

## CraA, a Major Facilitator Superfamily Efflux Pump Associated with Chloramphenicol Resistance in *Acinetobacter baumannii*<sup>∇</sup>

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***Acinetobacter baumannii* has been increasingly associated with hospital-acquired infections, and the presence of multidrug resistance strains is of great concern to clinicians. *A. baumannii* is thought to possess a great deal of intrinsic resistance to several antimicrobial agents, including chloramphenicol, although the mechanisms involved in such resistance are not well understood. In this work, we have identified a major facilitator superfamily efflux pump present in most *A. baumannii* strains, displaying strong substrate specificity toward chloramphenicol.**

*Acinetobacter baumannii* is an important opportunistic pathogen often associated with severe nosocomial infections, such as pneumonia, bacteremia, and secondary meningitis, especially in intensive care units. In the last few decades, *A. baumannii* infections have become a serious health problem due to the emergence of multidrug-resistant (MDR) phenotypes which, therefore, restrain antimicrobial therapy to just a few agents, mainly carbapenems (2, 22).

Antimicrobial resistance can be due to several distinct mechanisms, being that MDR phenotypes are related to the acquisition of genetic elements carrying different resistant determinants or to decreased membrane permeability, together with expression of active efflux pumps (21). To date, the following five efflux systems have been described in *A. baumannii*: AdeABC and AdeIJK, belonging to the resistance-nodulation-cell division family and conferring resistance to a wide spectrum of antimicrobial agents (3, 9); Tet(A) and Tet(B), from the major facilitator superfamily (MFS), involved in tetracycline and minocycline resistance (10, 11); and AbeM, included within the multidrug and toxic compound extrusion family, providing moderate resistance to several compounds (16).

The recent publication of up to six complete *A. baumannii* genomes (1, 7, 15, 18), however, accounts for the identification of novel putative efflux systems that may add to those already described in the literature, although experimental proof of their role in antimicrobial resistance is still needed. In this work, we have used homology search-based methods to identify an open reading frame (initially named *orf3*) encoding a putative efflux pump ortholog present in all fully sequenced *A. baumannii* strains (GenBank accession no. ABO13543.2). Its deduced amino acid sequence suggests that it consists of 409 residues and contains 12 transmembrane domains (Predict-

Protein server) (12, 13). It also showed sequence similarity (41% identity and 61% similarity) to the *Escherichia coli* MdfA protein (GenBank accession no. CAA69997.1), which has been previously described as an MFS efflux pump conferring resistance mainly to ciprofloxacin and chloramphenicol, among others (4). *Orf3* was 99% identical among all sequenced *Acinetobacter* strains and it was also detected by PCR analysis in 82 out of 82 *A. baumannii* clonally different clinical isolates from our culture collection.

To demonstrate the involvement of *Orf3* in MDR, the *orf3* gene from *A. baumannii* strain ATCC 19606 was cloned into the *Sma*I site of the suicide vector pEX100T (14) and disrupted by the insertion of a Km<sup>r</sup> cassette obtained from pUC4K (19). Insertion was at nucleotide position 457 after introducing a BamHI site using a two-step nested PCR mutagenesis. The resulting construct (designated pJV102) was mobilized from the *E. coli* strain S-17λpir to *A. baumannii* strain ATCC 19606 Rif<sup>r</sup> in order to knock out its cognate *orf3* gene by allelic replacement. Exconjugants were selected on LB agar plates containing 75 μg/ml rifampin (rifampicin) and 50 μg/ml kanamycin, and cells growing on these plates were further streaked on LB agar containing 50 μg/ml kanamycin and 2% sucrose, to ensure the loss of pJV102. *orf3* disruption within the resulting strain, designated JVAB01, was verified by PCR analysis.

Etest susceptibility assays to quinolones, tetracyclines, aminoglycosides, and imipenem (Table 1) showed identical MICs between ATCC 19606 and JVAB01, but a dramatic drop in chloramphenicol resistance was detected for the *Orf3* mutant strain (a MIC value of >256 μg/ml for ATCC 19606 compared to 2 μg/ml for JVAB01).

To check that the increased chloramphenicol susceptibility observed for the JVAB01 strain was in fact due to the lack of a functional *Orf3* protein, a 1,971-bp PCR fragment containing the *A. baumannii* ATCC 19606 *orf3* gene together with 520 bp upstream from the ATG codon was amplified by PCR and introduced into the *Eco*RI site of the shuttle vector pWH1266 (5), generating the plasmid pJV103. Electroporation of

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TABLE 1. MICs of antimicrobial agents in the investigated *A. baumannii* strains

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ ) for indicated strain		
	ATCC 19606	JVAB01 (Orf3 mutant)	JVAB01 + pJV103 (Orf3 <sup>+</sup> )
Tetracycline	6	6	96
Nalidixic acid	12	12	12
Ciprofloxacin	0.25	0.25	0.25
Norfloxacin	6	6	6
Imipenem	0.75	0.75	0.75
Clindamycin	>256	>256	>256
Erythromycin	1.5	1.5	1.5
Chloramphenicol	>256	2	192
Chloramphenicol + PA $\beta$ N	32	1.5	16

pJV103 into JVAB01 restored chloramphenicol resistance levels up to 192  $\mu\text{g/ml}$ , indicating that Orf3 was indeed responsible for the chloramphenicol-resistant phenotype. In addition, chloramphenicol resistance in the presence of the efflux pump inhibitor phenyl-arginine- $\beta$ -naphthylamide (PA $\beta$ N) was reduced at least eightfold for strain ATCC 19606 and the complemented strain while remaining barely unchanged in the mutant strain (Table 1).

Recent studies indicate that most *A. baumannii* isolates are intrinsically resistant to chloramphenicol, yet they fail to provide a mechanism responsible for such resistance (6, 8, 17). In a previous work, we analyzed 54 *A. baumannii* isolates highly resistant to chloramphenicol and demonstrated that they all lacked chloramphenicol acetyltransferase activity (20). Other studies have shown that resistance-nodulation-cell division efflux pumps might participate in chloramphenicol resistance to a certain extent, although they do not account for the high levels found in most isolates (3, 9). In this work, we have shown that *orf3* encodes an MFS efflux pump structurally related to the *E. coli* MdfA protein, although MdfA has been shown to exhibit an extraordinary broad spectrum of drug specificities (4) while Orf3 seems highly specific toward chloramphenicol. We believe this pump is responsible for the intrinsic chloramphenicol resistance described in *A. baumannii* strains, and therefore we suggest it be named CraA, for chloramphenicol resistance *Acinetobacter*.

The characterization of intrinsic resistance mechanisms within the genus *Acinetobacter* favors the potential development of inhibitors to be used in combination with antimicrobial agents in order to increase the available armamentarium against otherwise untreatable MDR strains.

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