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OXA-143, a Novel Carbapenem-Hydrolyzing Class D β-Lactamase in *Acinetobacter baumannii*▽

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A carbapenem-resistant *Acinetobacter baumannii* strain was isolated in Brazil in 2004 in which no known carbapenemase gene was detected by PCR. Cloning experiments, followed by expression in *Escherichia coli*, gave an *E. coli* recombinant strain expressing a novel carbapenem-hydrolyzing class D β-lactamase (CHDL). OXA-143 showed 88% amino acid sequence identity with OXA-40, 63% identity with OXA-23, and 52% identity with OXA-58. It hydrolyzed penicillins, oxacillin, meropenem, and imipenem but not expanded-spectrum cephalosporins. The $bla_{OXA-143}$ gene was located on a ca. 30-kb plasmid. After transformation into reference strain *A. baumannii* ATCC 19606, it conferred resistance to carbapenems. Analysis of the genetic environment of $bla_{OXA-143}$ revealed that it was associated with neither insertion sequences nor integron structures. However, it was bracketed by similar replicase-encoding genes at both ends, suggesting acquisition through a homologous recombination process. This study identified a novel class D β-lactamase involved in carbapenem resistance in *A. baumannii*. This enzyme is the first member of a novel subgroup of CHDLs whose prevalence remains to be determined.

Acinetobacter baumannii is a nosocomial pathogen that is characterized by its innate and acquired antimicrobial resistance (9, 20, 27). Carbapenems were considered to be the most active antimicrobials against *A. baumannii*. However, carbapenem resistance is rising and is often associated with a multidrug resistance phenotype (20, 25, 27). The main mechanisms of carbapenem resistance in *A. baumannii* correspond to efflux pumps, porin mutations, and the production of acquired carbapenem-hydrolyzing class D β-lactamases (CHDLs) (26–28). The impact of overexpression of the naturally occurring $bla_{OXA-51-like}$ gene remains to be determined, although recent data suggest its involvement in acquired resistance (12). Metallo-β-lactamases are still rarely identified in *A. baumannii* (25).

Some of the class D β-lactamases, also named oxacillinases (OXA), are able to weakly hydrolyze carbapenems. The genes that encode these enzymes are often associated with insertion sequences that provide additional promoter sequences, leading to their overexpression and ultimately to carbapenem resistance (26). To date, four main groups of CHDLs have been identified in *A. baumannii*, the intrinsic chromosomal OXA-51-like enzyme and the acquired OXA-23-like, OXA-40-like, and OXA-58-like enzymes (5). Acquired OXAs can be either chromosome or plasmid encoded (25). Here we report on a novel plasmid-mediated CHDL that could not be detected by any previous PCR techniques.

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MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility. *A. baumannii* clinical isolate 135040 was one of three blood culture isolates with identical repetitive-sequence PCR patterns isolated from the same Brazilian intensive care unit in 2004 (13, 17). Species identification was confirmed by *gyrB* multiplex PCR (18). *A. baumannii* ATCC 19606 and *Escherichia coli* strains Fusion blue and TOP10 were used for cloning experiments. Susceptibility to antimicrobials was evaluated by Etest (AB Biodisk, Solna, Sweden) by following the manufacturer's instructions and by disk diffusion and agar dilution in accordance with the guidelines of the Clinical and Laboratory Standards Institute (7).

PCR experiments. Detection of the known acquired CHDLs (bla_{OXA-23}, bla_{OXA-40}, bla_{OXA-58}) and metallo-β-lactamases (bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}) and the presence of the insertion sequence IS*Aba1* upstream of the intrinsic $bla_{OXA-51-like}$ gene was performed as previously described (10, 26, 28).

Plasmid analysis and cloning. Plasmid DNA was extracted from *A. baumannii* 135040 with a commercial kit (Qiagen, Hilden, Germany). Whole plasmid preparations were used to transform electrocompetent *A. baumannii* ATCC 19606 as previously described for *Pseudomonas aeruginosa* (6), and selection was performed with ticarcillin at 100 μg/ml.

Shotgun cloning of natural plasmid pPH98 DNA was performed. Briefly, DNA of plasmid pPH98 was extracted from *A. baumannii* 135040, digested with the restriction enzyme EcoRI, ligated into EcoRI-cut pBBR1MCS (19), and used to transform *E. coli* Fusion blue. The sizes of plasmid inserts were determined by restriction analysis. Transformants were selected onto 25 μg/ml ticarcillin-containing LB agar plates after overnight incubation, and recombinant plasmid pOXA-143 was retained for further analysis. In order to express the $bla_{OXA-143}$ gene at a high level, the corresponding gene was transformed into *E. coli* TOP10 (23) with the ZeroBluntTOPOPCR cloning kit (Invitrogen, Cergy-Pontoise, France), followed by selection on plates containing 50 μg/ml of amoxicillin (amoxicilline) and 30 μg/ml of kanamycin. The PCR amplicon encompassing the entire sequence of the $bla_{OXA-143}$ gene used for cloning was obtained with primers pre-OXA-143A (5′-AGTTAACTTTCAATAATTG-3′) and pre-OXA-143B (5′-TTGGAAAATTATATAATCCC-3′) from whole-cell DNA of *A. baumannii* 135040. Recombinant strain *E. coli* TOP10(pPH-1) expressing the $bla_{OXA-143}$ gene was retained for biochemical analysis and MIC determinations.

DNA sequencing. Recombinant strain *E. coli* Fusion blue(pOXA-143) was extracted and sequenced by primer walking (Eurofins MWG Operon, Ebersberg, Germany). The resulting sequence was analyzed with software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

TABLE 1. MICs determined by agar dilution of β-lactams for OXA-143-producing clinical isolate *A. baumannii* 135040, *A. baumannii* ATCC 19606(pPH98), *E. coli* TOP10(pPH-1), and reference strains *A. baumannii* ATCC 19606 and *E. coli* TOP

β-Lactam(s)	MIC (μg/ml) for:				
	A. baumannii 135040	*A. baumannii* ATCC 19606 (pPH98)	*A. baumannii* ATCC 19606	*E. coli* TOP10 (pPH-1)	*E. coli* TOP10
Amoxicillin	>256	>256	32	>512	4
Amoxicillin + CLA[a]	>256	>256	8	>512	4
Ticarcillin	>256	>256	4	128	4
Piperacillin	>256	16	4	8	1
Piperacillin + TZB[b]	>256	8	4	2	0.5
Ceftazidime	256	2	2	0.12	0.06
Cefotaxime	>256	16	8	0.12	0.12
Cefepime	4	2	1	0.12	0.06
Imipenem	>32	32	0.19	2	0.12
Meropenem	>32	32	0.19	2	0.12

[a] CLA, clavulanic acid at a fixed concentration of 4 μg/ml.
[b] TZB, tazobactam at a fixed concentration of 4 μg/ml.

IEF analysis. Isoelectric focusing (IEF) analysis was performed with an ampholine-polyacrylamide gel (pH 3.5 to 9.5) as described previously (22), by using culture extracts of *A. baumannii* 135040 and *E. coli* TOP10 harboring recombinant plasmid pPH-1.

β-Lactamase purification. A culture of *E. coli* TOP10 harboring recombinant plasmid pPH-1 that produced OXA-143 was grown overnight at 37°C in 4 liters of tryptic soy broth containing 100 μg/ml of amoxicillin and 30 μg/ml of kanamycin. The protein extracts obtained were purified as described previously (22). After sonication, the crude extract was treated with DNase and ultracentrifuged at 100,000 × *g*. The supernatant was filtered through a 0.45-μm filter and subjected to further purification steps, including fast protein liquid chromatography–ion-exchange chromatography with Q-Sepharose and 20 mM Tris-H_2SO_4 buffer (pH 8.5). The β-lactamase was recovered in the flowthrough. The extract was subsequently dialyzed in 20 mM diethanolamine-H_2SO_4 (pH 10.0) and loaded again onto the Q-Sepharose column equilibrated with the same buffer. The β-lactamase was retained in the column, and elution was performed with a K_2SO_4 gradient to prevent inhibition by NaCl. Finally, the fractions containing the highest β-lactamase activity were dialyzed against 100 mM phosphate buffer (pH 7.0). Enzyme purity was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinetic studies. Purified β-lactamases were used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0) (22). The k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial-rate conditions with a UV spectrophotometer as previously described (22). When biphasic hydrolysis was observed, reaction rates were measured at the steady state. The 50% inhibitory concentration (IC_{50}) of NaCl was determined (22). Specific activities of protein extracts and purified β-lactamase from a culture of *E. coli* TOP10(pPH-1) were determined as previously described (3). Protein content was determined by the Bio-Rad DC protein assay. One unit of enzyme activity was defined as the amount that hydrolyzed 1 μmol of imipenem/min/mg of protein.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence database under accession number GQ861437.

RESULTS

Antibiotic susceptibility, plasmid analysis, and cloning of the β-lactamase gene. Antimicrobial susceptibility results are shown in Table 1. *A. baumannii* 135040 was resistant to most β-lactams, including carbapenems. However, it was intermediately susceptible to cefepime and rifampin (rifampicin) and susceptible to ampicillin-sulbactam, colistin, tigecycline, and netilmicin (data not shown). PCR-based screening for the identification of known acquired carbapenemase genes remained negative. In addition, IS*Aba1* was not detected upstream of the intrinsic $bla_{OXA\text{-}51\text{-}like}$ gene. Plasmid analysis of *A. baumannii* 135040 identified a ca. 30-kb plasmid named pPH98. Transformation of *A. baumannii* ATCC 19606 with plasmid pPH98 led to resistance to imipenem and meropenem, in addition to piperacillin-tazobactam (Table 1). No other non-β-lactam resistance determinant was cotransferred. *E. coli* TOP10(pPH-1) expressing OXA-143 exhibited a resistance phenotype consistent with the expression of a CHDL. It exhibited reduced susceptibility to imipenem and meropenem and resistance to most penicillins that was not antagonized by β-lactamase inhibitors (Table 1).

Identification of the OXA-143 CHDL. Cloning of EcoRI-restricted plasmid DNA from *A. baumannii* 135040 into pBBR1MCS, followed by expression in *E. coli*, gave *E. coli*-(pOXA-143). DNA sequence analysis of plasmid pOXA-143 identified a 3.5-kb insert containing an 825-bp coding sequence termed $bla_{OXA\text{-}143}$, which encodes a novel class D β-lactamase of 275 amino acids (Fig. 1). Within the deduced protein sequence, a serine-threonine-phenylalanine-lysine tetrad (STFK) was found at positions DBL 70 to 73 (class D β-lactamase numbering) (8). A KSG element (positions 216 to 218) was found, as found in the sequences of the OXA-40-like and

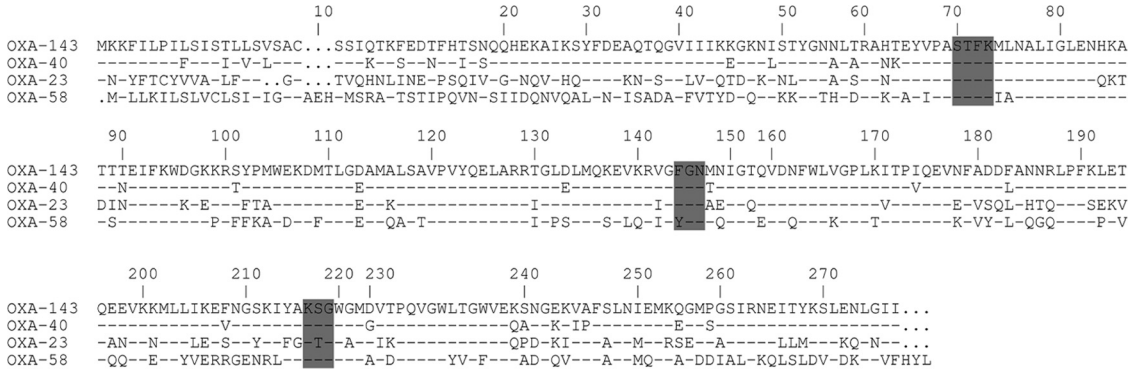

FIG. 1. Alignment of the OXA-143 amino acid sequence with those of OXA-40, OXA-23, and OXA-58. Conserved residues are shaded. β-Lactamases are numbered according to the DBL numbering system. Dashes represent conserved amino acids.

OXA-58-like CHDLs, whereas a KTG motif is present in the sequences of OXA-23-like enzymes and in most class D β-lactamases lacking any carbapenemase activity (Fig. 1). The class D β-lactamase structural element FGN at positions DBL 144 to 146 was conserved in OXA-143, as has been found in all of the CHDL sequences identified in *A. baumannii*, with the exception of those of the OXA-58 subgroup (24). The deduced amino acid sequence showed 88% identity with OXA-40, 63% identity with OXA-23, and 52% identity with OXA-58 (Fig. 1). The PCR primers used to detect the common bla_{OXA} genes in a multiplex PCR did not amplify $bla_{OXA-143}$. Thus, although showing significant similarities with the bla_{OXA-40} gene, $bla_{OXA-143}$ was undetectable with formerly proposed PCR systems (28). The two other Brazilian isolates tested positive by PCR for the presence of $bla_{OXA-143}$. Analysis of the genetic environment of $bla_{OXA-143}$ revealed that it was not associated with insertion sequences or integron located. Downstream of $bla_{OXA-143}$, a gene that encodes a mobilization protein was identified whose amino acid sequence shows 40% identity with that of *Acinetobacter* sp. strain ATCC 27244 (GenBank accession no. ZP_03823729). It was followed by a gene that encodes a replicase protein that shows 65% amino acid sequence identity with *A. baumannii* SDF (14). Interestingly, upstream of the $bla_{OXA-143}$ gene, the 3′ end of the same gene which encodes the replicase protein was present. Therefore, the $bla_{OXA-143}$ gene was bracketed by two copies of the same replicase gene, suggesting that its acquisition could have resulted from a homologous recombination process. It is noteworthy that the identification of that replicase gene in close proximity to $bla_{OXA-143}$ is in good agreement with a plasmid location.

IEF analysis. Culture extracts of *A. baumannii* 135040 analyzed by IEF gave two β-lactamases with pI values of 8.7 and 9.4, the latter corresponding to naturally produced AmpC (ADC) of *A. baumannii* (data not shown). A single pI value of 8.7 was obtained with a culture extract of *E. coli* TOP10 (pPH-1) expressing OXA-143.

Biochemical properties of β-lactamase OXA-143. After purification from extracts of *E. coli* TOP10(pPH-1), the specific activity of OXA-143 against benzylpenicillin was 4.8 U/mg of protein and its purification factor was 120-fold. The protein's purity was estimated to be >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown), and its apparent molecular mass was 29 to 30 kDa. OXA-143 has a narrow-spectrum hydrolysis profile that includes mostly penicillins (Table 2). The rates of imipenem and meropenem hydrolysis were low, whereas the MICs of both carbapenems for *E. coli* TOP10 expressing OXA-143 were increased by 16-fold (Table 1). In general, the catalytic activities of OXA-143 were similar to those of OXA-58 or OXA-40, taken as a reference for CHDL activity (24). Nevertheless, the k_{cat}/K_m values of imipenem were lower. Studies of activity inhibition, as measured by IC_{50} determination, showed that OXA-143 was inhibited by NaCl (IC_{50}, 25 mM).

TABLE 2. Kinetic parameters of purified β-lactamase OXA-143[a]

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ratio (s^{-1} μM^{-1})
Benzylpenicillin	5	80	0.063
Ampicillin	7	700	0.01
Ticarcillin	1	370	0.003
Piperacillin	1	40	0.025
Cephalothin	0.2	220	0.001
Oxacillin	1.2	450	0.003
Ceftazidime	<0.01	ND[b]	
Cefotaxime	<0.01	ND	
Cefepime	<0.01	ND	
Cefoxitin	<0.01	ND	
Aztreonam	<0.01	ND	
Imipenem	0.05	5	0.01
Meropenem	0.3	100	0.003

[a] Data are the means of three independent experiments. Standard deviations were within 10% of the means.
[b] ND, no detectable hydrolysis (<0.01 s^{-1}).

DISCUSSION

A novel carbapenem resistance determinant has been identified that was transferable at least between *A. baumannii* strains. This novel CHDL, OXA-143, is the first representative of a novel subclass of CHDLs, even though it is related to OXA-40-like enzymes. β-Lactamase OXA-143 hydrolyzed penicillins and carbapenems but did not significantly hydrolyze expanded-spectrum cephalosporins, as observed with other CHDLs. Similarly to the latter enzymes, OXA-143 shows a low K_m for the carbapenems but with low rates of hydrolysis (low k_{cat}) (1, 23, 24). Despite this weak hydrolysis, it is very likely that OXA-143 significantly contributes to resistance to imipenem and meropenem, as demonstrated previously with OXA-23, OXA-40, and OXA-58 (16).

The OXA-type enzymes are an ever-expanding group that includes more than 150 members (http://www.lahey.org/Studies/). Current investigations and detailed studies show that many of those enzymes possess carbapenemase properties and therefore contribute to antagonize the efficacy of those last-resort antimicrobials.

Evolution of OXA-51-like enzymes has been predicted by the stepwise charting of amino acid substitutions in a manner similar to that performed with SHV and TEM β-lactamases (4, 15), leading to a novel epidemiological tool (11). Less variation is found within the acquired OXA-23-like (11 substitutions), OXA-40-like (4 substitutions), and OXA-58-like (2 substitutions) enzymes. In contrast to OXA-40, OXA-143 exhibits 31 substitutions. This suggests that there are as-yet-undiscovered OXA enzymes that are intermediate between the OXA-40-like and OXA-143 enzymes. In addition, OXA-143 may have originated from another species and could be a recent acquisition by *A. baumannii*, as has been recently postulated for OXA-23 (21).

Interestingly, $bla_{OXA-143}$ was not associated with insertion sequence elements or with integron features. However, it was bracketed by two copies of the same replicase gene, which suggested a peculiar way of acquisition that might correspond to a homologous recombination process. Further experiments will be conducted to assess this hypothesis and to evaluate whether the plasmid carrying $bla_{OXA-143}$ is mobilizable and maybe self-conjugative, since a Mob protein showing 40% amino acid identity with that identified in *Bordetella bronchiseptica* was also identified (2).

In summary, OXA-143 is the first representative of a novel subgroup of CHDLs whose prevalence remains to be determined. It may, indeed, be quite prevalent, since resistance to

carbapenems in *A. baumannii* has not always been associated with known carbapenemases and previously designed primers could not detect this novel gene.

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