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Characterization of $bla_{OXA-143}$ Variants in *Acinetobacter baumannii* and *Acinetobacter pittii*

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The acquired carbapenem-hydrolyzing oxacillinase (OXA) OXA-143 has thus far been detected only in *Acinetobacter baumannii* isolates from Brazil. The aim of this study was to characterize three OXA-143 variants: OXA-231 and OXA-253 from carbapenem-resistant *A. baumannii* isolates and OXA-255 in a carbapenem-susceptible *Acinetobacter pittii* isolate originating from Brazil, Honduras, and the United States, respectively. The 5′ rapid amplification of cDNA ends (RACE) technique identified the same transcription initiation site for all $bla_{OXA-143-like}$ genes and revealed differences in the putative promoter regions. However, all cloned OXA-143 variants conferred carbapenem resistance on *A. baumannii* ATCC 17978 and OXA-255 conferred carbapenem resistance on *A. pittii* SH024, which was correlated with $bla_{OXA-255}$ gene expression. This is the first description of OXA-143-like outside *A. baumannii*. Detection of OXA-143-like in the United States and Honduras indicates its dissemination through the American continent.

Acinetobacter baumannii and *Acinetobacter pittii* are members of the "*A. baumannii* group," which, together with *A. nosocomialis*, comprise three phenotypically similar clinically relevant *Acinetobacter* species (1, 2). *A. baumannii* and *A. pittii* cause nosocomial infections and are associated with clinical outbreaks (3–5). While *A. pittii* is frequently found on both intact and diseased human skin and mucous membranes, it is prevalent on general wards and is usually susceptible to carbapenems. *A. baumannii* mainly affects patients in intensive care (6, 7). Carbapenems are considered the drugs of choice to treat infections caused by multidrug-resistant (MDR) *A. baumannii*. However, over the last decade carbapenem resistance has increased in *A. baumannii*, compromising available treatment options. The most common carbapenem resistance determinants in *Acinetobacter* spp. are carbapenem-hydrolyzing oxacillinases (OXA). *A. baumannii* isolates harbor the intrinsic OXA-51, of which >80 variants have been identified, and five groups of acquired OXA (OXA-23, -40, -58, -143, and -235) (8, 9). Of these, only OXA-23, OXA-40, and OXA-58 have been detected in other *Acinetobacter* species. For example, $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, and $bla_{OXA-58-like}$ have been described for carbapenem-nonsusceptible *A. pittii* isolates from China, Colombia, France, Germany, and the Irish Republic (10–13). bla_{OXA} genes are often associated with insertion sequences (IS) that mediate their mobility and overexpression, thereby leading to carbapenem resistance. However, bla_{OXA-40} and $bla_{OXA-143}$ seem to be exceptions to this (14). OXA-143 was first identified in 2009 in a carbapenem-resistant *A. baumannii* isolate and can be detected by multiplex PCR (15, 16). To date, OXA-143 has been reported only for *A. baumannii* isolates from certain states in Brazil (15, 17). However, outside Brazil, most bla_{OXA} screening is performed using the OXA multiplex PCR described by Woodford et al., which does not include $bla_{OXA-143-like}$ primers (18).

The aim of this study was to characterize $bla_{OXA-143}$ variants in two *A. baumannii* isolates and one *A. pittii* isolate.

MATERIALS AND METHODS

Bacterial strains, species identification, carbapenem susceptibility testing, and *A. baumannii* molecular typing. Carbapenem-resistant *A. baumannii* isolates AF81 and AF260 as well as carbapenem-susceptible *A. pittii* isolate AF726 were initially identified as $bla_{OXA-143-like}$ positive by multiplex PCR (Table 1) (16). AF81 originated from Brazil, AF260 originated from Honduras, and AF726 originated from the state of Indiana in the United States. Imipenem and meropenem susceptibility of *Acinetobacter* isolates and transformants was determined by Etest (bioMérieux, Nürtingen, Germany) according to standard protocols. To confirm species identification, *gyrB* multiplex PCR and *rpoB* sequencing were performed, as described previously (1). Isolates AF81 and AF260 were typed by repetitive sequence-based PCR (rep-PCR) (19).

PCR, sequencing, and cloning. The presence of bla_{OXA} genes was confirmed by multiplex PCR as described previously (8). Primers used for sequencing and cloning are shown in Table S1 in the supplemental material. $bla_{OXA-51-like}$ sequencing of isolates AF81 and AF260 was performed using primers OXA-69A and OXA-69B (20). $bla_{OXA-143-like}$ of isolate AF81 was amplified using primer pair OXA-231_F and OXA-231_R, cloned into pCR4-TOPO for sequencing (Invitrogen, Karlsruhe, Germany), and transferred into chemically competent *Escherichia coli* DH5α cells (New England BioLabs, Frankfurt am Main, Germany) according to the manufacturer's instructions. To sequence $bla_{OXA-143-like}$ from isolate AF260, total DNA was restricted by EcoRV endonuclease and shotgun cloned into pBBR1MCS, as previously described (21). $bla_{OXA-143-like}$-containing inserts of pBBR1MCS and pCR4-TOPO were amplified by PCR and sequenced by primer walking. To sequence $bla_{OXA-143-like}$ of AF726, total DNA was restricted by EcoRI endonuclease, self-ligated using Quick ligase (New England BioLabs), amplified by inverse PCR, and sequenced by primer walking. The $bla_{OXA-143}$ variants were numbered by the Lahey β-lactamase database (http://www.lahey.org/Studies/).

Assessment of $bla_{OXA-143-like}$ transferability. To determine the transferability of $bla_{OXA-143}$ variants, plasmids isolated from AF81, AF260, and AF726 were used for transformation. Electroporation was performed with

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TABLE 1 Characterization of clinical isolates AF81, AF260, and AF726[a]

Isolate	Country of origin	Date of isolation (mo/day/yr)	Carbapenem MIC (μg/ml)		Species identification	OXA-MPLX PCR	OXA-51 variant and rep-PCR type	OXA-143 variant	Replicon type and plasmid transfer
			IPM	MEM					
AF81	Brazil	01/04/2008	>32	>32	*A. baumannii*	51, 143	OXA-51, unclustered	231	GR 19, transferable
AF260	Honduras	02/01/2008	>32	>32	*A. baumannii*	51, 143	OXA-65, IC5	253	GR 12, transferable
AF726	USA (Indiana)	08/29/2007	0.75	1	*A. pittii*	143	ND	255	ND

[a] IPM, imipenem; MEM, meropenem; ND, not detected; MPLX, multiplex; IC, international clone; GR, group.

reference strains *A. baumannii* ATCC 17978 (plasmids of AF81 and AF260) and *A. pittii* SH024 (plasmid of AF726). Selection of *A. baumannii* transformants was performed on Mueller-Hinton agar (Oxoid, Wesel, Germany) supplemented with ticarcillin (150 μg/ml). Selection of *A. pittii* transformants was performed on Mueller-Hinton agar supplemented with 25, 40, 60, or 80 μg/ml of ticarcillin. The presence of $bla_{OXA-143-like}$ in the transformants was confirmed by PCR. In addition, plasmid replicon typing was performed with the clinical isolates, the reference strains, and the OXA transformants, as previously described (22).

Effect of OXA-143 variants on carbapenem susceptibility. To further characterize the impact of the three $bla_{OXA-143-like}$ variants on carbapenem susceptibility, the genes were amplified using primers listed in Table S1 in the supplemental material, cloned into the shuttle vector pWH1266, and transferred into *A. baumannii* reference strain ATCC 17978 by electroporation (23). Transformants were selected on Luria-Bertani agar (Oxoid) supplemented with 30 μg/ml of tetracycline. In addition, $bla_{OXA-143-like}$ of isolate AF726 was transferred into the *A. pittii* reference strain SH024 using the same selective medium.

Determination of $bla_{OXA-143-like}$ transcriptional initiation sites and gene expression. To identify the transcriptional start site of the three $bla_{OXA-143}$ variants, 5′ rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen). Primers used for 5′ RACE are shown in Table S1 in the supplemental material. Total RNA was prepared using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Amplicons of dC-tailed cDNA were purified and sequenced. In order to analyze differences in carbapenem susceptibility, $bla_{OXA-143-like}$ expression in the *A. pittii* strains was investigated based on independent experiments. Quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (8). $bla_{OXA-143-like}$ expression in the *A. pittii* transformant was compared to gene expression in AF726 and was normalized against expression of the *rpoB* reference gene. Standard curves for $bla_{OXA-143-like}$ and *rpoB* were included. Primers used for standard curves and qRT-PCR are shown in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The $bla_{OXA-231}$, $bla_{OXA-253}$, and $bla_{OXA-255}$ nucleotide sequences are available in GenBank under the following accession numbers: JQ326200 ($bla_{OXA-231}$), KC479324 ($bla_{OXA-253}$), and KC479325 ($bla_{OXA-255}$).

RESULTS AND DISCUSSION

Species identification, bla_{OXA} detection, OXA-51-like sequencing, and *A. baumannii* typing. *gyrB* multiplex PCR and *rpoB* sequencing confirmed AF81 and AF260 as *A. baumannii* and AF726 as *A. pittii*. Multiplex PCR confirmed the presence of $bla_{OXA-143-like}$ in all three isolates, as well as the presence of $bla_{OXA-51-like}$ in the two *A. baumannii* isolates. Sequencing of $bla_{OXA-51-like}$ identified bla_{OXA-51} and bla_{OXA-65} in isolates AF81 and AF260, respectively, which were not associated with IS*Aba1* (Table 1). Rep-PCR identified AF260 as international clone 5 (24), while AF81 did not cluster with any of the international clones. However, AF81 shared 98.5% similarity to *A. baumannii* 135040, the strain in which OXA-143 was first identified (15).

Identification of OXA-143-like-encoding genes. To amplify and sequence $bla_{OXA-143-like}$, flanking primers were designed based on $bla_{OXA-143}$ (GenBank accession no. GQ861437), which amplified the gene in AF81 but failed to amplify the gene in the other two isolates. Therefore, shotgun cloning was performed, and we cloned from AF260 an approximately 10-kb insert into pBBR1MCS, which was partially sequenced. Inverse PCR of AF726 revealed the presence of an approximately 3.5-kb amplicon which was also partially sequenced. Based on these sequences, $bla_{OXA-143-like}$ flanking primers specific for AF260 and AF726 were designed and used for cloning into pWH1266 (see Table S1 in the supplemental material). $bla_{OXA-143-like}$ sequencing revealed three OXA-143 variants which were assigned as OXA-231 (AF81), OXA-253 (AF260), and OXA-255 (AF726) by the Lahey β-lactamase database (Table 1). OXA-231 possessed one amino acid substitution compared to OXA-143 (D224→A) (Fig. 1A) and has been recently detected in another *A. baumannii* isolate from Brazil (25). OXA-253 shared 94% amino acid identity with OXA-143 (17 amino acid substitutions), while OXA-255 shared 92% amino acid identity with OXA-143 (21 amino acid substitutions). High similarity of OXA-253 and OXA-255 was also seen with OXA-182 (17 and 22 amino acid substitutions, respectively [Fig. 1A]). OXA-182 was detected in South Korea using $bla_{OXA-143-like}$ primers (26). Although OXA-253 and OXA-255 share similar amino acid identity with OXA-182 and OXA-143, they differ in their numbers of identical and positive (including conservative substitutions) amino acids. OXA-253 shares 258 of 275 identical amino acids with both enzymes, while the number of positive amino acids it shares with OXA-182 is higher than it shares with OXA-143 (266 and 262, respectively). In contrast, OXA-255 shares more identical amino acids with OXA-143 than OXA-182 (254 and 253, respectively) but fewer positive amino acids (261 and 264, respectively). This can be visualized in a phylogenetic tree of OXA variants (Fig. 1B). Thus, the OXA-143-like group shows large variations suggesting an ancient lineage, similar to OXA-51-like (27, 28).

***$bla_{OXA-143-like}$* transferability.** Clinical plasmids containing $bla_{OXA-231}$ and $bla_{OXA-253}$ were transferable into ATCC 17978. Replicon typing of ATCC 17978 transformants revealed that $bla_{OXA-231}$ and $bla_{OXA-253}$ were encoded on plasmids that harbored group (GR) 19 and GR 12 replicase genes, respectively (Table 1). Despite repeated attempts to transfer $bla_{OXA-255}$ and selection of transformants using ticarcillin concentrations as low as 25 μg/ml, the gene was not transferrable to either *A. pittii* or *A. baumannii* reference strains using plasmid preparations from AF726. Furthermore, plasmid replicon typing did not identify a known replicon in AF726. However, sequencing of $bla_{OXA-255}$ flanking regions identified sequences bracketing the $bla_{OXA-255}$ as plasmid sequences, which might indicate that this gene was initially plas-

(A)

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OXA-143   1 MKKFILPILSISTLLSVSACSSIQTKFEDTFHTSNQQHEKAIKSYFDEAQTQGVIIIKKGKNISTYGNNLTRAHTEYVPASTFKMLNALIGLENHKATTT 100
OXA-231     ----------------------------------------------------------------------------------------------------
OXA-253     --------F---I---L-------------SDI-D--QG-------------------E-----------A----------------------------
OXA-182     --------F---I---L---------------I---K---------------------E-----S-----V----------------------------N
OXA-255     --------F-------L----T--N---K-SDI-D-----------------------E----RI-----V----------------------------

OXA-143 101 EIFKWDGKKRSYPMWEKDMTLGDAMALSAVPVYQELARRTGLDLMQKEVKRVGFGNMNIGTQVDNFWLVGPLKITPIQEVNFADDFANNRLPFKLETQEE 200
OXA-231     ----------------------------------------------------------------------------------------------------
OXA-253     ----------------------------------------------------------------------------------------L-----------
OXA-182     ----------------------E------------D----I--N-------------------------I----------------L-----------
OXA-255     ------------------------------------------------------S-----N---------------A-----L--------------

OXA-143 201 VKKMLLIKEFNGSKIYAKSGWGMDVTPQVGWLTGWVEKSNGEKVAFSLNIEMKQGMPGSIRNEITYKSLENLGII 275
OXA-231     ----------------------A-----------------------------------------------------
OXA-253     ---------V---------------I-------------------P----L------S------------------
OXA-182     ---------V---------------S-------------------S----------S------------------
OXA-255     ---------V-----------------------------------P----L------S------------------
```

(B)

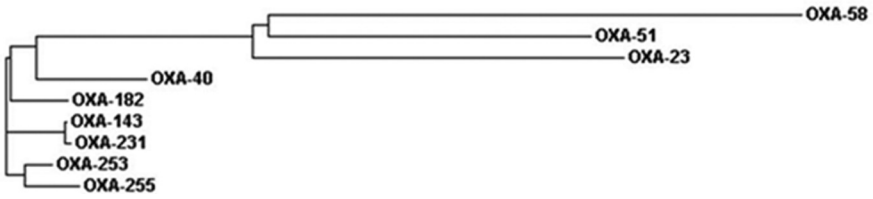

FIG 1 (A) Alignment of OXA-143-like amino acid sequences. Variants are sorted according to their amino acid identity with OXA-143. OXA-231 is closely related to OXA-143, with only one amino acid change at position 224. (B) Phylogenetic tree of OXA variants. This neighbor-joining tree was generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

mid located and subsequently integrated into the chromosome (see below).

Genetic environment of *bla*$_{\text{OXA-143-like}}$. Alignment of *bla*$_{\text{OXA}}$ and the surrounding sequences revealed high similarity of *bla*$_{\text{OXA-231}}$ flanking regions compared to *bla*$_{\text{OXA-143}}$ (GenBank accession no. GQ861437). The degrees of similarity were 98% for 193 bp upstream of *bla*$_{\text{OXA-231}}$ and 99% for 198 bp downstream of the gene. Accordingly, the start codon of a replicase gene was detected downstream of *bla*$_{\text{OXA-231}}$ (see Fig. S2 in the supplemental material). Flanking regions of the other two *bla* genes were not conserved, which explains why we were initially unable to amplify the whole genes from AF260 and AF726.

Downstream of *bla*$_{\text{OXA-255}}$, a putative peptidase gene was detected which showed 77% similarity to a peptidase encoded on *A. baumannii* plasmid p3ABSDF (GenBank accession no. CU468233) (see Fig. S2 in the supplemental material). Upstream of the *bla*$_{\text{OXA-255}}$ gene, an open reading frame coding for 136 amino acids of a putative TonB-dependent receptor plug domain was detected. Conserved domains of the plug superfamily function as gates within channel-forming TonB-dependent receptors, which are important for the iron uptake in Gram-negative bacteria (29). A BLAST search revealed 51% amino acid identity with a hypothetical protein previously described for *Acinetobacter* species NIPH1867 (locus tag WP_005210788), containing a TonB-dependent siderophore receptor domain.

In contrast, the sequence upstream of *bla*$_{\text{OXA-253}}$ showed 91% similarity with the sequence upstream of *bla*$_{\text{OXA-40-like}}$ in *A. baumannii* plasmid pAC92 (GenBank accession no. JN982952), containing a putative inner membrane protein and an XerC/D recombination site (see Fig. S2 in the supplemental material). Analysis of the partial protein sequence revealed only one amino acid difference compared to another *A. baumannii* membrane protein (locus tag WP_000465837). The XerC/D site (5′-ACTTCGTATA ATATCCATTATGTTAAAT-3′) was located 74 bp upstream of *bla*$_{\text{OXA-253}}$. In addition, another putative XerC/D recombination site (5′-ATATTGTATAACCTATATTATGTTATTT-3′) was identified 111 bp downstream of the gene. XerC/D recombination sites are often associated with *bla*$_{\text{OXA-40-like}}$ genes. However, our results indicate that the three OXA-143 variants described in this report might have evolved from different progenitors. This might also include OXA-182, as a putative transposase gene has been detected downstream of the *bla* gene, which is not part of the flanking regions of the other variants. Interestingly, the available upstream sequence of *bla*$_{\text{OXA-182}}$ (20 bp) is 100% identical with the same region upstream of *bla*$_{\text{OXA-143}}$, which further indicates relatedness of this OXA to the OXA-143 group.

Impact of OXA-231, OXA-253, and OXA-255 on carbapenem susceptibility. Cloning of *bla*$_{\text{OXA-143-like}}$ into pWH1266 and transfer into *A. baumannii* ATCC 17978 conferred carbapenem resistance. Imipenem and meropenem MICs increased from 0.25 μg/ml in the reference strain to >32 μg/ml in all transformants (see Table S3 in the supplemental material). Furthermore, OXA-255conferred carbapenem resistance on *A. pittii* SH024, with imipenem and meropenem MICs increasing from 0.25 and 0.5 μg/ml to 16 and >32 μg/ml, respectively (see Table S3 in the supplemental material).

Identification of *bla*$_{\text{OXA-143-like}}$ transcription initiation sites and *bla*$_{\text{OXA-255}}$ expression. In order to identify the transcriptional initiation site of *bla*$_{\text{OXA-143-like}}$ and deduce the promoter region, 5′ RACE was performed. We identified the same transcriptional ini-

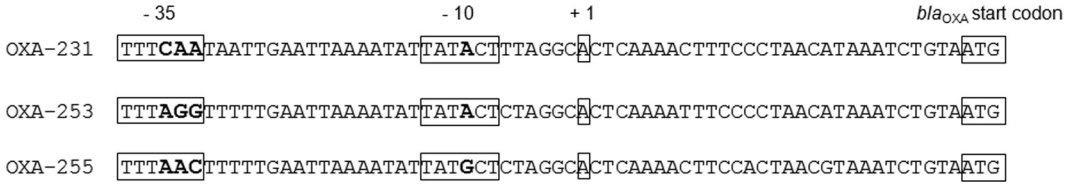

FIG 2 Alignment of $bla_{OXA-143-like}$ predicted promoter sequences. The start codon, the transcription initiation site, and the −10 and −35 boxes are boxed. Differences in the −10 and −35 boxes are marked by bold type.

tiation site for all $bla_{OXA-143-like}$ genes 30 bp upstream of the start codon (Fig. 2). Six base pairs upstream of the transcriptional initiation site, the same −10 box was identified in AF81 and AF260 (TATACT), while a substitution was present in AF726 (TATGCT). Interestingly, sequence alignment of the $bla_{OXA-231}$ and $bla_{OXA-143}$ upstream regions revealed the presence of the same putative promoters. The spacer region between the −10 and −35 boxes had an optimal size of 17 bp. Putative −35 boxes harbored 3 nucleotide differences (Fig. 2). Based on carbapenem MICs of ATCC 17978 transformants harboring recombinant pWH1266, OXA-143-like variants and their different promoter sequences did not seem to significantly influence carbapenem susceptibility (see Table S3 in the supplemental material). However, because OXA-255 did not confer carbapenem resistance on the clinical isolate AF726, while OXA-255-transformed ATCC 17978 and *A. pittii* SH024 were carbapenem resistant, we investigated expression of $bla_{OXA-255}$ in *A. pittii*. Expression analysis revealed that although $bla_{OXA-255}$ was expressed in AF726, it was overexpressed 24-fold in the SH024 transformant, which correlated with carbapenem resistance. This suggests that the $bla_{OXA-255}$ promoter is functioning and that there may be other regulatory mechanisms that affect its overexpression in the clinical isolate.

Conclusion. To date, the OXA-143 subclass has predominantly been described as occurring in carbapenem-resistant *A. baumannii* isolates from Brazil (17, 25, 30, 31). This study constitutes the first detection of OXA-143 variants in Honduras and the United States and indicates the spread of this carbapenemase in the Western Hemisphere. The occurrence of OXA-255 conferring carbapenem resistance on *A. baumannii* and on *A. pittii* highlights the potential of this OXA to spread within the genus *Acinetobacter*.

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