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Role of AxyZ Transcriptional Regulator in Overproduction of AxyXY-OprZ Multidrug Efflux System in *Achromobacter* Species Mutants Selected by Tobramycin

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ABSTRACT AxyXY-OprZ is an RND-type efflux system that confers innate aminoglycoside resistance to *Achromobacter* spp. We investigated here a putative TetR family transcriptional regulator encoded by the *axyZ* gene located upstream of *axyXY-oprZ*. An in-frame *axyZ* gene deletion assay led to increased MICs of antibiotic substrates of the efflux system, including aminoglycosides, cefepime, fluoroquinolones, tetracyclines, and erythromycin, indicating that the product of *axyZ* negatively regulates expression of *axyXY-oprZ*. Moreover, we identified an amino acid substitution at position 29 of AxyZ (V29G) in a clinical *Achromobacter* strain that occurred during the course of chronic respiratory tract colonization in a cystic fibrosis (CF) patient. This substitution, also detected in three other strains exposed *in vitro* to tobramycin, led to an increase in the *axyY* transcription level (5- to 17-fold) together with an increase in antibiotic resistance level. This overproduction of AxyXY-OprZ is the first description of antibiotic resistance acquisition due to modification of a chromosomally encoded mechanism in *Achromobacter* and might have an impact on the management of infected CF patients. Indeed, tobramycin is widely used for aerosol therapy within this population, and we have demonstrated that it easily selects mutants with increased MICs of not only aminoglycosides but also fluoroquinolones, cefepime, and tetracyclines.

KEYWORDS *Achromobacter*, AxyZ, AxyXY-OprZ, tobramycin, cystic fibrosis, efflux

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Achromobacter spp. belong to the Gram-negative nonfermenters respiratory pathogens that are emerging in cystic fibrosis (CF) patients (1). The *Achromobacter* genus includes 18 species: *A. xylosoxidans* (2); *A. piechaudii* and *A. ruhlandii* (3); *A. denitrificans* (4), *A. spanius*, and *A. insolitus* (5); *A. marplatensis* (6); *A. animicus*, *A. mucicolens*, and *A. pulmonis* (7, 8); *A. insuavis*, *A. aegrifaciens*, *A. anxifer*, and *A. dolens* (9); *A. agilis*, *A. pestifer*, *A. kerstersii*, and *A. deleyi* (10). The most prevalent species in CF are *A. xylosoxidans*, *A. ruhlandii*, and *A. insuavis* (11, 12). The pathogenic role of *Achromobacter* in CF remains unclear, but during the course of chronic lung infection, it might cause a level of inflammation similar to caused by *Pseudomonas aeruginosa* (13). Some guidelines suggest that patients chronically infected with *Achromobacter* or patients with clinical degradation should be treated (14). Nevertheless, *Achromobacter* displays not only innate but also frequently acquired multidrug resistance, particularly in the case of chronic colonization, making treatment a real challenge (15). The mechanisms of acquired resistance are unknown; only a few β-lactamase-encoding genes located on mobile genetic elements have been reported thus far (16–20).

Two resistance-nodulation-cell division (RND)-type efflux systems have been de-

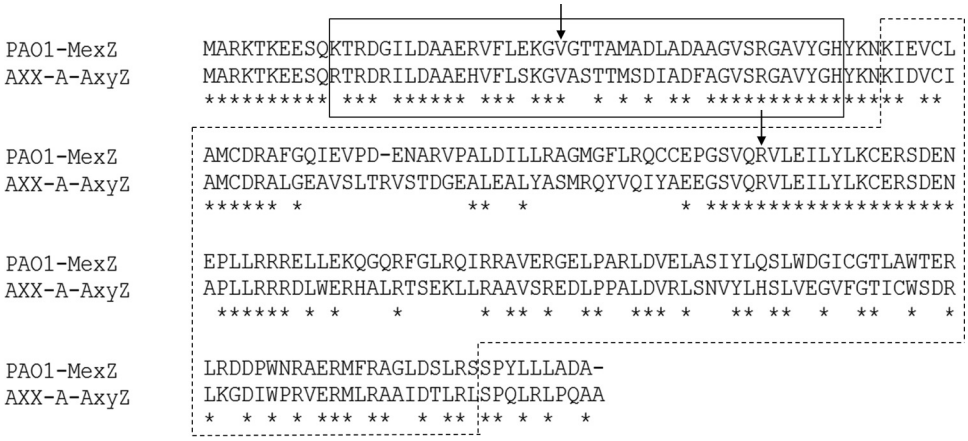

FIG 1 Alignment of MexZ of *Pseudomonas aeruginosa* (PAO1 strain) and AxyZ of *Achromobacter insuavis* (AXX-A strain) (Clustal Omega). The area boxed with the solid line delimits the DNA-binding domain, and the area boxed with the dashed line delimits the C-terminal domain (41). Amino acid sequence similarity was as follows: overall, 59%; DNA-binding domain, 76%; C-terminal domain, 51%. Stars indicate identical residue positions; arrows indicate positions of substitutions detected in laboratory mutants, i.e., valine at position 29 and arginine at position 105.

scribed to date in *Achromobacter* and contribute to its intrinsic antibiotic resistance: AxyABM and AxyXY-OprZ (21, 22). The latter system is encoded by three chromosomal genes: *axyX*, *axyY*, and *oprZ*. The first gene encodes the membrane fusion protein, the second encodes the RND pump, and the third encodes the outer membrane protein. AxyXY-OprZ is responsible for the innate high-level aminoglycoside (AG) resistance of the *Achromobacter* species that are the most prevalent in CF patient airways (23). The antibiotic substrate range of AxyXY-OprZ is wide and includes, in addition to AG, cefepime, fluoroquinolones, tetracyclines, and, to a lesser extent, carbapenems, ceftazidime, and erythromycin. Several of these antibiotics (ceftazidime, carbapenems, ciprofloxacin, tobramycin, and amikacin) are major compounds commonly used to treat pulmonary infections in CF patients (14).

AxyX and AxyY are orthologs of the well-described MexX and MexY in *Pseudomonas aeruginosa* (24). AxyXY-OprZ and MexXY/OprM share a similar antibiotic substrate range. It has been demonstrated that MexXY/OprM plays an important role in multidrug resistance acquisition in *P. aeruginosa*, particularly in CF patients (25, 26). Its expression level is controlled by multiple pathways, including local and global regulatory factors (27, 28). Among these factors the local repressor MexZ that belongs to the TetR family has been widely studied (29). Various mutations have been described within the *mexZ* gene as well as within the intergenic sequence between *mexZ* and *mexX* (25, 30). *mexZ* mutations lead to overexpression of *mexXY* and an increase in the MICs of antibiotic substrates (31). It has been demonstrated that mutations in *mexZ* can be selected by exposure of *P. aeruginosa* to AG (30). In addition, MexZ activity is downregulated in the presence of ribosome inhibitor antibiotics (32, 33).

We have previously described upstream of *axyXY-oprZ* and in an inverted orientation an open reading frame, named *axyZ*, encoding a putative TetR family transcription repressor (22). AxyZ shares 59% amino acid similarity with MexZ of *P. aeruginosa* (Fig. 1). In the present study, we examined the role of *axyZ* in the expression of *axyXY-oprZ*. Furthermore, we looked for *axyZ* mutations in CF isolates that acquired antibiotic resistance during chronic colonization and in laboratory mutants selected by tobramycin exposure. Finally, we tested the inducibility of *axyXY-oprZ* by doxycycline and tobramycin, which are ribosome-targeting antibiotics.

RESULTS

Consequences of in-frame *axyZ* gene deletion. In the AXX-A strain with a deletion of *axyZ* (AXX-A-ΔZ) the MICs of all antibiotic substrates of AxyXY-OprZ except for carbapenems and ceftazidime were increased (Table 1). MICs of antibiotics were from 1.3-fold to more than 10-fold higher in the AXX-A-ΔZ strain than in AXX-A. In

TABLE 1 MICs of AxyXY-OprZ antibiotic substrates for *Achromobacter* isolates and their mutants

	MIC (μg/ml)[a]										
Drug	AXX-A	AXX-A-ΔZ	ACH-CF-490	ACH-CF-911	CIP-102236	CIP-102236$_{V29G}$	CIP-102236$_{R105S}$	ACH-NCF-39	ACH-NCF-39$_{V29G}$	ACH-CF-842	ACH-CF-842$_{V29G}$
Tobramycin	16	128 (8)	32	64 (2)	32	256 (8)	>256 (>8)	24	96 (4)	8	32 (4)
Amikacin	256	>256	≥256	>256	>256	>256	>256	192	>256 (>1.3)	32	>256 (>8)
Gentamicin	48	>256 (>5.3)	48	256 (5.3)	192	>256 (>1.3)	>256 (>1.3)	48	>256 (>5.3)	12	≥256 (≥21)
Netilmicin	24	>256 (>10.7)	64	≥256 (≥4)	>256	>256	>256	64	>256 (>4)	16	>256 (>16)
Ceftazidime	4	4	4	4	1.5	4 (2.6)	3 (2)	1.5	3 (2)	1.5	1.5
Cefepime	16	128 (8)	192	>256 (>1.3)	48	>256 (>5.3)	>256 (>5.3)	24	>256 (>10)	8	32 (4)
Imipenem	1	1	2	4 (2)	2	2	2	6	8 (1.3)	2	4 (2)
Meropenem	0.064	0.064	0.064	6 (94)	0.094	0.125 (1.3)	0.125 (1.3)	0.094	0.125 (1.3)	0.75	0.75
Doripenem	0.25	0.25	0.25	2 (8)	0.38	0.5 (1.3)	0.5 (1.3)	0.25	0.38 (1.5)	1	1
Ofloxacin	2	12 (6)	≥32	>32	16	>32 (>2)	>32 (>2)	6	>32 (>5.3)	24	>32 (>1.3)
Levofloxacin	1	4 (4)	3	>32 (>10)	3	12 (4)	32 (>10)	1.5	6 (4)	4	8 (2)
Ciprofloxacin	0.75	2 (2.6)	1.5	>32 (>21)	2	8 (4)	>32 (>16)	1.5	8 (5.3)	2	6 (3)
Tetracycline	48	64 (1.3)	192	>256 (>1.3)	96	128 (1.3)	>256 (>2.7)	192	≥256 (≥1.3)	48	192 (4)
Doxycycline	8	12 (1.5)	24	96 (4)	12	16 (1.3)	32 (2.7)	16	24 (1.5)	6	8 (1.3)
Tigecycline	4	8 (2)	3	3	2	2	3 (1.5)	1.5	2 (1.3)	0.38	0.5 (1.3)
Erythromycin	128	256 (2)	192	>256 (>1.3)	96	>256 (>2.7)	>256 (>2.7)	32	128 (4)	>256	>256

[a]Values in parentheses represent relative MIC increases (ratio of the MIC for the mutant isolate to the MIC for the original isolate).

TABLE 2 Relative expression levels of *axyY* genes in mutant versus parental strains

Parental strain	Mutant	Relative *axyY* expression (CV)[a]
AXX-A	AXX-A-ΔZ	9.18 (0.43)
ACH-CF-490	ACH-CF-911	4.98 (0.39)
CIP-102236	CIP-102236$_{V29G}$	5.17 (0.37)
CIP-102236	CIP-102236$_{R105S}$	9.16 (0.1)
ACH-NCF-39	ACH-NCF-39$_{V29G}$	17.32 (0.44)
ACH-CF-842	ACH-CF-842$_{V29G}$	6.44 (0.33)

[a]Fold increase in the mutant compared to the level in the parental strain. CV, coefficient of variation.

addition, the level of *axyY* expression was 9.18-fold higher in AXX-A-ΔZ than in AXX-A (mean value from five independent experiments with a coefficient of variation of 0.43) (Table 2).

***axyZ* sequencing in clinical isolates.** Two mutations were detected in strain ACH-CF-911 compared to the sequence of ACH-CF-490. The first one occurred in the *axyZ-axyX* intergenic sequence (G → A substitution at nucleotide position 169 upstream of the start codon of *axyZ*). The second one, which occurred within *axyZ* (86T → G), was a nonsynonymous substitution, exchanging a valine for a glycine at amino acid position 29 of AxyZ (V29G). From 2004 (isolation year of ACH-CF-490) to 2012 (isolation year of ACH-CF-911), we collected successively 13 isogenic isolates from this patient and sequenced the *axyZ* genes. Substitution V29G has been found in 11 out of the 13 isolates, with the first one being isolated in 2007.

For the other pairs of clinical isolates, we did not detect any mutation in either *axyZ* or the *axyZ-axyX* intergenic sequence when we compared the sequence of the second isolate to that of the first.

***axyZ* mutation assays (laboratory mutants).** One-step mutants were obtained for CIP-102236, ACH-NCF-39, and ACH-CF-842 on medium containing tobramycin concentrations ranging from 2× to 8× the MIC of the parental strains. Mutation frequency was approximately 10^{-7}. *axyZ* was sequenced in all the mutants that harbored increased resistance to antibiotic substrates of AxyXY-OprZ.

In some mutants for which the antibiotic MICs were increased, no mutation was detected. Nevertheless, in other mutants we detected two amino acid substitutions within AxyZ that were stable after successive subcultures (Fig. 1). The first one, R105S, was encountered in a single mutant, obtained from CIP-102236, and it was named CIP-102236$_{R105S}$. Interestingly, the second one, V29G (already detected in the clinical isolate ACH-CF-911) was found in several mutants, named CIP-102236$_{V29G}$, ACH-NCF-39$_{V29G}$, and ACH-CF-842$_{V29G}$. MICs of aminoglycosides, cefepime, fluoroquinolones, tetracyclines, and erythromycin were higher in R105S and V29G mutants than in parental strains (Table 1). The increase in the MICs was similar to that observed between the AXX-A and AXX-A-ΔZ strains. MICs of ceftazidime, meropenem, and doripenem were slightly increased in three of the four mutants.

Relative gene expression measurement of *axyY*. *axyY* relative transcript levels in AXX-CF-911, CIP-102236$_{V29G}$, CIP-102236$_{R105S}$, ACH-NCF-39$_{V29G}$, and ACH-CF-842$_{V29G}$ were, respectively, 4.98-, 5.17-, 9.16-, 17.32-, and 6.44-fold higher than in parental strains. Use of either the *rpsL* or *rpoD* housekeeping gene led to similar results. Furthermore, interexperiment reproducibility showed that using reverse transcription-quantitative PCR (RT-qPCR) with the present protocol is a robust method for measuring gene expression levels in *Achromobacter*.

Induction assay by ribosome-targeting antibiotics. *axyY* expression was increased (6.52-fold) in AXX-A after exposure to doxycycline (1 μg/ml). Exposure to tobramycin led to increased *axyY* expression depending on the drug concentration: 1.86-fold for 4 μg/ml, 5.93-fold for 8 μg/ml, and 6.35-fold for 16 μg/ml.

DISCUSSION

In the present work we have confirmed that *axyZ* encodes a transcriptional repressor of *axyXY-oprZ*. Indeed, in-frame *axyZ* gene deletion experiments led to an increased

transcript level of *axyY* as well as increased MICs of antibiotic substrates of AxyXY-OprZ. Two types of mutants of *axyZ* were obtained by tobramycin exposure. The substitution V29G that resulted in overexpression of *axyXY-oprZ* is particularly interesting. Indeed, it has been detected in not only a clinical isolate but also three laboratory mutants. On the one hand, these clinical and laboratory mutants displayed an antibiotic resistance evolution similar to that observed between AXX-A and AXX-A-ΔZ. On the other hand, all mutants overexpressed *axyY*. It is likely that the V29G substitution alters AxyZ, preventing the repression of *axyXY-oprZ*. It is noteworthy that most of the mutations reported in *mexZ* of *P. aeruginosa* occur either in the DNA-binding domain or in the C-terminal domain (28, 34). Position 29 in MexZ belongs to the DNA-binding domain of MexZ (Fig. 1). Furthermore, comparison of amino acid sequences of AxyZ from *A. insuavis* AXX-A, MexZ from *P. aeruginosa* PAO1, and AmrR (ortholog of MexZ) from *Burkholderia pseudomallei* 1026b (35) revealed that valine at position 29 is a conserved residue, suggesting its probable importance in the function of the members of the TetR family.

In some laboratory mutants selected on tobramycin, we did not detect any mutation in either *axyZ* or the *axyZ-axyX* intergenic sequence, whereas these genes harbored increased resistance levels to AxyXY-OprZ antibiotic substrates. Moreover, the increase of *axyY* expression was variable among the V29G mutants (5- to 17-fold higher than in parental strains). These observations strongly suggest that *axyXY-oprZ* expression depends on not only *axyZ* but also other regulatory pathways, as previously described in *P. aeruginosa* (27). Among them, the PA5471 gene indirectly activates *mexXY* by repressing *mexZ* when bacteria are exposed to antibiotics that target the ribosome (33). We observed that *axyY* expression was induced in AXX-A in the presence of subinhibitory concentrations of doxycycline or tobramycin. An ortholog of the PA5471 gene is present in most of the *Achromobacter* published genomes but, surprisingly, not in that of AXX-A. The adaptation of efflux expression in response to antibiotic exposure probably results from multiple regulatory mechanisms.

As exposure to tobramycin can induce mutations in *axyZ*, one might wonder about potential consequences of AG therapy among CF patients. Indeed, analysis of medical data of a patient chronically colonized by the resistant mutant ACH-CF-911 revealed that the patient received tobramycin aerosol therapy on two occasions, in 2005 and 2008. Although the first mutant isolate had been detected after the first tobramycin cure, we could not correlate the mutation event with tobramycin treatment because the patient also received other, different, antibiotics. Therefore, further studies should be conducted on a larger number of clinical isolates from patients treated or not by AG. We observed that the V29G substitution is stable *in vivo*. Indeed, it has been detected in all isolates obtained from 2007 to 2012. In some cases, mutant and nonmutant isogenic subpopulations coexisted in the respiratory airways.

In conclusion, our results strongly suggest that *axyZ* encodes the local negative regulator of *axyXY-oprZ* in *Achromobacter* spp. Some stable mutations in *axyZ* that can be selected by tobramycin led to overexpression of the efflux system and increased MICs of aminoglycosides, fluoroquinolones, cefepime, and tetracyclines. The prevalence of this mechanism in clinical isolates and the potential role of tobramycin aerosol therapy in selecting resistant mutants have to be studied.

MATERIALS AND METHODS

Bacterial strains. *Achromobacter* species strains used in this work are listed and described in Table 3. Species-level identifications were performed by *nrdA* sequencing (11).

The AXX-A strain, initially assigned to the species *A. xylosoxidans*, was reclassified as *A. insuavis* when the multilocus sequence typing (MLST) identification scheme for *Achromobacter* was published (9, 23, 36). AXX-A has been used in previous studies to characterize AxyABM and AxyXY-OprZ RND-type efflux systems (21, 22). It was used in the present work to examine the role of *axyZ* in the expression of *axyXY-oprZ* by gene deletion.

Five pairs of *Achromobacter* isolates from CF sputum samples (four pairs of *A. xylosoxidans* and one pair of *A. insuavis*) from five different patients were included. Each pair of *Achromobacter* isolates of the CF sputum samples had the following characteristics: both isolates were recovered from the same patient at different periods (interval ranging from 5 to 11 years), they shared the same pulsed-field gel

TABLE 3 Isolates used in the study

Isolate	Species	Origin	*axyZ* application
AXX-A	*A. insuavis*	Ear discharge	Inactivation
ACH-CF-490, ACH-CF-911[a]	*A. xylosoxidans*	CF sputum	Sequencing
CIP-102236	*A. xylosoxidans*	Sputum	Mutation assays
ACH-NCF-39	*A. xylosoxidans*	Catheter insertion site	Mutation assays
ACH-CF-842	*A. xylosoxidans*	CF sputum	Mutation assays

[a]Isogenic isolates.

electrophoresis profile (isogenic isolates [data not shown]), and the second isolate of each pair harbored antibiotic resistance levels higher than those of the first one, indicating resistance acquisition during chronic colonization.

Three other *A. xylosoxidans* strains were included for *axyZ* mutation assays: CIP-102236 from the Institut Pasteur collection, ACH-NCF-39 recovered from a catheter insertion site skin swab, and ACH-CF-842 from a CF patient sputum sample.

This study was performed in accordance with the principles of good clinical practice and the need for ethics committee approval was waived.

MICs determinations. MICs of tobramycin, amikacin, gentamicin, netilmicin, ceftazidime, cefepime, imipenem, meropenem, doripenem, ofloxacin, levofloxacin, ciprofloxacin, tetracycline, doxycycline, tigecycline, and erythromycin were measured by the Etest method (bioMérieux) as recommended by the manufacturer and as previously described (21, 22). Mueller-Hinton agar plates (Mueller-Hinton E medium; bioMérieux) were inoculated by swabbing the surfaces of the plates with a bacterial suspension adjusted to a 0.5 McFarland turbidity standard, and MICs were recorded after a 24-h incubation at 37°C.

Bacterial DNA preparation, amplification, and sequencing of *axyZ*. Bacterial DNA templates were prepared by heat lysis of cells. A bacterial suspension in 300 μl of distilled water was boiled for 10 min and then centrifuged (10,000 $\times$ *g* for 5 min). The supernatant was used as the DNA template. The *axyZ*-flanking degenerate primers axyZ-F and axyZ-R (Table 4) were designed for sequencing the whole *axyZ* gene as well as the *axyZ-axyX* intergenic sequence. PCR was carried out in reaction mixtures containing deoxynucleoside triphosphates (dNTP; 0.2 mM), axyZ-F and axyZ-R primers (0.25 μM each), *Taq* polymerase (2.5 U; Fermentas) with the supplied buffer, $MgCl_2$ (1.5 mM), dimethyl sulfoxide (5%, vol/vol), and DNA template (1 μl), adjusted with water to a final volume of 50 μl. The cycling parameters were 94°C for 10 min, followed by 30 cycles of 94°C for 60 s, 60°C for 90 s, and 72°C for 90 s, with a final step at 72°C for 10 min. PCR products were purified with a Millipore centrifugal filter unit (Amicon Microcon PCR kit; Millipore). Double-strand sequencing was performed using BigDye, version 1.1, Terminator chemistry and a 3130XL genetic analyzer (Applied Biosystems).

In-frame *axyZ* gene deletion. An in-frame *axyZ* gene deletion in AXX-A was constructed as previously described (27, 37). A DNA fragment of the 700 nucleotides upstream of the *axyZ* gene and the 700 nucleotides downstream of *axyZ* were synthesized (GeneArt; Thermo Fisher Scientific). This fragment was cloned into the pKNG101 vector in *Escherichia coli* CC118 *λpir*. The recombinant plasmid pINA-axyZ was transferred into AXX-A by conjugation using the helper strain *E. coli* HB101 that contains the pRK2013 vector. Recombinant AXX-A strains were selected on *Pseudomonas* isolation agar containing 5 mg/ml streptomycin and cultivated on Luria-Bertani (LB) agar containing 5% sucrose in order to force the recombination event leading to gene deletion. The deletion was confirmed by sequencing (primers Screen-deltaAxyZ-F, Screen-deltaAxyZ-R, Seq-deltaAxyZ-1, Seq-deltaAxyZ-2, Seq-deltaAxyZ-3, and Seq-deltaAxyZ-4) (Table 4). The knockout clone was named AXX-A-ΔZ.

Selection of one-step mutants. CIP-102236, ACH-NCF-39, and ACH-CF-842 were grown by plating 100 μl of a bacterial suspension adjusted to a 0.5 McFarland turbidity standard on Mueller-Hinton agar containing tobramycin at concentrations of 2×, 4×, 8×, and 16× the MIC of each strain. Such

TABLE 4 Primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]
axyZ-F	ATGCGCTTGGACAGYGACGA
axyZ-R	CGGATRCGARTGCGAGCGTT
Screen-deltaAxyZ-F	TTGGAAGGAATATGGCAACG
Screen-deltaAxyZ-R	ACGTAGATCGGGTCGAGTTG
Seq-deltaAxyZ-1	CCCTCAAGCAACTCGAAGC
Seq-deltaAxyZ-2	GCTTCGAGTTGCTTGAGGG
Seq-deltaAxyZ-3	CGCCAGCATACTTTTGAACA
Seq-deltaAxyZ-4	TGTTCAAAAGTATGCTGGCG
axyY-qPCR-F3	TGTATTCGGTCAACGGCTTC
axyY-qPCR-R4	GCGCGAACACCATCAGGTT
rpsl-qPCR-1F	ACGCACTTTGGCAACCTTAC
rpsl-qPCR-1R	GCGAAGTCAGCATCATCAAA
rpoD-qPCR-F1	AAGGACGGCTACAAGTCGGA
rpoD-qPCR-R1	GCCAGCTTCTCGACCATCTT

[a]The nucleotide base code for degenerate primers is as follows: Y is C or T; R is A or G.

concentrations were previously chosen by Islam et al. and by Westbrock-Wadman et al. to select *Pseudomonas aeruginosa* aminoglycoside-resistant mutants that overexpressed *mexXY* (30, 38). Plates were incubated for 48 to 72 h at 37°C. For each mutant we performed antibiotic susceptibility testing and compared the profile of the mutant to that of the parental strain. In the case of acquired resistance, the whole *axyZ* gene was sequenced.

Relative gene expression measurement of *axyY*. The transcript level of *axyY* was determined by RT-qPCR as follows.

(i) RNA extraction. Strains were inoculated into Luria-Bertani broth (10 g/liter tryptone [Difco Laboratories], 5 g/liter yeast extract [Oxoid], and 10 g/liter sodium chloride [VWR]) at 37°C overnight and subcultured in 20 ml of LB broth at 37°C with shaking until mid-exponential phase (optical density at 600 nm [OD_{600}] of 1 to 1.2). Culture (0.5 ml) was added to 1 ml of RNAprotect bacteria reagent (Qiagen), bacterial cells were pelleted (5,000 × *g*, 10 min), and total RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions and eluted twice in 30 μl of RNase-free water. Residual genomic DNA was removed by 10 units of DNase treatment (RQ1 RNase-Free DNase; Promega) for 30 min at 37°C. RNA was purified by ethanol (2 volumes) and sodium acetate (1/10 volume) precipitation. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

(ii) Reverse transcription. One microgram of RNA was used for total cDNA synthesis using an ImProm-II reverse transcription system (Promega) with the random hexamer primer protocol. A control without reverse transcriptase was performed and then included in the PCR step to ensure total genomic DNA removal. Aliquots of serial dilutions in DNA-free water (1:10, 1:100, and 1:1,000) of each cDNA were conserved at −20°C until use in the PCR experiment.

(iii) Quantitative PCR. qPCRs were performed on a LightCycler 2.0 instrument (Roche) in reaction mixtures containing 4 μl of LightCycler FastStart DNA Master Plus SYBR green I master mix (Roche), forward and reverse primers (0.5 μM each), and cDNA (1 μl), adjusted with DNase-free water to a final volume of 20 μl. The cycling parameters were the following: 95°C for 10 min (denaturation); 40 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 20 s (amplification, single fluorescence acquisition mode); 64°C to 95°C with a heating slope of 0.1°C per s (melting curve, continuous fluorescence acquisition mode); and a final cooling step at 40°C for 30 s. Primers were designed in conserved sequences of the three targeted genes: *axyY* and two reference housekeeping genes, *rpsL* and *rpoD*. *rpsL*, which encodes a ribosomal protein, is commonly used for measurement of relative gene expression in *P. aeruginosa* (25). In the absence of any available data about a gene expression measurement method in *Achromobacter*, it was necessary to confirm our results by using another housekeeping gene. For this purpose, we chose *rpoD*, encoding one of the sigma factors that confer promoter-specific transcription initiation on RNA polymerase (39). All the PCRs targeting *axyY*, *rpsL*, and *rpoD* of each mutant and the respective parental strain were performed in duplicate within the same qPCR experiment, and each experiment was repeated at least twice (from two independently RNA extractions). Each qPCR experiment included 1:10, 1:100, and 1:1,000 cDNA dilutions of the parental strain in order to calculate the real-time PCR efficiency of each targeted gene. For calculating relative expression, we used the method described by Pfaffl (40).

Induction assay by ribosome-targeting antibiotics. We performed induction assays of *axyXY-oprZ* in AXX-A by cultivating the strain in the presence of subinhibitory concentrations of doxycycline (1 μg/ml) or tobramycin (4, 8, and 16 μg/ml), followed by measurement of the *axyY* expression level by RT-qPCR.

Accession number(s). Nucleotide sequences of the *axyZ* genes in the following strains have been submitted to the GenBank under the indicated accession numbers: ACH-CF-490, KY630178; ACH-CF-911, KY630179; CIP-102236, KY630180; CIP-102236_{V29G}, KY630181; CIP-102236_{R105S}, KY630182; ACH-NCF-39, KY630183; ACH-NCF-39_{V29G}, KY630184; ACH-CF-842, KY630185; ACH-CF-842_{V29G}, KY630186.

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