

Contribution of Efflux Pumps, Porins, and β -Lactamases to Multidrug Resistance in Clinical Isolates of *Acinetobacter baumannii*

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We investigated the mechanisms of resistance to carbapenems, aminoglycosides, glycolcyclines, tetracyclines, and quinolones in 90 multidrug-resistant clinical strains of *Acinetobacter baumannii* isolated from two genetically unrelated *A. baumannii* clones: clone PFGE-ROC-1 (53 strains producing the OXA-58 β -lactamase enzyme and 18 strains with the OXA-24 β -lactamase) and clone PFGE-HUI-1 (19 strains susceptible to carbapenems). We used real-time reverse transcriptase PCR to correlate antimicrobial resistance (MICs) with expression of genes encoding chromosomal β -lactamases (AmpC and OXA-51), porins (OmpA, CarO, Omp33, Dcap-like, OprB, Omp25, OprC, OprD, and OmpW), and proteins integral to six efflux systems (AdeABC, AdeIJK, AdeFGH, CraA, AbeM, and AmvA). Overexpression of the AdeABC system (level of expression relative to that by *A. baumannii* ATCC 17978, 30- to 45-fold) was significantly associated with resistance to tigecycline, minocycline, and gentamicin and other biological functions. However, hyperexpression of the AdeIJK efflux pump (level of expression relative to that by *A. baumannii* ATCC 17978, 8- to 10-fold) was significantly associated only with resistance to tigecycline and minocycline (to which the TetB efflux system also contributed). TetB and TetA(39) efflux pumps were detected in clinical strains and were associated with resistance to tetracyclines and doxycycline. The absence of the AdeABC system and the lack of expression of other mechanisms suggest that tigecycline-resistant strains of the PFGE-HUI-1 clone may be associated with a novel resistance-nodulation-cell efflux pump (decreased MICs in the presence of the inhibitor Phe-Arg β -naphthylamide dihydrochloride) and the TetA(39) system.

Acinetobacter baumannii is an important pathogen that causes nosocomial infections associated with high morbidity and mortality (1). Multidrug-resistant (MDR) strains of *A. baumannii* have emerged in the last few decades as a result of the combination of two main factors: (i) a high level of genomic plasticity (2) and (ii) mutation of endogenous genes, alteration of which is associated with antimicrobial resistance, such as overexpression of the chromosomally encoded ADC β -lactamase (AmpC) (3) and the OXA-51-like β -lactamase (4), loss of expression of porins (CarO and Omp33) (5, 6), mutation in the *gyrA* and *parC* genes (7), and overexpression of efflux systems (8).

Overexpression of the OXA-51-like β -lactamase has been associated with resistance to carbapenems and decreased expression of CarO and Omp33 (5, 6, 9).

Efflux pumps have multifactorial roles. These mechanisms are important for detoxification of intracellular metabolites, bacterial virulence (in both animal and plant hosts), intercellular signaling and trafficking, and cell homeostasis (10). Three resistance-nodulation-cell division (RND) systems, AdeABC, AdeIJK, and AdeFGH, have been characterized and reported to cause MDR in *A. baumannii* (8). AdeABC is the RND system most frequently involved in MDR in clinical strains; it has been found in approximately 80% of clinical isolates (the rates reported vary from 53% to 97%) (11) but was not detected in 32 environmental isolates (12). AdeRS is a two-component system that regulates AdeABC expression (13). Mutations in this system and the presence of an IS*Aba1* insertion sequence in this system can lead to overexpression of the AdeABC operon (13–15). However, strains of *A. bau-*

mannii that express AdeABC without mutations have been found in association with AdeRS (16, 17). Recently, the *adeN* gene has been found to be associated with the regulation of the AdeIJK system (18), and mutations in the *adeL* gene have been associated with overexpression of the AdeFGH pump (11). Three other types of efflux systems have been described in *A. baumannii*: CraA (a major facilitator superfamily [MFS] pump), which confers intrinsic chloramphenicol resistance (19); AbeM (a member of the multidrug and toxic compound extrusion [MATE] family of pumps), which extrudes several antimicrobials and biocides (20); and AmvA (an MFS pump), which confers resistance to detergents, disinfectants, dyes, and erythromycin. Overexpression of the AmvA efflux pump has been associated with increased drug resistance in *A. baumannii* clinical isolates (21). Finally, several tetracycline efflux pumps (systems acquired from the MFS superfamily) have been described in *A. baumannii*. The most prevalent of these are TetA, which is associated with resistance to tetracycline, and TetB, which is implicated in resistance to tetracycline, doxy-

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cycline, and minocycline (8). TetA(39) is an important tetracycline resistance mechanism in clinical strains (22).

Because of the complexity of clinical strains of *A. baumannii*, many researchers have used ATCC reference strains to investigate the mechanisms of resistance. However, very few studies have analyzed the combinations of mechanisms and their interrelation in clinical isolates of *Acinetobacter baumannii*. Here, we studied the interplay between the mechanisms of multidrug resistance in clinical *A. baumannii* strains, particularly those involving efflux pumps, the influx of antimicrobials, and chromosomally encoded β -lactamases.

MATERIALS AND METHODS

Bacterial isolates and molecular typing. In 2010, 444 strains of *A. baumannii* were isolated (from 273 patients) in 42 participating hospitals and identified as part of the second multicenter study on this pathogen in Spain (GEIH-REIPI-2010-Ab) (23). The strains were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (24) and amplified rRNA gene restriction analysis (ARDRA) (25). Species identification was confirmed by detection of the OXA-51 gene by PCR (26) and also by detection of the *bsp* gene (a novel target) by real-time quantitative PCR (27, 28).

The clonal relationship between all strains displaying various levels of antibiotic susceptibility ($n = 71$) from a hospital in southern Spain and a hospital in the Canary Islands ($n = 19$) was determined by pulsed-field gel electrophoresis (PFGE) (29, 30) of samples of chromosomal DNA digested with *Apa*I (Roche, Mannheim, Germany) and embedded in low-melting point agarose. The restriction fragments thus obtained were separated in a CHEF DR-III system (Bio-Rad, Hercules, CA). FPQuest II software, version 4.5 (Bio-Rad), was used to analyze the band patterns in the agarose gel electrophoresis images (cutoff = 85%). Strains of both clones were analyzed by multilocus sequence typing, according to the system developed by Nemeč and coworkers (31).

Susceptibility testing. The antibiotic susceptibility profile was determined according to CLSI recommendations (23). In strains of the PFGE-HUI-1 clone, we determined MICs in the presence of Phe-Arg β -naphthylamide dihydrochloride (PAbetaN; a commonly assumed inhibitor of the RND efflux pump) (32).

DNA amplifications studies. We used PCR to detect the genes coding for common aminoglycoside-modifying enzymes (*AacA4*, *AacC1*, *AacC2*, *AadB*, *AadA1*, *AphA1*, *AphA6*, and *AadA2*) (33); CHDL enzymes (*OXA-23*, *-24*, *-51*, *-58*, and *-143*) (34, 35); MBL enzymes (*IMP*, *VIM*, *SPM-1*, *GIM-1*, *SIM-1*, *BIC*, *DIM*, and *NDM*) (36); extended-spectrum β -lactamases (ESBLs), such as GES enzymes; and carbapenemases, such as KPC enzymes. We sequenced the *gyrA* and *parC* genes to study the presence of the mutations. Finally, we analyzed the *tet* genes most commonly detected in isolates of *A. baumannii* [*tetA*, *tetB*, *tetA*(34), and *tetA*(39)] (37, 38).

Real-time RT-PCR studies. We used real-time reverse transcriptase PCR (RT-PCR) to examine all isolates for expression of *adeB*, *adeJ*, *adeG*, *abeM*, *craA*, and *amvA* (genes belonging to efflux pumps systems); *oprC*, *oprD*, *ompW*, *ompA*, *carO*, *omp33*, *dcap*-like, *oprB*, and *omp25* (genes harboring porins or outer membrane protein); and finally, the *OXA-51* and *ampC* β -lactamase genes. We obtained DNase-treated RNA from late-log-phase cultures (optical density = 0.4 to 0.6 absorbance units) by using a High Pure RNA isolation kit (Roche, Germany) and 50 ng of RNA. Analysis of controls without reverse transcriptase confirmed the absence of contaminating DNA in the samples. We used a LightCycler 480 RNA master hydrolysis probe (Roche, Germany) for the RT-PCR studies. The Universal Probe Library (UPL) TaqMan probes (Roche, Germany) and primers used are listed in Table 1. All were designed from conserved regions of DNA after the alignment of the genomes of the following strains of *A. baumannii*: AB 307-0294, AB 0057, ACICU, SDF, AYE, and ATCC 17978. We adjusted the concentrations of the samples to achieve efficiencies of 90% to 110% and performed all experiments in triplicate from

three RNA extractions. For each strain, we normalized the levels of expression of all genes relative to those of the single-copy housekeeping genes *rpoB* and *gyrB*. We then calibrated the normalized expression of each gene of interest relative to its expression by *A. baumannii* ATCC 17978, which was assigned a value of 1.0.

Sequencing of the genes regulating AdeABC and AdeIJK efflux pumps. We sought mutations in the regulatory genes *adeR-adeS* and *adeN*, which have previously been associated with upregulation of the AdeABC and AdeIJK efflux systems, respectively. We amplified the genes by using the primers listed in Table 1. We used the MacroGen program (MacroGen Europe, Amsterdam, Netherlands) for DNA sequencing and the NCBI BLAST (www.ncbi.nlm.gov/BLAST) and CLUSTAL (www.ebi.ac.uk/Tools/msa/clustalw2/) programs for posterior analyses.

Statistical analysis. We categorized the strains into two groups according to antimicrobial susceptibility (not following the CLSI or EUCAST clinical breakpoints). We worked with the Student's *t* test to compare differences in gene expression between groups and thus evaluate any associations with antibiotic resistance. Differences were considered significant at a *P* value of <0.05 .

Nucleotide sequence accession numbers. The nucleotide sequences of the *adeR*, *adeS*, and *adeN* genes from strains of the PFGE-ROC-1 clone were submitted to the GenBank database and have been assigned accession numbers [KF147860](https://doi.org/10.1093/nar/kfz147), [KF147861](https://doi.org/10.1093/nar/kfz147), and [KF147862](https://doi.org/10.1093/nar/kfz147), respectively.

RESULTS

MICs, typing, and PCR detection of genes of the isolates. To study the expression levels of efflux pump systems, porins, and chromosomal β -lactamases, we selected clonally related strains with different antibiotic susceptibilities (39). In Spain, OXA-type enzymes are prevalent in carbapenem-resistant strains of *A. baumannii* (40, 41). Isolates of *A. baumannii* from two hospitals in Spain that showed some clonal relation were designated clone PFGE-ROC-1 (sequence type 2 [ST2]) ($n = 71$; Fig. 1) and clone PFGE-HUI-1 (ST79) ($n = 19$; Fig. 2). Moreover, 53 strains of the PFGE-ROC-1 clone carried the OXA-58 β -lactamase gene (designated PFGE-ROC-1_{OXA-58}; imipenem MICs, 8 to 64 mg/liter; meropenem MICs, 8 to 16 mg/liter) and 18 isolates carried the OXA-24 β -lactamase gene (designated PFGE-ROC-1_{OXA-24}; imipenem MICs, ≥ 64 mg/liter; meropenem MICs, 32 to 64 mg/liter). The isolates of clone PFGE-HUI-1 ($n = 19$) were susceptible to carbapenems. We studied the variability in the MICs of glycolcyclines, aminoglycosides, tetracyclines, rifampin, and doripenem for all isolates, with the following results: (i) for PFGE-ROC-1_{OXA-58} (Table 2) tigecycline MICs were ≤ 0.25 to 2 mg/liter, gentamicin MICs were 1 to >64 mg/liter, amikacin MICs were <2 to 64 mg/liter, doxycycline MICs were 16 to >64 mg/liter, minocycline MICs were 1 to 8 mg/liter, tetracycline MICs were >64 mg/liter, netilmicin MICs were 1 to >64 mg/liter, rifampin MICs were 1 to 64 mg/liter, tobramycin MICs were <0.5 to 64, and doripenem MICs were 4 to 8 mg/liter. (ii) For PFGE-ROC-1_{OXA-24} (Table 3), tigecycline MICs were ≤ 0.25 to 1 mg/liter, gentamicin MICs were 2 to >64 mg/liter, amikacin MICs were <2 to 64 mg/liter, doxycycline MICs were 16 to 32 mg/liter, minocycline MICs were <0.5 to 4 mg/liter, tetracycline MICs were >64 mg/liter, netilmicin MICs were 64 to >64 mg/liter, rifampin MICs were <0.5 to 4 mg/liter, tobramycin MICs were 4 to 64 mg/liter, and doripenem MICs were 64 to >64 mg/liter. (iii) For PFGE-HUI-1 (Table 4), tigecycline MICs were 1 to 2 mg/liter, gentamicin MICs were 16- to >64 mg/liter, amikacin MICs were 4 to 64 mg/liter, doxycycline MICs were <0.5 to 8 mg/liter, minocycline MICs were <0.5 to 1 mg/liter, tetracycline MICs were 4 to >64 mg/liter, netilmicin MICs were 4 to >64 mg/liter, rifampin MICs were 2 to 32 mg/liter, tobramycin MICs were 8 to 64 mg/liter, and doripenem MICs were <0.5 to 2 mg/liter.

TABLE 1 Primers used in this study

Analysis and gene	Orientation	Primer sequence (5'–3')	UPL probe ^a	Reference or source
RT-PCR analysis				
<i>rpoB</i>	Forward	CGTGTATCTGCGCTTGG	131	This study
	Reverse	CGTACTTCGAAGCCTGCAC		
<i>gyrB</i>	Forward	TGGTGGAACGTGGTCATATTT	76	This study
	Reverse	TGCTCTTGCTTACCCCTTTTTG		
<i>adeB</i>	Forward	CGAGTGGCACAACACTAGCATC	61	This study
	Reverse	CCTTGTCTGGCTGCACTCT		
<i>adeJ</i>	Forward	CCTATTGCACAATATCCAACGA	119	This study
	Reverse	AGGATAAGTCGCAGCAATCG		
<i>adeG</i>	Forward	GTCTGAAAATGGTCGTTCTG	43	This study
	Reverse	AGCTTCTGCTTGGCTAGATGA		
<i>craA</i>	Forward	TTCATTGCTTGC GCCTTT	125	This study
	Reverse	CCAGTGCCATGAAACATAATCA		
<i>abeM</i>	Forward	AGGGACGTATTATGGCGAAA	165	This study
	Reverse	CTGCTGTGCTTAGACCAATTTTT		
<i>amvA</i>	Forward	GCAGAGAAATTTTGC ACTTTGG	10	This study
	Reverse	CGACTAATGGACCAAAAAGCTG		
<i>ompA</i>	Forward	GGTATTTCAGATAATTTTTTCAGCAACTT	129	This study
	Reverse	AACAAATCAAACATCAAAGACCAA		
<i>ompW</i>	Forward	GCCTTATTTGCTCTGCCAAC	60	This study
	Reverse	CGTTTGAAACCATCACCATCT		
<i>dcap-like</i>	Forward	TGATCGACTTCTCGACAACA	77	This study
	Reverse	GTGTAGTTGGGCTAGTTTGTAGTT		
<i>oprC</i>	Forward	ACTCGATACAAAGCGGTGGA	9	This study
	Reverse	TTTAATACGTGAACCAAACATACCTC		
<i>oprB</i>	Forward	GCCCCACACTTCTTGAACAG	67	This study
	Reverse	ATGGGCAATCGCTTTCTG		
<i>omp25</i>	Forward	CGAACGTGAAATCGACAACA	128	This study
	Reverse	CGTAACCTTTAACACCTAGAGCAAG		
<i>omp33-omp36</i>	Forward	CAAGTGTGCTAACCAATTCGCT	FAM-CCAACTGCTGCTATCCAAA CGACCAA-BBQ	This study
	Reverse	GTTTTCTTGACCGAATGCACC		
<i>carO</i>	Forward	TGTTTCATGACAGCTATGCATTGATA	FAM-CGCTCGTGTGCTGAAGTAGGTAC TACAGGTT-BBQ	This study
	Reverse	CCCAATGCTAAACCTACATATGGGT		
Sequencing analysis				
<i>adeR</i>	Forward	ACTACGATATTGGCGACATT		13–15
	Reverse	GCGTCAGATTAAGCAAGATT		
<i>adeS</i>	Forward	TTGGTTAGCCACTGTTATCT		13–15
	Reverse	AGTGGACGTTAGGTCAAGTT		
<i>adeN</i>	Forward	GCTGTTAGGTTGGGGTCGTA		18
	Reverse	CGTGACCAAAAAGTACGAATCA		

^a FAM, 6-carboxyfluorescein; BBQ, BlackBerry Quencher.

We detected *tet* genes in both clones: the *tetB* gene in all strains of PFGE-ROC-1 and the *tetA*(39) gene in strains of the PFGE-HUI-1 clone (except for strains 421, 422, 423, 424, and 426).

In both *A. baumannii* clones, we detected the AacC1/AphA1/AadB combination of acetylases in strains displaying some resistance to aminoglycosides. We also detected mutations in the *gyrA* (Ser₈₃ → Leu) and *parC* (Ser₈₀ → Leu) genes in strains showing resistance to quinolones.

Relative gene expression. The levels of expression of the efflux pump genes in the isolates relative to that by *A. baumannii* ATCC 17978 (relative expression ([RE] values) are shown in Tables 2 to 4. For clone PFGE-ROC-1, we applied statistical analysis to genes with RE values higher than 8 (i.e., genes *adeB* and *adeJ*) to determine how gene expression was related to the antibiotic MICs (for strains carrying the OXA-58 β-lactamase gene, see Fig. 3; for strains carrying the OXA-24 β-lactamase gene, see Fig. 4). However, we were not able to analyze the *adeB* gene in strains of clone

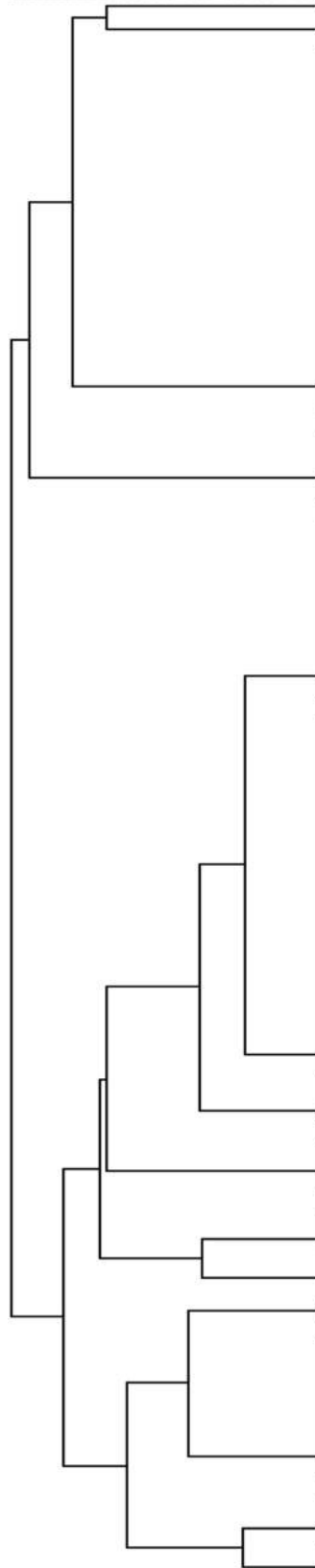
PFGE-HUI-1, because the internal and external primers used did not amplify the genes in the AdeABC operon of these strains. Moreover, the relative expression of *adeJ* in this clone was not higher than 2.

The RE values of *adeG*, *craA*, *abeM*, and *amvA* in all strains ranged from 0.003 to 1.

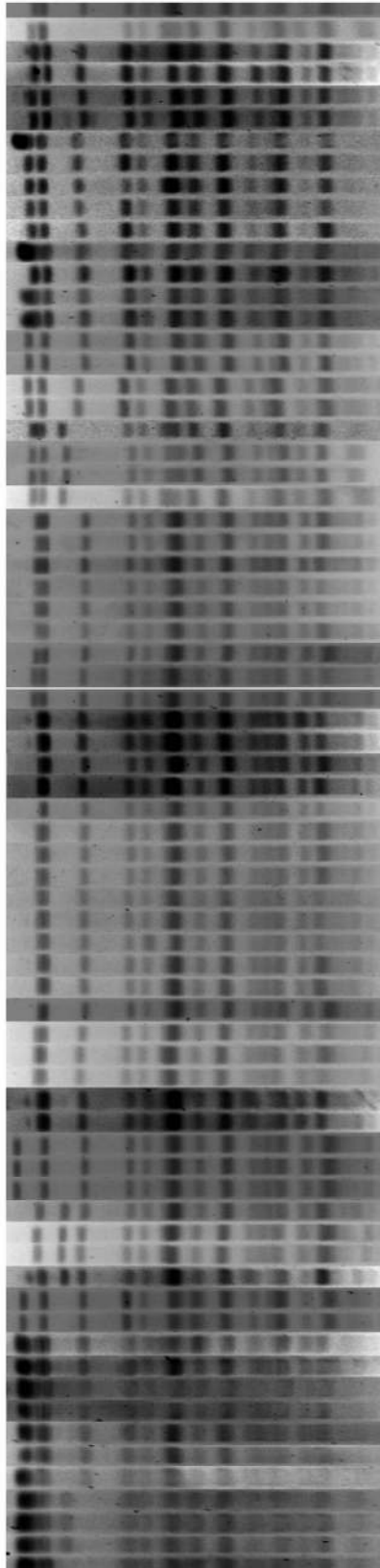
Porin expression was not significantly related to antibiotic resistance in strains of clone PFGE-ROC-1 or PFGE-HUI-1. However, in strains of clone PFGE-ROC-1_{OXA-24} the RE values of the *carO* and *omp25* genes were lower than those in strains of clone PFGE-ROC-1_{OXA-58} (Fig. 5). However, the RE values of the OXA-51 and *ampC* β-lactamase genes were similar among the isolates.

Polymorphisms of the regulatory genes of the AdeABC and AdeIJK efflux pumps. Strains of the PFGE-ROC-1 clone that overexpressed the AdeABC efflux pump had three mutations in the *adeS* gene (Ala₉₄ → Val, Gly₁₈₆ → Val, and Phe₂₁₄ → Leu) and

Dice (D pt 0.5 0%) (T d 1.0 % 1.0%) (K > 0.0% S > 0.0%) [D 0% 100.0%]
PFGE Apal



PFGE Apal



STRAIN	REP-PCR	PFGE	OXA
118	R24	PFGE-ROC-1M	OXA-58
126	R24	PFGE-ROC-1N	OXA-58
40	R24	PFGE-ROC-1D	OXA-24
44	R24	PFGE-ROC-1D	OXA-24
46	R24	PFGE-ROC-1D	OXA-24
48	R24	PFGE-ROC-1D	OXA-24
60	R24	PFGE-ROC-1D	OXA-24
61	R24	PFGE-ROC-1D	OXA-24
62	R24	PFGE-ROC-1D	OXA-24
63	R24	PFGE-ROC-1D	OXA-24
67	R24	PFGE-ROC-1D	OXA-24
72	R24	PFGE-ROC-1D	OXA-24
73	R24	PFGE-ROC-1D	OXA-24
74	R24	PFGE-ROC-1D	OXA-24
75	R24	PFGE-ROC-1D	OXA-24
95	R24	PFGE-ROC-1D	OXA-24
96	R24	PFGE-ROC-1D	OXA-24
45	R24	PFGE-ROC-1D	OXA-24
47	R24	PFGE-ROC-1D	OXA-24
64	R24	PFGE-ROC-1H	OXA-58
85	R24	PFGE-ROC-1H	OXA-58
87	R24	PFGE-ROC-1H	OXA-58
65	R24	PFGE-ROC-1H	OXA-58
88	R24	PFGE-ROC-1E	OXA-58
89	R24	PFGE-ROC-1E	OXA-58
90	R24	PFGE-ROC-1E	OXA-58
91	R24	PFGE-ROC-1E	OXA-58
93	R24	PFGE-ROC-1E	OXA-58
94	R24	PFGE-ROC-1E	OXA-58
111	R24	PFGE-ROC-1E	OXA-58
119	R24	PFGE-ROC-1E	OXA-58
120	R24	PFGE-ROC-1E	OXA-58
36	R24	PFGE-ROC-1C	OXA-58
38	R24	PFGE-ROC-1C	OXA-58
51	R24	PFGE-ROC-1C	OXA-58
55	R24	PFGE-ROC-1C	OXA-58
92	R24	PFGE-ROC-1C	OXA-58
99	R24	PFGE-ROC-1C	OXA-58
100	R24	PFGE-ROC-1C	OXA-58
101	R24	PFGE-ROC-1C	OXA-58
106	R24	PFGE-ROC-1C	OXA-58
107	R24	PFGE-ROC-1C	OXA-58
108	R24	PFGE-ROC-1C	OXA-58
109	R24	PFGE-ROC-1C	OXA-58
110	R24	PFGE-ROC-1C	OXA-58
112	R24	PFGE-ROC-1C	OXA-58
125	R24	PFGE-ROC-1C	OXA-58
56	R24	PFGE-ROC-1C	OXA-58
57	R24	PFGE-ROC-1C	OXA-58
35	R24	PFGE-ROC-1B	OXA-58
37	R24	PFGE-ROC-1B	OXA-58
113	R24	PFGE-ROC-1K	OXA-58
114	R24	PFGE-ROC-1K	OXA-58
115	R24	PFGE-ROC-1K	OXA-58
102	R24	PFGE-ROC-1G	OXA-58
124	R24	PFGE-ROC-1G	OXA-58
58	R24	PFGE-ROC-1G	OXA-58
41	R24	PFGE-ROC-1F	OXA-58
116	R24	PFGE-ROC-1L	OXA-24
117	R24	PFGE-ROC-1L	OXA-24
32	R24	PFGE-ROC-1A	OXA-24
34	R24	PFGE-ROC-1A	OXA-58
50	R24	PFGE-ROC-1A	OXA-24
53	R24	PFGE-ROC-1A	OXA-58
78	R24	PFGE-ROC-1A	OXA-58
83	R24	PFGE-ROC-1A	OXA-58
84	R24	PFGE-ROC-1A	OXA-58
79	R24	PFGE-ROC-1I	OXA-58
81	R24	PFGE-ROC-1I	OXA-58
82	R24	PFGE-ROC-1I	OXA-58
80	R24	PFGE-ROC-1J	OXA-58

FIG 1 Pulsed-field electrophoresis of strains of the PFGE-ROC-1 clone. REP-PCR, repetitive element palindromic PCR.

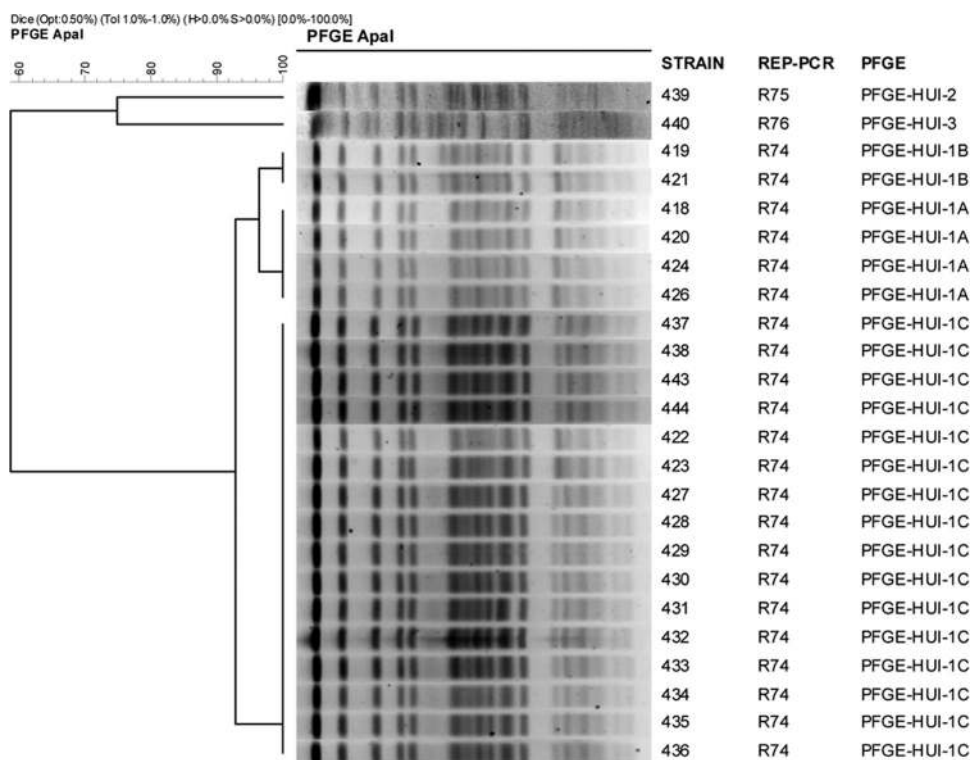


FIG 2 Pulsed-field electrophoresis of strains of the PFGE-HUI-1 clone.

one mutation in the *adeR* gene (Ala₁₃₆ → Val). Only two strains of this clone had mutations in the *adeN* regulatory gene in the AdeIJK efflux pump (His₁₁₁ → Pro, Ile₁₁₂ → Phe). The *adeS* and *adeR* genes in strains of clone PFGE-HUI-1 were not successfully amplified. In all strains of this clone, the *adeN* gene had one mutation (Pro₁₆ → Lys).

Relation between relative gene expression and MICs. Possible interactions between the mechanisms of resistance of the clinical strains of the two clones are summarized in Table 5.

(i) **Carbapenems.** For the carbapenems (imipenem, meropenem, and doripenem), resistance was associated with the presence of the OXA-type enzymes (OXA-24 and OXA-58 β-lactamases) in strains of clone PFGE-ROC-1.

(ii) **Aminoglycosides.** In gentamicin-resistant strains (MICs > 8 mg/liter) of clone PFGE-ROC-1, the AdeABC system was overexpressed and/or acetylases (AacC1/AphA1/AadB) were present. Moreover, in strains of both clones (PFGE-ROC-1 and PFGE-HUI-1), the presence of acetylases (AacC1/AphA1/AadB) was associated with resistance to netilmicin (MICs > 16 mg/liter), tobramycin (MICs > 8 mg/liter), and amikacin (MICs > 16 mg/liter).

(iii) **Glycylcyclines.** Resistance to tigecycline (MICs > 0.5 mg/liter) was associated with overexpression of the RND systems (AdeIJK efflux pump in PFGE-ROC-1_{OXA58} [Fig. 3] and AdeABC efflux pump in PFGE-ROC-1_{OXA24} [Fig. 4]) and the TetA(39) efflux pump. The presence of PAbetaN (an inhibitor of the RND system) in strains of clone PFGE-HUI-1 was associated with decreased resistance to tigecycline. In the strains of this clone with no TetA efflux pump (strains 421, 422, 423, 424, and 426), the tigecycline MIC decreased from 1 to ≤0.25 mg/liter (with PAbetaN). In those strains of clone PFGE-HUI-1 with a TetA system,

the tigecycline MIC decreased from 2 to 1 mg/liter in the presence of PAbetaN. The AdeIJK and AdeABC efflux pumps (in strains of clone PFGE-ROC-1) were associated with resistance to minocycline (MICs > 2 mg/liter). However, PFGE-ROC_{OXA-58} displayed resistance to this antibiotic, possibly because of overexpression of the AdeIJK and TetB efflux pumps.

(iv) **Tetracyclines.** Resistance to tetracyclines was associated with TetB and TetA(39) efflux pumps. In doxycycline-resistant isolates (strains of PFGE-ROC-1_{OXA-58} with MICs of >16 mg/liter), AdeIJK was overexpressed together with Tet systems.

(v) **Quinolones.** Finally, mutations of the *gyrA* and *parC* genes conferred resistance to ciprofloxacin without any variations in MICs.

DISCUSSION

The impact of the interplay between different mechanisms of antimicrobial resistance in the susceptibility or resistance to antibiotics has been addressed in previous studies. Here, we focused on two of these studies in relation to the present study. One of these studies involved clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients (representative of the Liverpool epidemic strain) (39), and the other involved strains isolated from bloodstream infections (40). In the present study, we attempted to determine if similar conclusions can be applied to clinical strains of *A. baumannii* in which resistance is associated with a multifactorial mechanism. We analyzed strains of two different clones, PFGE-ROC-1 and PFGE-HUI-1. The PFGE-ROC-1 clone included 53 strains carrying the OXA-58 β-lactamase gene (PFGE-ROC-1_{OXA-58}) and 18 strains carrying the OXA-24 carbapenemase gene (PFGE-ROC-1_{OXA-24}). The enzymes encoded by both of these genes are highly prevalent in isolates of *A. baumannii* in

TABLE 2 MICs and RE of genes harboring efflux pumps^c

Strain ^a	MIC ^d (mg/liter)										RE ^b					
	TIG	GEN	AMK	DOX	MIN	NET	TET	RIF	TOB	DOR	<i>adeB</i>	<i>adeJ</i>	<i>adeG</i>	<i>craA</i>	<i>abeM</i>	<i>amvA</i>
65	≤0.25	8	<2	16	2	1	>64	2	1	4	1.13	3.10	1.00	0.43	0.21	0.002
34	0.5	2	32	32	2	>64	>64	64	32	8	27.73	0.55	0.70	1.31	0.60	0.003
35	0.5	4	16	32	2	>64	>64	64	64	8	31.97	0.40	0.82	1.12	0.66	0.003
37	0.5	4	16	32	2	>64	>64	64	32	8	28.10	0.42	0.70	1.25	0.78	0.003
41	0.5	4	32	16	2	>64	>64	64	64	4	26.89	0.57	0.66	1.34	0.50	0.003
51	0.5	4	16	32	4	>64	>64	64	64	8	16.21	1.62	0.41	0.65	0.70	0.004
53	0.5	4	16	32	4	>64	>64	64	32	8	17.03	1.06	0.49	0.70	0.48	0.003
55	0.5	4	16	32	4	>64	>64	64	64	8	18.25	1.38	0.85	0.64	0.37	0.004
57	0.5	4	16	32	4	>64	>64	64	64	8	10.43	4.69	0.37	0.70	0.78	0.003
78	0.5	32	<2	16	2	4	>64	64	<0.5	4	23.88	5.44	0.96	1.14	0.40	0.007
88	0.5	4	32	16	2	>64	>64	32	32	4	11.77	6.60	0.92	0.73	0.24	0.003
118	0.5	32	16	32	2	>64	>64	32	64	4	35.08	0.61	1.31	0.64	0.27	0.003
126	0.5	16	<2	2	4	4	>64	32	<0.5	4	1.77	1.06	1.27	0.64	0.32	0.004
36	1	4	16	32	2	>64	>64	64	64	4	32.25	0.61	0.80	1.03	0.69	0.004
38	1	4	32	32	2	>64	>64	64	64	8	30.51	1.34	0.78	1.14	0.51	0.002
58	1	4	32	32	4	>64	>64	64	64	8	26.93	2.20	0.44	0.85	0.45	0.005
83	1	2	32	32	4	>64	>64	64	32	4	33.76	7.98	1.37	0.80	0.19	0.002
84	1	2	32	32	4	>64	>64	64	64	4	13.45	10.02	1.08	0.85	0.24	0.003
86	1	64	8	32	4	32	>64	2	2	4	22.39	6.85	0.97	0.69	0.19	0.004
87	1	64	4	64	4	16	>64	2	2	4	43.48	6.82	0.92	0.67	0.08	0.003
89	1	>64	4	64	4	32	>64	64	64	4	7.25	5.61	1.17	0.89	0.21	0.003
90	1	2	16	64	4	>64	>64	64	32	8	20.86	10.33	1.00	0.72	0.23	0.004
110	1	2	16	16	4	>64	>64	32	64	4	30.22	5.18	1.25	0.31	0.30	0.003
111	1	2	16	16	2	>64	>64	32	32	4	9.86	5.39	1.55	0.31	0.21	0.003
112	1	2	8	16	2	>64	>64	32	32	8	11.45	3.06	1.23	0.21	0.18	0.36
113	1	2	<2	32	2	4	>64	32	<0.5	4	24.40	4.27	0.89	0.31	0.25	0.006
114	1	1	<2	32	2	4	>64	32	1	4	7.01	3.87	0.77	0.27	0.26	0.004
115	1	1	<2	16	2	4	>64	16	<0.5	4	30.32	3.13	0.82	0.75	0.19	0.003
119	1	32	16	32	2	>64	>64	32	32	4	43.01	0.60	1.23	0.54	0.33	0.003
120	1	32	16	16	2	>64	>64	32	32	4	35.43	0.66	0.75	0.65	0.19	0.003
124	1	4	64	64	4	>64	>64	32	64	4	46.58	0.72	0.79	0.63	0.30	0.004
125	1	4	16	32	8	>64	>64	64	32	4	32.77	8.97	0.45	0.70	0.30	0.005
56	2	2	<2	32	4	8	>64	64	1	8	21.05	5.82	0.48	0.87	0.38	0.003
64	2	64	4	32	2	16	>64	2	4	4	68.98	2.94	0.36	0.62	0.21	0.004
79	2	>64	4	16	1	16	>64	2	2	8	37.66	12.58	1.57	0.59	0.22	0.004
80	2	>64	8	32	4	32	>64	1	2	8	32.70	10.77	0.97	0.63	0.19	0.002
81	2	64	4	32	4	16	>64	2	2	4	46.54	11.16	0.92	0.86	0.18	0.002
82	2	64	<2	32	2	16	>64	1	1	4	27.63	10.04	1.00	0.97	0.15	0.002
101	2	2	16	32	4	>64	>64	32	32	4	12.44	4.13	1.25	0.24	0.13	0.003
102	2	2	16	32	4	>64	>64	32	32	4	8.91	7.26	1.17	0.36	0.28	0.005
106	2	2	8	64	8	>64	>64	64	32	4	12.06	10.55	1.38	0.76	0.31	0.006
107	2	2	32	64	8	>64	>64	64	32	4	16.38	10.80	0.91	0.25	0.32	0.004
108	2	4	32	64	8	>64	>64	64	32	8	23.92	10.94	1.07	0.21	0.29	0.002
109	2	4	32	64	8	>64	>64	64	32	8	23.37	6.32	0.68	0.32	0.22	0.003

^a Strains are ranked according to the MIC of tigecycline.

^b Increased gene RE values of ≥2 are indicated in bold.

^c The reference strain used was *A. baumannii* ATCC 17978. RNA (50 μg/ml) was from strains of the PFGE-ROC-1_{OXA58} clone.

^d TIG, tigecycline; GEN, gentamicin; AMK, amikacin; DOX, doxycycline; MIN, minocycline; NET, netilmicin; TET, tetracycline; RIF, rifampin; TOB, tobramycin; DOR, doripenem.

the Iberian Peninsula (41, 42). Only strains of the PFGE-HUI-1 clone ($n = 19$ strains) have previously shown susceptibility to carbapenems (23).

Overexpression of the AdeABC system (RE, 30- to 45-fold) was significantly associated with resistance to gentamicin (MICs > 8 mg/liter) in strains of PFGE-ROC-1 (which produce the OXA-58 and OXA-24 β-lactamases) (8). Moreover, in strains of PFGE-ROC-1_{OXA-24}, resistance to tigecycline (MICs > 0.5 mg/liter) and minocycline (MICs > 2 mg/liter) was also significantly associated

with expression of this efflux pump, as previously reported (8). All strains of the PFGE-ROC-1 clone had mutations in the *adeR* (Ala₁₃₆ → Val) and *adeS* (Ala₉₄ → Val, Gly₁₈₆ → Val, and Phe₂₁₄ → Leu) genes. Hornsey et al. associated the Ala₉₄ → Val substitution with overexpression of the AdeABC efflux pump in *A. baumannii* strains representative of the prevalent United Kingdom lineage, OXA-23 clone 1 (16, 43). However, the other mutations have not previously been described. Peleg et al. (17) reported that increased (40- to 54-fold) expression of the *adeB* gene was

TABLE 3 MICs and RE of genes harboring efflux pumps^c

Strain ^a	MIC ^d (mg/liter)										RE ^b					
	TIG	GEN	AMK	DOX	MIN	TET	NET	RIF	TOB	DOR	<i>adeB</i> ^b	<i>adeJ</i>	<i>adeG</i>	<i>craA</i>	<i>abeM</i>	<i>amvA</i>
44	≤0.25	2	32	16	1	>64	>64	2	32	64	26.60	1.69	0.68	1.47	0.39	0.002
45	≤0.25	2	32	16	1	>64	>64	1	64	>64	15.00	1.21	0.67	0.71	0.43	0.001
47	≤0.25	4	16	16	1	>64	>64	1	64	64	19.50	1.29	0.80	0.53	0.45	0.002
61	≤0.25	4	16	16	4	>64	>64	2	64	64	23.25	1.24	0.34	0.99	0.15	0.005
62	≤0.25	2	32	16	1	>64	>64	1	64	>64	20.19	1.67	0.49	0.49	0.17	0.002
95	≤0.25	4	32	16	1	>64	>64	<0.5	32	>64	33.95	1.33	1.06	0.32	0.22	0.002
40	0.5	4	32	16	1	>64	>64	1	64	64	12.88	0.25	0.59	0.91	0.36	0.001
46	0.5	2	32	16	1	>64	>64	1	64	>64	10.82	1.70	0.84	1.18	0.38	0.002
48	0.5	8	32	16	1	>64	>64	1	64	64	10.28	1.13	1.04	0.81	0.33	0.002
50	0.5	2	64	16	<0.5	>64	>64	2	64	64	11.91	1.25	0.44	0.70	0.41	0.002
60	0.5	4	64	16	1	>64	>64	1	64	64	29.20	1.52	0.36	1.18	0.41	0.003
72	0.5	2	32	16	<0.5	>64	>64	1	32	>64	24.94	1.19	0.87	0.73	0.23	0.004
73	0.5	2	32	16	<0.5	>64	>64	2	64	>64	7.53	1.21	0.60	0.72	0.17	0.004
74	0.5	>64	<2	32	4	>64	64	4	4	>64	22.19	1.08	0.51	0.68	0.17	0.003
75	0.5	>64	4	16	4	>64	64	4	4	>64	13.95	0.84	0.49	0.52	0.17	0.002
96	0.5	4	32	16	1	>64	>64	1	64	64	8.55	1.46	0.97	0.31	0.22	0.002
63	1	4	16	16	1	>64	>64	1	32	64	43.11	1.87	0.41	0.59	0.23	0.005
67	1	64	32	16	<0.5	>64	>64	1	64	>64	44.95	0.81	0.63	0.45	0.19	0.002

^a Strains are ranked according to the MIC of tigecycline.^b Increased gene RE values of ≥2 are indicated in bold.^c The reference strain used was *A. baumannii* ATCC 17978. RNA (50 μg/ml) was from strains of the PFGE-ROC-1_{OXA24} clone.^d TIG, tigecycline; GEN, gentamicin; AMK, amikacin; DOX, doxycycline; MIN, minocycline; TET, tetracycline; NET, netilmicin; RIF, rifampin; TOB, tobramycin; DOR, doripenem.

associated with tigecycline MICs of 4 to 16 mg/liter in *A. baumannii*. We found that tigecycline-susceptible strains (MIC = 0.5 mg/liter) were associated with increased expression of the *adeB* gene (about 20- to 30-fold), which could indicate the role of the AdeABC efflux pump in others functions necessary for the pathogenesis of clinical strains of *A. baumannii*, such as colonization, infection, and the persistence of microorganisms in the host (10).

We did not detect the AdeABC efflux pump or regulator genes in clinical strains of *A. baumannii* clone PFGE-HUI-1 (susceptible to carbapenems). As mentioned above, this efflux pump is present in 80% (range, 53% to 97%) of clinical isolates studied so far (8).

Increased expression of the AdeIJK efflux pump (RE, 8- to 10-fold) was significantly associated with tigecycline resistance (MICs > 0.5 mg/liter) and minocycline resistance (MICs > 2

TABLE 4 MICs and RE of efflux pumps^c

Strain ^a	MIC ^d (mg/liter)										RE ^b					
	TIG	GEN	AMK	DOX	MIN	TET	NET	RIF	TOB	DOR	<i>adeB</i>	<i>adeJ</i>	<i>adeG</i>	<i>craA</i>	<i>abeM</i>	<i>amvA</i>
421	1	>64	64	<0.5	<0.5	4	64	2	64	1	NA	1.44	1.23	1.20	0.04	0.01
422	1	16	16	<0.5	<0.5	8	8	2	8	<0.5	NA	1.37	2.11	0.82	0.05	0.02
423	1	16	16	<0.5	<0.5	8	8	2	16	1	NA	1.21	1.16	0.63	0.07	0.02
424	1	16	64	1	1	4	8	16	32	<0.5	NA	1.41	0.89	0.68	0.04	0.02
426	1	64	32	1	1	8	64	16	32	<0.5	NA	1.69	1.00	0.45	0.04	0.02
427	2	16	16	8	<0.5	>64	8	4	8	1	NA	1.46	0.83	0.42	0.04	0.02
428	2	16	16	4	<0.5	>64	4	2	8	1	NA	1.64	0.89	0.49	0.05	0.02
429	2	64	4	4	<0.5	>64	8	4	8	1	NA	1.75	0.91	0.48	0.04	0.01
430	2	32	16	8	<0.5	>64	16	4	16	1	NA	1.22	0.76	0.48	0.04	0.02
431	2	64	4	8	1	>64	32	32	32	1	NA	1.28	1.00	0.44	0.04	0.02
432	2	16	16	4	<0.5	>64	4	4	16	2	NA	1.61	0.90	0.53	0.04	0.02
433	2	16	16	8	<0.5	>64	8	4	16	2	NA	1.51	0.89	0.54	0.04	0.02
434	2	16	16	4	<0.5	>64	8	4	8	2	NA	1.82	1.09	0.50	0.04	0.02
435	2	32	32	4	<0.5	>64	8	2	32	1	NA	1.27	0.90	0.34	0.05	0.01
436	2	16	16	8	1	>64	16	4	8	1	NA	1.32	0.79	0.43	0.04	0.01
437	2	16	16	4	1	>64	4	4	16	<0.5	NA	0.72	1.18	0.78	0.03	0.03
438	2	32	16	8	1	>64	8	4	16	<0.5	NA	1.25	1.51	0.60	0.03	0.02
443	2	16	16	4	<0.5	>64	8	4	8	1	NA	0.91	0.80	0.52	0.04	0.01

^a Strains are ranked according to the MIC of tigecycline.^b Primers for *adeA* and *adeC* genes were also used. NA, not applicable.^c The reference strain used was *A. baumannii* ATCC 17978. RNA (50 μg/ml) was from strains of the PFGE-HUI-1 clone (susceptible to carbapenems).^d TIG, tigecycline; GEN, gentamicin; AMK, amikacin; DOX, doxycycline; MIN, minocycline; TET, tetracycline; NET, netilmicin; RIF, rifampin; TOB, tobramycin; DOR, doripenem.

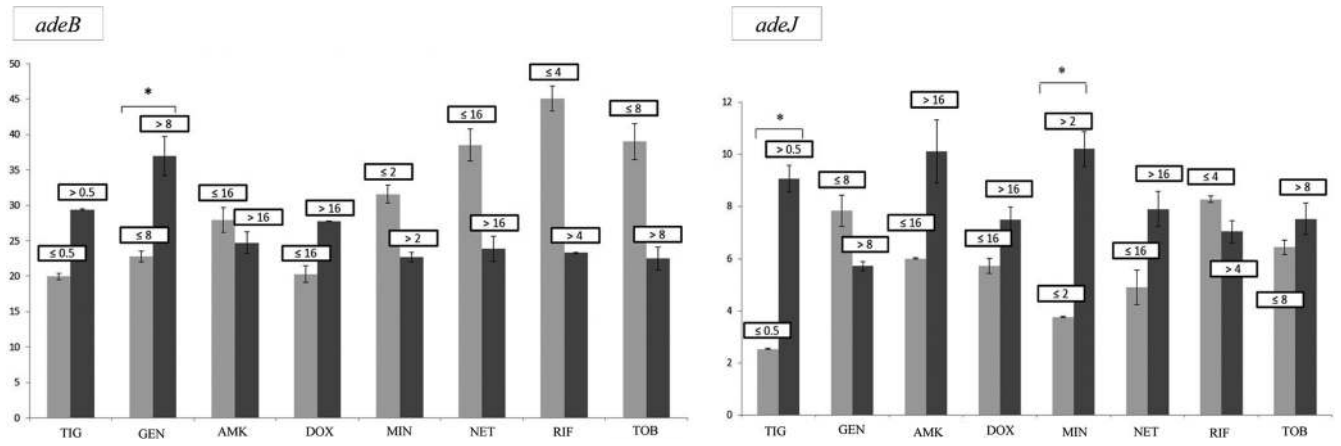


FIG 3 Relative expression of the *adeB* and *adeJ* genes from strains of the PFGE-ROC-1_{OXA-58} clone in relation to the MICs of different antibiotics. *, $P < 0.05$ (Student's *t* test). Light gray bars, strains susceptible to several antibiotics (grouped by MIC [in mg/liter]); dark gray bars, strains resistant to several antibiotics (grouped by MIC [in mg/liter]); TIG, tigecycline; GEN, gentamicin; AMK, amikacin; DOX, doxycycline; MIN, minocycline; NET, netilmicin; RIF, rifampin; TOB, tobramycin.

mg/liter) in strains of PFGE-ROC-1_{OXA-58}. However, this system was not significantly associated with resistance to netilmicin or tobramycin (aminoglycosides). These results are consistent with those obtained by Coyne et al. (44). These authors also noted that overexpression of this pump is always lower than that of the AdeABC system. These results could confirm the theory that high-level expression of the AdeIJK efflux pump is toxic to the host cell (45). The *adeJ* gene was not overexpressed in strains of the PFGE-ROC-1_{OXA-24} and PFGE-HUI-1 clones. Only two strains of the PFGE-ROC-1_{OXA-58} clone had two new mutations in a gene regulating the AdeIJK pump (*adeN*; His₁₁₁ → Pro, Ile₁₁₂ → Phe), and all strains of PFGE-HUI-1 had a Pro₁₆ → Lys substitution in the *adeN* gene. None of these mutations have been associated with

overexpression of AdeIJK, although other possible mechanisms of regulation cannot be ruled out (18).

Expression of *adeG* (AdeFGH), *craA*, *abeM*, and *amvA* was not increased (RE, 0.003 to 1) in strains of the PFGE-ROC-1 or PFGE-HUI-1 clone.

Gram-positive bacteria are the origin of *tet* genes detected in Gram-negative bacteria, such as *A. baumannii* (22, 38). Here, we detected the *tetB* gene in strains of the PFGE-ROC-1 clone, all of which were resistant to tetracycline (MICs = 16 to 64 mg/liter) and doxycycline (MICs = 16 to 64 mg/liter). Moreover, in some strains (PFGE-ROC-1_{OXA-58}), overexpression of AdeIJK together with the presence of this acquired efflux pump was possibly associated with resistance to minocycline (MICs = 2 to 4 mg/liter). In

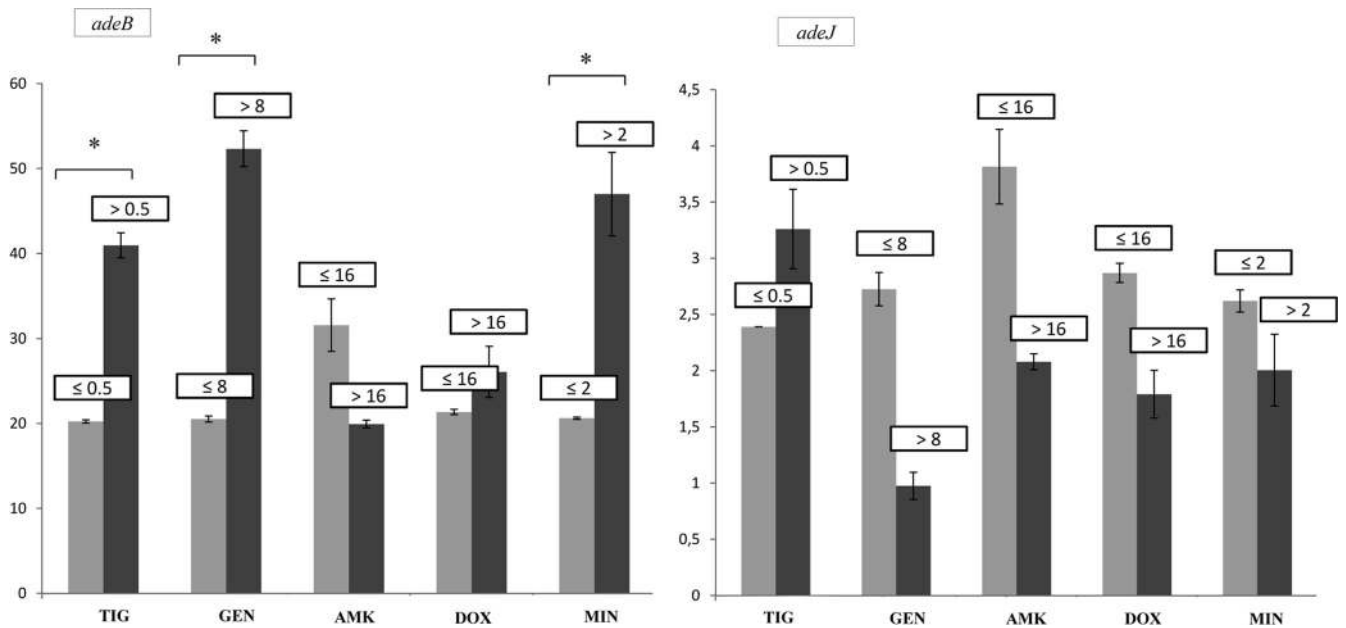


FIG 4 Relative expression of the *adeB* and *adeJ* genes from strains of the PFGE-ROC-1_{OXA-24} clone in relation to the MICs of different antibiotics. *, $P < 0.05$ (Student's *t* test). Light gray bars, strains susceptible to several antibiotics (grouped by MIC [in mg/liter]); dark gray bars, strains resistant to several antibiotics (grouped by MIC [in mg/liter]); TIG, tigecycline; GEN, gentamicin; AMK, amikacin; DOX, doxycycline; MIN, minocycline.

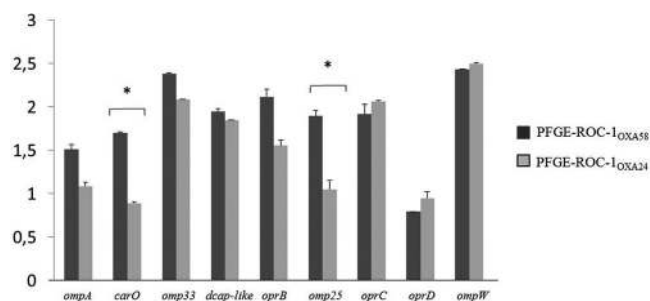


FIG 5 Relative expression of porin genes by strains of the PFGE-ROC-1_{OXA-58} and PFGE-ROC-1_{OXA-24} clones. *, $P < 0.05$ (Student's *t* test).

other pathogens, such as *Escherichia coli*, the combination of AcrAB-TolC and TetA has been associated with a high degree of resistance to tetracycline (46). Our results showed that detection of the *tetA*(39) gene in all strains of this clone was associated with resistance to tetracycline and doxycycline. Agersø and Guardabassi (47) analyzed the presence of this gene in *A. baumannii* strains. These authors located the gene in both environmental and clinical strains, and they found the *tetA*(39) gene in 33 tigecycline-resistant strains (MICs ≥ 16 mg/liter). We noted that in strains of PFGE-HUI-1 harboring the *tetA*(39) gene, the tigecycline MIC was lower (2 to 1 mg/liter) in the presence of PAbetaN (an RND efflux pump inhibitor), and the MIC decreased from 1 to 0.25 mg/liter in the *A. baumannii* strains without this gene. This suggests the involvement of a new RND efflux pump, together with the TetA(39) system, in the resistance to tigecycline.

In relation to porins and unlike in other pathogens, such as *Pseudomonas aeruginosa*, in which OprD expression plays an important role in resistance to carbapenem antibiotics (39), we found that decreased expression of a porin was significantly associated with antimicrobial resistance. We observed decreased RE only of the *carO* and *omp25* genes, on comparing strains of PFGE clone-ROC-1_{OXA-58} and PFGE clone-ROC-1_{OXA-24}. This decrease was not associated with resistance to carbapenems, which is known to be associated with the presence of the β -lactamases (9). Moreover, the carbapenem resistance was not associated with expression of the OXA-51 and AmpC chromosomal β -lactamases.

Overall, our data revealed that the presence of OXA-type enzymes (OXA-24 and/or OXA-58) is sufficient to confer resistance to carbapenem in the *A. baumannii* strains under study, as previously found (41, 42). Moreover, resistance to doripenem was also associated with the presence of the β -lactamases OXA-58 (MICs = 4 to 8 mg/liter) and OXA-24 (MICs = 64 to >64 mg/liter) (compared with MICs for strains of the PFGE-HUI-1 clone of 0.5 to 1 mg/liter). Marti and colleagues (48) analyzed the activity of doripenem against clinical isolates of *A. baumannii* and concluded that doripenem was more active than imipenem and meropenem in strains carrying the OXA-58 β -lactamase gene. However, in the present study, doripenem, imipenem, and meropenem MICs were high for the clinical strains producing the OXA-24 enzyme.

Quinolone resistance did not vary between the strains under study and was associated with previously reported mutations in *gyrA* and *parC* (7). Aminoglycoside-resistant isolates of clones PFGE-ROC-1 and PFGE-HUI-1 showed acetylases known to be common in *A. baumannii* strains (AacC1, AphA1, and AadB) (49).

In conclusion, (i) the clinical strains of *Acinetobacter baumannii* under study possess efflux systems and other mechanisms (possibly connected) that enable them to develop resistance to various antimicrobials and that also have other functions necessary in bacterial pathogenesis. (ii) Overexpression of the AdeABC system was found to be associated with resistance to glycylycylcine (tigecycline and minocycline) and aminoglycosides (gentamicin), and possibly other biological functions, in the clinical strains under study. (iii) Hyperexpression of the AdeIJK efflux pump was significantly associated with resistance to tigecycline and minocycline but did not appear to be involved in other functions related to the pathogenesis of the bacterium. This efflux pump may be related to the TetB system and, thus, to minocycline resistance. (iv) Porins, AmpC β -lactamases, and OXA-51 were not involved in the antimicrobial resistance observed in the present study in the presence of OXA-type enzymes (OXA-24 and OXA-58). (v) The OXA-24 and OXA-58 β -lactamases were associated with resistance to meropenem, doripenem, and imipenem (especially the OXA-24 β -lactamase). (vi) The presence of the Tet efflux pumps in *A. baumannii* isolates was associated with resistance to tetracyclines and doxycycline. (vii) Finally, a new RND efflux pump may

TABLE 5 Interplay of mechanisms of resistance to several antibiotics of the strains of clones PFGE-ROC-1 and PFGE-HUI-1 under study

Antibiotic(s)	Mechanism of resistance		
	PFGE-ROC-1 _{OXA-58}	PFGE-ROC-1 _{OXA-24}	PFGE-HUI-1
Tigecycline	Overexpression of AdeIJK	Overexpression of AdeABC	New RND efflux system/TetA(39) efflux pump ^c
Gentamicin	Overexpression of AdeABC/acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)	Overexpression of AdeABC/acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)	Acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)
Minocycline	Overexpression of AdeIJK/TetB efflux pumps	Overexpression of AdeABC ^b	
Netilmicin, tobramycin, and amikacin	Acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)	Acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)	Acetylases (<i>aacC1</i> , <i>aphA1</i> , <i>aadB</i>)
Imipenem, meropenem, and doripenem	OXA-58 β -lactamase	OXA-24 β -lactamase	
Ciprofloxacin	Mutations in <i>gyrA</i> and <i>parC</i>	Mutations in <i>gyrA</i> and <i>parC</i>	Mutations in <i>gyrA</i> and <i>parC</i>
Doxycycline	Overexpression of AdeIJK ^a /TetB efflux pumps	TetB efflux pump	TetA(39) efflux pump ^c
Tetracyclines	TetB efflux pump	TetB efflux pump	TetA(39) efflux pump ^c

^a Nonsignificantly increased expression relative to strains with doxycycline resistance.

^b Only three isolates, 61, 74, and 75.

^c Except for strains 421, 422, 423, 424, and 426.

act in combination with the TetA(39) system to confer resistance to tigecycline in the absence of the AdeABC efflux pump and overexpression of the other systems in *A. baumannii* clinical strains susceptible to carbapenems.

The main limitation of the study was that we were not able to study the complex mechanisms of resistance to carbapenems in strains that did not produce OXA-type enzymes.

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REFERENCES

1. Peleg AY, Seifert H, Paterson DL. 2008. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* 21:538–582.
2. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51:3471–3484.
3. Bou G, Martínez-Beltrán J. 2000. Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 44:428–432.
4. Brown S, Young HK, Amyes SG. 2005. Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. *Clin. Microbiol. Infect.* 11:15–23.
5. del Mar Tomás M, Beceiro A, Pérez A, Velasco D, Moure R, Villanueva R, Martínez-Beltrán J, Bou G. 2005. Cloning and functional analysis of the gene encoding the 33- to 36-kilodalton outer membrane protein associated with carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 49:5172–5175.
6. Limansky AS, Mussi MA, Viale AM. 2002. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. *J. Clin. Microbiol.* 40:4776–4778.
7. Hujer KM, Hujer AM, Endimiani A, Thomson JM, Adams MD, Goglin K, Rather PN, Pennella TT, Massire C, Eshoo MW, Sampath R, Blyn LB, Ecker DJ, Bonomo RA. 2009. Rapid determination of quinolone resistance in *Acinetobacter* spp. *J. Clin. Microbiol.* 47:1436–1442.
8. Coyne S, Courvalin P, Périchon B. 2011. Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob. Agents Chemother.* 55:947–953.
9. Poirel L, Nordmann P. 2006. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin. Microbiol. Infect.* 12:826–836.
10. Martínez JL, Sanchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33:430–449.
11. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Périchon B. 2010. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54:4389–4393.
12. Huys G, Cnockaert M, Nemeč A, Swings J. 2005. Sequence-based typing of adeB as a potential tool to identify intraspecific groups among clinical strains of multidrug-resistant *Acinetobacter baumannii*. *J. Clin. Microbiol.* 43:5327–5331.
13. Magnet S, Courvalin P, Lambert T. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob. Agents Chemother.* 45:3375–3380.
14. Sun JR, Chan MC, Chang TY, Wang WY, Chiueh TS. 2010. Overexpression of the adeB gene in clinical isolates of tigecycline-nonsusceptible *Acinetobacter baumannii* without insertion mutations in adeRS. *Antimicrob. Agents Chemother.* 54:4934–4938.
15. Sun JR, Perng CL, Chan MC, Morita Y, Lin JC, Su CM, Wang WY, Chang TY, Chiueh TS. 2012. A truncated AdeS kinase protein generated by ISAbA1 insertion correlates with tigecycline resistance in *Acinetobacter baumannii*. *PLoS One* 7:e49534. doi:10.1371/journal.pone.0049534.
16. Hornsey M, Ellington MJ, Doumith M, Thomas CP, Gordon NC, Wareham DW, Quinn J, Lolans K, Livermore DM, Woodford N. 2010. AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 65:1589–1593.
17. Peleg AY, Adams J, Paterson DL. 2007. Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 51:2065–2069.
18. Rosenfeld N, Bouchier C, Courvalin P, Périchon B. 2012. Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter*

- baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrob. Agents Chemother.* 56:2504–2510.
19. Roca I, Marti S, Espinal P, Martinez P, Gibert I, Vila J. 2009. CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53:4013–4014.
 20. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. 2005. AbeM, an H⁺-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob. Agents Chemother.* 49:4362–4364.
 21. Rajamohan G, Srinivasan VB, Gebreyes WA. 2010. Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 65:1919–1925.
 22. Akers KS, Mende K, Yun HC, Hospenthal DR, Beckius ML, Yu X, Murray CK. 2009. Tetracycline susceptibility testing and resistance genes in isolates of *Acinetobacter baumannii*-*Acinetobacter calcoaceticus* complex from a U.S. military hospital. *Antimicrob. Agents Chemother.* 53:2693–2695.
 23. Fernández-Cuenca F, Tomás-Carmona M, Caballero-Moyano F, Bou G, Martínez-Martínez L, Vila J, Pachón J, Cisneros JM, Rodríguez-Baño J, Pascual A, Grupo del Proyecto GEIH-REIPI-Ab 2010. 2013. In vitro activity of 18 antimicrobial agents against clinical isolates of *Acinetobacter* spp.: multicenter national study GEIH-REIPI-Ab 2010. *Enferm. Infecc. Microbiol. Clin.* 31:4–9. (In Spanish.)
 24. Espinal P, Seifert H, Dijkshoorn L, Vila J, Roca I. 2012. Rapid and accurate identification of genomic species from the *Acinetobacter baumannii* (Ab) group by MALDI-TOF MS. *Clin. Microbiol. Infect.* 18:1097–1103.
 25. Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos P, Claeys G, Verschraegen G. 1995. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* 33:11–15.
 26. Turton JF, Woodford N, Glover J, Yarde S, Kaufmann ME, Pitt TL. 2006. Identification of *Acinetobacter baumannii* by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *J. Clin. Microbiol.* 44:2974–2976.
 27. Zander E, Higgins PG, Fernández-González A, Seifert H. 2013. Detection of intrinsic bla(OXA-51-like) by multiplex PCR on its own is not reliable for the identification of *Acinetobacter baumannii*. *Int. J. Med. Microbiol.* 303:88–89.
 28. Zhang L, Ding G, Wei L, Pan X, Mei L, Zhang Y, Lu Y. 2011. Establishment of a novel target-based real-time quantitative PCR method for *Acinetobacter baumannii* detection. *Diagn. Mol. Pathol.* 20:242–248.
 29. Seifert H, Dolzani L, Bressan R, van der Reijden T, van Strijen B, Stefanik D, Heersma H, Dijkshoorn L. 2005. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J. Clin. Microbiol.* 43:4328–4335.
 30. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233–2239.
 31. Nemeč A, Krizová L, Maixnerová M, Diancourt L, van der Reijden TJ, Brisse S, van den Broek P, Dijkshoorn L. 2008. Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant strains of European clone II. *J. Antimicrob. Chemother.* 62:484–489.
 32. Pannek S, Higgins PG, Steinke P, Jonas D, Akova M, Bohnert JA, Seifert H, Kern WV. 2006. Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenylarginine-beta-naphthylamide. *J. Antimicrob. Chemother.* 57:970–974.
 33. Akers KS, Chaney C, Barsoumian A, Beckius M, Zera W, Yu X, Guymon C, Keen EF, III, Robinson BJ, Mende K, Murray CK. 2010. Aminoglycoside resistance and susceptibility testing errors in *Acinetobacter baumannii*-*calcoaceticus* complex. *J. Clin. Microbiol.* 48:1132–1138.
 34. Walther-Rasmussen J, Høiby N. 2006. OXA-type carbapenemases. *J. Antimicrob. Chemother.* 57:373–383.
 35. Higgins PG, Poirel L, Lehmann M, Nordmann P, Seifert H. 2009. OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 53:5035–5038.
 36. Ellington MJ, Kistler J, Livermore DM, Woodford N. 2007. Multiplex PCR for rapid detection of genes encoding acquired metallo-beta-lactamases. *J. Antimicrob. Chemother.* 59:321–322.
 37. Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC. 2003. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob. Agents Chemother.* 47:883–888.
 38. Ribera A, Ruiz J, Vila J. 2003. Presence of the Tet M determinant in a clinical isolate of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 47:2310–2312.
 39. Tomás M, Doumith M, Warner M, Turton JF, Beceiro A, Bou G, Livermore DM, Woodford N. 2010. Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* 54:2219–2224.
 40. Cabot G, Ocampo-Sosa AA, Tubau F, Macía MD, Rodríguez C, Moya B, Zamorano L, Suárez C, Peña C, Martínez-Martínez L, Oliver A, Spanish Network for Research in Infectious Diseases (REIPI). 2011. Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. *Antimicrob. Agents Chemother.* 55:1906–1911.
 41. Merino M, Acosta J, Poza M, Sanz F, Beceiro A, Chaves F, Bou G. 2010. OXA-24 carbapenemase gene flanked by XerC/XerD-like recombination sites in different plasmids from different *Acinetobacter* species isolated during a nosocomial outbreak. *Antimicrob. Agents Chemother.* 54:2724–2727.
 42. Ruiz M, Marti S, Fernandez-Cuenca F, Pascual A, Vila J. 2007. High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain. *Clin. Microbiol. Infect.* 13:1192–1198.
 43. Hornsey M, Loman N, Wareham DW, Ellington MJ, Pallen MJ, Turton JF, Underwood A, Gaulton T, Thomas CP, Doumith M, Livermore DM, Woodford N. 2011. Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *J. Antimicrob. Chemother.* 66:1499–1503.
 44. Coyne S, Guignon G, Courvalin P, Périchon B. 2010. Screening and quantification of the expression of antibiotic resistance genes in *Acinetobacter baumannii* with a microarray. *Antimicrob. Agents Chemother.* 54:333–340.
 45. Damier-Piolle L, Magnet S, Bremont S, Lambert T, Courvalin P. 2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 52:557–562.
 46. de Cristóbal RE, Vincent PA, Salomón RA. 2006. Multidrug resistance pump AcrAB-TolC is required for high-level, Tet(A)-mediated tetracycline resistance in *Escherichia coli*. *J. Antimicrob. Chemother.* 58:31–36.
 47. Agersø Y, Guardabassi L. 2005. Identification of Tet 39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. *J. Antimicrob. Chemother.* 55:566–569.
 48. Marti S, Sánchez-Céspedes J, Alba V, Vila J. 2009. In vitro activity of doripenem against *Acinetobacter baumannii* clinical isolates. *Int. J. Antimicrob. Agents* 33:181–182.
 49. Asadollahi K, Taherikalani M, Maleki A, Alizadeh E, Valadbaigi H, Soroush S, Maleki H, Asadollahi P, Emancini M. 2011. Diversity of aminoglycoside modifying enzyme genes among multidrug resistant *Acinetobacter baumannii* genotypes isolated from nosocomial infections in Tehran hospitals and their association with class 1 integrons. *Acta Microbiol. Immunol. Hung.* 58:359–370.