Characterization of the Carbapenem-Hydrolyzing Oxacillinase Oxa-58 in an *Acinetobacter* Genospecies 3 Clinical Isolate[▽]

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Based on imipenem resistance in an *Acinetobacter* genospecies 3 clinical isolate, we were able to identify, for the first time in this genomic species, a plasmid-encoded *bla*_{OXA-58} gene that was 100% homologous to the same gene in *Acinetobacter baumannii*.

Since 1986 members of the genus *Acinetobacter* are determined by DNA-DNA hybridization. Genospecies 1 (*Acinetobacter calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU are genetically closely related and are commonly known as the *A. calcoaceticus*-*A. baumannii* complex. With the exception of genospecies 1, the other members of this complex have been involved in nosocomial infections and have the ability to spread in hospitals (3, 9, 19, 23, 25, 26). Treatment of these nosocomial infections is becoming a problem because increasing resistance to antibiotics, especially in the case of *A. baumannii*. In the last decade, carbapenem resistance in *Acinetobacter* spp. has been reported worldwide (3, 16, 23), mostly associated with the synthesis of carbapenem-hydrolyzing β -lactamases, reduced outer membrane permeability and, occasionally, modification of penicillin-binding proteins (7, 16, 22, 27). The most prevalent carbapenemases in *Acinetobacter* spp. are the carbapenem-hydrolyzing class D β -lactamases, which are divided into four phylogenetic subgroups: OXA-23, OXA-24, and OXA-58 with all their variants and the OXA-51 family, which is intrinsic to *A. baumannii* (16, 27).

OXA-58 confers reduced susceptibility to carbapenems, but it produces high-level resistance to carbapenems when additional efflux mechanisms are expressed (12, 15). It was first identified in France in 2003 and, at present, is found worldwide in *A. baumannii* isolates (13, 16, 20, 21), as well as in *A. junii* isolates from Romania and Australia (12, 14).

The clinical isolates Ac057 (*Acinetobacter* sp. strain G3) and Ac058 (*A. baumannii*) were obtained from the same hospital in November 2000 and were identified by amplified ribosomal DNA restriction analysis (ARDRA) (6, 24). The epidemiological difference was corroborated by pulsed-field gel electrophoresis (PFGE) with ApaI (Promega, Madrid, Spain) under conditions described elsewhere (11).

Antimicrobial susceptibility analysis was performed by Etest according to the manufacturer's instructions (AB Biodisk, Sölna, Sweden) and determined that both strains had an imipenem MIC of >32 μ g/ml (Table 1). The breakpoints for imipenem were those proposed by the Clinical and Laboratory Standards Institute (5).

PCR analysis with specific primers for all class D β -lactamases (Table 2) determined the presence of the *bla*_{OXA-58} gene in both strains; *A. baumannii* strain Ac058 was also positive for the *bla*_{OXA-51} gene. Additional primers were designed at the beginning and end of the *bla*_{OXA-58} gene (Table 2) to amplify the whole fragment. This gene presented 100% homology with the *bla*_{OXA-58} gene from *A. baumannii* listed in GenBank.

Plasmid DNA identification was attempted by using genomic mapping with I-CeuI (10) and by digestion with the S1 nuclease (1). I-CeuI cuts a 26-bp site in the *rrl* gene (23S rRNA), shearing the bacterial genome into an analyzable number of fragments (10). The S1 nuclease transforms supercoiled plasmids into linear molecules (1). Digested genomic DNA and plasmids were sepa-

TABLE 1. MICs for the clinical isolates used in this study

Strain	MIC (μ g/ml) ^a																
	AMP	PIP	CEF	FOX	CAZ	FEP	SAM	IMP	MEM	CIP	GEN	TOB	AMK	DOX	AZM	RIF	PMB
Ac057	256	512	256	256	8	8	8	>32	8	32	<1	0.25	0.5	<0.5	4	2	2
Ac058	256	512	256	256	256	64	64	>32	8	64	16	64	256	16	128	2	1

Abbreviations: AMP, ampicillin; PIP, piperacillin; CEF, cephalothin; FOX, cefoxitin; CAZ, ceftazidime; FEP, cefepime; SAM, ampicillin-sulbactam; IMP, imipenem; MEM, Meropenem; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; DOX, doxycycline; AZM, azithromycin; RIF, rifampin; PMB, polymyxin B.

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TABLE 2. Oligonucleotide sequences used in this study

Nucleotide	Sequence (5'-3')	Size (bp)	Use
OXA51-1	AACAAGCGCTATTTTATTTTCAG	641	Detection <i>bla</i> _{OXA-51} variants
OXA51-2	CCCATCCCCAACCCTTTT		
OXA23-1	GATGTGTCATAGTATTCGTCGT	641	Detection <i>bla</i> _{OXA-23} variants
OXA23-2	TCACAACAACATAAAAGCACTGT		
OXA24-1	ATGAAAAAATTATCTTCTATATTCAGC	825	Detection <i>bla</i> _{OXA-24} variants
OXA24-2	TTAAATGATTCCAAGATTTTCTAGC		
OXA58-1	AGTATGCGGGCTTGTGCT	453	Detection <i>bla</i> _{OXA-58} variants
OXA58-2	AACTTCCGTGCCTATTTG		Southern blot probe ^a
OXA58-1TOT	ATGAAATTATTAAAAATATTG	843	Total gene amplification
OXA58-2TOT	TTATAAATAATGAAAAACACC		Total gene amplification
OXA58-inv1	GAGCGCAGAGGGGAGAATCGTC		Genetic surrounding
OXA58-inv2	CTCAGCACAAAGCCCCAATACT		Genetic surrounding
OXA58-inv3	AAGCCATGCAAGCATCTACA		Genetic surrounding
OXA58-inv4	CATCTCTTTCACCTGTGTCTGAA		Genetic surrounding

^a Primers OXA58-1 and OXA58-2 were used for detecting the *bla*_{OXA-58} gene and also to generate the probe for Southern blot analysis.

rated by PFGE (Fig. 1). Probes were marked with the PCR DIG probe synthesis kit (Roche, Barcelona, Spain), and detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase and the color substrates NBT/BCIP (Roche) according to the manufacturer's instructions. In Fig. 1a, the most intense bands would represent fragments of genomic DNA, and the faded bands represent plasmid DNA. Hybridization with probes for the *bla*_{OXA-58} gene (Fig. 1c) and the 23S rRNA gene (Fig. 1b) suggest that in both isolates the *bla*_{OXA-58} gene is present in a plasmid. With the S1 nuclease (Fig. 2a), the highest band would be the genomic DNA and the remaining bands would be linear plasmids. Hybridization with the probe for the OXA-58 gene

(Fig. 2c) gives the same pattern as obtained with I-CeuI. The hybridization signal with the probe for the 23S rRNA gene was only observed in the undigested genomic DNA (Fig. 2b). Although conjugation experiments did not show any plasmid transfer between strains, Southern blot analysis suggests that the *bla*_{OXA-58} gene could be present in a plasmid in both strains, and the plasmid from *A. baumannii* is possibly different from the plasmid in the *Acinetobacter* genospecies 3 isolate.

In order to determine the genetic structure surrounding of the *bla*_{OXA-58} gene, DNA from both isolates was digested with MspI "C*CGG" (Promega). The fragments obtained were autoligated overnight at 16°C using a T4 DNA ligase (Promega).

FIG. 1. Plasmid identification by genomic mapping with I-CeuI. (a) PFGE gel. (b) Hybridization with probe for the 23S rRNA gene. (c) Hybridization with probe for the OXA-58. Lane 1, *A. baumannii* strain Ac058; lane 2, *Acinetobacter* genospecies 3 strain Ac057.

FIG. 2. Plasmid identification by digestion with S1 nuclease. (a) PFGE gel. (b) Hybridization with probe for the 23S rRNA gene. (c) Hybridization with probe for the OXA-58. Lane 1, *A. baumannii* strain Ac058; lane 2, *Acinetobacter* genospecies 3 strain Ac057.

←ISABA3.....TTTCTTTATACTATCACTGAGGCAGGTTGGACAT
 TTGATTGCTAGAGTTATTTGCA1TTCTCTATTTATCAAAATCCAA
 TCGGCTTTTCTCTCAGCATACTTTTGGAAACACTACCAATTTTAAA
 GTTGATATCATGAAATTATTAATAATATTGAGTTTAGTTTGCTT
 AAGCATAAGTATTGGGGCTTGCTGAGCATAGTATGAGTCGAG
 CAAAAACAAGTACAATTCCACAAGTGAATAACTCAATCATCGAT
 CAGAATGTTCAAGCGCTTTTAAATGAAATCTCAGCTGATGCTGT
 GTTGTGCATATGATGGTCAAAATATTAATAATATGGCAGCG
 ATTTAGACCGAGCAAAACAGCTTATATTCCTGCATCTACATTTA
 AAATTGCCAATGCACATAATTGGTTTGAATAATCATAAAGCAACA
 TCTACAGAAATATTTAAGTGGGATGGAAGCCACGTTTTTTAA
 AGCATGGGACAAAGATTTACTTTGGGCGAAGCCATGCAAGCAT
 CTACAGTCCTGTATATCAAGAATTGGCACGTCGTATTGGTCCA
 AGCTAATGCAAAAGTGAATTGCAACGTATTGGTTATGGCAATAT
 GCAATAGGCACGGAAGTTGATCAATTTTGGTTGAAAGGGCCTT
 TGACAATTACCTTATACAAGAAGTAAAGTTTGTGTATGATTTAG
 CCCAAGGGCAATTGCCTTTTAACTGAAAGTTCAGCAACAAGTG
 AAAGAGATGTTGTATGTAGAGCGCAGAGGGGAGAATCGTCTAT
 ATGCTAAAAGTGGCTGGGGAATGGCTGTAGACCCGCAAGTGGG
 TTGGTATGTGGGTTTGTGTTGAAAAGGCAGATGGGCAAGTGGTG
 GCATTTGCTTTAAATATGCAAAATGAAAGCTGGTGTATGATTTGC
 TCTACGTAACAATTTGCTTTAGATGTGCTAGATAAGTTGGGTG
 TTTTTCATTATTTAAGAATTAGAAGTTTGGAGTTATCTATTTT
TTGGTAGTGTTC4AAAAGTATGCTGAAGAA4AAGCCGATTGGATT
TGATAAATAGAGAAATGCAAACTCTAGCAATCAAAATGTCCA
 ACCTGCCTCAGTGTATAGTATAAAGAAAATGGTATCAAAAGTAGA
 TGGGAAACAAACTAT.....ISABA3→

TTTATC: -35 motif of the promoter

TTTCTT: -10 motif of the promoter

CATACTTTTGAACACTACCA44 – IRL ISABA3

FIG. 3. Structure of the genetic surrounding in *Acinetobacter* genospecies 3 strain Ac057, which is structurally identical to the one described by Poirel et al. (17).

The fragment of DNA containing the *bla*_{OXA-58} gene was used as a template for a PCR with inverse primers designed from the *bla*_{OXA-58} gene sequence (Table 2). All PCR fragments were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom) and analyzed in an automatic DNA sequencer (3100 Genetic Analyzer; Applied Biosystems).

Analysis of the genetic surrounding confirms that both plasmids are different (Fig. 3). In Ac057, the *bla*_{OXA-58} gene is surrounded by two copies of the Insertion Sequence ISABA3; the copy downstream has the same direction as the *bla*_{OXA-58} gene, and the upstream copy has the opposite direction (Fig. 3). This structure has already been described in *A. baumannii* by Poirel et al. (17, 18).

The presence of the OXA-58 alone does not account for the level of resistance to imipenem of these isolates (MIC of >32 µg/ml). Further work is needed to determine whether additional efflux pumps or porin modifications are involved.

A. baumannii is certainly the most frequently isolated species in hospitals and also the microorganism of greatest clinical interest in this genus. However, *Acinetobacter* genospecies 3 and 13 are also nosocomial pathogens, and they should be considered in hospital settings. Previous studies in *Acinetobacter* genospecies 3 have revealed the presence of AmpC (2), IMP-4 (4), and *bla*_{VIM-2} (28). In addition to these previously described enzymes, we report here, for the first time, the presence of the *bla*_{OXA-58} in this microorganism. The main reason for the lack of interest on non-*baumannii* *Acinetobacter* isolates is probably their susceptibility to antimicrobial agents (9). However, as suggested by Horrevorts et al. (8), the clinical significance of genospecies 3 can be underestimated because

the resistant strains can be erroneously classified as *A. baumannii*.

Nucleotide sequence accession number. The GenBank accession number for the *bla*_{OXA-58} in *Acinetobacter* genospecies 3 is EU642594.

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