Characterization of bla\textsubscript{OXA-143} variants in \textit{Acinetobacter baumannii} and \textit{Acinetobacter pittii}

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Running title: \textit{bla}_{OXA-143}\textsubscript{-like} in \textit{Acinetobacter} spp.
The acquired carbapenem-hydrolysing oxacillinase (OXA) OXA-143 has thus far only been detected 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 USA, respectively. 5′-RACE technique identified the same transcription initiation site for all *bla*OXA-143-like and revealed differences in the putative promoter regions. However, all cloned OXA-143 variants conferred carbapenem resistance in *A. baumannii* ATCC 17978 and OXA-255 conferred carbapenem resistance in *A. pittii* SH024, which was correlated with *bla*OXA-255 gene expression. This is the first description of OXA-143-like outside of *A. baumannii*. Detection of OXA-143-like in the USA 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, comprises 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-hydrolysing 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, blaOXA-23-like, blaOXA-40-like and blaOXA-58-like have been described in carbapenem non-susceptible A. pittii isolates from China, Columbia, France, Germany and the Irish Republic (10-13). blaOXA genes are often associated with insertion sequences (IS) that mediate their mobility and overexpression, thereby leading to carbapenem resistance. However, blaOXA-40 and blaOXA-143 seem to be exceptions of 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 only been reported in A. baumannii isolates from certain states in Brazil (15, 17). However, outside of Brazil most blaOXA screening is performed using the OXA-multiplex PCR described by Woodford et al. that does not include blaOXA-143-like primers (18).
The aim of this study was to characterize \textit{blaOXA-143} variants in two \textit{A. baumannii} isolates and one \textit{A. pittii} isolate.

**MATERIALS AND METHODS**

**Bacterial strains, species identification, carbapenem susceptibility testing, and \textit{A. baumannii} molecular typing.** Carbapenem-resistant \textit{A. baumannii} isolates AF81 and AF260, as well as carbapenem-susceptible \textit{A. pittii} isolate AF726 were initially identified as \textit{blaOXA-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, USA. Imipenem and meropenem susceptibility of \textit{Acinetobacter} isolates and transformants was determined by Etest (bioMérieux, Nürtingen, Germany) according to standard protocols. To confirm species identification, \textit{gyrB} multiplex PCR and \textit{rpoB} sequencing were performed, as described previously (1). Isolates AF81 and AF260 were typed by rep-PCR (19).

**PCR, sequencing and cloning.** Presence of \textit{blaOXA} 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. \textit{blaOXA-51}-like sequencing of isolates AF81 and AF260 was performed using primers OXA-69A and OXA-69B (20). \textit{blaOXA-143}-like of isolate AF81 was amplified using primer pair OXA-231\textsubscript{F} and OXA-231\textsubscript{R}, cloned into pCR4-TOPO for sequencing (Invitrogen, Karlsruhe, Germany) and transferred into chemically competent \textit{E. coli} DH5\textalpha{} cells (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer’s instructions. To sequence \textit{blaOXA-143}-like from isolate AF260, total DNA was restricted by EcoRV endonuclease and shotgun cloned into pBBR1MCS, as previously described (21). \textit{blaOXA-143}-like containing inserts of pBBR1MCS and pCR4-TOPO were amplified by PCR and sequenced by primer walking. To sequence \textit{blaOXA-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 blaOXA-143 variants were numbered by the Lahey β-lactamase database (http://www.lahey.org/Studies/).

**Assessment of blaOXA-143-like transferability.** To determine the transferability of blaOXA-143 variants, plasmids isolated from AF81, AF260 and AF726 were used for transformation. Electroporation was performed with 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 ticarcillin. Presence of blaOXA-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 blaOXA-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 tetracycline. In addition, blaOXA-143-like of isolate AF726 was transferred into the *A. pittii* reference strain SH024 using the same selective medium.

**Determination of blaOXA-143-like transcriptional initiation sites and gene expression.** To identify the transcriptional start site of the three blaOXA-143 variants 5′-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, blaOXA-143-like expression in the A. pittii strains was investigated based on independent experiments. Reverse transcription and qRT-PCR were performed as described previously (8). blaOXA-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 blaOXA-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 blaOXA-231, blaOXA-253 and blaOXA-255 nucleotide sequences are available at GenBank under the following accession numbers: JQ326200 (blaOXA-231), KC479324 (blaOXA-253) and KC479325 (blaOXA-255).

**RESULTS AND DISCUSSION**

Species identification, blaOXA 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 blaOXA-143-like in all three isolates, as well as the presence of blaOXA-51-like in the two A. baumannii isolates. Sequencing of blaOXA-51-like identified blaOXA-51 and blaOXA-65 in isolates AF81 and AF260, respectively, which were not associated with ISAba1 (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 blaOXA-143-like, flanking primers were designed based on blaOXA-143 (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\textsubscript{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\textsubscript{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 \textit{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\textsubscript{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 number 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 with OXA-143 (266 vs. 262, respectively). In contrast, OXA-255 shares more identical amino acids with OXA-143 than OXA-182 (254 vs. 253, respectively), but fewer positive amino acids (261 vs. 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 plasmid-located and subsequently integrated into the chromosome (see below).

**Genetic environment of bla**OXA-143-like. Alignment of *bla*OXA and the surrounding sequences revealed high similarity of *bla*OXA-231 flanking regions compared to *bla*OXA-143 (accession no. GQ861437). Similarity was 98% for 193 bp upstream of *bla*OXA-231 and 99% for 198 bp downstream of the gene. Accordingly, the start codon of a replicase gene was detected downstream of *bla*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*OXA-255 a putative peptidase gene was detected which showed 77% similarity to a peptidase encoded on *A. baumannii* plasmid p3ABSDF (accession no. CU468233) (see Fig. S2 in the supplemental material). Upstream of the *bla*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). BLAST search revealed 51% amino acid identity with a hypothetical
protein previously described in *Acinetobacter* species NIPH1867 (accession no. WP_005210788), containing a TonB-dependent siderophore receptor domain.

In contrast, the sequence upstream of *bla*\textsubscript{OXA-253} showed 91% similarity with the sequence upstream of *bla*\textsubscript{OXA-40-like} in *A. baumannii* plasmid pAC92 (accession no. JN982952), containing a putative inner membrane protein and a 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 (accession no. WP_000465837). The XerC/D site (5´-ACTTCGTATAATATCCATTATGTTAAAT-3´) was located 74 bp upstream of *bla*\textsubscript{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*\textsubscript{OXA-40-like} genes. However our results indicate that the three OXA-143 variants described in this study 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*\textsubscript{OXA-182} (20 bp) is 100% identical with the same region upstream of *bla*\textsubscript{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*\textsubscript{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-255 conferred carbapenem resistance in *A. pittii* SH024, with imipenem
and meropenem MICs raising 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<sub>OXA-143</sub>-like transcription initiation sites and bla<sub>OXA-255</sub> expression.** In order to identify the transcriptional initiation site of bla<sub>OXA-143</sub>-like and deduce the promoter region, 5'-RACE was performed. We identified the same transcriptional initiation site for all bla<sub>OXA-143</sub>-like genes 30 bp upstream of the start codon (Fig. 2). Six bp upstream of the transcriptional initiation site the same -10 box was identified in AF81 and AF260 (TATACT), while a substitution was present in AF726 (TATG<sub>CT</sub>). Interestingly, sequence alignment of bla<sub>OXA-231</sub> and bla<sub>OXA-143</sub> 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 in the clinical isolate AF726, while OXA-255-transformed ATCC 17978 and *A. pittii* SH024 were carbapenem-resistant, we investigated expression of bla<sub>OXA-255</sub> in *A. pittii*. Expression analysis revealed that although bla<sub>OXA-255</sub> was expressed in AF726, it was overexpressed 24-fold in the SH024 transformant, which correlated with carbapenem resistance. This suggests that the bla<sub>OXA-255</sub> 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 in carbapenem-resistant *A. baumannii* isolates from Brazil (17, 25, 30, 31). This report constitutes the first detection of OXA-143 variants in Honduras and the USA and indicates
the spread of this carbapenemase in the western hemisphere. The occurrence of OXA-255
conferring carbapenem resistance in *A. baumannii* and in *A. pittii* highlights the potential of
this OXA to spread within the genus *Acinetobacter*.

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References


Table 1: Characterization of clinical isolates AF81, AF260 and AF726.

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<th>Date of isolation</th>
<th>Carbapenem MIC [µg/ml]</th>
<th>Species identification</th>
<th>OXA-MPLX PCR</th>
<th>OXA-51 variant and repPCR type</th>
<th>OXA-143 variant</th>
<th>Replicon typing and plasmid transfer</th>
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<td>01/04/2008</td>
<td>&gt;32</td>
<td>A. baumannii</td>
<td>51</td>
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<td>143</td>
<td>GR19 transferable</td>
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<tr>
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<td>02/01/2008</td>
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<td>143</td>
<td>GR12 transferable</td>
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<td>A. pittii</td>
<td>143</td>
<td>nd</td>
<td>255</td>
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nd, not detected; IC, international clone; GR, group
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 neighbour-joining tree was generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).
FIG 2: Alignment of *bla*OXA-143-like predicted promoter sequences. The start codon, the transcription initiation site, -10 and -35 boxes are highlighted with black boxes. Differences in the -10 and -35 boxes are marked in larger font.
<p>| OXA-143 | 1 | MKFILPILSITLLSLSACSSIGTKEFDTFHTSNQCHEARAISYFYDEAQTVGVIIIIKKQWNYSTYGNLLTRANEYVYPASTFKMLNALIGLENHNATTI 100 |
| OXA-231 | | F---I--L--SDI--D--QG--E---S---A-|
| OXA-253 | | F---I--L--I--K--E---S---V---|
| OXA-255 | | F---I--L--T--N--K--SDI--D--E---R---V--|
| OXA-143 | 101 | EIFKWGKKRYSYMKEKGDGLAMALSAYPYPYQELARRGLGLMNQEVKRVGQMNIGTQVDNFKLVQFLKITPIQEVNFADDFAHRLPFKLETQE 200 |
| OXA-231 | | E---D---I---N---|
| OXA-253 | | E---D---I---N---|
| OXA-255 | | E---D---I---N---|
| OXA-143 | 201 | YKHMILLIKFNGSKYIAKSGWDVTQPGWLEWSEKSGEKVAFSLNIMKQPMGSIRNEITYKSENHLGII 275 |
| OXA-231 | | A---|
| OXA-253 | | A---|
| OXA-255 | | A---|
| OXA-143 | | V---|
| OXA-231 | | A---|
| OXA-253 | | A---|
| OXA-182 | | V---|
| OXA-255 | | V---|</p>
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<td>OXA-255</td>
<td>TTTAACCCATTGAATTAAATTTATACTCTAGGCCCTCAAAACTTTCCACTAAGATCTGTAATG</td>
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