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ORIGINAL ARTICLE

Dissemination of $bla_{OXA-370}$ is mediated by IncX plasmids and the Tn6435 transposon

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Abstract

In *Enterobacteriaceae*, the $bla_{OXA-48-like}$ genes have been identified on plasmids in different regions of the world. The OXA-370 is a plasmid-encoded OXA-$_{48-like}$ enzyme reported in two distinct regions of Brazil. Recently, we demonstrate that the $bla_{OXA-370}$ gene is disseminated among several *Enterobacteriaceae* species and clones, indicating a high potential for dissemination. In this work, we described for the first time the complete nucleotide sequence of six plasmids harboring the $bla_{OXA-370}$ gene. Complete DNA sequencing using the Illumina platform and annotation of the plasmids showed that they belonged to incompatibility groups IncX and had in average 70 kbp. The $bla_{OXA-370}$ gene is located in a composite transposon containing four genes encoding transposases, named Tn6435. In this study, highly similar plasmids were detected in different *Enterobacteriaceae* genera.

Keywords OXA-370 · *Enterobacteriaceae* · Plasmids · Brazil · Tn6435

Introduction

OXA-type β-lactamases are currently classified in three different subgroups based on their hydrolytic profile [1]. The group 2df includes enzymes able to hydrolyze carbapenems, including OXA-48. OXA-370, a plasmid-encoded OXA-48-like enzyme, differs from the former by a single amino acid substitution and was firstly reported from Porto Alegre, south of Brazil [2] and subsequently reported from Rio de Janeiro, on the southeast of Brazil [3]. Dissemination of $bla_{OXA-370}$ has been demonstrated to occur by clonal dissemination [3], but recently the detection of this gene was demonstrated in different enterobacterial species [4]. However, there is no data regarding the complete sequence of plasmids harboring the $bla_{OXA-370}$ gene so far. The aim of this work was to determine the complete nucleotide of plasmids involved in the dissemination of the $bla_{OXA-370}$ gene among different species of *Enterobacteriaceae*.

Alexandre P. Zavascki and Jorge L. M. Sampaio are both senior authors and contributed equally to the work.

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Methods

Strains used in this study and species identification

The *Enterobacteriaceae* isolates in this study are listed in Table 1. All strains were isolated from rectal swab samples in the same hospital in Porto Alegre, RS, Brazil. Identification to the species level was achieved by partial sequencing of the *rrs* gene [5]. *Enterobacter* strains were also identified at the species level by partial sequencing of the *hsp60* gene, as previously described [6, 7], with modifications proposed by Campos et al. [8].

Plasmid extraction, transformation, and conjugation assays

Extraction of plasmid DNA from wild-type strains was carried out as previously described by Birnboim and Doli [9], except that sodium acetate solution was refrigerated. Plasmid DNA solutions were used to transform electrocompetent *E. coli* TOP10 (Thermo) using *E. coli* Pulser (BioRad). Transformants were selected on LB agar containing ampicillin (100 mg/L). Plasmids were subsequently extracted from transformants grown in LB broth containing ampicillin (100 mg/L). Conjugation experiments were performed using the wild-type strains as donors and *E. coli* J53 as the recipient, as described previously [10]. Transconjugants were selected on LB agar containing ampicillin (100 mg/L) plus sodium azide (125 mg/L). The presence of the $bla_{\text{OXA-370}}$ gene in transformants and transconjugants was confirmed by PCR as previously described by Poirel et al. [11] with modifications proposed by Campos et al. [8]. The presence of plasmids was confirmed by 0.7% agarose gel electrophoresis applying 2.5 V/cm during 7 h. *E. coli* 39R861 was used as a reference for estimating plasmid size [12]. Plasmid bands were visualized under UV transillumination after GelRed (Biotium) staining.

Antimicrobial susceptibility profile of transformants

Antimicrobial susceptibility profiles for carbapenems were determined by broth microdilution [13] using cation-adjusted Mueller-Hinton broth (Becton-Dickinson). *E. coli* ATCC 25922 was used as a control. Results were interpreted according to the M100-S25 document from CLSI [13].

Complete plasmid sequencing, assembly, annotation, and analysis

Plasmid DNA samples were tagmented using the Nextera DNA sample preparation kit before fragments of ~ 2000 bp were captured, purified, and sequenced using a MiSeq Reagent kit, v2 (500 cycles), in MiSeq system (Illumina). Sequences were assembled de novo in contigs using the SeqMan NGen program and subsequently aligned using SeqMan Pro, both in version 14.1.0 (DNAStar). Open reading frames (ORFs) were predicted and annotated using RAST (http://rast.nmpdr.org/) [14]. Manual curation and sequence similarity searches directed against the GenBank database were carried out using the ARTEMIS [15] genome browser and annotation tool. Insertion sequences were manually reviewed, directing searches against the IS Finder database (https://www-is.biotoul.fr/) [16]. The full plasmid sequences were compared to those available at GenBank using BLAST. Plasmid incompatibility group was evaluated using the Plasmid Finder software (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [17]. Plasmid nucleotide sequences were aligned using BioEdit [18].

Results

The plasmids carrying the $bla_{\text{OXA-370}}$ gene were successfully transferred by conjugation from wild-type strains to *E. coli* J53. Carbapenem MICs for the transformants carrying the $bla_{\text{OXA-370}}$ gene are described in Table 2. A total of six plasmids had the complete sequence determined. They were all circular and their GC content varied from 46.7 to 47.2%. All sequenced plasmids belonged to the IncX incompatibility group and had in average 70 kbp (Table 1). When the full nucleotide sequences of the 70-kbp plasmids were analyzed using the PlasmidFinder 1.3 Server, no plasmid replicons were found even using a 50.0% threshold. Contrasting to this

Table 1 OXA-370-producing *Enterobacteriaceae* isolates and plasmids information

WT strain	Species	Date	PFGE clone	Plasmid	Plasmid size (bp)	GC (%) content	Number of ORFs
844	*K. pneumoniae*	Aug/3013s	Kp1	p844	69,218	46.8	94
1032	*K. pneumoniae*	Oct/2013	Kp2	p1032	67,392	46.8	94
1182	*K. pneumoniae*	Oct/2013	Kp2	p1182	67,063	46.7	93
1233	*E. hormaechei*	Oct/2013	Eh1	p1233	69,606	46.9	95
1368	*E. hormaechei*	Nov/2013	Eh1	p1368	71,680	47.2	100
2048	*K. pneumoniae*	Feb/2014	Kp2	p2048	68,390	46.8	94

Table 2 Minimal inhibitory concentration (MIC) for the transformants harboring $bla_{OXA-370}$

WT strain	Transformants[a]	MIC (mg/L) for carbapenems		
		Imipenem	Meropenem	Ertapenem
844	Tf 844	1.0	0.06	0.25
1032	Tf 1032	0.5	0.06	0.06
1182	Tf 1182	0.25	0.06	0.25
1368	Tf 1368	0.5	0.03	0.125
2048	Tf 2048	0.5	0.03	0.5
E. coli TOP10		0.25	0.03	0.015

[a] Tf 1233 was not included in the table since it co-harbored $bla_{OXA-370}$ and bla_{NDM-1}

finding, annotation revealed the presence of genes encoding replication-associated proteins (*pir*, *bis*, *parA*, *hns*, and *topB*), characteristically found in IncX plasmids. These genes are highlighted in blue color in Figure 1S.

The largest IncX plasmid was designated p1368 and all others had their nucleotide sequences compared to it. Similarity indexes among them varied from 98 to 99%.

The p1368 plasmid, as well as the others described in this study, has a *virB* operon-encoding proteins of a type IV secretion apparatus implicated in conjugal transfer, in which *virB3* is fused with *virB4*. *virB7* is absent, but there is a gene immediately upstream, *virB8,* encoding for a 42 amino acid hypothetical protein 89% similar to PilX7 protein from *E. coli* (GenBank EIQ73345.1). It has *vapC*-like and *vapD*-like genes, known to encode a toxin-antitoxin system implicated in plasmid stability [19]. The plasmid also has the *frmA*, *frmB*, and *frmR* genes implicated in formaldehyde metabolism and tolerance [20]. Concerning antimicrobial resistance genes, only genes encoding β-lactamases were found. The $bla_{CTX-M-8}$ and the $bla_{OXA-370}$ genes were found located 24 kbp apart from each other (Figure 1S).

The $bla_{OXA-370}$ gene is located in a 3,710-bp composite transposon containing four genes encoding transposases; it is bracketed by IS*26* at the 5′ and *tnpA* gene at 3′ (Figure 2S). This transposon was registered as Tn6435 at the Transposon Registry Database (http://transposon.lstmed.ac.uk). This mobile element is present in all IncX plasmids sequenced in this study and also an IncF plasmid p87F from *Enterobacter hormaechei*, which partial sequence was previously described by our group (GenBank accession KJ488493.1). When the Tn6435 complete nucleotide sequence was compared to other sequences available at GenBank, the maximum query cover was 63%.

When the p1368 nucleotide complete nucleotide sequence was compared to that available at GenBank, the maximum query cover (52%) and similarity index (99%) were observed with GenBank deposit CP011588.1, an isolate of *Enterobacter asburiae* from human sample detected in VA, USA, in 2008.

The IncX plasmids harboring $bla_{OXA-370}$ differ from one another by insertion sequences. In the p1368 plasmid, isolated from *E. hormaechei* in October 2013, the *uvrB* gene is interrupted by the IS*Kpn24*, but this interruption is absent in the other five plasmids sequenced (Figure 1S). The p1233 plasmid, isolated from *E. hormaechei* in October 2013, has a IS*Kpn40* between two genes encoding hypothetical proteins but this insertion is absent in all other plasmids. In plasmids p1032 and p1182, the IS*5075*, observed downstream the Tn6435 in p1368, p1233, p844, and p2048, is absent (Figure 1S). Of note, highly similar plasmids (99%) were detected in different *Enterobacteriaceae* genera.

Discussion

In this paper, we described for the first time the complete nucleotide sequence of six plasmids harboring the $bla_{OXA-370}$ gene. All of them belong to the IncX incompatibility group. IncX plasmids have been described to harbor different carbapenemase genes and to be responsible for the dissemination of these genes in different countries around the world, as is the case for bla_{NDM-1} [8, 21, 22], bla_{KPC-2} [23–26], bla_{KPC-3} [27], and bla_{KPC-4} and bla_{KPC-5} [28]. More recently, these groups of plasmids have been described to harbor the *mcr-1* gene [29, 30]. There is a report of $bla_{OXA-370}$ in plasmids of variable sizes in *Klebsiella pneumoniae* from Rio de Janeiro [3], but the complete nucleotide sequences and the incompatibility groups have not been reported.

All plasmid sequenced had a *virB* operon with *virB3* fused with *virB4*, as previously reported in plasmids from *Campylobacter* [31] and apparently lacked the *virB7* gene. The *virB* operon encodes a type IV secretion system (T4SS) that enables plasmid transfer through conjugation. If we consider that plasmids harboring $bla_{OXA-370}$ were successfully transferred by conjugation from wild-type strains to *E. coli* J53, that the small VirB7 protein is essential for T4SS function and there are more than ten VirB7 homologues [32], it is possible that the hypothetical protein encoded by the gene located immediately upstream the *virB8* gene is in fact a new Virb7 homologue.

When we compared the carbapenem MICs obtained for transformants containing different plasmids described in this study, they differed by one or two dilutions. Consequently, transposon position on the plasmid does not seem to interfere in carbapenem MICs.

The plasmids that we sequenced in this work were detected in isolates cultivated from rectal swabs during the period from August 2013 to February 2014. In a 6-month period, we observed various occurrences of insertion sequences in the same *K. pneumoniae* and *E. hormaechei* clonal groups Kp1 and Eh1. These findings suggest a high frequency of genetic events in a plasmid in a short period of time.

We found that the same Tn6435 was present in plasmids pertaining to IncX and IncF incompatibility groups. This finding evidences the capacity of this transposon to be transferred from one plasmid to another.

The complete sequence of IncX plasmids containing the $bla_{\text{OXA-370}}$ gene showed that $bla_{\text{CTX-M-8}}$ was also present. Considering that OXA-370 is a weak carbapenemase that is not active against third- and fourth-generation cephalosporins [2], the presence of the $bla_{\text{CTX-M-8}}$ may facilitate the dissemination of clones harboring the IncX plasmids we sequenced.

In summary, we demonstrate that the $bla_{\text{OXA-370}}$ gene is disseminated among *Enterobacteriaceae* both by plasmid transfer and mobilization by a newly describe transposon herein designated Tn6435.

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Compliance with ethical standards

Conflict of interest A. P. Z. has received honoraria for speaking engagements and consultancy from Merck, AstraZeneca, Pfizer, and United Pharmaceuticals. All other authors have no conflicts of interest to declare.

Ethical approval The study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre (14-0046).

Informed consent Not applicable.

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