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Innate Aminoglycoside Resistance of *Achromobacter xylosoxidans* Is Due to AxyXY-OprZ, an RND-Type Multidrug Efflux Pump

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Achromobacter xylosoxidans **is an innately multidrug-resistant pathogen which is emerging in cystic fibrosis (CF) patients. We characterized a new resistance-nodulation-cell division (RND)-type multidrug efflux pump, AxyXY-OprZ. This system is responsible for the intrinsic high-level resistance of *A. xylosoxidans* to aminoglycosides (tobramycin, amikacin, and gentamicin). Furthermore, it can extrude cefepime, carbapenems, some fluoroquinolones, tetracyclines, and erythromycin. Some of the AxyXY-OprZ substrates are major components widely used to treat pulmonary infections in CF patients.**

Achromobacter xylosoxidans is an opportunistic human pathogen (1, 2) that is increasingly isolated from the respiratory tract of cystic fibrosis (CF) patients (3–5). Recent studies underscore its probable involvement in the inflammatory response and in the decline of the lung function (6, 7). This species exhibits innate resistance to many antibiotics, including cephalosporins (except ceftazidime), aztreonam, and aminoglycosides (1, 8–10). Clinical strains frequently harbor acquired resistances, especially to ceftazidime, ciprofloxacin, and carbapenems. We have recently described the first resistance-nodulation-cell division (RND)-type multidrug efflux pump in *A. xylosoxidans*, AxyABM (11). This system shares some properties with the *Pseudomonas aeruginosa* MexAB-OprM efflux pump: AxyABM can extrude cephalosporins (except cefepime), fluoroquinolones, and chloramphenicol. Moreover, AxyABM plays a major role in the innate resistance to aztreonam. Nevertheless, the mechanism(s) leading to aminoglycoside and cefepime resistance remain(s) unknown. It is likely that other efflux systems contribute to the antibiotic resistance of *A. xylosoxidans*, since the use of reserpine, an efflux pump inhibitor, leads to decreased MICs of tetracyclines, which are not substrates of AxyABM (11).

We have determined the whole-genome shotgun sequence of the *A. xylosoxidans* AXX-A strain (GenBank accession number AFRQ01000000). We examined this sequence looking for homology with *mexX* and *mexY* from *P. aeruginosa*, given that the MexXY/OprM efflux system has been shown to extrude aminoglycosides, cefepime, tetracyclines, fluoroquinolones, macrolides, and chloramphenicol (12–15). The MexY RND transporter from *P. aeruginosa* interacts with the periplasmic protein MexX and the outer membrane channel OprM that is encoded by the *mexAB-oprM* multidrug efflux operon. The expression of *mexXY* is complex and governed by several regulatory mechanisms. One of them is negative regulation by the product of the *mexZ* gene located upstream from *mexX* (12, 16). By using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), we detected 3 putative genes in the AXX-A genome (contig 71, GenBank accession number AFRQ01000061.1), designated *axyZ*, *axyX*, and *axyY*, sharing 67, 67, and 77% nucleotide similarity with *mexZ*, *mexX*, and *mexY*, respectively. Downstream from *axyY*, we found another open reading frame (ORF), *oprZ*, encoding a probable outer membrane protein. The aim of our study was to evaluate the involvement of this putative RND-type efflux pump in the multidrug resistance of *A. xylosoxidans*.

In this work, we studied three clinical isolates of *A. xylosoxidans*: AXX-A, AXX-D, and AXX-H. The strains were identified by using the API20NE system (bioMérieux, Marcy l'Etoile, France) and by sequencing of the *rrs* gene. Susceptibility testing was performed by Etest (bioMérieux, Marcy l'Etoile, France) and interpreted according to the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical_breakpoints/). AXX-A harbors a wild-type antibiotic resistance phenotype, AXX-D an acquired resistance to ceftazidime and fluoroquinolones, and AXX-H an acquired resistance to ceftazidime, fluoroquinolones, and carbapenems (Table 1). In these strains, we have inactivated *axyY*, encoding the transporter component. For this purpose, the primers INA-axyY-F and INA-axyY-R (Table 2) were designed to amplify a 755-bp region of *axyY* (nucleotide positions from 1696 to 2450). The PCR product was cloned into the pUC19 vector by using the In-Fusion HD cloning kit (Clontech Laboratories, Mountain View, CA) as recommended by the manufacturer. Newly constructed plasmids (pINA-axyY-AXX-A, pINA-axyY-AXX-D, and pINA-axyY-AXX-H) were used as suicide vectors. They were introduced into each strain by electroporation. Recombinant clones (*axyY*::Tic) named AXX-A-ΔY, AXX-D-ΔY, and AXX-H-ΔY were selected on Mueller-Hinton agar plates containing 50 μg/ml of ticarcillin. The disruption of *axyY* in each strain was confirmed by PCR and DNA sequencing (primer pairs V-INA-axyY-F/M14R and M14F/V-INA-axyY-R).

The *axyY* inactivation led to decreased MICs of aminoglycosides, carbapenems, cefepime, some fluoroquinolones, tetracyclines, erythromycin, and to a lesser extent, ceftazidime (Table 1). The activities of all aminoglycosides tested were substantially enhanced. Susceptibility to tobramycin, amikacin, netilmicin, and gentamicin was restored for all strains. The activities of carbapenems were slightly enhanced in the mutants AXX-A-ΔY and AXX-D-ΔY compared with their activities in the original strains,

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TABLE 1 MICs of 22 antibiotics for clinical strains and *axyY*::Tic mutants

Antibiotic	MIC (μg/ml) for strain[a]:					
	AXX-A	AXX-A-ΔY	AXX-D	AXX-D-ΔY	AXX-H	AXX-H-ΔY
Ticarcillin[b]	0.25	>256	0.25	>256	0.25	>256
Tobramycin	16	1.5 (10)	24	0.5 (48)	32	1 (32)
Amikacin	256	2 (128)	96	0.25 (384)	>256	3 (>85)
Netilmicin	32	2 (16)	64	0.75 (85)	192	1 (192)
Gentamicin	48	1 (48)	12	0.094 (127)	48	0.75 (64)
Kanamycin	32	2 (16)	>256	3 (>85)	>256	12 (>21)
Ceftazidime	4	2 (2)	48	24 (2)	48	24 (2)
Cefepime	16	8 (2)	>256	24 (>10)	>256	>256
Imipenem	1	0.5 (2)	1.5	1 (1.5)	4	1 (4)
Meropenem	0.094	0.047 (2)	0.064	0.032 (2)	12	2 (6)
Doripenem	0.19	0.047 (4)	0.25	0.19 (1.3)	16	1.5 (10)
Tetracycline	48	12 (4)	>256	32 (>8)	>256	>256
Doxycycline	8	3 (2.7)	48	12 (4)	128	24 (5)
Tigecycline	4	0.75 (5.3)	8	1 (8)	6	2 (3)
Nalidixic Acid	24	12 (2)	>256	>256	>256	>256
Norfloxacin	8	4 (2)	>256	24 (>10)	>256	128 (>2)
Ofloxacin	2	1.5 (1.3)	>32	24 (>1.3)	>32	>32
Levofloxacin	0.75	0.75 (1)	16	4 (4)	>32	8 (>4)
Moxifloxacin	1.5	1 (1.5)	>32	2 (>16)	>32	24 (>1.3)
Ciprofloxacin	0.75	0.5 (1.5)	>32	3 (>10)	>32	8 (>4)
Chloramphenicol	12	8 (1.5)	>256	>256	>256	192 (>1.3)
Erythromycin	64	16 (4)	>256	6 (>42)	>256	>256

[a] Values in parentheses represent the relative MIC decreases (ratio of the MIC for the parent strain to the MIC for the mutant strain).
[b] The ticarcillin resistance observed in the mutants is caused by the production of the penicillinase encoded by the *bla* gene from the pUC19 vector.

AXX-A and AXX-D. Interestingly, the MICs of meropenem and doripenem were decreased 6-fold and 10-fold, respectively, after *axyY* disruption in the carbapenem-resistant strain AXX-H. This suggests that AxyXY-OprZ might lead to acquired resistance to carbapenems. Nevertheless, the MIC values of meropenem and doripenem for AXX-H-ΔY, 2 and 1.5 μg/ml, respectively, were still more elevated than those for AXX-A and AXX-D. It is likely that other mechanisms are involved in the residual carbapenem resistance of AXX-H-ΔY. Concerning the cephalosporins, we observed that *axyY* inactivation resulted in a 2-fold decrease of the ceftazidime MIC, whatever the resistance level in parent strains. The activity of cefepime was partially restored in AXX-D-ΔY but not in AXX-H-ΔY, suggesting the association of various mechanisms of resistance. Finally, AxyXY-OprZ can also extrude tetracyclines, some fluoroquinolones, and erythromycin, which are also substrates of MexXY/OprM. The restoration of the original drug resistance phenotypes was observed in spontaneous revertants obtained by culturing mutant strains without ticarcillin.

TABLE 2 Primers used in the study

Primer	Nucleotide sequence (5′–3′)
INA-axyY-F	CGGTACCCGGGGATCCAGGGCAGCTTCATGGCCAT[a]
INA-axyY-R	CGACTCTAGAGGATCGGGAAGCCGTTGTAGCGGTT[a]
V-INA-axyY-F	CGCCTGACCACGCGCTATA
M14F[b]	CCAGGGTTTTCCCAGTCACGA
M14R[b]	GCGGATAACAATTTCACACAGGA
V-INA-axyY-R	CGGACAGCCGCTCTTCGTA
oprZ-F	CACGCTCAAGCTGACCCA
oprZ-R	CCTCCAGCATCTCCAGGT

[a] Underlined sequences comprise 15 bases of homology with the ends of the linearized vector pUC19.
[b] M14F and M14R are primers designed in suicide plasmids.

AxyXY-OprZ from *A. xylosoxidans* and MexXY/OprM from *P. aeruginosa* have common substrates. This is consistent with the high amino acid sequence similarity observed between the transporters AxyY and MexY (73%), the transporter component being responsible for substrate recognition of the RND-type efflux systems (17). Nevertheless, AxyXY-OprZ confers a much higher level of resistance to aminoglycosides than MexXY-OprM in wild strains. It has recently been reported for some aminoglycoside-resistant strains of *P. aeruginosa* that *mexX* and *mexY* are linked to the *oprA* gene in the same operon and that MexXY can utilize either OprM or OprA to form drug efflux complexes (18). Such an operon, including an *oprA* gene, has been also described in *Burkholderia pseudomallei*, a species intrinsically highly resistant to aminoglycosides due to expression of the AmrAB-OprA efflux pump (19). The *oprZ* gene that we detected in the three strains studied (primers oprZ-F and oprZ-R) seems to be a homologue of the *oprA* genes from *P. aeruginosa* and *B. pseudomallei* (71% nucleotide identity and 57% amino acid identity). We plan to assess the contribution of OprZ to the high level of resistance to aminoglycosides.

In conclusion, we have demonstrated that AxyXY-OprZ confers on *A. xylosoxidans* a broad spectrum of antimicrobial agent resistance. The most interesting finding is that AxyXY-OprZ confers on *A. xylosoxidans* its intrinsic high level of resistance to aminoglycosides. Our results suggest the involvement of AxyXY-OprZ in acquired resistance to carbapenems and fluoroquinolones that are major antimicrobial components for the treatment of pulmonary infections in CF patients. This will be supported by further studies that will include more clinical isolates.

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