Multicopy *bla*_{OXA-58} Gene as a Source of High-Level Resistance to Carbapenems in *Acinetobacter baumannii*[†]

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The mechanisms at the origin of heterogeneous carbapenem resistance levels observed among *Acinetobacter baumannii* isolates collected in 2005 in a large University Hospital of Rome, Italy, were investigated. These isolates were related and possessed similar plasmids carrying the carbapenem-hydrolyzing oxacillinase gene *bla*_{OXA-58} but showed variable levels of resistance to carbapenems. Analysis of sequences surrounding the *bla*_{OXA-58} gene showed genetic variability, with the presence in several isolates of multiple copies of the *bla*_{OXA-58} gene; this extra copy number was likely related to an IS26-mediated transposition or recombination process.

Acinetobacter baumannii is an important opportunistic pathogen responsible for a variety of nosocomial infections particularly affecting critically ill patients in intensive care units, where it is associated with significantly increased mortality (12, 18). In recent years, several outbreaks of nosocomial infections caused by *A. baumannii* strains resistant to a wide range of antibiotics, including broad-spectrum β -lactams, aminoglycosides, and fluoroquinolones, have been documented (5, 19, 30). While carbapenems are the drugs of choice for treating *Acinetobacter* infections, carbapenem resistance is now increasing (6, 9, 10, 16, 19, 27, 28, 34).

Acinetobacter spp. may develop resistance to carbapenems through various mechanisms, including decreased permeability (mostly related to porin modifications), overexpression of efflux pumps, and production of carbapenemases (25, 32). The carbapenemases may be metallo- β -lactamases or carbapenem-hydrolyzing oxacillinases (33). Four groups of carbapenem-hydrolyzing oxacillinases have been identified in *A. baumannii*, represented by the OXA-23, OXA-24, OXA-51, and OXA-58 enzymes (4, 15, 24, 26). The *bla*_{OXA-51}-like genes occur naturally in that species (15) and are usually weakly expressed. However, insertion sequence IS*Aba1* may enhance the expression of *bla*_{OXA-51}-like genes by providing promoter sequences, indicating that OXA-51-like enzymes might play a role in reduced susceptibility to carbapenems (29). Plasmid-borne *bla*_{OXA-23} and *bla*_{OXA-58} genes have been shown to contribute significantly to carbapenem resistance in *A. baumannii* (15). The *bla*_{OXA-58} gene has been detected in carbapenem-resistant *A. baumannii* isolates from different parts of the world (1, 4, 13, 23, 26). It has been shown that the *bla*_{OXA-58} gene is often associated with insertion sequences (IS*Aba1*, IS*Aba2*, IS*Aba3*, or IS*I8*) involved in its expression, and likely in its acquisition

in the case of IS*Aba3* (23). In addition, it has been shown that a homologous recombination process was at the origin of *bla*_{OXA-58} acquisition in a French *A. baumannii* isolate (22). Recently, the *bla*_{OXA-58} gene was identified in six clonally related and carbapenem-resistant *A. baumannii* isolates recovered in 2005 at the University Hospital Policlinico Umberto I in Rome, Italy (1). These isolates carried a plasmid-borne *bla*_{OXA-58} gene (the plasmid was designated pOUR, for OXA-58 Umberto I Rome) and were resistant to carbapenems, according to the current CLSI criteria, with variable MICs of imipenem and meropenem. Thus, our goal was to elucidate the mechanism(s) at the origin of this resistance level heterogeneity.

MATERIALS AND METHODS

Bacterial strains and MIC determinations. Three carbapenem-resistant *A. baumannii* isolates were analyzed in this study. They were recovered in 2005 from patients hospitalized in the intensive care unit of the Policlinico Umberto I, a large university hospital in Rome, Italy (Table 1) (1). They showed identical pulsed-field gel electrophoresis (PFGE) patterns and were positive for the *bla*_{OXA-58} gene. *A. baumannii* MAD, recovered from France and carrying the *bla*_{OXA-58} gene, was used as the reference strain (24). Imipenem MICs of >32 μ g/ml have been evaluated by the dilution technique on solid media according to the CLSI guidelines (3); the other MICs were the result of an Etest interpretation (31).

β -Lactamase activity. Crude enzymatic extracts were obtained by sonication as described previously. The hydrolytic activities of the β -lactamase extracts against imipenem and ceftazidime were determined by UV spectrophotometry as described previously and were expressed in units (1 unit was the amount of enzyme hydrolyzing 1 μ mol of substrate per min per mg of protein) (20).

Isoelectric focusing analysis was performed with a pH 3.5 to 9.5 ampholin polyacrylamide gel (GE Healthcare, Saclay, France) for 2.5 h at 25 W, 25 mA, and 2,000 V in a flatbed Multiphor II apparatus with culture extracts of the *A. baumannii* isolates obtained as mentioned above (14).

PCR experiments. Total DNAs of *A. baumannii* isolates were extracted by using the Wizard Genomic DNA purification kit (Promega, Milan, Italy) according to the manufacturer's procedure. The DNAs were used as templates under standard PCR conditions with a series of primers. Amplification of the *bla*_{OXA-58} gene was performed with primers OXA-58A–OXA-58B (15) and that of the naturally occurring *bla*_{OXA-51/69} gene with primers OXA-69A–OXA-69B (15). The *bla*_{OXA-20} gene was identified using the primers OXA-20F (5'-AGA ATA GCA CGC GCA ATT GC-3') and OXA-20B (5'-CTG TTG TAC TTG TCT CTC TTG G-3'). The putative colinearity between insertion sequence IS*Aba1*

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TABLE 1. Characteristics of the clonally related *A. baumannii* clinical isolates and their transformants

Strain ^a	Copy no. of <i>bla</i> _{OXA-58} gene	MIC (mg/ml) ^b			Hydrolytic sp act ^c	
		IMP	MEM	CAZ	IMP	CAZ
BM4547	0	0.5	0.5	4	<0.01	<0.01
183	1	16	4	512	0.165	0.075
T183	1	6	3	8	0.303	<0.01
193	2	32	8	512	0.227	0.077
T193	2	16	4	8	0.365	<0.01
186	3	128	12	512	0.270	0.069
T186	3	64	8	8	0.388	<0.01

^a The transformants (T) were obtained with purified self-circularized EcoRI fragments carrying the *bla*_{OXA-58} region and the replication sequences from the respective plasmids, transforming the BM4547 susceptible *A. baumannii* strain.

^b IMP, imipenem; MEM, meropenem; CAZ, ceftazidime.

^c Specific activities are expressed in U/mg of protein, and standard deviations were within 10% (mean of three independent determinations).

and the naturally occurring *bla*_{OXA-51}-like gene was searched for by using primers preABprom+ and OXA-69B, as reported previously (14, 15).

Plasmid analysis. Plasmids were purified using the PureLink HiPure Plasmid Midiprep Kit (Invitrogen, Milan, Italy). SacI- and HindIII-generated fragments were separated on 1% agarose gels, transferred onto nylon membranes (Nylon Membranes Positively Charged; Roche Diagnostics, Monza, Italy), and hybridized with PCR-generated probes specific for *bla*_{OXA-58} labeled with digoxigenin (DIG DNA Labeling and Detection kit; Roche Diagnostics, Monza, Italy).

Cloning and sequencing. PCR products were ligated into the pCR2.1 vector (Invitrogen, Milan, Italy) and used to transform competent *Escherichia coli* INVαF' cells (INVαF' Chemically Competent *Escherichia coli*; Invitrogen, Milan, Italy), and selection of the transformants was performed on LB agar plates containing ampicillin (100 µg/ml).

SacI libraries were obtained from isolates 183 and 186, and EcoRI libraries were obtained from isolates 183, 186, and 193 in the pZerO-2 vector (Invitrogen, Milan, Italy). The libraries were used to transform competent *E. coli* DH5α cells (MAX Efficiency DH5α Chemically Competent Cells; Invitrogen, Milan, Italy), and selection of the transformants was performed on LB agar plates containing kanamycin (40 µg/ml), ampicillin (20 µg/ml), and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The nucleotide sequences of the SacI-generated inserts were fully determined on both strands with an ABI Prism 377 sequencer (Applied Biosystems) with universal and internal primers. The EcoRI-generated inserts were partially sequenced using universal and internal primers.

The EcoRI restriction fragments of 17, 13.5, and 10 kb from the 186, 193, and 183 plasmids, respectively, were separated by and eluted from agarose gels with the QIAGEN Gel Extraction Kit (QIAGEN, Courtaboeuf, France); 40 ng of each eluted band was self-ligated in 50 µl of reaction mixture with T4 DNA ligase (Roche Diagnostic, Meylan, France). Ligation mixtures were electroporated into *A. baumannii* strain BM4547 by electroporation (14), and transformants were selected on tetracycline-containing plates (50 µg/ml).

Quantification of the *bla*_{OXA-58} gene by real-time PCR amplification. Real-time experiments were performed with three independent 10-fold dilution series for each strain tested. They were repeated at least three times in different PCR runs. The *repAciI* gene was chosen as the reference point to control the plasmid copy number. The PCR assay for the *repAciI* gene was performed with primers repAFW (5'-GAG AGA TTT AGT TGT AAA GGA CAA TGC-3') and repARV (5'-CGA CTC ATA ACA TTT CGG ATA TTC CCA TTA-3').

The quantity of each target in each isolate was expressed as the difference in threshold cycle values between the *repAciI* and the *bla*_{OXA-58} genes. PCR efficiency was approximately 90% for both targets. Real-time PCR was performed by an initial denaturation at 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s, using primers OXA-58A and OXA-58B plus repAFW and repARV. The real-time PCR was performed in a volume of 20 µl containing 20 ng of purified genomic DNA, 2 µl of reaction buffer (Faststart DNA Master SYBER Green I; Roche Diagnostics, Monza, Italy), 4 mM MgCl₂, 0.25 µM of each primer. Data were analyzed with LightCycler software, version 4.2.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in the study for isolates 183 and 186 have been assigned accession numbers EF138631 and EF138630, respectively, in the EMBL-GenBank database.

RESULTS AND DISCUSSION

Variability of the MICs. MICs of imipenem, meropenem, and ceftazidime for *A. baumannii* isolates are presented in Table 1. These isolates were previously demonstrated to be *bla*_{OXA-58} positive, but isoelectric focusing analysis revealed the production of additional β-lactamases. A band at pI 7.2 corresponded to OXA-58, a band at pI 7.4 to the narrow-spectrum oxacillinase OXA-20 (whose gene was further identified by PCR), and a band at pI 9.2 to the natural AmpC (data not shown). The three *A. baumannii* isolates included in this study showed variable MICs of carbapenems and variable hydrolytic specific activities using imipenem as a substrate (Table 1). Isolate 183 showed lower MICs of imipenem and meropenem than isolates 186 and 193. In addition, compared to isolate 183, imipenem hydrolysis rates were 1.6-fold and 1.3-fold higher for isolates 186 and 193, respectively. For comparison, specific hydrolytic activities for ceftazidime (a β-lactam that is not a substrate of OXA-58) were almost identical, ruling out possible differences in AmpC expression in some isolates. PCR assays were performed (14) and showed the absence of colinearity between IS*AbaI* and the *bla*_{OXA-51}-like gene in all the isolates tested (data not shown), likely ruling out any involvement of that naturally occurring carbapenem-hydrolyzing oxacillinase as a source of variable phenotypes.

Detailed analysis of the plasmids carrying the *bla*_{OXA-58} gene. Restriction by SacI and HindIII enzymes revealed that plasmids from isolates 186 and 193 showed similar restriction patterns, differing slightly from a plasmid of isolate 183 (Fig. 1). Hybridization with a *bla*_{OXA-58}-specific probe performed on SacI-restricted fragments gave an approximately 6.8-kb positive signal and also an additional 4.5-kb positive signal for plasmids from isolates 186 and 193, whereas the latter signal was not detected in a plasmid from isolate 183. Due to a HindIII site within the *bla*_{OXA-58} coding sequence, the HindIII restriction generated two fragments for each *bla*_{OXA-58} gene copy. The results from the *bla*_{OXA-58}-specific hybridization

FIG. 1. Plasmid localization of the *bla*_{OXA-58} gene. Shown is agarose (1%) gel electrophoresis in 1× Tris-acetate-EDTA buffer of SacI- or HindIII-digested plasmids from *A. baumannii* isolates, stained with ethidium bromide and visualized under UV light, and Southern blot hybridization with the *bla*_{OXA-58} probe. M is a 1-kb Plus Ladder, and M₁ is the HindIII-digested lambda DNA (Invitrogen, Milan, Italy).

FIG. 2. Consensus map of the *bla*_{OXA-58} gene region. The insertion sequences identified within the resistance regions are shown as boxes, and the arrows indicate the directions of transcription of the genes. The *bla*_{OXA-58} gene is represented by the heavy black arrow. Restriction sites for *EcoRI*, *HindIII*, and *SacI* are indicated. The bars below the map represent the positions of the constructs obtained from the libraries and sequenced. The dashed lines show the insertion site of the second copy of the *IS26-ISAb2-bla*_{OXA-58}-*ISAb3* module in isolate 193 with respect to isolate 183. The brackets indicate the presence of two *IS26-ISAb2-bla*_{OXA-58}-*ISAb3* modules in isolate 186.

performed on the *HindIII*-restricted plasmids identified a probable single gene copy (two positive signals) for plasmid 183, whereas multiple *bla*_{OXA-58} copies (three *HindIII*-positive fragments) were identified with plasmids from isolates 186 and 193.

Cloning and characterization of the *bla*_{OXA-58} surrounding regions. A *SacI* library was constructed from isolates 183 and 186, and *SacI* inserts containing the *bla*_{OXA-58} gene were cloned and fully sequenced (Fig. 2). An identical 6,860-bp *SacI* fragment was obtained from plasmids from isolates 186 and 183 (giving rise to recombinant plasmids p186-6.8 and p183-6.8 [Fig. 2]) and showed 99% nucleotide identity with the sequence identified in *A. baumannii* MAD (positions 2051 to 5257; GenBank no. AY665723) (22), containing *ISAb2*, *bla*_{OXA-58}, and *ISAb3*. The *ISAb3* element was followed by insertion sequence *IS26*, a structure differing from that identified in *A. baumannii* MAD, and then by a second *ISAb2*-like element, whose *InsB* transposase unit exhibited 93% amino acid identity with that of *ISAb2*, presenting a stop codon in its center region as a result of a 1-bp insertion.

Recombinant plasmid p186-4.5, containing a 4,542-bp *SacI*

fragment, was obtained from plasmid DNA from isolate 186, which possessed a second copy of the *bla*_{OXA-58} gene flanked by the *ISAb2*, *ISAb3*, and *IS26* elements. This sequence revealed a perfect duplication of the *IS26-ISAb2-bla*_{OXA-58}-*ISAb3* structure. Recombinant plasmids p186EcoRI, p193EcoRI, and p183EcoRI carrying *EcoRI* fragments of 17, 13.5, and 10 kb were obtained from isolates 186, 193, and 183, respectively. DNA sequencing and PCR mapping demonstrated the colinearity of the two *bla*_{OXA-58} copies in a head-to-tail orientation (Fig. 2).

The presence of *IS26* elements in these duplicated regions indicates that the insertion sequences could have mediated the duplication of the *bla*_{OXA-58} locus by imperfect duplicative transposition, starting with the cointegrate fusion and duplication of the elements but lacking the resolution of the cointegrate (7). A very similar *IS26*-mediated amplification of resistance genes was described in VIM-1-producing *Klebsiella pneumoniae* strains isolated in Greek hospitals (17). In that case, the *K. pneumoniae* isolates exhibited highly similar pulsed-field gel electrophoresis patterns but MICs of imipenem varying from 2 to 64 µg/ml. It was shown that the IncN plasmid mediating the higher MIC of imipenem carried two

copies of the In-541 integron carrying the *bla*_{VIM-1} gene flanked by IS26 elements (17). Also, a relationship between the IS26-*bla*_{SHV} gene copy number and levels of resistance to ceftazidime, cefotaxime, and aztreonam was observed for a collection of *K. pneumoniae* clinical isolates from Brisbane, Australia (11).

Identification of a novel replicon on *bla*_{OXA-58} positive plasmids. Sequence analysis of the EcoRI-made recombinant plasmids obtained from isolates 186, 193, and 183 identified an open reading frame whose corresponding gene showed 63% nucleotide identity with the *repM* gene of plasmid pMAC, a 9.5-kb plasmid from *A. baumannii* (EMBL no. AAT09649) harboring genes involved in organic peroxide resistance (8). This *rep* gene, designated *repAciI*, was flanked at its 5' end by four 23-bp-long direct repeats (5'-ATATGTCCACGTTTAC CTTGCA-3'), namely, the iterons controlling the plasmid replication rate. Further upstream, the origin of plasmid replication (*oriV*) was also identified. A quantitative determination of the *bla*_{OXA-58} gene copies was performed for the *repAciI* gene, assumed to be one copy per plasmid. This experiment confirmed that isolate 186 possessed three copies, isolate 193 two copies, and isolate 183 only one copy of the *bla*_{OXA-58} gene (Fig. 3).

The increase in the carbapenem resistance level is linked to increased production of OXA-58. Although the amount of β -lactamase production can be critical in determining levels of resistance to its substrates, it is likely that the increase in the carbapenem resistance level was linked to an increased production of OXA-58 by the multiplication of the IS26-IS4ba2-*bla*_{OXA-58}-IS4ba3 locus on plasmids carrying an identical replicase gene. To better assess the role of the reiterated copies of the IS26-IS4ba2-*bla*_{OXA-58}-IS4ba3 structure in the levels of *A. baumannii* resistance to carbapenems, the EcoRI inserts from the 186, 193, and 183 plasmids, containing the *bla*_{OXA-58} locus but also the *oriV* and the *repAciI* gene (and thus a potentially functional replicon), were self-circularized and transferred into a carbapenem-susceptible *A. baumannii* strain. The circularized EcoRI bands were detected extrachromosomally in the transformants, thus demonstrating the functional activity of the replication system within this plasmid locus. The MICs of imipenem, meropenem, and ceftazidime measured for the transformants obtained with the self-circularized EcoRI band are reported in Table 1. The MICs were consistent with the *bla*_{OXA-58} gene dosages in the three clones analyzed. The highest MICs of imipenem and meropenem were obtained for the *A. baumannii* transformants carrying the EcoRI insert with three *bla*_{OXA-58} gene copies (Table 1). Since the levels of resistance to several substrates (including carbapenems) were higher for original strains than for the transformants, it is clear that additional resistance mechanisms were expressed in the clinical isolates. Interestingly, the specific activity of imipenem was higher in these transformants than in the original strains. These results suggest a higher level of expression of the *bla*_{OXA-58} gene or an increased replication rate of the self-circularized EcoRI band in the *A. baumannii* BM4547 recipient strain compared to the original plasmids.

The identification of a novel replication system associated with the plasmids carrying the *bla*_{OXA-58} gene provides an important source of information that may be used for further screening of *bla*_{OXA-58}-positive plasmids of different origins.

FIG. 3. Determination of the *bla*_{OXA-58} gene copy number by real-time PCR amplification. Shown are amplification curves obtained in quantitative real-time PCR experiments performed on the *repAciI* gene (one copy per plasmid) and the *bla*_{OXA-58} gene measured for the isolates 183 (one copy per plasmid), 193 (two copies per plasmid), and 186 (three copies per plasmid).

This screening can be useful to trace the spread of common plasmids carrying this relevant resistance gene in *A. baumannii*, as shown with enterobacterial isolates for other relevant carbapenem resistance determinants, such as metallo- β -lactamase VIM-1 (2).

Whereas it has already been demonstrated that increased resistance to carbapenems in *A. baumannii* could be due to porin modifications, efflux system overexpression, or acquisition of carbapenem-hydrolyzing β -lactamases and also increased expression of naturally occurring *bla*_{OXA-51}-like genes (21), there is evidence that the presence of insertion sequences can mediate the amplification of the region containing the β -lactamase gene, significantly increasing the level of resistance in the clinical strain. This mechanism adds novel insights into the diversity of mechanisms at the origin of carbapenem resistance in *A. baumannii*.

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