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A Short-Form Paper for Antimicrobial Agents and Chemotherapy

Title: Functional characterization of AbaQ, a novel efflux pump mediating quinolone resistance in *Acinetobacter baumannii*.

Running title: The *A. baumannii* AbaQ efflux pump

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ABSTRACT

Acinetobacter baumannii has emerged as an important multidrug-resistant nosocomial pathogen. In previous work, we identified a putative MFS transporter, AU097_RS17040, involved in the pathogenicity of *A. baumannii* (Pérez-Varela *et al*., Infect Immun. 2017). In this study, we analyzed the susceptibility to diverse antimicrobial agents of *A. baumannii* cells defective in this transporter, referred to herein as AbaQ. Our results showed that AbaQ is mainly involved in the extrusion of quinolone-type drugs in *A. baumannii*.

Acinetobacter baumannii is a multidrug-resistant (MDR) pathogen that causes hospital-acquired infections (1). In previous work, we identified a new, putative major facilitator superfamily (MFS) transporter in *A. baumannii* strain ATCC 17978, AU097_RS17040. This transporter, referred to herein as AbaQ (*A. baumannii* quinolone resistance transporter), is involved in surface-associated motility as well as the virulence of *A. baumannii* (2).

Sequence analysis of AbaQ, annotated as a MFS transporter in *A. baumannii* strain ATCC 17978 (accession number WP_000345069), indicated an ORF of 1305 nucleotides. According to the deduced amino acid sequence, the protein consists of 434 residues and has a molecular mass of 47.8 kDa and a theoretical isoelectric point (pI) of 9.27. Based on predictions of its secondary structure and transmembrane topology, AbaQ is composed of 12 α-helical transmembrane segments, with both the N- and C-termini located in the cytoplasm (Fig. 1A). Support for this structure came from an independent analysis that revealed the 3D structure of the protein (Fig. 1B). These data indicated that AbaQ is a drug H^+ antiporter-1 (DHA1), which differs from DHA2-type MFS drug transporters by the presence of 12 rather than 14 transmembrane segments (3). The predicted product of the *abaQ* gene exhibited low amino acid identity and similarity (< 24% and < 38%, respectively) with other MFS transporters involved in drug efflux in *A. baumannii* (Table 1).

To determine whether a lack of AbaQ alters antimicrobial susceptibilities, the response of two *A. baumannii abaQ* mutants obtained in previous work (2) to antimicrobials of different classes was tested. Compared to the wild-type (WT) parental strain, *A. baumannii* ATCC 17978, the *abaQ* mutant had a 2- to 4-fold higher susceptibility to trimethoprim and novobiocin (Table 2). The highest susceptibilities (approximately 8- to 32-fold) occurred in response to the quinolone-type antibiotics ciprofloxacin,

48 levofloxacin, and nalidixic acid (Table 2). By contrast, the mutant and its WT parent did
49 not differ in their susceptibilities to β-lactams (meropenem and ampicillin),
50 aminoglycosides (amikacin and gentamycin), macrolides (erythromycin), and
51 polymixines (colistin) or to other antimicrobials (chloramphenicol, tetracycline,
52 minocycline, and rifampin) (Table 2). To further corroborate the relevance of this MFS
53 in other *A. baumannii* strains, we analyzed the *abaQ* knockout mutant derived from *A.*
54 *baumannii* strain MAR002, a biofilm-hyperproducing strain recently isolated from a
55 wound sample collected from a patient at the Hospital del Mar in Barcelona (2, 4, 5).
56 Assays of the mutant indicated that inactivation of the AbaQ homologue in *A.*
57 *baumannii* MAR002 (99% amino acid identity) also caused the highest reduction
58 (approximately 8- to 32-fold) in the minimum inhibitory concentration (MIC) of
59 quinolone-type antibiotics (data not shown).

60 To complement the mutants, the AbaQ gene, including its own promoter, was amplified
61 from the genome of *A. baumannii* strain ATCC 17978 using the AbaQFXbaI and
62 AbaQRXbaI oligonucleotides (5'-ACTGTCTAGAGGAATATCACAGCTTGCAGCG
63 and 5'-ACTGTCTAGATTACAAAGGCTTTTGAATATTC, respectively), cloning
64 them into the *Xba*I restriction site of the pET-RA vector (6). Complementation of the
65 *abaQ*-mutant derived from *A. baumannii* ATCC 17978 completely restored
66 antimicrobial susceptibility to the same levels determined in the WT parental strain
67 (Table 2). The recovery of the WT phenotype was also observed in the complemented
68 *abaQ* MAR002 mutant derivative (data not shown). These results provided clear
69 evidence of the critical role of AbaQ in the efflux of quinolone-type antibiotics in *A.*
70 *baumannii*.

71 To assess whether AbaQ confers resistance to quinolones through an active efflux
72 mechanism, the MIC of ciprofloxacin in the presence of the efflux pump inhibitor

carbonyl cyanide 3-chlorophenylhydrazone CCCP (Sigma) was determined in *A. baumannii* strains carrying (WT and AbaQ$^-$ + pET-RA+AbaQ) or lacking (AbaQ$^-$ and AbaQ$^-$ + pET-RAØ) the *abaQ* gene. The addition of CCCP (20 mg/L) decreased the ciprofloxacin MIC 16-fold in AbaQ-carrying cells (from 0.25 to 0.015 mg/L) but only 4-fold in cells lacking the *abaQ* gene (from 0.0078 to 0.0019 mg/L). The addition of CCCP alone did not inhibit bacterial cell growth in any of the *A. baumannii* strains, indicating that the above results were not due to the toxicity of CCCP itself. Accordingly, when ampicillin, which is not a substrate of the AbaQ transporter (Table 2), was used instead of ciprofloxacin, there was no difference in antimicrobial susceptibility in the presence or absence of CCCP in any of the cultured *A. baumannii* strains (data not shown). These results unequivocally demonstrated that the *abaQ* gene product confers decreased susceptibility to quinolones, by encoding an active efflux transporter.

The majority of the MFS transporters involved in drug-efflux described thus far in *A. baumannii* mediate chloramphenicol efflux, with a smaller number mediating the efflux of other antimicrobials, such as erythromycin, tetracycline, minocycline, fosfomycin, and colistin (Table 1). Among the latter group of transporters is AmvA, which participates in the efflux of erythromycin and different classes of disinfectants, detergents, and dyes but also confers modest resistance to quinolone-type antimicrobials (7). In *amvA* mutant strains, susceptibility to both ciprofloxacin and norfloxacin is 2-fold higher than in the WT strain whereas susceptibility to nalidixic acid is unchanged (7). Other transporters belonging to different families and involved in the extrusion of a wide range of antimicrobials, including quinolones, have been described in *A. baumannii*: AdeABC (8), AdeDE (9), AdeFGH (10), AdeIJK (11), AdeM (12), and AdeT (13), all of them belonging to the resistance/nodulation/division (RND)

superfamily; AbeM, AbeM2, and AbeM4 transporters, belonging to the MATE (multiple antimicrobial toxin extrusion) family (12, 14); and AbeS, belonging to the small multidrug resistance (SMR) family (15). No role in quinolone efflux has been detected in the only ATP-binding cassette (ABC) transporter described so far in *A. baumannii* (A1S_1503) (16) or in any of the transporters that make up the most recently discovered family of efflux pumps: the proteobacterial antimicrobial compound efflux (PACE) transporters (17).

To our knowledge, AbaQ, which is widely present in *A. baumannii* clinical isolates and involved in both surface-associated motility and virulence (2), is the first MFS efflux-pump shown to play an important role in the extrusion of quinolone-type antimicrobials in this MDR nosocomial pathogen.

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121 **Table 1.** Major facilitator superfamily (MFS) transporters described in *A. baumannii*

MFS	Accession number	Main antimicrobial exported	Identity[a]	Similarity[a]
AmvA (7)	ACQ82816	Erythromycin	17.8%	31.0%
TetA (18)	AAO38186	Tetracycline	17.6%	28.5%
TetB (19)	AFV67369	Minocycline	19%	33.8%
CraA (20)	ABO13543	Chloramphenicol	19%	36.9%
FloR (21)	AQT19056	Chloramphenicol	16.9%	28.2%
CmlA (22)	AMD83513	Chloramphenicol	17.5%	28.3%
AbaF (23)	ABO11759	Fosfomycin	23.7%	37.6%
EmrB (24)	ABO12199	Colistin	17.2%	30.7%
AedC (25)	ABO11341	Tetracycline/ Chloramphenicol	18.5%	32.7%

122 [a]With respect to AbaQ of *A. baumannii* strain ATCC 17978, using the Basic Local
123 Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov) and Clustal Omega
124 (http://www.ebi.ac.uk/Tools/msa/clustalo/).

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Table 2. Minimum inhibitory concentrations (MICs, mg/L) of various antimicrobials in wild-type *A. baumannii* ATCC 17978 (WT) and the *abaQ* mutant derivative (AbaQ$^-$). The MICs for the *abaQ* mutant carrying the empty pET-RA plasmid (pET-RAØ) and the mutant carrying the pET-RA+AbaQ plasmid (complemented mutant) are also shown.

	WT	AbaQ$^-$	Fold change[a]	AbaQ$^-$ + pET-RAØ	AbaQ$^-$ + pET-RA +AbaQ	Fold change[b]
Ciprofloxacin	0.25	0.0078	32	0.0078	0.25	32
Levofloxacin	0.125	0.0078	16	0.0078	0.125	16
Nalidixic acid	8	1	8	1	8	8
Trimethoprim	16	4	4	4	16	4
Novobiocin	8	4	2	4	8	2
Meropenem	2	2	1	2	2	1
Ampicillin	16	16	1	16	16	1
Amikacin	1	1	1	1	1	1
Gentamicin	0.5	0.5	1	0.5	0.5	1
Erythromycin	4	4	1	4	4	1
Colistin	0.5	0.5	1	0.5	0.5	1
Chloramphenicol	16	16	1	16	16	1
Tetracycline	4	4	1	4	4	1
Minocycline	0.062	0.062	1	0.062	0.062	1
Rifampin	4	4	1	NA[c]	NA[c]	NA[c]

[a]Ratio of the MICs of the WT vs. AbaQ$^-$.
[b]Ratio of the MICs for AbaQ$^-$+pET-RA+AbaQ vs. AbaQ$^-$+ pET-RAØ.
[c]NA, not applicable (the pET-RA plasmid carries rifampin resistance).

FIGURE LEGEND

Fig. 1. (A) Prediction of the structure of AbaQ using Protter (10). The putative protein is shown parallel to the cytoplasmic membrane. **(B)** 3D representation of AbaQ viewed along the plane of the membrane from the periplasmic side using RaptorX Structure Prediction (26) and visualized using PyMOL software (27). The 12 transmembrane α-helices are numbered (1–12); both the N- and C-termini are located in the cytoplasm.

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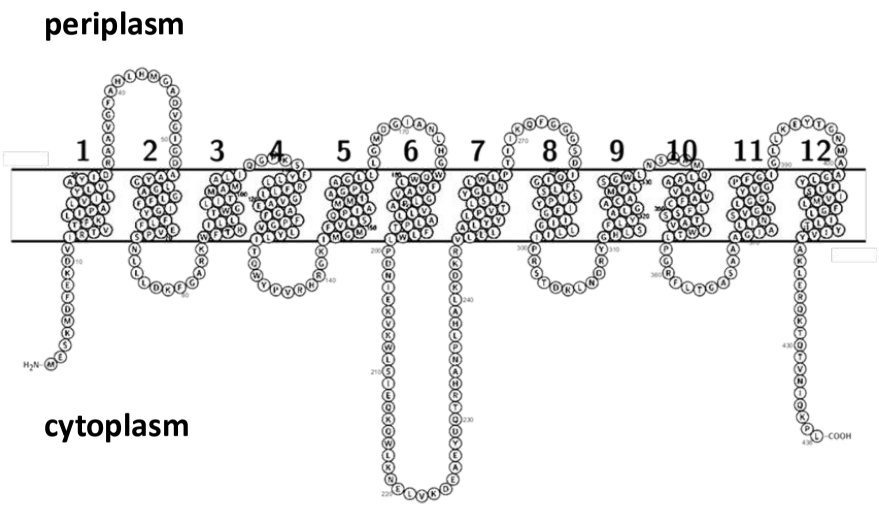
Fig. 1

(A)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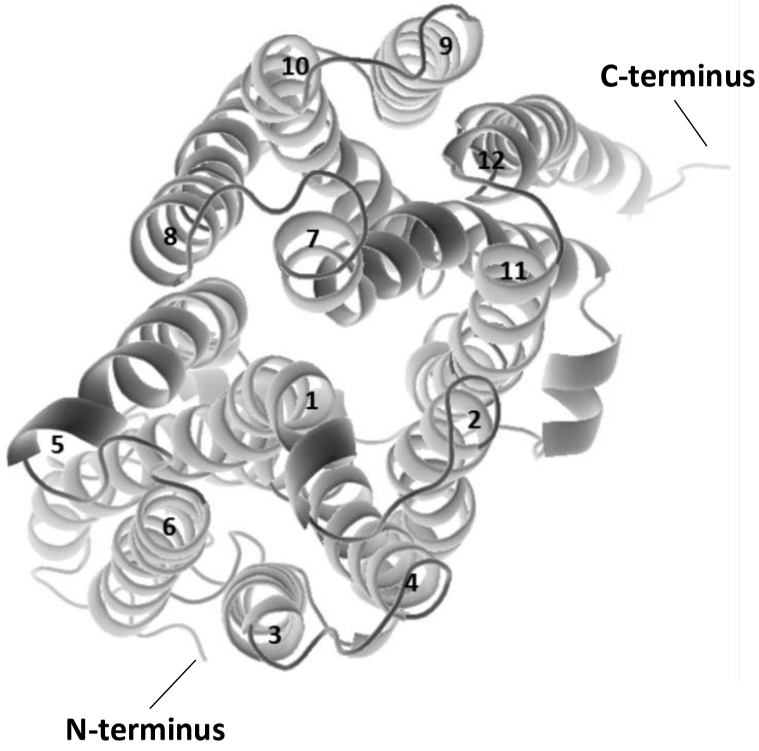
C-terminus
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