 10.1101/cshperspect.a025395

Gade, S. S., Norskov-Lauritsen, N., and Ridderberg, W. (2017). Prevalence and species distribution of Achromobacter sp. cultured from cystic fibrosis patients

attending the Aarhus centre in Denmark. *J. Med. Microbiol.* 66, 686–689. doi: 10.1099/jmm.0.000499

Hoyle, N., Zhvaniya, P., Balarjishvili, N., Bolkvadze, D., Nadareishvili, L., Nizharadze, D., et al. (2018). Phage therapy against Achromobacter xylosoxidans lung infection in a patient with cystic fibrosis: a case report. *Res. Microbiol.* 169, 540–542. doi: 10.1016/j.resmic.2018.05.001

Hu, Y., Zhu, Y., Ma, Y., Liu, F., Lu, N., Yang, X., et al. (2015). Genomic insights into intrinsic and acquired drug resistance mechanisms in Achromobacter xylosoxidans. *Antimicrob. Agents Chemother.* 59, 1152–1161. doi: 10.1128/AAC.04260-14

Isler, B., Kidd, T. J., Stewart, A. G., Harris, P., and Paterson, D. L. (2020). Achromobacter Infections and Treatment Options. *Antimicrob. Agents Chemother.* 64, e1025–e1020. doi: 10.1128/AAC.01025-20

Kosowska-Shick, K., Clark, C., Credito, K., Dewasse, B., Beachel, L., Ednie, L., et al. (2008). *In vitro* capability of faropenem to select for resistant mutants of Streptococcus pneumoniae and *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* 52, 748–752. doi: 10.1128/AAC.01389-07

Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., et al. (2001). Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob. Agents Chemother.* 45, 105–116. doi: 10.1128/AAC.45.1.105-116.2001

Magallon, A., Amoureux, L., Garrigos, T., Sonois, M., Varin, V., Neuwirth, C., et al. (2022). Role of AxyABM overexpression in acquired resistance in Achromobacter xylosoxidans. *J. Antimicrob. Chemother* 13:dkab479. doi: 10.1093/jac/dkab479

Magallon, A., Roussel, M., Neuwirth, C., Tetu, J., Cheiakh, A. C., Boulet, B., et al. (2021). Fluoroquinolone resistance in Achromobacter spp.: substitutions in QRDRs of GyrA, GyrB, ParC and ParE and implication of the RND efflux system AxyEF-OprN. *J. Antimicrob. Chemother.* 76, 297–304. doi: 10.1093/jac/dkaa440

Marsac, C., Berdah, L., Thouvenin, G., Sermet-Gaudelus, I., and Corvol, H. (2021). Achromobacter xylosoxidans airway infection is associated with lung disease severity in children with cystic fibrosis. *ERJ. Open. Res.* 7, 00076–02021. doi: 10.1183/23120541.00076-2021

Maseda, H., Yoneyama, H., and Nakae, T. (2000). Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 44, 658–664. doi: 10.1128/AAC.44.3.658-664.2000

Mendes, R. E., Deshpande, L. M., Costello, A. J., and Farrell, D. J. (2012). Molecular epidemiology of Staphylococcus epidermidis clinical isolates from U.S. hospitals. *Antimicrob. Agents Chemother.* 56, 4656–4661. doi: 10.1128/AAC.00279-12

Morita, Y., Nakashima, K. I., Nishino, K., Kotani, K., Tomida, J., Inoue, M., et al. (2016). Berberine Is a Novel Type Efflux Inhibitor Which Attenuates the MexXY-Mediated Aminoglycoside Resistance in *Pseudomonas aeruginosa*. *Front. Microbiol.* 7:1223. doi: 10.3389/fmicb.2016.01223

Mustafa, M. H., Khandekar, S., Tunney, M. M., Elborn, J. S., Kahl, B. C., Denis, O., et al. (2017). Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients. *Eur. Respir. J.* 49:1601847. doi: 10.1183/13993003.01847-2016

Nei, M., and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford, UK: Oxford University, 1–348.

Nielsen, S. M., Penstoft, L. N., and Norskov-Lauritsen, N. (2019). Motility, Biofilm Formation and Antimicrobial Efflux of Sessile and Planktonic Cells of Achromobacter xylosoxidans. *Pathogens.* 8:14. doi: 10.3390/pathogens8010014

Nordmann, P., Dortet, L., and Poirel, L. (2012a). Rapid detection of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae*. *J. Clin. Microbiol.* 50, 3016–3022.

Nordmann, P., Poirel, L., and Dortet, L. (2012b). Rapid detection of carbapenemase-producing *Enterobacteriaceae*. *Emerg. Infect. Dis.* 18, 1503–1507.

Ocaktan, A., Yoneyama, H., and Nakae, T. (1997). Use of fluorescence probes to monitor function of the subunit proteins of the MexA-MexB-oprM drug extrusion machinery in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 272, 21964–21969. doi: 10.1074/jbc.272.35.21964

Okoliegbe, I. N., Hijazi, K., Cooper, K., Ironside, C., and Gould, I. M. (2020). Longitudinal surveillance and combination antimicrobial susceptibility testing of multidrug-resistant Achromobacter spp. From cystic fibrosis patients. *Antimicrob. Agents Chemother.* 64, e1467–e1420. doi: 10.1128/AAC.01467-20

Papalia, M., Steffanowski, C., Traglia, G., Almuzara, M., Martina, P., Galanternik, L., et al. (2020). Diversity of Achromobacter species recovered from patients with cystic fibrosis, in Argentina. *Rev. Argent Microbiol.* 52, 13–18. doi: 10.1016/j.ram.2019.03.004

Poole, K., Lau, C. H.-F., Gilmour, C., Hao, Y., and Lam, J. S. (2015). Polymyxin Susceptibility in *Pseudomonas aeruginosa* Linked to the MexXY-OprM Multidrug Efflux System. *Antimicrob. Agents Chemother.* 59, 7276–7289. doi: 10.1128/AAC.01785-15

Raidt, L., Idelevich, E. A., Dubbers, A., Kuster, P., Drevinek, P., Peters, G., et al. (2015). Increased Prevalence and Resistance of Important Pathogens Recovered from Respiratory Specimens of Cystic Fibrosis Patients During a Decade. *Pediatr. Infect. Dis. J.* 34, 700–705. doi: 10.1097/INF.0000000000000714

Raso, T., Bianco, O., Grosso, B., Zucca, M., and Savoia, D. (2008). Achromobacter xylosoxidans respiratory tract infections in cystic fibrosis patients. *APMIS* 116, 837–841. doi: 10.1111/j.1600-0463.2008.00995.x

Saiman, L., Chen, Y., Tabibi, S., San Gabriel, P., Zhou, J., Liu, Z., et al. (2001). Identification and antimicrobial susceptibility of Alcaligenes xylosoxidans isolated from patients with cystic fibrosis. *J. Clin. Microbiol.* 39, 3942–3945. doi: 10.1128/JCM.39.11.3942-3945.2001

Silva, I. N., Santos, P. M., Santos, M. R., Zlosnik, J. E. A., Speert, D. P., Buskirk, S. W., et al. (2016). Long-Term Evolution of Burkholderia multivorans during a Chronic Cystic Fibrosis Infection Reveals Shifting Forces of Selection. *mSystems* 1, e29–e16. doi: 10.1128/mSystems.00029-16

Somayaji, R., Stanojevic, S., Tullis, D. E., Stephenson, A. L., Ratjen, F., and Waters, V. (2017). Clinical Outcomes Associated with Achromobacter Species Infection in Patients with Cystic Fibrosis. *Ann. Am. Thorac. Soc.* 14, 1412–1418. doi: 10.1513/AnnalsATS.201701-071OC

Spilker, T., Vandamme, P., and LiPuma, J. J. (2012). A multilocus sequence typing scheme implies population structure and reveals several putative novel Achromobacter species. *J. Clin. Microbiol.* 50, 3010–3015. doi: 10.1128/JCM.00814-12

Steinberg, J. P., and Del Rio, C. (2005). "Other gram-negative and gram-variable bacilli," in *Principles and practices of infectious diseases, 6th ed*, eds G. L. Mandell, J. E. Bennett, and R. Dolin (London, UK: Churchill Livingstone), 2751–2768.

Swenson, C. E., and Sadikot, R. T. (2015). Achromobacter respiratory infections. *Ann. Am. Thorac. Soc.* 12, 252–258.

Takenouchi, T., Sakagawa, E., and Sugawara, M. (1999). Detection of gyrA mutations among 335 *Pseudomonas aeruginosa* strains isolated in Japan and their susceptibilities to fluoroquinolones. *Antimicrob. Agents Chemother.* 43, 406–409. doi: 10.1128/AAC.43.2.406

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38, 3022–3027. doi: 10.1093/molbev/msab120

Tetart, M., Wallet, F., Kyheng, M., Leroy, S., Perez, T., Le Rouzic, O., et al. (2019). Impact of Achromobacter xylosoxidans isolation on the respiratory function of adult patients with cystic fibrosis. *ERJ. Open. Res.* 5, 00051–2019. doi: 10.1183/23120541.00051-2019

Traglia, G. M., Almuzara, M., Merkier, A. K., Adams, C., Galanternik, L., Vay, C., et al. (2012). Achromobacter xylosoxidans: an emerging pathogen carrying different elements involved in horizontal genetic transfer. *Curr. Microbiol.* 65, 673–678. doi: 10.1007/s00284-012-0213-5

Wang, M., Ridderberg, W., Hansen, C. R., Hoiby, N., Jensen-Fangel, S., Olesen, H. V., et al. (2013). Early treatment with inhaled antibiotics postpones next occurrence of Achromobacter in cystic fibrosis. *J. Cyst. Fibros.* 12, 638–643. doi: 10.1016/j.jcf.2013.04.013

Yabuuchi, E., and Oyama, A. (1971). Achromobacter xylosoxidans n. sp. from human ear discharge. *Jpn. J. Microbiol.* 15, 477–481. doi: 10.1111/j.1348-0421.1971.tb00607.x

Yoshida, H., Bogaki, M., Nakamura, M., and Nakamura, S. (1990). Quinolone resistance-determining region in the DNA gyrase gyrA gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* 34, 1271–1272. doi: 10.1128/AAC.34.6.1271

Zeiser, E. T., Becka, S. A., Barnes, M. D., Taracila, M. A., LiPuma, J. J., and Papp-Wallace, K. M. (2019). Resurrecting Old beta-Lactams: Potent Inhibitory Activity of Temocillin against Multidrug-Resistant Burkholderia Species Isolates from the United States. *Antimicrob. Agents Chemother.* 63, e2315–e2318. doi: 10.1128/AAC.02315-18

Zhao, Y., Niu, W., Sun, Y., Hao, H., Yu, D., Xu, G., et al. (2015). Identification and characterization of a serious multidrug resistant *Stenotrophomonas* maltophilia strain in China. *Biomed. Res. Int.* 2015:580240. doi: 10.1155/2015/580240

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Supplementary Material

Role of efflux and target mutations in antibiotic resistance of *Achromobacter xylosoxidans* and *A. insuavis* isolates from patients with cystic fibrosis

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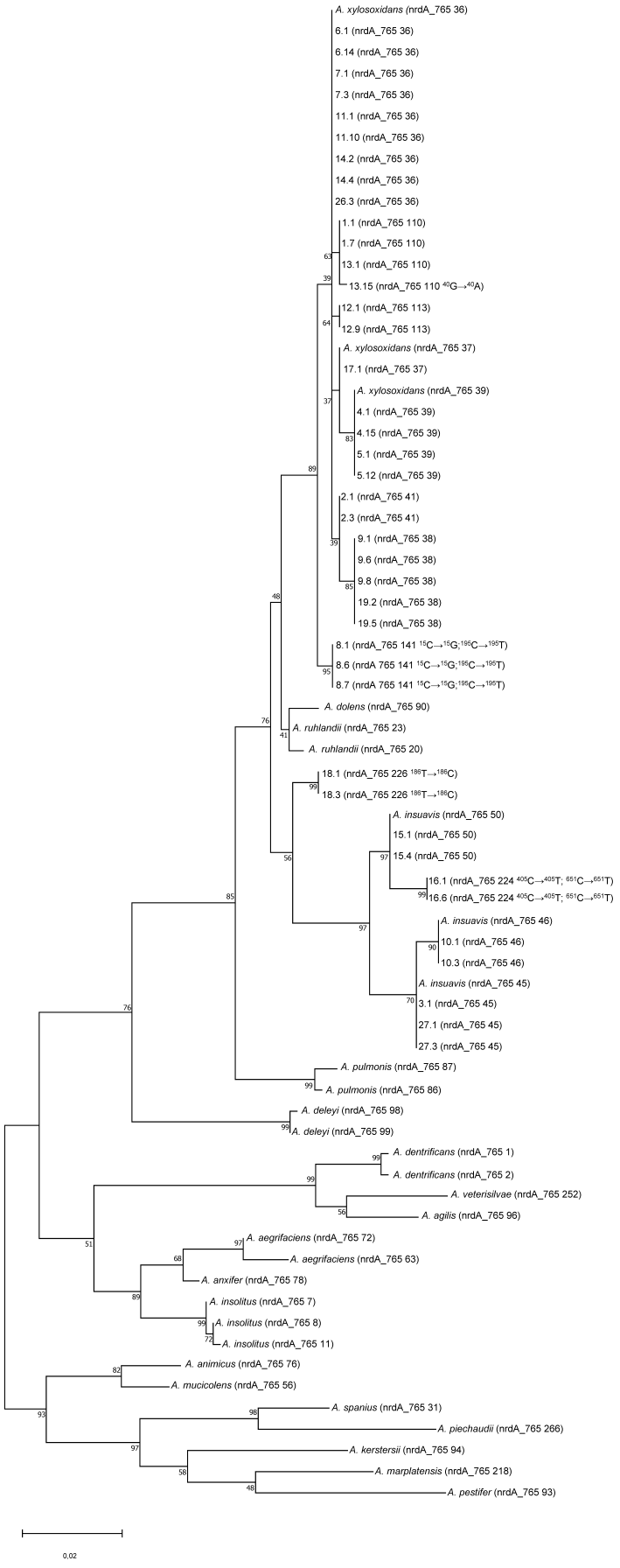

A. xylosoxidans (nrdA_765 36)
6.1 (nrdA_765 36)
6.14 (nrdA_765 36)
7.1 (nrdA_765 36)
7.3 (nrdA_765 36)
11.1 (nrdA_765 36)
11.10 (nrdA_765 36)
14.2 (nrdA_765 36)
14.4 (nrdA_765 36)
26.3 (nrdA_765 36)
1.1 (nrdA_765 110)
1.7 (nrdA_765 110)
13.1 (nrdA_765 110)
13.15 (nrdA_765 110 40G→40A)
12.1 (nrdA_765 113)
12.9 (nrdA_765 113)
A. xylosoxidans (nrdA_765 37)
17.1 (nrdA_765 37)
A. xylosoxidans (nrdA_765 39)
4.1 (nrdA_765 39)
4.15 (nrdA_765 39)
5.1 (nrdA_765 39)
5.12 (nrdA_765 39)
2.1 (nrdA_765 41)
2.3 (nrdA_765 41)
9.1 (nrdA_765 38)
9.6 (nrdA_765 38)
9.8 (nrdA_765 38)
19.2 (nrdA_765 38)
19.5 (nrdA_765 38)
8.1 (nrdA_765 141 15C→15G;195C→195T)
8.6 (nrdA 765 141 15C→15G;195C→195T)
8.7 (nrdA 765 141 15C→15G;195C→195T)
A. dolens (nrdA_765 90)
A. ruhlandii (nrdA_765 23)
A. ruhlandii (nrdA_765 20)
18.1 (nrdA_765 226 186T→186C)
18.3 (nrdA_765 226 186T→186C)
A. insuavis (nrdA_765 50)
15.1 (nrdA_765 50)
15.4 (nrdA_765 50)
16.1 (nrdA_765 224 405C→405T; 651C→651T)
16.6 (nrdA_765 224 405C→405T; 651C→651T)
A. insuavis (nrdA_765 46)
10.1 (nrdA_765 46)
10.3 (nrdA_765 46)
A. insuavis (nrdA_765 45)
3.1 (nrdA_765 45)
27.1 (nrdA_765 45)
27.3 (nrdA_765 45)
A. pulmonis (nrdA_765 87)
A. pulmonis (nrdA_765 86)
A. deleyi (nrdA_765 98)
A. deleyi (nrdA_765 99)
A. dentrificans (nrdA_765 1)
A. dentrificans (nrdA_765 2)
A. veterisilvae (nrdA_765 252)
A. agilis (nrdA_765 96)
A. aegrifaciens (nrdA_765 72)
A. aegrifaciens (nrdA_765 63)
A. anxifer (nrdA_765 78)
A. insolitus (nrdA_765 7)
A. insolitus (nrdA_765 8)
A. insolitus (nrdA_765 11)
A. animicus (nrdA_765 76)
A. mucicolens (nrdA_765 56)
A. spanius (nrdA_765 31)
A. piechaudii (nrdA_765 266)
A. kerstersii (nrdA_765 94)
A. marplatensis (nrdA_765 218)
A. pestifer (nrdA_765 93)
0.02

Figure S1a: Phylogenetic analysis of *Achromobacter* strains using the *nrdA*_765 typing scheme.

The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-3092,47) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,1236)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 70 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 765 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). Allelic match according to the nrdA_765 scheme is shown in brackets. If no exact match could be identified the nearest partial match is displayed with altered nucleobases. Closely related and all other *Achromobacter* species, for which *nrdA* typings are available are added to help better describing this collection.

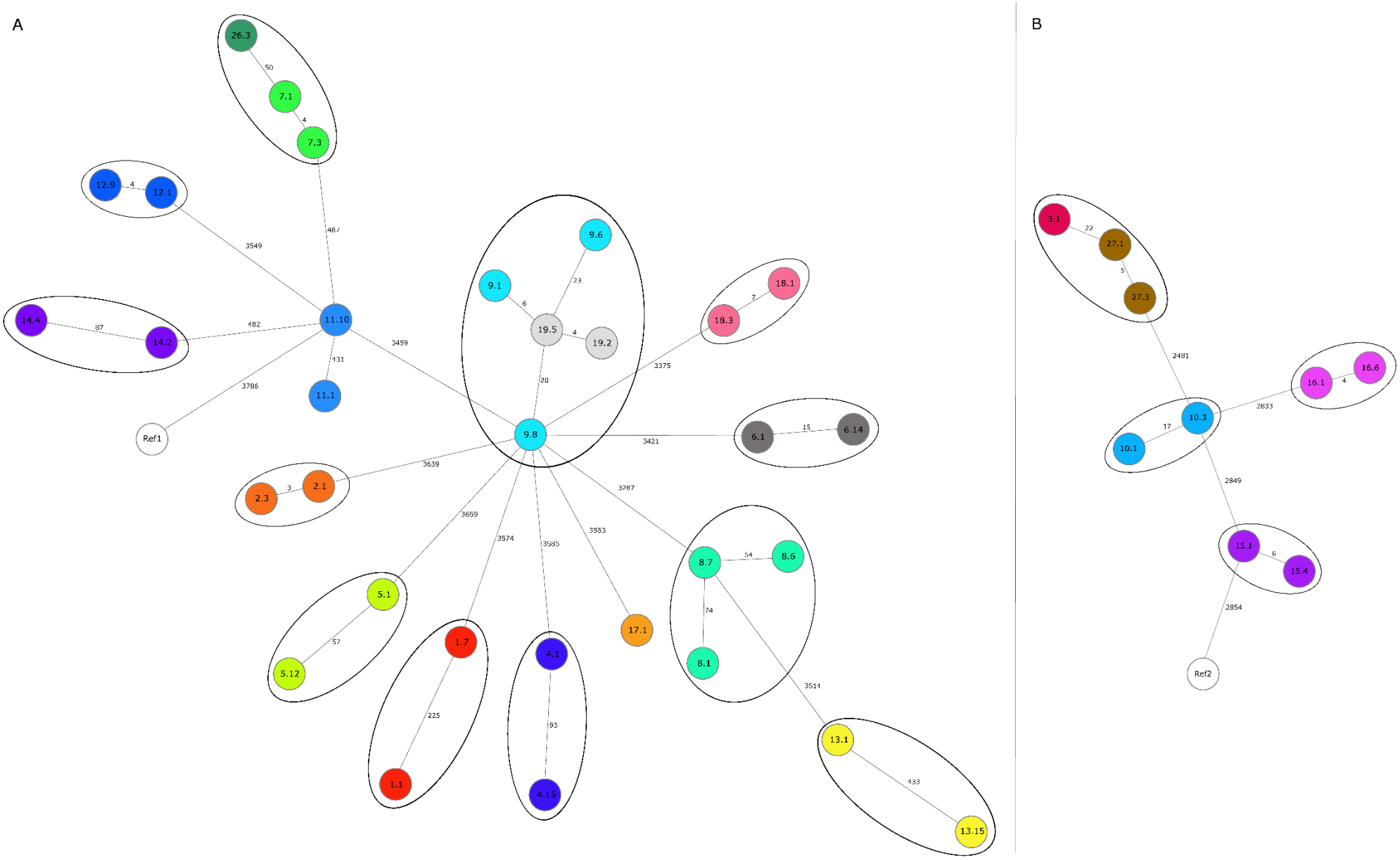

A
26.3
50
7.1
4
7.3
487
12.9
4
12.1
3549
14.4
97
14.2
482
11.10
131
11.1
3786
Ref1
3459
9.6
23
9.1
6
19.5
4
19.2
20
18.1
7
18.3
3375
9.8
3421
6.1
15
6.14
3639
2.3
3
2.1
3659
3574
3585
3553
3787
5.1
57
5.12
1.7
225
1.1
4.1
93
4.15
17.1
8.7
54
8.6
74
8.1
3514
13.1
433
13.15
B
3.1
22
27.1
5
27.3
2481
16.1
4
16.6
2833
10.3
17
10.1
2849
15.1
6
15.4
2854
Ref2

Figure S1b: Minimum spanning tree of 32 *A. xylosoxidans* (A) and 9 *A. insuavis* (B) strains isolated from 21 CF patients. Strains are displayed based on 5,778 cgMLST target genes, pairwise ignoring missing values and compared genetically to *A. xylosoxidans* (=Ref1; LN831029.1) and *A. insuavis* (=Ref2; GCA_003096315.1) reference genomes. Each circle stands for one genotype. Connecting lines indicate allelic differences between neighbour genotypes. Black borders surrounding genotypes point out genetic clusters based on allele changes over time. The same colour of circles indicates isolation of *Achromobacter* species of the same patient. Number on the circles indicate chronology.

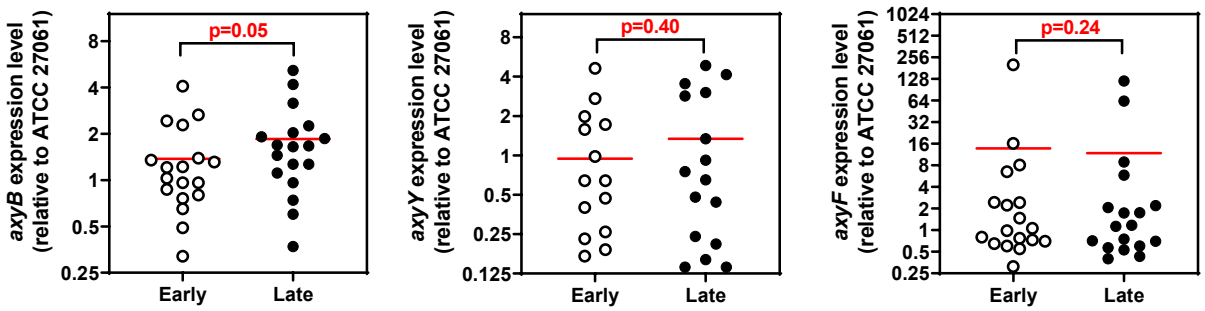
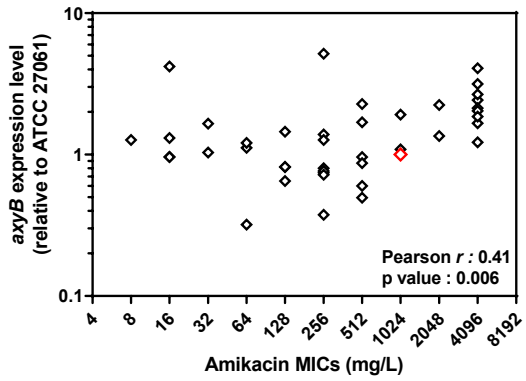
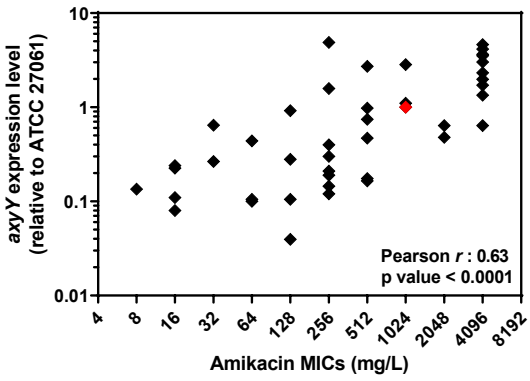
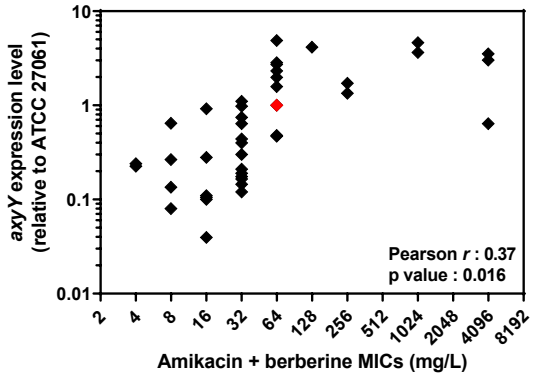
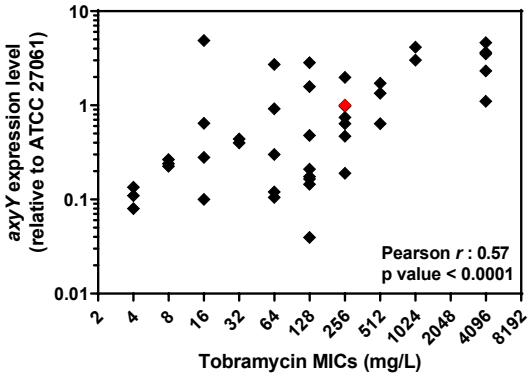


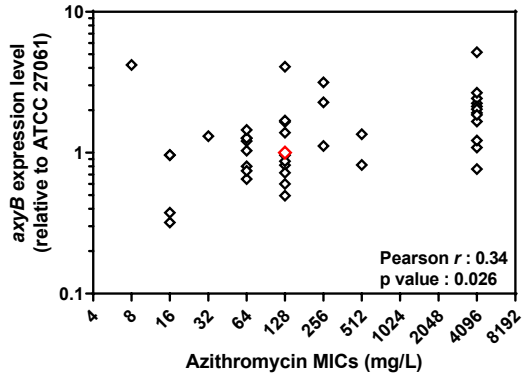
Figure S2: comparison of the expression level of *axyB*, *axyY*, or *axyF* genes in the early and late isolates from the 18 pairs collected successively in the same patients with an interval time of 1 to 11 years. Data are expressed as the expression level ($\Delta\Delta C_T$ target/gene/housekeeping gene) for each clinical isolate in comparison with the expression level measured in the reference strain ATCC 27061 (value set at 1). The red horizontal line corresponds to the mean value. Statistical analysis: Wilcoxon matched-pairs signed-rank test.

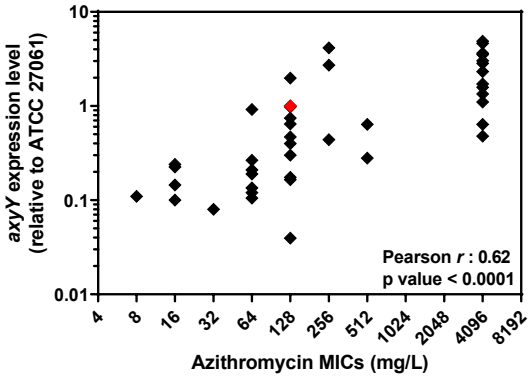

axyB expression level
(relative to ATCC 27061)
Pearson r : 0.41
p value : 0.006
Amikacin MICs (mg/L)

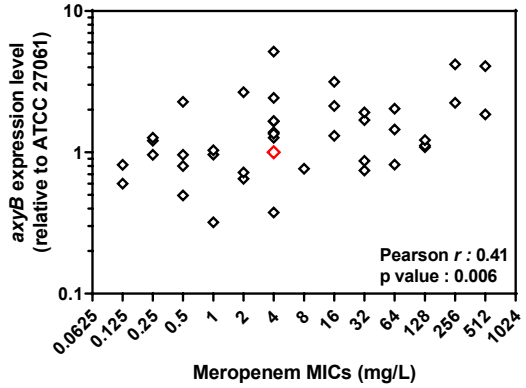

axyY expression level
(relative to ATCC 27061)
Pearson r : 0.63
p value < 0.0001
Amikacin MICs (mg/L)


axyY expression level
(relative to ATCC 27061)
Pearson r : 0.37
p value : 0.016
Amikacin + berberine MICs (mg/L)


axyY expression level
(relative to ATCC 27061)
Pearson r : 0.57
p value < 0.0001
Tobramycin MICs (mg/L)


axyB expression level
(relative to ATCC 27061)
Pearson r : 0.34
p value : 0.026
Azithromycin MICs (mg/L)


axyY expression level
(relative to ATCC 27061)
Pearson r : 0.62
p value < 0.0001
Azithromycin MICs (mg/L)


axyB expression level
(relative to ATCC 27061)
Pearson r : 0.41
p value : 0.006
Meropenem MICs (mg/L)

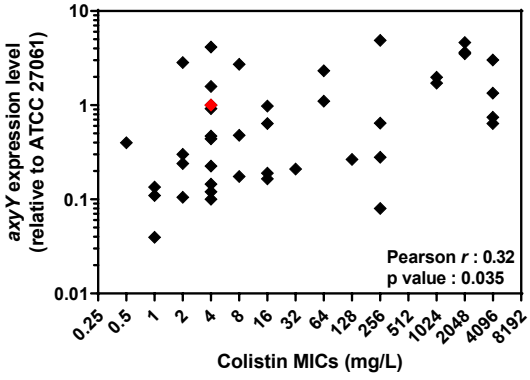

axyY expression level
(relative to ATCC 27061)
Pearson r : 0.32
p value : 0.035
Colistin MICs (mg/L)

Figure S3: Correlation between the expression level of *axyB, axyY* or *axyF* and the MIC of antibiotics. These graphs show only those data for which significant correlations were evidenced. See Supplementary Table S4 for the whole set of data. Correlations with *axyB* levels are shown with open symbols; those with *axyY,* with closed symbols; those with *axyF*, with grey symbols. Data are shown as the expression level (ΔΔCT target gene/housekeeping gene) for each clinical isolate in comparison with the expression level measured in the reference strain ATCC 27061 (value set at 1; illustrated in the graphs by the red symbols).

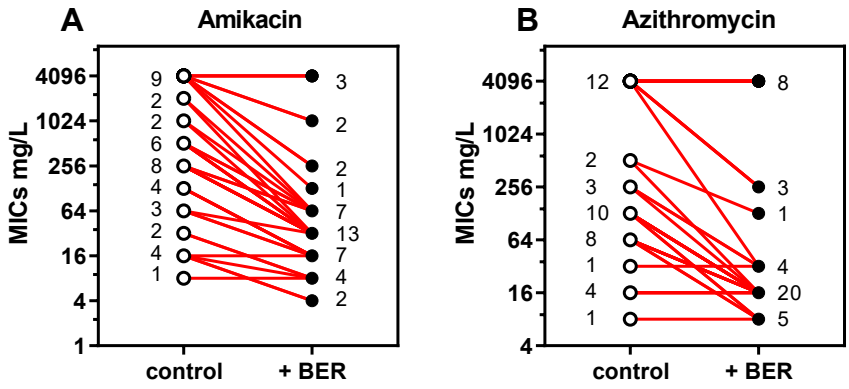


Figure S4: Influence of active efflux on amikacin, tobramycin and azithromycin activity. MICs of amikacin (A) and azithromycin (B) in the absence (control) of in the presence of 128 mg/L berberine (BER) for the 41 clinical isolates. The red lines joint data points for the same isolates. Figures on the left and on the right show the number of isolates presenting each MIC value.

Table S1: Primers and conditions* used for real-time PCR

Efflux systems	
axyB-Fwd	5'- AGGTGATCGAGCAGCAGATG -3'
axyB -Rev	5'- AACGTCAGCGTGATGGACATG -3'
axyY-Fwd	5'- TGGTGTTCTGCGTGATGTAC-3'
axyY-Rev	5'- ACATCGTCAGCACGTTGATC -3'
axyF-Fwd	5'- TTCGCTGCTGGACAACAAG-3'
axyF-Rev	5'- TCGTATTCGATGCGGTATTCC-3'
Housekeeping genes	
16S-Fwd	5'- ACAAGCGGTGGATGATGTG -3'
16S-Rev	5'- ATCTCTTCGGCATTCCAGACATG-3'

*Two min of cDNA denaturation at 98 °C, followed by 2 steps (39 cycles) of 10 s at 98 °C, 60 s at 64.4 °C. A melt curve was run at the end of the real-time PCR cycles, to check for the presence of a unique PCR reaction product.

Table S2: Relationship between efflux genes expression (mean of n=2) and antibiotic MICs in 41 *Achromobacter* CF isolates (versus the reference strain ATCC 27061)

Parameter **for AMK MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression levels**
Number of XY Pairs	42	42	42
Pearson r	0.4155	0.6289	-0.2044
95% confidence interval	0.1276 to 0.6388	0.4018 to 0.7832	-0.4787 to 0.1062
P value (two-tailed)	0.0062	P<0.0001	0.1940
P value summary	**	***	ns
Is the correlation significant? (alpha=0.05)	Yes	Yes	No
R squared	0.1726	0.3956	0.04180

Parameter **for AMK+BER MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.09429	0.3720	-0.1039
95% confidence interval	-0.2159 to 0.3872	0.07670 to 0.6073	-0.3954 to 0.2066
P value (two-tailed)	0.5526	0.0153	0.5124
P value summary	ns	*	ns
Is the correlation significant? (alpha=0.05)	No	Yes	No
R squared	0.008890	0.1384	0.01080

Parameter for **AZI MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.3422	0.6241	-0.1839
95% confidence interval	0.04260 to 0.5853	0.3950 to 0.7801	-0.4620 to 0.1273
P value (two-tailed)	0.0266	P<0.0001	0.2438
P value summary	*	***	ns
Is the correlation significant? (alpha=0.05)	Yes	Yes	No
R squared	0.1171	0.3894	0.03380

Parameter for **AZI+BER MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.05779	0.1873	-0.1428
95% confidence interval	-0.2506 to 0.3555	-0.1238 to 0.4648	-0.4282 to 0.1685
P value (two-tailed)	0.7162	0.2350	0.3670
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.003340	0.03507	0.02039

Parameter for **TMO MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.1170	0.2069	-0.2559
95% confidence interval	-0.1939 to 0.4065	-0.1036 to 0.4807	-0.5195 to 0.05220
P value (two-tailed)	0.4607	0.1886	0.1019
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No

Parameter **for PIP-TZB (4 mg/L) MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.1345	0.03159	-0.1301
95% confidence interval	-0.1767 to 0.4213	-0.2751 to 0.3324	-0.4176 to 0.1811
P value (two-tailed)	0.3958	0.8426	0.4116
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.01809	0.0009978	0.01692

Parameter **for MEM MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.4150	0.1463	-0.08230
95% confidence interval	0.1270 to 0.6384	-0.1651 to 0.4311	-0.3769 to 0.2274
P value (two-tailed)	0.0063	0.3553	0.6043
P value summary	**	ns	ns
Is the correlation significant? (alpha=0.05)	Yes	No	No
R squared	0.1722	0.02139	0.006774

Parameter **for CIP MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.09325	0.09895	0.2489
95% confidence interval	-0.2169 to 0.3863	-0.2114 to 0.3912	-0.05957 to 0.5140
P value (two-tailed)	0.5569	0.5330	0.1119
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.008696	0.009791	0.06197

Parameter **for TIC MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.2834	0.03582	-0.1719
95% confidence interval	-0.02250 to 0.5408	-0.2711 to 0.3362	-0.4523 to 0.1394
P value (two-tailed)	0.0689	0.8218	0.2764
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.08034	0.001283	0.02955

Parameter **for TIC-AVI (32 mg/L) MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.03222	0.09607	-0.1608
95% confidence interval	-0.2745 to 0.3330	-0.2142 to 0.3887	-0.4432 to 0.1505
P value (two-tailed)	0.8395	0.5450	0.3089
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.001038	0.009229	0.02587

Parameter **for CAZ MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	-0.04817	0.1270	-0.1215
95% confidence interval	-0.3471 to 0.2596	-0.1841 to 0.4150	-0.4103 to 0.1895
P value (two-tailed)	0.7619	0.4230	0.4435
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.002321	0.01612	0.01475

Parameter **for TOB MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.1731	0.5752	-0.1341
95% confidence interval	-0.1381 to 0.4533	0.3286 to 0.7483	-0.4209 to 0.1772
P value (two-tailed)	0.2728	P<0.0001	0.3973
P value summary	ns	***	ns
Is the correlation significant? (alpha=0.05)	No	Yes	No
R squared	0.02998	0.3308	0.01797

Parameter **for CST MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	0.1765	0.3338	-0.1353
95% confidence interval	-0.1347 to 0.4561	0.03312 to 0.5790	-0.4219 to 0.1760
P value (two-tailed)	0.2634	0.0308	0.3930
P value summary	ns	*	ns
Is the correlation significant? (alpha=0.05)	No	Yes	No
R squared	0.03116	0.1114	0.01830

Parameter **for CHL MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42
Pearson r	-0.1932	-0.2655	0.6610
95% confidence interval	-0.4695 to 0.1177	-0.5269 to 0.04179	0.4468 to 0.8035
P value (two-tailed)	0.2203	0.0892	<0.0001
P value summary	ns	ns	***
Is the correlation significant? (alpha=0.05)	No	No	Yes
R squared	0.03731	0.07050	0.4369

Parameter **for DOX MICs versus gene expression levels**	***axyB* expression levels**	***axyY* expression levels**	***axyF* expression level**
Number of XY Pairs	42	42	42

Pearson r	0.04138	-0.01640	-0.1684
95% confidence interval	-0.2697 to 0.3446	-0.3224 to 0.2927	-0.4526 to 0.1469
P value (two-tailed)	0.7973	0.9189	0.2926
P value summary	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No
R squared	0.001712	0.0002690	0.02836

Table S3: Resistance mechanisms to azithromycin, including ribosomal mutations in *rpl4, rpl22, rrl* genes and expression of efflux pumps. *A. insuavis* AXX-A (MIC, 64 mg/L) and *A. xylosoxidans* ATCC27061 (MIC, 128 mg/L) are used as references.

Strain identification[a]		AZI MIC (mg/L)		Ribosomal mutations[b]			Gene expression level	
Strain	**Isolation year**	**-BER[c]**	**+BER**	***rpl4***	***rpl22***	***rrl***	***axyB*[g]**	***axyY*[g]**
ATCC 27061		128	16	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, A2792G	1	1
16.1[h]	2016	16	16	Silent mutation (C219G)	-	A1385G, T1402C, A1471G, T1519C	0.96	0.23
16.6 [h]	2017	16	16	Silent mutation (C219G)	-	Missing data	0.96	0.24
15.1[h]	2009	16	16	-	-	Missing data	0.32	0.10
15.4[h]	2013	16	16	-	-	Missing data	0.37	0.14
6.1	2006	128	8	Silent mutations (T96C, T123C, C444T)	T134C (V45A) Silent mutations (T78C, T189C)	C141G, G148C, T275C, A371G, G1012A, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, A2792G	4.08	1.98
6.14	2017	8	8	Silent mutations (T96C, T123C, C444T)	T134C (V45A) Silent mutations (T78C, T189C)	C141G, G148C, T275C, A371G, G1012A, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, A2792G	4.19	0.11
13.1	2009	128	16	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C	T275C, G371A, G1012A, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, A2792G	0.87	0.98
27.1[h]	2010	64	16	Silent mutations (T411C)	T134C (V45A)	A120G, G1012A, T1178_A1179del, A1385G, T1402C	1.21	0.11
27.3[h]	2013	64	16	Silent mutations (T411C)	T134C (V45A)	A120G, G1012A, T1178_A1179del, A1385G, T1402C	1.27	0.12
10.1[h]	2010	32	32	Silent mutations (T411C)	T134C (V45A)	A120G, G1012A, T1178_A1179del, A1385G, T1402C, A1471G	1.31	0.08
10.3[h]	2011	256	32	Silent mutations (T411C)	T134C (V45A)	A120G, G1012A, T1178_A1179del, A1385G, T1402C, A1471G	1.11	0.44
1.1	2011	256	32	Silent mutations (T96C,	T134C (V45A) Silent mutations	C144T, T275C, A371G, G1012A, T1178_A1179del, A1385G, T1402C,	2.28	2.72

				T123C, T411C)	(T78C, T189C)	A1471G, T1519C, A2792G		
1.7	2015	256	16	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	T275C, A371G, G1012A, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, A2792G	3.16	4.15
7.1	2014	128	16	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, A1385G, T1402C, A2792G	0.96	0.47
26.3	2017	128	16	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, A1385G, T1402C, A2792G	0.72	0.30
17.1	2014	>2048	>2048	Silent mutations (T96C, T123C, T411C, C414T)	T134C (V45A) Silent mutations (T78C, T189C)	T275C, A371G, G1012A, T1175A, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, A2792G	1.09	1.11
4.1	2010	>2048	>2048	Silent mutations (T96C, T123C, T411C, C414T)	T134C (V45A) Silent mutations (T78C, T189C)	T275C, A371G, G1012A, C1170T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **A2043T**[f], A2792G,	2.43	1.72
4.15	2017	>2048	>2048	Silent mutations (T96C, T123C, T411C, C414T)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1170T, T1178_A1179del, A1385G, T1402C, A1471G, T1519C, **A2043T**, A2792G	1.67	1.34
5.1	2007	512	128	Silent mutations (T96C, T123C, T411C, C414T) **A194G (Q65R) uncharged to charged**	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, A2792G	1.35	0.64
5.12	2015	>2048	>2048	Silent mutations (T96C, T123C, T411C, C414T)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **A2043G**[f], A2792G	2.25	0.48

9.1	2010	64	8	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, A2792G	1.03	0.26
9.6	2012	512	16	Silent mutations (T96C, T123C, T411C), **G205C (G69R[d]) uncharged to charged**	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, A2792G	0.82	0.28
9.8	2013	>2048	32	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **A2044G[f],** A2792G	5.17	4.87
8.1	2010	>2048	256	Silent mutation (T96C, T123C, C330T, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **C2596T[e],** A2792G.	2.66	4.63
8.6	2014	>2048	256	Silent mutation (T96C, T123C, C330T, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **C2596T[e],** A2792G.	2.13	3.64
8.7	2015	>2048	256	Silent mutation (T96C, T123C, C330T, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, C1172T, T1178_A1179del, G1180A, A1385G, T1402C, A1471G, T1519C, **C2596T[e],** A2792G.	2.03	3.53
11.1	2010	>2048	>2048	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, **T1325C**, A1385G, T1402C, **A2043G,** A2792G	1.22	0.64
11.10	2015	>2048	>2048	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, **A1284G**, A1385G, T1402C, **A2044G,** A2792G	1.86	3.02

14.2	2014	>2048	>2048	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, A1385G, T1402C, **A2043G,** A2792G	0.76	1.58
14.4	2016	>2048	>2048	Silent mutations (T96C, T123C, T411C)	T134C (V45A) Silent mutations (T78C, T189C)	C144T, T275C, A371G, G1012A, T1175A, T1178_A1179del, A1385G, T1402C, **A2043G,** A2792G	1.91	2.84

[a]strain numbering: first figure, patient identification number; second figure, isolate number in this patient (late isolate over the whole period of sampling; see also Table S3). The strains gathered in the same quadrant without inside borders are successive isolates from the same patient. They are classified in order to show close from one another those which share the same mutations, ordered by increasing MICs.
[b] Reference sequence is that of *A. insuavis* AXX-A. (-) : same sequence as AXX-A. Mutations highlighted in blue are found in *A. xylosoxidans* ATCC 27061 when comparing its sequence with that *of A. insuavis* AXX-A which has a AZI MIC of 64 mg/L. Mutations in green have not been previously reported; mutations in red are found only in isolates with MIC ≥ 512 mg/L.
[c] used at 128 mg/L
[d] previously reported in macrolide-resistant *Streptococcus pneumoniae* (Clark et al., 2007; Kosowska-Shick et al., 2008) in linezolid-resistant *Staphylococcus epidermidis* (Mendes et al., 2012) and in macrolide-resistant *Burkholderia multivorans* form patient with CF (corresponding to position 70, G70R)(Silva et al., 2016).
[e] the sequence of AXX-A is numbered based on its alignment with that of the corresponding sequence in *P. aeruginosa* to facilitate the identification of previously described mutations.
[f] A2043T, A2043G, A2044G induced higher levels of resistance to azithromycin than C2596T in *P. aeruginosa* (Mustafa et al., 2017).
[g] as determined by qPCR.
[h]*A. insuavis*

Table S4: Resistance mechanisms to ciprofloxacin, including expression levels of *axyF* and mutations in *gyrA*, *gyrB*, *parC*, and *parE*. *A. insuavis* AXX-A (MIC, 1 mg/L) and *A. xylosoxidans* ATCC27061 (MIC, 4-8 mg/L) are used as references.

Sample identification[a]		CIP MIC (mg/L)[b]	*axyF* expression levels[c]	Mutations[d]			
Strains	Isolation year			*gyrA*	*gyrB*	*parC*	*parE*
ATCC 27061	ref	4-8	1	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384A, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
3.1	2012	1	0.7	G879A	Y64W, A258S, R639K	G281C, N392H, F508Y	-
15.1 [e]	2009	4	0.6	-	N592S, A631T, A633S, A634T, R639K	-	-
16.1	2016	16	201	**L454M**	-	-	-
18.1-18.3	2009-2011	4-8	1.1	T204S, I222V, T527S, D531E	N11T, G12S, N188T, I190V, A258T, A387G, N592G, S609A, E629D, S630T, R639K, E662A, V689I, R699K, R711Q, E720D, N726T, E759D	H7Q, V382I, V384A, N392H, K421N, V437A, E441Q, E447D, A474E, S482T, V536I, S591A, L693V, V741A, K764R	V11I, Y27H, Q32L, V41I, F132Y, A150T, S335A, S463T, N507H, A555S, S557A, T593S
9.1	2010	4	6.5	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, V417L, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
19.2	2009	32	16.2	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, V417L, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
17.1	2014	16	0.9	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f], **T881M**	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384A, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A -

4.1	2010	4	2.2	T204S, I222V, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
4.15	2017	16	2.2	**Q83L** T204S, I222V, D531E, ins 866_868, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
8.1	2010	8	0.7	T204S, I222V, A491T, D531E, M706V, ins 866_868, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, N392H K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, N507H, A555S, S557A
8.6	2014	32	0.7	**D87N**, T204S, I222V, A491T, D531E, M706V, ins 866_868, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, N392H K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, N507H, A555S, S557A
2.1	2016	4	0.8	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, S463T, N507H, A555S, S557A
5.1	2007	16	0.7	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, **I683V**, V689I, R699K, R711Q, A714V, E720D, N726T, E759D	H7Q, G281C, V382I, V384T, K421N, V437A, E447D, A474E, S482A, F508Y, V536I, S591A, L693V, V741A	V11I, Y27H, Q32L, F132Y, E219Q, S335A, N507H, A555S, S557A
5.12	2015	32	0.7	T204S, I222V, V488I, A491T, D531E, 866_868insGQE, D869E[f]	N188T, I190V, A258T, A387G, S581A, E629D, S630T, R639K, V649I, E662A, **I683V**, V689I,	H7Q, G281C, V382I, V384T, K421N, V437A, E447D, A474E, S482A, F508Y,	V11I, Y27H, Q32L, F132Y, E219Q, S335A, N507H, A555S, S557A

					R699K, R711Q, A714V, E720D, N726T, E759D	V536I, S591A, L693V, V741A
10.1	2010	4	0.7	G879A[f]	Y64W, A258S, R639K	G281C, N392H, F508Y -
10.3[e]	2011	128	63	G879A[f]	Y64W, A258S, R639K	G281C, N392H, F508Y -

[a]strain numbering: first figure, patient identification number; second figure, isolate number in this patient (late isolate over the whole period of sampling; see also Table S3). The strains are classified in order to show close from one another those which share the same mutations, ordered by increasing MICs. Strains gathered in the same quadrant without inside borders can be compared as showing additional mutations associated with higher MICs in the presence or absence of *axyF* overexpression.
[b] CLSI breakpoints (mg/L) for CIP: S ≤ 1; [c] as determined by qPCR.
[d] Reference sequence is that of *A. insuavis* AXX-A. **(-)**: same sequence as AXX-A. Mutations found in isolates with CIP MIC ≤ 1mg/L, ≥ 4, or 16 mg/L are highlighted in blue, green, or red colour respectively. Several mutations highlighted in green are also found in *A. xylosoxidans* ATCC 27061 when comparing its sequence with that *of A. insuavis* AXX-A which has a CIP MIC of 1 mg/L and could be associated with resistance.
[e]*A. insuavis*
[f] numbering based on the ATCC 27061 sequence (because located after the insertion of 3 aminoacids in this strain as compared to AXX-A)

References

Clark, C. L., Kosowska-Shick, K., Ednie, L. M., and Appelbaum, P. C. (2007). Capability of 11 antipneumococcal antibiotics to select for resistance by multistep and single-step methodologies. *Antimicrob. Agents Chemother.* 51, 4196-4201.

Kosowska-Shick, K., Clark, C., Credito, K., Dewasse, B., Beachel, L., Ednie, L., and Appelbaum, P. C. (2008). In vitro capability of faropenem to select for resistant mutants of Streptococcus pneumoniae and Haemophilus influenzae. *Antimicrob. Agents Chemother.* 52, 748-752.

Mendes, R. E., Deshpande, L. M., Costello, A. J., and Farrell, D. J. (2012). Molecular epidemiology of Staphylococcus epidermidis clinical isolates from U.S. hospitals. *Antimicrob. Agents Chemother.* 56, 4656-4661.

Mustafa, M. H., Khandekar, S., Tunney, M. M., Elborn, J. S., Kahl, B. C., Denis, O., Plesiat, P., Traore, H., Tulkens, P. M., Vanderbist, F., and Van Bambeke, F. (2017). Acquired resistance to macrolides in Pseudomonas aeruginosa from cystic fibrosis patients. *Eur. Respir. J.* 49, 1601847.

Nei, M. and Kumar, S. (2000). Molecular Evolution and Phylogenetics. 1-348.

Silva, I. N., Santos, P. M., Santos, M. R., Zlosnik, J. E. A., Speert, D. P., Buskirk, S. W., Bruger, E. L., Waters, C. M., Cooper, V. S., and Moreira, L. M. (2016). Long-Term Evolution of Burkholderia multivorans during a Chronic Cystic Fibrosis Infection Reveals Shifting Forces of Selection. *mSystems.* 1, e00029-16.

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38, 3022-3027.