

Role of Common *bla*_{OXA-24/OXA-40}-Carrying Platforms and Plasmids in the Spread of OXA-24/OXA-40 among *Acinetobacter* Species Clinical Isolates

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The spread of OXA-24/OXA-40 (OXA-24/40)-producing *Acinetobacter* spp. in the Iberian Peninsula has been strongly influenced by clonal expansion, but the role of horizontal gene transfer has scarcely been explored. *bla*_{OXA-24/40}-carrying plasmids and genetic environments were characterized in representative ($n = 15$) *Acinetobacter* species clinical isolates (obtained between 2001 and 2007) by *Acinetobacter baumannii* PCR-based replicon typing, sequencing, hybridization, and restriction fragment length polymorphism. Besides the identification of *bla*_{OXA-24/40} within the chromosomes of some isolates, the circulation of common *bla*_{OXA-24/40}-carrying plasmids (30-kb *repA_AB*; 10-kb *aci2*) and genetic backbones among *Acinetobacter* spp. was demonstrated.

Carbapenem resistance in *Acinetobacter* spp. has been mostly associated with the production of OXA-23, OXA-24/OXA-40 (OXA-24/40), and OXA-58 carbapenem-hydrolyzing class D β -lactamases (1, 3, 5–12). Whereas OXA-23- and OXA-58-producing *Acinetobacter baumannii* are widely disseminated (3, 6, 12), OXA-24/40 producers have been reported mainly as endemic in the Iberian Peninsula, where the ongoing spread of OXA-24/40 since 1995 has been explained by clonal expansion (6, 10). In addition, there have been reports of sporadic cases in Italy and France and of outbreaks in the United States (5, 7, 8). Recently, some studies demonstrated the mobilization of *bla*_{OXA-24/40} by lateral gene transfer events (1, 5, 9, 10). In this study, the characterization of the diversity of OXA-24/40-encoding plasmids and the genetic environment of the *bla*_{OXA-24/40} genes was conducted in *A. baumannii* and *Acinetobacter haemolyticus* clinical isolates from Portugal.

Fifteen representative OXA-24/40-producing *Acinetobacter* spp. isolates (13 *A. baumannii* and 2 *A. haemolyticus*) were selected from a collection of 157 carbapenem-resistant *Acinetobacter* species clinical isolates from two geographically distant Portuguese hospitals (obtained between 2001 and 2007), (i) Hospital Geral de Santo António (HGSA) and (ii) Centro Hospitalar da Cova da Beira (CHCB) (Table 1) (6). Isolates were identified with the Vitek 2 system and 16S rRNA gene sequencing. Detection of the *bla*_{OXA-24/40} gene was performed as previously described (6, 10). Antibiotic susceptibility testing was done by Etest and the disk diffusion method following CLSI guidelines (4). Isolates were chosen on the basis of their ApaI pulsed-field gel electrophoresis (PFGE) patterns (4 subtypes arbitrarily designated A [$n = 4$; obtained between 2001 and 2004 from HGSA], A1 [$n = 3$; obtained between 2006 and 2007 from CHCB], A3 [$n = 5$; obtained in 2006 from HGSA], and A4 [$n = 1$; obtained in 2003 from HGSA]) and plasmid content (1 to 3 plasmids ranging from 10 kb to 90 kb). They all belonged to the ST98 lineage (6) according to the multilocus sequence typing (MLST) scheme proposed by Bartual et al. (2) (<http://pubmlst.org/abaumannii/>). This sequence type (ST) is a double-locus variant of the worldwide-disseminated ST92, which

is endemic in Portuguese hospitals and has been identified in other European countries associated with the production of different OXA types (6, 12). Conjugative transfer (solid, 37°C) and transformation by electroporation were conducted in a subset of isolates using *A. baumannii* ATCC 19606, *Escherichia coli* K-12 BM21 (rifampin and nalidixic acid resistant, plasmid free), or *Acinetobacter baylyi* ADP1 as the recipient strain, as described previously (5). Transconjugants and transformants were selected using tryptic soy agar (TSA) plates supplemented with rifampin (100 μ g/ml) and/or ampicillin (30 μ g/ml) or imipenem (0.5 to 1 μ g/ml), as reported. The location (chromosome/plasmid) of *bla*_{OXA-24/40} was assessed by hybridization of I-CeuI and S1 gels with specific probes (*bla*_{OXA-24/40}, 16S rRNA genes) in the wild-type and transformant strains. Plasmid characterization was further accomplished by the recently proposed PCR-based replicon typing scheme for *A. baumannii* plasmids (AB-PBRT), sequencing and hybridization with replicase (*rep*) probes (3), and restriction fragment length polymorphism (RFLP) (EcoRI, BamHI, or HindIII).

An *in silico* comparative analysis of six fully sequenced and one partially sequenced *Acinetobacter* species *bla*_{OXA-24/40}-carrying plasmids deposited in the GenBank database (pABVA01, pMCMCU3, pMCMCU1, pMCMCU2, pMMD, pAB02, and pMMA2, with the respective accession numbers NC_012813.1, GQ904227.1, GQ342610.1, NC_013506.1, GQ904226.1, AY228470.1, and GQ377752.1) was performed at the NCBI website using the BLAST software and the nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Based on those sequences, the vicinity of the *bla*_{OXA-24/40} gene was characterized

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TABLE 1 Epidemiology and plasmid characterization of representative OXA-24/40-producing *Acinetobacter* species clinical isolates from Portugal

Species	PFGE subtype (no. of isolates)	Yr(s) of isolation	Hospital ^a	<i>bla</i> _{OXA-24/40} plasmid	<i>rep</i> content ^b (plasmid size [kb])	Antibiotic resistance profile ^d
				size(s) (kb) ^b (RFLP profile) (no. of isolates)		
<i>A. baumannii</i>	A (4)	2001–2004	HGSA	90, 30 (A) (3)	<u><i>repA_AB</i> (30), <i>repAci2</i>, <i>repAci6</i></u>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, TOB, TET, MIN
		2001–2004	HGSA	70, 30 (ND) (1)	<i>repA_AB</i> (30), <i>repAci2</i> , <i>repAci6</i>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP
	A1 (3)	2006–2007	CHCB	10^c (B2) (3)	<i>repA_AB</i> , <u><i>repAci2</i> (10)</u> , <i>repAci6</i>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP
	A3 (5)	2006	HGSA	90, 10 (B2)^c (1)	<i>repA_AB</i> , <u><i>repAci2</i> (10)</u> , <i>repAci6</i>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, (TOB)
	A4 (1)	2003	HGSA	30 (A1) (4)	<u><i>repA_AB</i> (30), <i>repAci2</i>, <i>repAci6</i></u>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, (TOB)
				30 (ND), 10 (B)^c (1)	<i>repA_AB</i> , <u><i>repAci2</i> (10)</u>	CAZ, ATM, IPM, MEM, PIP, FEP, CIP, MIN
<i>A. haemolyticus</i>	(2)	2002	HGSA	90, 30 (A1)^c (2)	<u><i>repA_AB</i> (30), <i>repAci2</i></u>	IPM, MEM, PIP, CIP, TOB, TET

^a HGSA, Hospital Geral de Santo António (Northern region); CHCB, Centro Hospitalar da Cova da Beira (Center region).

^b Plasmid number, size, type, and *rep* content were determined by RFLP, PCR, and hybridization of S1- and I-CeuI-digested genomic DNA with specific probes (*bla*_{OXA-24/40}, *repAci2*, *repA_AB*, and *repAci6*). ND, not done. Plasmids harboring *bla*_{OXA-24/40} appear in bold. Plasmid transfer by transformation is shown as underlined.

^c The *bla*_{OXA-24/40} probe additionally hybridized in a chromosomal band.

^d Parentheses indicate variability of a given resistance determinant. Resistance among transformants is represented by underlining. Abbreviations: CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; PIP, piperacillin; FEP, cefepime; CIP, ciprofloxacin; TOB, tobramycin; TET, tetracycline; MIN, minocycline.

in representative plasmids from our collection by a PCR mapping strategy and further sequencing (Fig. 1).

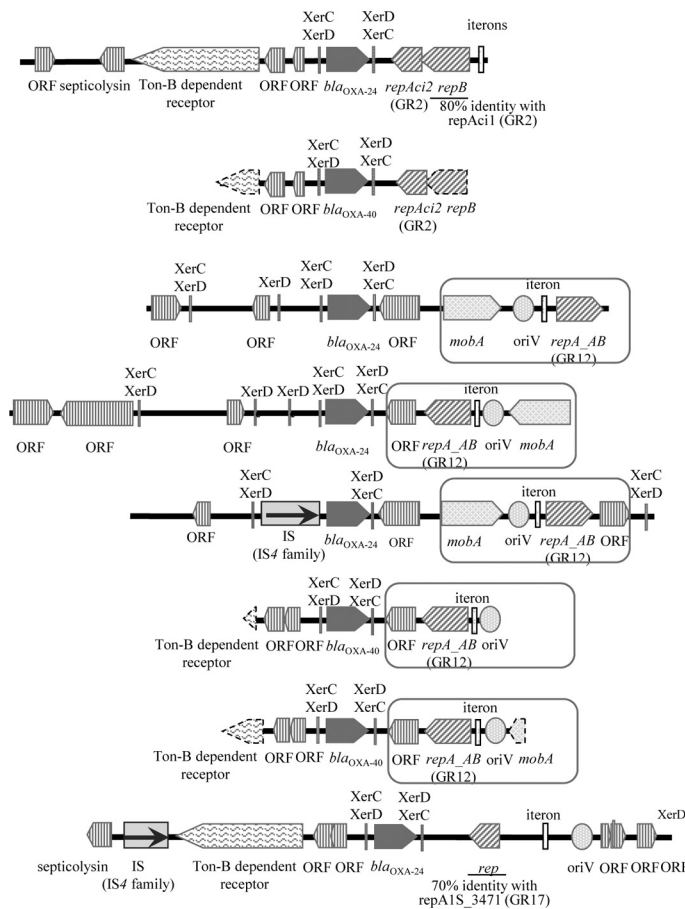
A. baumannii isolates were resistant to almost all β -lactams, showing variable susceptibility to aztreonam, whereas *A. haemolyticus* isolates ($n = 2$) exhibited resistance to all β -lactams except cefepime, ceftazidime, and aztreonam. All isolates were resistant to ciprofloxacin, whereas susceptibility to aminoglycosides and tetracycline was variable, according to CLSI guidelines (Table 1) (4). As consistently observed in other studies, attempts to obtain OXA-24/40-producing transconjugants were unsuccessful (9). Transformants were obtained for *A. baumannii* isolates belonging to PFGE type A ($n = 1$) and PFGE type A4 ($n = 1$) and one *A. haemolyticus* isolate by using *A. baylyi* ADP1 as the receptor strain, and they acquired resistance to all β -lactams except ceftazidime, aztreonam, and cefepime. Resistance to other groups of antibiotics was not cotransferred. Variable plasmid size (90 kb, 70 kb, 30 kb and/or 10 kb) and *rep* content (GR2 group *aci2* and/or *aci6* and/or GR12 group *repA_AB*) were observed among the different species and PFGE types. The *bla*_{OXA-24/40} gene was identified within distinct plasmids (70 to 90 kb and 30 kb, 90 kb and 10 kb, 30 kb, or 10 kb) and additionally within the chromosomes of some *A. baumannii* and *A. haemolyticus* isolates (Table 1).

Highly related 30-kb *bla*_{OXA-24/40}-carrying plasmids (arbitrarily designated RFLP pattern A) were identified in *A. baumannii* (PFGE subtypes A and A3) and *A. haemolyticus* isolates collected within the same hospital (obtained between 2001 and 2006) (Table 1). These plasmids belonged to the GR12 group (3) and showed *rep* content (100% identity to *repA_AB*) and sequences surrounding *bla*_{OXA-24/40} identical to those of the partially sequenced prototype pAB02 plasmid (GenBank accession number AY228470.1) (Fig. 1). A module consisting of an open reading frame (ORF) of unknown function, the replicase *repA_AB*, *oriV*, and *mobA* (represented within a rectangle in Fig. 1) was also found within that structure. Interestingly, it has been previously identified in other 10-kb *repA_AB* plasmids and seems to be a recurrent platform in plasmids (10 kb/30 kb) from different *A. baumannii* clones and *Acinetobacter* species in several European countries (Portugal, Spain, and France) (Fig. 1) (1, 9). The presence of *mob*

genes probably facilitates plasmid mobilization by self-transmissible plasmids and may ultimately explain their occurrence in non-*A. baumannii* species (3, 9, 10).

A 10-kb OXA-24/40-encoding plasmid (arbitrarily designated RFLP pattern B) was also frequently identified among *A. baumannii* isolates (PFGE subtypes A1, A3, and A4) collected from different hospitals (obtained between 2003 and 2007) (Table 1). This plasmid belonged to the GR2 group (3), with a *rep* sequence identical to that of *aci2* and a *bla*_{OXA-24/40} genetic environment closely related to those from the pABVA01 and pMMCU3 plasmids (GenBank accession numbers NC_012813 and GQ904227.1, respectively) identified in *A. baumannii* isolates from Italy and Spain, respectively (1, 5, 9).

The same *rep* genes (GR2, GR6, and GR12) have been presumptively associated with other OXA-24/40-producing *A. baumannii* plasmids from Portugal and Spain by AB-PBRT, thus suggesting the circulation of a common plasmid pool (3, 12). Both *bla*_{OXA-24/40} genes and larger contiguous modules are flanked by XerC/XerD-like binding sites (Fig. 1), which might be responsible for their mobilization (5, 9) and may explain the identification of *bla*_{OXA-24/40} and/or common modules in different plasmid scaffolds or in chromosomes. Furthermore, the variability observed in the number and location of the *orf* of unknown function, *oriV*, and iteron sequences suggests frequent recombinatorial events (5, 9). This study not only confirms the horizontal transmission of common resistance modules between *Acinetobacter* plasmids but also highlights interspecies plasmid transfer. Although conjugative transfer was not detected, the *aci6* replicase (GR6; 100% identity to that of plasmid pACICU2 [GenBank accession number NC_010606]) was able to mediate horizontal transfer of antibiotic resistance plasmids, as previously suggested (3, 12). Moreover, a recently described mechanism involving the release of outer membrane vesicles harboring *bla*_{OXA-24/40}-carrying plasmids might also shed some light on this process (11). It is interesting to highlight that the *bla*_{OXA-24/40} genetic environment and plasmid types are different from those observed for *bla*_{OXA-23} or *bla*_{OXA-58}, thus suggesting independent acquisition and subsequent selection events (3, 12).



Plasmid (Accession number)	Size (bp)	Species (Country)	Date	Sequence type	Reference(s)
pABVA01 (NC_012813)	8963	<i>A. baumannii</i> (Italy)	2000	Unknown	5
pMMC3 (GQ904227.1)		(Spain)	2006-2008	Unknown	1, 9
pAC92	~10000 (partial sequence)	<i>A. baumannii</i> (Portugal)	2003-2007	ST98	This study
pMMCU1 (GQ342610.1)	8771	<i>A. calcoaceticus</i> (Spain)	2006-2008	Unknown	1, 9
pMMCU2 (NC_013506.1)	10270	<i>A. baumannii</i> (Spain)	2006-2008	Unknown	1, 9
pMMD (GQ904226.1)	9964	<i>A. baumannii</i> (Spain)	2006-2008	Unknown	1, 9
pAB02 (AY228470.1)	4162 (partial sequence)	<i>A. baumannii</i> (France)	(2004)	Unknown	Unpublished
pAC63	~30000 (partial sequence)	<i>A. haemolyticus</i> <i>A. baumannii</i> (Portugal)	2001-2006	NA ST98	This study
pMMA2 (GQ377752.1)	10679	<i>A. baumannii</i> (Spain)	2006	ST56	1, 9

FIG 1 *bla*_{OXA-24/40} genetic surroundings observed in clinical *Acinetobacter* spp. from different countries that were identified in this and previous studies. Parentheses around a year indicate that the year of submission to GenBank is given because the isolation date is unknown. Discontinuous lines represent partial sequences. NA, not applicable; ORF, open reading frame.

In this study, common *bla*_{OXA-24/40}-carrying plasmids (30-kb *repA_AB* and 10-kb *aci2*) were identified among representative ST98 *A. baumannii* and *A. haemolyticus* clinical isolates collected over a large period of time (2001 to 2007). In addition, with a systematic *in silico* analysis, we were able to identify the same plasmid types and closely related *bla*_{OXA-24/40} genetic surroundings in OXA-24/40-producing isolates from different European countries. These findings, together with some previous evidence for horizontal gene transfer of *bla*_{OXA-24/40}-carrying modules, strongly supports the role of common platforms and plasmids in the dissemination process of OXA-24/40 among *Acinetobacter* spp.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study that correspond to the representative 30-kb *repA_AB* (pAC63) and 10-kb *aci2* (pAC92) OXA-24/40-encoding plasmids have been submitted to the GenBank database (GenBank accession numbers [JN982951](#) and [JN982952](#), respectively).

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