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Contribution of Acquired Carbapenem-Hydrolyzing Oxacillinases to Carbapenem Resistance in *Acinetobacter baumannii*

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Carbapenem-hydrolyzing oxacillinases are reported increasingly in *Acinetobacter baumannii*. Since they hydrolyze carbapenems at low levels, the roles of carbapenem-hydrolyzing oxacillinases OXA-23, OXA-40, and OXA-58 in *A. baumannii* were determined. The bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} genes were inserted in broad-host-range plasmid pAT801 and transformed in *Escherichia coli* DH10B and in *A. baumannii* CIP 70.10 and its point mutant derivative *A. baumannii* BM4547, which overexpresses the efflux pump AdeABC. Natural plasmids harboring the bla_{OXA-23} and bla_{OXA-58} genes were also transformed in *A. baumannii* CIP 70.10. In addition, the bla_{OXA-40} gene was inactivated at its chromosome location in *A. baumannii* CLA-1. Intermediate levels of resistance or reduced susceptibilities to carbapenems were observed for *A. baumannii* transformants expressing OXA-23, OXA-40, and OXA-58. The inactivation of bla_{OXA-40} in *A. baumannii* CLA-1 yielded reduced susceptibilities to carbapenems. Carbapenem-hydrolyzing oxacillinases OXA-23, OXA-40, and to a lesser extent OXA-58 play a role in carbapenem resistance in *A. baumannii*, and overexpression of efflux pump AdeABC may also contribute to higher levels of resistance to β-lactams, including carbapenems.

Acinetobacter baumannii is frequently associated with nosocomial infections (3). Its acquired resistance to many antibiotics may complicate significantly the choice for antibiotic treatment (7, 17). *A. baumannii* naturally produces a cephalosporinase (5) that may be overexpressed due to insertion of insertion sequence (IS) IS*Aba1* that brings promoter sequences for its high level of expression (10, 29; L. Poirel, C. Héritier, and P. Nordmann, Abstr. 6th Int. Symp. Biol. *Acinetobacter*, abstr. pC4, 2004). However, overproduction of AmpC alone contributes to ceftazidime resistance but not to carbapenem resistance (5, 10, 29). Nevertheless, *A. baumannii* may acquire additional β-lactam resistance phenotypes, including carbapenem resistance.

Although carbapenem resistance may be due in part to an impaired permeability related to porin changes or to penicillin binding protein modifications (9, 13, 30), recent reports indicated that carbapenem-hydrolyzing β-lactamases may play a significant role (27). Carbapenem-hydrolyzing oxacillinases (Ambler class D β-lactamases) and metallo-β-lactamases (Ambler class B β-lactamases) have been reported in *A. baumannii* (27, 31).

Seven oxacillinases with weak carbapenemase activity have been characterized in *A. baumannii*, including OXA-23, -24, -25, -26, -27, -40, and -58 from *A. baumannii* isolates in Scotland, Brazil, Spain, Belgium, Singapore, Portugal, and France (1, 4, 6, 11, 12, 14, 15, 19, 23, 26). These oxacillinases may be divided into three different groups. The β-lactamases OXA-23 and -27 share 99% amino acid identity, whereas they share 60% identity with a second group of oxacillinases consisting of OXA-24, -25, -26, and -40 that differ by a few amino acid substitutions (1, 6, 15). β-Lactamase OXA-58 that by itself constitutes a third group of carbapenem-hydrolyzing oxacillinases is weakly related to the other oxacillinases sharing 48 and 47% amino acid identity with OXA-23 and OXA-24, respectively (26). Recently, a novel carbapenem-hydrolyzing oxacillinase, OXA-51, has been described in *A. baumannii* clinical isolates from Argentina (8). This oxacillinase shares less than 62 and 56% amino acid identity with OXA-40 and OXA-23, respectively (8). Its clinical role in carbapenem resistance of *A. baumannii* remains to be determined.

Since studies published on these oxacillinases reported mostly analyses of clinical isolates, it is difficult to compare the precise roles of these β-lactamases in resistance to carbapenems in *A. baumannii* (1, 15, 23, 26).

Thus, the aim of our study was to evaluate the roles of OXA-23, OXA-40, and OXA-58 taken as representatives of the three major groups of carbapenem-hydrolyzing oxacillinases for providing carbapenem resistance in *A. baumannii*, using reference and isogenic strains. Gene manipulations and expression studies have been performed not only in *Escherichia coli* but also in *A. baumannii*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. baumannii* clinical isolates FER and CLA-1 (15) were isolated at the Hôpital de Bicêtre (Le Kremlin-Bicêtre, France) in 2004 and 2001, respectively (Table 1). *A. baumannii* clinical isolate MAD was isolated in 2003 at the Rangueil hospital (Toulouse, France) (26) (Table 1). These isolates were identified by the API 20NE system (bioMérieux, Marcy l'Etoile, France) and by sequencing of 16S rRNA genes (data not shown).

Escherichia coli reference strain DH10B, *A. baumannii* CIP 70.10 (Pasteur Institute, Paris, France) and its point mutant derivative, *A. baumannii* BM4547 (which overexpresses the AdeABC efflux pump [20]), and shuttle plasmid pAT801 (16) were used for cloning experiments. *A. baumannii* CIP70.10 and *A. baumannii* BM4547 were also used in transformation experiments. Plasmid pCR-Blunt II-TOPO (Invitrogen, Cergy Pontoise, France) was used in inactivation experiments.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been referenced elsewhere (2, 24). Antibiotic-containing disks

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TABLE 1. Strains and transformants used in this study

Strain	Origin	β-Lactamase content	Reference
A. baumannii FER	Clinical strain	OXA-23 and AmpC	This study
A. baumannii CLA-1	Clinical strain	OXA-40 and AmpC	15
A. baumannii MAD	Clinical strain	OXA-58 and AmpC	26
E. coli DH10B[a]	Reference strain	AmpC (*E. coli*)	15
A. baumannii CIP 70.10	Reference strain	AmpC	20
A. baumannii BM4547	Reference strain	AmpC	20
E. coli DH10B(pOXA-23)	Transformant	OXA-23	This study
E. coli DH10B(pOXA-40)	Transformant	OXA-40	This study
E. coli DH10B(pOXA-58)	Transformant	OXA-58	This study
A. baumannii CIP 70.10(pOXA-23)	Transformant	OXA-23 and AmpC	This study
A. baumannii CIP 70.10(pOXA-40)	Transformant	OXA-40 and AmpC	This study
A. baumannii CIP 70.10(pOXA-58)	Transformant	OXA-58 and AmpC	This study
A. baumannii BM4547(pOXA-23)	Transformant	OXA-23 and AmpC	This study
A. baumannii BM4547(pOXA-40)	Transformant	OXA-40 and AmpC	This study
A. baumannii BM4547(pOXA-58)	Transformant	OXA-58 and AmpC	This study
A. baumannii CIP 70.10(pFER)	Transformant	OXA-23 and AmpC	This study
A. baumannii CIP70.10(pMAD)	Transformant	OXA-58 and AmpC	26
A. baumannii BM4547(pFER)	Transformant	OXA-23 and AmpC	This study
A. baumannii BM4547(pMAD)	Transformant	OXA-58 and AmpC	This study
A. baumannii CLA-1 (ΔOXA-40)	Recombinant strain	AmpC	This study

[a] As usual in *E. coli*, its naturally occurring *ampC* gene is not expressed.

were used for detection of antibiotic susceptibility with Mueller-Hinton agar plates and a disk diffusion assay (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France) (www.sfm.fr). MICs were determined by an agar dilution technique as previously reported (24), and results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards [NCCLS]) (22).

Plasmid analysis and transformation. Extraction of plasmid DNA from *A. baumannii* FER and *A. baumannii* MAD was attempted using the Kieser method (18). Plasmid suspensions were used for transformation experiments in *A. baumannii* CIP 70.10 using a Gene Pulser II electroporator (Bio-Rad, Ivry-sur-Seine, France) as previously described for *E. coli* (25). Electrotransformation products were selected on ticarcillin (50 μg/ml)-containing plates.

PCR experiments. PCR experiments were performed as previously described (2, 28). The entire bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} genes, without their natural promoter and ribosome binding site (RBS) sequences, were amplified using combinations of primers OXA-23A and OXA-23B, OXA-40A and OXA-40B, and preOXA-58A and preOXA-58B, respectively (Table 2). The sizes of the corresponding PCR products were 840 bp, 846 bp, and 859 bp for the bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} genes, respectively. A 495-bp internal fragment of the bla_{OXA-40} gene was generated with primers OXA-IMP1 and OXA-IMP2 for inactivation experiments (see below). Primers RA-1 and RA-2 (Table 2) were used to amplify a 603-bp fragment corresponding to the entire *arr-2* gene using the genomic DNA of *E. coli* MG-1 as the template (21). All PCR products were sequenced with an Applied Biosystems sequencer (ABI 3100).

Cloning experiments. The low-copy-number plasmid pAT801 conferring resistance to ampicillin was used as a shuttle vector able to replicate in *A. baumannii* and *E. coli*; it consists of part of pWH1266 and pUC18 (16). Inactivation of the bla_{TEM-1} gene of pAT801 was made using a PCR product corresponding to the entire *arr-2* gene inserted into the ScaI-restricted plasmid pAT801, giving rise to pAT801-RA conferring resistance to rifampin. PCR products corresponding to either the bla_{OXA-23}, bla_{OXA-40}, or bla_{OXA-58} gene then were inserted into the EcoRI- and BamHI-restricted plasmid pIM-1-RA under the control of the *lacZ* RBS and promoter sequences. This gave rise to recombinant plasmids pOXA-23, pOXA-40, and pOXA-58, which were transformed in *E. coli* DH10B, as described previously (25). Recombinant plasmids, extracted using the QIAGEN maxiprep kit (QIAGEN, Courtaboeuf, France), were transformed in *A. baumannii* CIP 70.10 and in *A. baumannii* BM4547. Transformants were selected on ticarcillin (50 μg/ml)- and rifampin (25 μg/ml)-containing plates.

Gene inactivation. Kanamycin-resistant plasmid pCR-BluntII-TOPO (Invitrogen, Cergy-Pontoise, France) unable to replicate in *A. baumannii* was used as a suicide vector. An internal fragment of the bla_{OXA-40} gene, generated as described above, was inserted in the pCR-BluntII-TOPO using the Zero Blunt TOPO PCR Cloning kit (Invitrogen, Cergy Pontoise, France), giving rise to plasmid pTOPO-OXA-40. This plasmid was introduced in the kanamycin-susceptible *A. baumannii* CLA-1 strain by electrotransformation. Selection of *A. baumannii* CLA-1(pTOPO-OXA-40) was made on kanamycin (30 μg/ml)-containing plates.

Inactivation of the bla_{OXA-40} gene by insertion of pTOPO-OXA-40 was checked by PCR amplification using primers M13 reverse and OXA-40A (Table 2). The lack of production of β-lactamase OXA-40 in *A. baumannii* CLA-1 (ΔOXA-40) was also verified by isoelectric focusing as previously described (15).

In order to complement *A. baumannii* CLA-1 (ΔOXA-40) isolate, recombinant plasmid pOXA-40 was transformed in *A. baumannii* CLA-1 (ΔOXA-40) as described previously (25). Selection of *A. baumannii* CLA-1 (ΔOXA-40)

TABLE 2. Sequences of primers designed for this study

Primer	Sequence (5′→3′)[a]	Location and/or primer type
RA-1	GCAGCCAAATCCCAACAATTAAGG	*arr-2*
RA-2	CGCCGCCATAAACGGCGACAGG	*arr-2*, reverse primer
OXA-23A	GGAATTCCATGAATAAATATTTTACTTGC	bla_{OXA-23}
OXA-23B	CGGGATCCCGTTAAATAATATTCAGGTC	bla_{OXA-23}, reverse primer
OXA-40A	GGAATTCCATGAAAAAATTTATACTTCC	bla_{OXA-40}
OXA-40B	CGGGATCCCGTTAAATGATTCCAAGATTTTCTAGCG	bla_{OXA-40}, reverse primer
preOXA-58A	GGAATTCCATGAAATTATTAAAATTGAGTTTAG	bla_{OXA-58}
preOXA-58B	CGGGATCCCGTTATAAATAATGAAAAACACCC	bla_{OXA-58}, reverse primer
OXA-IMP1	GCAAATAMAGAATATGTSCC	bla_{OXA-40}, internal primer
OXA-IMP2	CTCMACCCARCCRGTCAACC	bla_{OXA-40}, internal reverse primer
M13 reverse	GTCCTTTGTCGATACT	pCR-BluntII-TOPO reverse sequencing primer

[a] M is A or C, S is G or C, and R is A or G.

TABLE 3. MICs of β-lactams for *A. baumannii* strains

β-Lactam[a]	MIC (μg/ml) of β-lactam for *A. baumanii* strain[b]:										
	FER (OXA-23)	CIP 70.10 (pFER)	BM4547 (pFER)	CLA-1 (OXA-40)	CLA-1 (ΔOXA-40)	CLA-1 (ΔOXA-40) (pOXA-40)	MAD (OXA-58)	CIP 70.10 (pMAD)	BM4547 (pMAD)	CIP 70.10	BM4547
AMX	>256	>256	>256	>256	>256	>256	>256	>256	>256	32	64
TIC	>256	>256	>256	>256	128	>256	>256	>256	>256	4	8
CAZ	>32	4	4	>32	>32	>32	>32	2	4	2	4
CTX	>32	16	>32	>32	>32	>32	32	8	>32	8	>32
FEP	>32	>32	>32	>32	32	>32	>32	1	>32	1	>32
CPO	>32	>32	>32	>32	>32	>32	>32	8	>32	2	>32
ATM	128	64	64	128	128	128	32	16	16	16	16
IPM	>32	16	>32	>32	2	>32	32	2	32	0.25	0.5
MEM	>32	16	>32	>32	4	>32	>32	2	32	0.25	0.5

[a] AMX, amoxicillin; TIC, ticarcillin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem; MEM, meropenem.
[b] MICs of β-lactams for *A. baumannii* FER (OXA-23), *A. baumannii* CIP 70.10 and *A. baumannii* BM4547 harboring natural plasmid pFER, *A. baumannii* CLA-1 (OXA-40), *A. baumannii* CLA-1 (ΔOXA-40) and *A. baumannii* CLA-1 (ΔOXA-40) harboring recombinant plasmid pOXA-40, *A. baumannii* MAD (OXA-58), *A. baumannii* CIP 70.10 and *A. baumannii* BM4547 harboring natural plasmid pMAD, and *A. baumannii* CIP 70.10 and *A. baumannii* BM4547 reference strains.

(pOXA-40) was made on ticarcillin (50 μg/ml)-, rifampin (25 μg/ml)-, and kanamycin (30 μg/ml)-containing plates.

RESULTS AND DISCUSSION

Antibiotic susceptibility and plasmid analyses. *A. baumannii* clinical isolates FER, CLA-1, and MAD produced the β-lactamases OXA-23, OXA-40, and OXA-58, respectively (15, 26). They were resistant to all β-lactams, including carbapenems (Table 3).

Plasmid extractions of *A. baumannii* FER and *A. baumannii* MAD revealed a ca. 70-kb plasmid and a 30-kb plasmid (26), respectively, whereas no plasmid was detected in *A. baumannii* CLA-1 (15). These plasmids were extracted and then electrotransformed in *A. baumannii* CIP 70.10 and in its point mutant derivative, *A. baumannii* BM4547, which overexpresses the AdeABC efflux pump. *A. baumannii* CIP 70.10(pFER) and *A. baumannii* BM4547(pFER) transformants, were resistant to ticarcillin, imipenem, and meropenem, with a higher level of carbapenem resistance for *A. baumannii* BM4547(pFER) (Table 3). *A. baumannii* CIP 70.10(pMAD) transformant, was resistant to ticarcillin and had a reduced susceptibility to imipenem and meropenem, whereas the *A. baumannii* BM4547(pMAD) transformant was resistant to imipenem and meropenem (Table 3). These results indicated that overexpression of the AdeABC efflux pump and expression of OXA-23 or OXA-58 led to higher levels of carbapenem resistance.

Cloning and expression of the carbapenem-hydrolyzing oxacillinase genes in *E. coli*. After PCR amplification, the bla_{OXA-23}, bla_{OXA-40}, and bla_{OXA-58} genes were inserted into the rifampin-resistant vector pAT801-RA under the control of the *lacZ* RBS and promoter sequences and expressed in *E. coli* DH10B. Antibiotic susceptibility testing revealed that these *E. coli* recombinant strains had a β-lactam resistance that include reduced susceptibility to imipenem (Table 4).

Expression of the carbapenem-hydrolyzing oxacillinase genes in *A. baumannii*. Recombinant plasmids pOXA-23, pOXA-40, and pOXA-58 were then electrotransformed in *A. baumannii* CIP 70.10. High-level resistance to ticarcillin and susceptibility to ceftazidime was observed for all transformants, as expected for this type of oxacillinases (15, 23, 26). Reduced susceptibilities to imipenem and meropenem were observed for *A. baumannii* CIP 70.10(pOXA-23) and *A. baumannii* CIP 70.10(pOXA-40), and *A. baumannii* CIP 70.10(pOXA-58) was susceptible to these antibiotics (Table 4). Thus, OXA-58 conferred only very weak reduced susceptibilities to carbapenems once expressed in *A. baumannii*, by contrast to OXA-23 and OXA-40. Recombinant plasmids pOXA-23, pOXA-40, and pOXA-58 were then electrotransformed in *A. baumannii* BM4547, which overexpresses the efflux pump AdeABC. *A. baumannii* BM4547(pOXA-23) and *A. baumannii* BM4547(pOXA-40) had intermediate levels of resistance to imipenem and meropenem (Table 4), whereas only a slight increase of carbapenem resistance in *A. baumannii* BM4547(pOXA-58) was observed (Table 4). These results showed that overexpression of the AdeABC efflux pump associated with expression of these oxacillinases induced a higher level of carbapenem resistance.

These discrepancies suggested that the kinetics of these oxacillinases were more complex, since they are supposed to have similar hydrolytic activities for these substrates (15, 23, 26). Note that since bla_{OXA-23} and bla_{OXA-58} have been found in association with IS elements, it is likely that these structures provided higher levels of expression for OXA-23 and OXA-58 in *A. baumannii*.

Inactivation of bla_{OXA-40}. In order to determine the precise role of the chromosomally located β-lactamase OXA-40 in carbapenem resistance of *A. baumannii* CLA-1, inactivation of the bla_{OXA-40} gene was performed. After transformation of the pTOPO-OXA-40 recombinant plasmid into *A. baumannii* CLA-1 and selection on kanamycin-containing plates, *A. baumannii* CLA-1 (ΔOXA-40) was obtained. We checked that *A. baumannii* CLA-1 (ΔOXA-40) did not express OXA-40 by isoelectric focusing compared to results obtained with culture of *A. baumannii* CLA-1. A culture extract of *A. baumannii* CLA-1 subjected to isoelectric focusing gave two β-lactamases with pIs of 8.6 and 9.4, which correspond to the pIs of OXA-40 and AmpC, respectively, whereas a culture extract of *A. baumannii* CLA-1 (ΔOXA-40) gave only one β-lactamase with a pI of 9.4, which corresponds to the pI of AmpC (data not shown). Inactivation of the bla_{OXA-40} gene resulted in susceptibility to carbapenems (Table 3), thus showing the contributive role of OXA-40 in carbapenem resistance. This result was

confirmed, as resistance to carbapenems was restored in *A. baumannii* CLA-1 (ΔOXA-40) (pOXA-40) by complementing the phenotype with the expression of OXA-40 (Table 3).

Conclusion. This study demonstrated that carbapenem-hydrolyzing oxacillinases contribute significantly to resistance to carbapenems in *A. baumannii*. *A. baumannii* transformants with natural plasmid pFER or pMAD, producing either β-lactamase OXA-23 or OXA-58, had higher levels of carbapenem resistance than *A. baumannii* transformants with recombinant plasmid pOXA-23 or pOXA-58 producing the same oxacillinase. Thus, in vivo association of bla_{OXA-23} and bla_{OXA-58} genes with insertion sequence elements IS*Aba1* and IS*Aba3*, respectively (10, 26, 29), were likely responsible for their higher levels of expression in *A. baumannii* by providing strong promoter sequences. Finally, overexpression of efflux pump AdeABC of *A. baumannii* contributed significantly to the high level of resistance to most β-lactams, including carbapenems. Further work may determine whether this effect is direct or indirect through changes of outer membrane proteins.

TABLE 4. MICs of β-lactams for *E. coli* and *A. baumannii* strains

β-Lactam[a]	MIC (μg/ml) of β-lactam for strain[b]:											
	E. coli						*A. baumannii*					
	DH10B (pOXA-23)	DH10B (pOXA-40)	DH10B (pOXA-58)	DH10B (pAT801-RA)	CIP 70.10 (pOXA-23)	CIP 70.10 (pOXA-40)	CIP 70.10 (pOXA-58)	CIP 70.10 (pAT801-RA)	BM4547 (pOXA-23)	BM4547 (pOXA-40)	BM4547 (pOXA-58)	BM4547 (pAT801-RA)
AMX	>256	>256	>256	4	>256	>256	>256	32	>256	>256	>256	64
TIC	>256	>256	>256	4	>256	>256	>256	4	>256	>256	>256	8
CAZ	0.06	0.06	0.06	0.06	4	4	4	2	4	4	4	4
CTX	0.12	0.12	0.12	0.12	16	16	16	8	>32	>32	>32	>32
FEP	0.12	0.12	0.12	0.06	2	2	2	1	>32	>32	>32	>32
CPO	0.12	0.12	0.12	0.06	4	4	2	2	>32	>32	>32	>32
ATM	0.12	0.12	0.12	0.12	16	16	16	16	16	8	16	16
IPM	0.25	0.5	0.25	0.06	4	4	0.5	0.25	8	8	1	0.5
MEM	0.06	0.06	0.06	0.06	4	4	0.5	0.25	8	8	1	0.5

[a] AMX, amoxicillin; TIC, ticarcillin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem; MEM, meropenem.

[b] MICs of β-lactams for *E. coli* DH10B harboring recombinant plasmid pOXA-23, pOXA-40, or pOXA-58; *E. coli* DH10B reference strain harboring plasmid pAT801-RA; *A. baumannii* CIP 70.10 harboring recombinant plasmid pOXA-23, pOXA-40, or pOXA-58; *A. baumannii* CIP 70.10 reference strain harboring plasmid pAT801-RA; *A. baumannii* BM4547 harboring recombinant plasmid pOXA-23, pOXA-40 or pOXA-58; and *A. baumannii* BM4547 reference strain harboring plasmid pAT801-RA.

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