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Carbapenem-Resistant *Acinetobacter baumannii* Isolates from Tunisia Producing the OXA-58-Like Carbapenem-Hydrolyzing Oxacillinase OXA-97$^{\nabla}$

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The basis of the β-lactam resistance of 39 multidrug-resistant *Acinetobacter baumannii* isolates recovered from hospitalized patients was studied. These isolates were collected from 2001 to 2005 at the Sahloul Hospital in Sousse, Tunisia. They belonged to two distinct clones. One clone that grouped 19 isolates produced a carbapenem-hydrolyzing oxacillinase, OXA-97, that differed from OXA-58 by a single amino acid substitution and conferred the same β-lactam resistance profile as OXA-58. The *bla*$_{OXA-97}$ gene was located on plasmids that varied in size in 18 isolates and was chromosomally located in a single isolate. Cloning and sequencing identified genetic structures surrounding the *bla*$_{OXA-97}$ gene similar to those reported to be adjacent to the *bla*$_{OXA-58}$ gene. In addition, the novel IS*Aba8* element (which is of the IS*21* family) was identified. This is the first report of the nosocomial spread of carbapenemase producers in *A. baumannii* isolates in Africa.

Acinetobacter baumannii is an opportunistic pathogen that is increasingly being reported to be a cause of nosocomial infections (9, 16, 17). Carbapenem resistance is now observed worldwide in *A. baumannii* isolates, leading to limited therapeutic options. Several mechanisms are responsible for resistance to carbapenems in *A. baumannii*. These are reduced outer membrane permeability, penicillin binding protein changes, and mostly, the production of carbapenemases (21).

The carbapenem-hydrolyzing β-lactamases in *A. baumannii* are either metallo-β-lactamases (MBLs) (31) or oxacillinases (carbapenem-hydrolyzing class D β-lactamases [CHDLs]) (21). Three major subgroups of acquired CHDLs have been identified in *A. baumannii* and are represented by the OXA-23, OXA-24/OXA-40, and OXA-58 β-lactamases. The *bla*$_{OXA-58}$ gene has been identified in France, Italy, Belgium, the United Kingdom, Austria, Turkey, Greece, Kuwait, Brazil, Argentina, and Australia (1, 4, 5, 8, 10, 23, 25).

The OXA-58 β-lactamase was first identified in Europe from a carbapenem-resistant *A. baumannii* isolate recovered in France in 2003 that was at the origin of a nosocomial outbreak (24). The *bla*$_{OXA-58}$ gene was located on a plasmid, and its activity was inhibited by NaCl (14, 24). Genetic investigations showed that the *bla*$_{OXA-58}$ gene was bracketed by insertion sequences (ISs), which were likely the origin of its acquisition and expression (22).

The aim of the study described here was to analyze multidrug-resistant *A. baumannii* isolates that were resistant to carbapenems and that were recovered from the same hospital over 5 years for their β-lactamase contents. This work constitutes the first analysis of an outbreak of CHDL-producing *A. baumannii* strains in Africa.

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MATERIALS AND METHODS

Bacterial isolates. The 39 *A. baumannii* clinical isolates were identified by using the API 32GN system (bioMérieux SA, Marcy l'Etoile, France) and sequencing of the *rpoB* gene, as described previously (7). Electrocompetent *Escherichia coli* TOP10 and *A. baumannii* CIP7010 were used as recipient strains in the transformation experiments, as described previously (14). *A. baumannii* MAD, which carries the *bla*$_{OXA-58}$ gene, was used as an OXA-58-producing reference strain (24). *E. coli* NCTC 50192, which harbors four plasmids of 154, 66, 38, and 7 kb, respectively, was used as a size marker.

Susceptibility testing and screening for MBL-producing strains. The antibiotic susceptibilities of the *A. baumannii* isolates were first determined by the disk diffusion method on Mueller-Hinton agar plates with β-lactam antibiotic- and non-β-lactam antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), according to the guidelines of the Clinical and Laboratory Standards Institute (3). MBL production was evaluated by using Etest strips with imipenem and EDTA (AB Biodisk, Solna, Sweden) for all strains studied. The susceptibility to colistin and tigecycline was also evaluated by Etest (AB Biodisk) (31). The breakpoints used for tigecycline susceptibility testing were those recommended by the EUCAST for members of the family *Enterobacteriaceae*, with susceptibility being an MIC $\leq$1 μg/ml and resistance being an MIC $>$2 μg/ml.

PCR amplification for detection of CHDL genes and sequencing. Under standard PCR conditions (26), a series of primers was used for detection of CHDL-encoding genes, including *bla*$_{OXA-23}$, *bla*$_{OXA-24/OXA-40}$, and *bla*$_{OXA-58}$, as reported previously (14). Primers for the detection of genetic structures identified at the 5′ or 3′ end of *bla*$_{OXA-58}$ (including the IS*Aba2* and IS*Aba3* elements) were used in combination with primers OXA-58A and OXA-58B (24). Additionally, the naturally occurring *bla*$_{OXA-51/OXA-69}$ gene of *A. baumannii* was amplified (with primers OXA-69A and OXA-69B) (12). PCR combinations were per-

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formed with bla_{OXA-69}-specific primers on one side and IS*Aba1*-specific primers on the other side (2, 30). Sequencing reactions were performed with an automated sequencer (ABI 3130; Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed with software available over the Internet (http://www.ncbi.nlm.hih.gov).

Cloning experiments and analysis of recombinant plasmids. Cloning into *E. coli* TOP10 was performed as described previously (19) by using EcoRI-digested whole-cell DNA of bla_{OXA-97}-positive *A. baumannii* strain A10 ligated into EcoRI-restricted plasmid pBK-CMV (Stratagene, La Jolla, CA), followed by selection on plates containing 50 μg/ml of amoxicillin and 30 μg/ml of kanamycin.

In addition, PCR amplicons encompassing the entire sequence of the bla_{OXA-97} gene and bla_{OXA-58} were obtained with primers pre-OXA-58 prom+ and pre-OXA-58B (22) from whole-cell DNA of *A. baumannii* isolates A1 and MAD, respectively, and were subsequently cloned by use of a ZeroBlunt TOPO PCR cloning kit (Invitrogen, Cergy-Pontoise, France). Recombinant strains *E. coli* TOP10(pOXA-97) and *E. coli* TOP10(pOXA-58) were used for MIC and specific activity comparisons (24).

PFGE and Southern hybridization. Pulsed-field gel electrophoresis (PFGE) analysis was done, and the results were interpreted as described previously (18, 29). DNA-DNA hybridizations were performed as described by Sambrook et al. (26) with a probe consisting of a 528-bp PCR fragment internal to bla_{OXA-97} generated from *A. baumannii* A1 (24). Labeling of the probe and signal detection were carried out with a nonradioactive enhanced chemiluminescence labeling and detection kit according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Transformation and plasmid DNA content analysis. Transformation experiments were performed with clinical isolates *A. baumannii* A1 and *A. baumannii* A2 as plasmid donors and *A. baumannii* CIP7010 as the recipient strain (14). Transformants were selected on Trypticase soy agar plates containing 50 μg/ml of ticarcillin.

To assess the chromosomal location of the β-lactamase gene, we used the homing endonuclease I-CeuI (Ozyme; New England Biolabs) (15) and separated the fragments by PFGE, as reported previously (18). After transfer onto a nylon membrane, the DNA was UV cross-linked (Stratalinker; Stratagene) and hybridized with two different probes: a 1,504-bp PCR-generated probe specific for the 16S and 23S rRNA genes (18) and a 528-bp probe specific for bla_{OXA-97}, as indicated above.

Measurement of β-lactamase specific activities. Crude cell extracts were obtained by sonication from *A. baumannii* clinical isolates and *E. coli* TOP10 recombinant strains expressing β-lactamases OXA-97 and OXA-58, respectively, as described previously (13, 20). The β-lactamase specific activities were determined for imipenem, meropenem, cephalothin, ceftazidime, oxacillin, and benzylpenicillin, which were each used at a final concentration of 100 μM, as described previously (13).

Nucleotide sequence accession number. The nucleotide sequence data reported in this work have been deposited in the GenBank nucleotide database under accession no. EF102240.

RESULTS AND DISCUSSION

Epidemiology of the carbapenem-resistant *A. baumannii* isolates. From October 2001 to September 2005, 445 nonrepetitive *A. baumannii* isolates were identified at the Sahloul Hospital, Sousse, Tunisia; 222 (50%) of these isolates were resistant to carbapenems. Among these isolates, all 39 nonrepetitive isolates that had been kept frozen were retained for this study. A single isolate was recovered in 2001, 5 isolates were recovered in 2002, 8 isolates were recovered in 2003, 7 isolates were recovered in 2004, and 18 isolates were recovered in 2005. These isolates had been recovered from different specimens (mostly blood cultures and pus) of patients hospitalized in five wards. Two main antibiotic resistance phenotypes were observed. Nineteen isolates (isolates A1 to A19) exhibited phenotype I (resistance to all β-lactams, including carbapenems, fluoroquinolones, tigecycline, and gentamicin, and susceptibility to tobramycin and colistin). Twenty isolates exhibited phenotype II (resistance to ceftazidime, carbapenems, and fluoroquinolones; intermediate susceptibility to ticarcillin, cefepime, and tigecycline; and susceptibility to gentamicin, tobramycin, and colistin). PFGE analysis showed that the 19 *A. baumannii* isolates of phenotype I were clonally related, whereas the other carbapenem-resistant isolates (phenotype II) were distinct but clonally related (data not shown). The isolates belonging to phenotype II were recovered in 2004 (3 of 7 isolates) and 2005 (17 of 18 isolates). Hydrolytic assays showed that isolates of phenotype I had weak carbapenemase activities, whereas those of phenotype II did not (Table 1).

Identification of CHDL-encoding *A. baumannii* isolates. The results obtained with Etest strips combining imipenem and EDTA did not identify MBL production by the phenotype I or II isolates. PCR experiments with primers specific for CHDL-encoding genes were performed with all 39 clinical isolates; and bla_{OXA-58}-like genes were identified only in isolates exhibiting phenotype I, but no bla_{OXA-23}- or bla_{OXA-40}-like gene was detected in the whole collection. The carbapenem resistance in the phenotype II isolates was therefore not mediated by β-lactamase and may be related to permeability defects and/or the overexpression of efflux. Overall, the isolates positive for the bla_{OXA-58}-like gene were recovered from multiple wards and were detected during the entire study period.

Sequencing of the bla_{OXA-58}-positive amplicons obtained from all isolates of phenotype I identified a gene with a single base pair substitution with respect to the sequence of bla_{OXA-58}. This substitution gave rise to OXA-97 with an Ala-to-Gly substitution at position DBL35 (corresponding to amino acid 53 of the premature OXA-58 sequence) (24). Cloning experiments were performed in identical plasmid and strain backgrounds to compare the OXA-97 and the OXA-58 hydrolysis spectra toward β-lactams. Analysis of the MICs of β-lactams and measurement of the specific activities with imipenem and benzylpenicillin as the substrates showed very similar results for both recombinant strains, *E. coli* DH10B(pOXA-97) and *E. coli* DH10B(pOXA-58), suggesting an identical hydrolysis spectrum (Table).

Screening of the naturally occurring $bla_{OXA-51/OXA-69}$ gene of *A. baumannii* was performed with the bla_{OXA-97}-positive isolates that gave positive results. PCR was also performed to search for the IS*Aba1* element and gave a positive result for all these isolates, in agreement with previous observations showing the ubiquity of that IS element in *A. baumannii* (11, 28). However, PCR experiments did not identify IS*Aba1* upstream of the bla_{OXA-97} gene, thus ruling out the IS*Aba1*-mediated overexpression of this naturally occurring CHDL gene.

Genetic location of bla_{OXA-97}. Plasmid analysis identified two or three plasmids with different sizes in the 19 bla_{OXA-97}-positive isolates. Hybridization with a bla_{OXA-97}-specific probe gave a single positive signal, corresponding to a 60-kb plasmid for 12 isolates, a 50-kb plasmid for 4 isolates, and a 45-kb plasmid for 2 isolates; no hybridization signal was obtained for isolate A2. The chromosomal location of the bla_{OXA-97} gene in isolate A2 was confirmed by use of the I-CeuI technique. Thus, β-lactamase OXA-97 was plasmid encoded in all except one of the isolates; in the latter isolate, integration of the bla_{OXA-97}-positive plasmid or mobilization of this gene from the plasmid to the chromosome may have occurred.

Transfer of the ticarcillin resistance marker was successful by use of all the bla_{OXA-97}-positive *A. baumannii* clinical iso-

TABLE 1. MICs of β-lactams and specific activities[a] against different β-lactams for clinical isolates of *A. baumannii* belonging to antibiotic resistance phenotypes I and II, *A. baumannii* MAD, *E. coli* TOP10(pOXA-58), *E. coli* TOP10(pOXA-97), *A. baumannii* CIP7010, *A. baumannii* CIP7010(pOXA-97), *E. coli* DH10B, and *E. coli* DH10B(pOXA-97)

β-Lactam	MIC (μg/ml)									Sp act (mU/mg of protein)[c]								
	A. baumannii (phenotype I)	*A. baumannii* (phenotype II)	*A. baumannii* MAD	*E. coli* TOP10 (pOXA-58)	*E. coli* TOP10 (pOXA-97)	*A. baumannii* CIP7010	*A. baumannii* CIP7010 (pOXA-97)	*E. coli* DH10B	*E. coli* DH10B (pOXA-97)	*A. baumannii* (phenotype I)	*A. baumannii* (phenotype II)	*A. baumannii* MAD	*E. coli* TOP10 (pOXA-58)	*E. coli* TOP10 (pOXA-97)	*A. baumannii* CIP7010	*A. baumannii* CIP7010 (pOXA-97)	*E. coli* DH10B	*E. coli* DH10B (pOXA-97)
Ampicillin	>512	>512	>512	>512	>512	>512	>512	4	>512									
Ampicillin + CLA[b]	>512	>512	>512	128	128	512	>512	4	128									
Ticacillin	>512	64	>512	>512	>512	4	>512	4	>512									
Ceftazidime	>512	>512	128	0.06	0.06	2	2	0.06	0.12	1	0.4	2	1.5	0.5	—	—	ND	—
Cefepime	64	256	256	0.06	0.06	1	1	0.06	0.12									
Aztreonam	128	128	32	0.06	0.12	64	64	0.12	0.06									
Imipenem	32	16	32	2	2	0.25	2	0.06	1	5.2	ND	4.5	3	4	—	—	—	—
Meropenem	16	16	>64	0.12	0.12	0.25	2	0.06	0.5	1.7	ND	1.5	1	1	—	—	ND	—
Cephalothin										20	10	15	15	20	—	—	ND	—
Oxacillin										230	ND	230	210	220	—	—	ND	—
Penicillin G										220	210	170	150	220	—	—	—	—

[a] Data are the means of three independent experiments. Standard deviations were within 10% of the means.
[b] CLA, clavulanic acid (4 μg/ml).
[c] ND, not detectable; —, not determined.

lates as donors (except for isolate A2) and an *A. baumannii* reference strain as the recipient but not an *E. coli* strain as the recipient. *A. baumannii* CIP7010 transformants harboring the *bla*$_{OXA-97}$-positive natural plasmids exhibited a β-lactam resistance pattern consistent with that resulting from the expression of OXA-58/OXA-97, with MICs identical to those already obtained with natural plasmid pMAD (14). The *bla*$_{OXA-97}$-positive plasmids did not confer additional resistance to antibiotics, except for resistance to tetracycline for plasmids obtained from four isolates.

Genetic structures surrounding the *bla*$_{OXA-97}$ gene. Cloning of the EcoRI-restricted DNA of *A. baumannii* A1 gave rise to recombinant plasmid pA1WM, which expressed the *bla*$_{OXA-97}$ gene. Sequencing of the 13,122-bp insert revealed that an IS*Aba3*-like element was located 20 bp upstream of *bla*$_{OXA-97}$, as previously identified in other structures upstream of the *bla*$_{OXA-58}$ gene (23, 24). PCR mapping showed that this IS*Aba3*-like element was not truncated by an IS*26*, IS*Aba1*, or IS*Aba2* element, as opposed to what has previously been found (2, 24, 25). An entire copy of IS*Aba3* was identified downstream of the *bla*$_{OXA-97}$ gene, followed by the *araC1* and the *lysE* genes, as observed in natural plasmid pMAD (25). A sequence similar to what has been defined as the Re27-2 structure in *A. baumannii* MAD was identified downstream of the *lysE* gene (3 nucleotides of those 27 bp). That structure was likely involved in a homologous recombination process at the origin of the acquisition of the *bla*$_{OXA-58}$ gene in the latter strain (22). An *araC2*-like gene was identified in *A. baumannii* MAD (Fig. 1). A gene encoding a putative regulator was found downstream of *araC2*, followed by an *orf* corresponding to a putative 160-amino-acid protein and by a gene encoding a putative transposase. The latter gene was truncated by the insertion of a novel IS element named IS*Aba8* (see below). An operon containing genes involved in macrolide resistance was identified downstream of the putative transposase gene and was identical to that found on pRSB105, a plasmid isolated from sewage water treatment plants (27). It included a gene encoding a 491-amino-acid efflux protein also identified in *E. coli* and *Citrobacter freundii* (GenBank accession numbers AAN87714.1 and ABG33795.1, respectively) and a 294-amino-acid macrolide 2′-phosphotransferase. The gene encoding a 203-amino-acid resolvase/site-specific recombinase identified in pRSB105 was also found (Fig. 1). Overall, the genetic structures surrounding the *bla*$_{OXA-97}$ gene were similar to those identified in association with the *bla*$_{OXA-58}$ gene in a series of isolates identified from France, Italy, and Greece (2, 22, 23), indicating that a common structure has been at the origin of the dissemination of the *bla*$_{OXA-58}$ and *bla*$_{OXA-97}$ genes.

Identification of IS*Aba8*, a novel IS. IS*Aba8* is 1,867 bp and belongs to the IS*21* family (http://www-is.biotoul.fr/). It possesses 27-bp inverted repeats, and its transposition has likely generated the observed 4-bp target site duplication (CCAT in the structure identified). IS*Aba8* contains two open reading frames (Orf1 and Orf2) encoding proteins of 339 and 257 amino acids, respectively. The deduced amino acid sequences of the Orf1 and Orf2 proteins had 72% and 79% amino acid identities to the transposase subunits of IS*Psy4* and IS*Rso19*, respectively.

Conclusion. This work identified carbapenemase-producing *A. baumannii* isolates as sources of nosocomial infections in

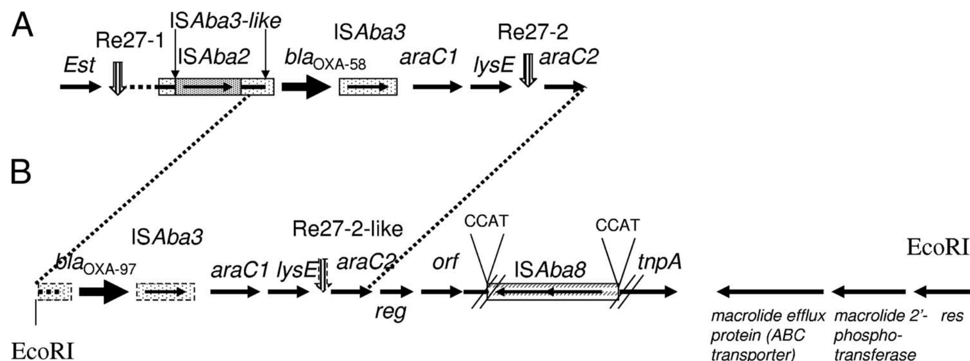

FIG. 1. Schematic map of the genetic structure containing the bla_{OXA-58} and bla_{OXA-97} genes. (A) The structure identified in bla_{OXA-58}-positive isolate MAD (22, 24); (B) the structure identified in *A. baumannii* A1 (this study). The genes and their corresponding transcription orientations are indicated by horizontal arrows. The transcription regulator genes (*araC1* and *araC2*), the threonine efflux protein gene (*lysE*), the esterase gene (*est*), the putative regulatory gene (*reg*), the gene encoding an open reading frame of unknown function (*orf*), the putative transposase gene (*tnpA*), and the putative resolvase gene (*res*) are indicated. Vertical arrows are for the Re27-like sequences. The genetic structure which is highly similar between panels A and B is indicated with the dotted lines. The EcoRI restriction sites bracketing the insert of pA1WM are indicated. The figure is not to scale.

Tunisia. An identical OXA-97-producing *A. baumannii* isolate was identified from 2001 to 2005, indicating its persistence in the hospital and its environment. This novel β-lactamase, OXA-97, is the second member of the OXA-58 subgroup of CHDLs, whose production confers the same β-lactam resistance profile as OXA-58. This report constitutes the first report of the nosocomial dissemination of a CHDL-producing *A. baumannii* strain in Africa, after the identification of single OXA-23-producing *A. baumannii* isolates from Algeria, Libya, and South Africa (6, 28). The current worldwide emergence of multiresistant *A. baumannii* isolates is mostly associated with carbapenemase producers. Therefore, such carbapenemases may be considered the main targets in the development of inhibitors.

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