A proteogenomic view on antibiotic resistance in pathogenic Enterobacter species

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Chapter 5

Antibiotic resistance plasmids cointegrated into a megaplasmid harbouring the blaOXA-427 carbapenemase gene

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Abstract

OXA-427 is a new class D carbapenemase encountered in different species of *Enterobacteriaceae* in a Belgian hospital. To study the dispersal of this gene, we performed a comparative analysis of two plasmids containing the $bla_{OXA-427}$ gene isolated from a *Klebsiella pneumoniae* and an *Enterobacter cloacae* complex strain. The two IncA/C2 plasmids containing $bla_{OXA-427}$ share the same backbone, however, in the *K. pneumoniae* strain, this plasmid is cointegrated into an IncFIB plasmid, forming a 321-kb megaplasmid with multiple multi-resistance regions.

Introduction

The dissemination of carbapenem resistance in *Enterobacteriaceae* is a major public health concern, as carbapenem antibiotics are a last resort therapy for these common pathogens. The new OXA-427 carbapenemase presents a profile of wide resistance to β-lactams, including broad spectrum penicillins, extended-spectrum cephalosporins, and carbapenems [1]. The $bla_{OXA-427}$ gene was detected in different *Enterobacteriaceae* species (n=6) and in different patients (n=9) hospitalised at a university hospital in Belgium, in different hospital wards, between March 2012 and June 2014 [1]. This suggests a high level of interspecies transmission of this resistance gene, probably associated with horizontal gene transfer of a specific plasmid. Recent studies, based on long-read sequencing data, suggest that mobility of resistance genes occurs at multiple nested genetic levels with transmission of the strains between individuals, transfer of plasmids between strains and transposition of the resistance gene between plasmids [2,3]. We report the comparative analysis of two plasmids containing the $bla_{OXA-427}$ gene, for future study of the potential mechanism of interspecies transmission of the $bla_{OXA-427}$ gene.

Two OXA-427 producing isolates, one of *Klebsiella pneumoniae* (KLPN57), and second of *Enterobacter cloacae* complex (ENCL58), were cultured from clinical samples of two different patients in January 2013 and October 2013, respectively. Genomic DNA was extracted from these isolates and PacBio sequencing was performed at the Genomics Core laboratory (Leuven, Belgium) according to the protocol used by Conlan et al. [4]. Genomic DNA was sheared to 10–15 kb fragments and converted into SMRTbell template libraries. Each strain was sequenced on a single SMRT cell on the PacBio RSII using P6 polymerase binding and C4 sequencing kits. The sequencing coverage for both plasmids ranged from 160-fold to 400-fold coverage. Genome assemblies were performed using HGAP version 3 as part of SMRTAnalysis version 2.3.0, annotation of the genes was perfomred by using RAST (http://rast.nmpdr.org), and the functional assignments for the predicted ORFs confirmed using BLASTP in the Protein Data Bank (PDB) from NCBI (http://www.ncbi.nlm.nih.gov). IS finder and ResFinder were used for identification of mobile elements and antibiotic resistance genes, respectively [5,6]. PubMLST was used for multilocus sequence typing (MLST)[7]. The

plasmid map was drawn using Snapgene software (http://www. snapgene.com). The genomic content of the *E. cloacae* strain, ENCL58, consists of a 5.0 Mb genome with sequence type (ST-171) and three plasmids (177 kb, 121 kb and 84 kb). The 177,037 bp plasmid pENCL58_01, carrying the $bla_{OXA-427}$ gene, is an IncA/C2 plasmid of plasmid multilocus sequence type 3, 100% identical to KX869741.1[1] (Figure 1). The other two plasmids of IncFIb-, and IncFII-type carry no known resistance genes. The IncA/C2 plasmid shows overall high levels of homology with other IncA/C2 plasmids found in *Enterobacter aerogenes* (FO203354.1; 99% sequence identity, 87% coverage).

K. pneumoniae (Gene bank accession number JQ010984.1; 99% sequence identity, 86% coverage) and *Salmonella enterica* (KM670336.1; 99% sequence identity, 75% coverage). The plasmid pENCL58_01 contains 223 coding sequences including three functional antibiotic resistance genes ($bla_{OXA-427}$, bla_{TEM-1B} and *strB*) and two truncated resistance genes (*strA* and *sul2*). The bla_{TEM-1} and the *strB-strA* gene combination is located adjacent to the $bla_{OXA-427}$ resistance transposon in the direction away from *repA*, and both are associated with the presence of IS15 elements, making it a resistance island (RI). In this plasmid, two transfer regions were identified, located from position 49313 to 57324 and including 3 *tra* genes and *kfrA* (for plasmid maintenance) and one from positions 145665 to 173831 that includes 12 *tra* genes and *trhF*.

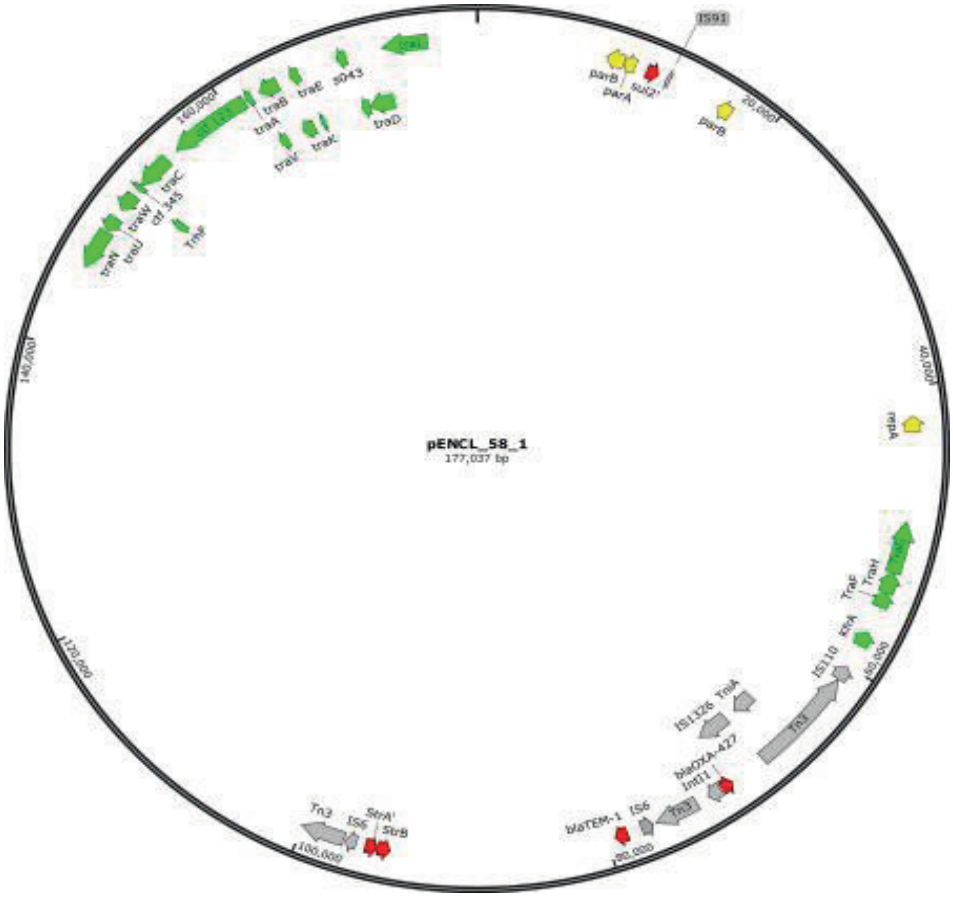

Figure 1. Circular representation of pENCL58_01, harbouring $bla_{OXA-427.}$ Only genes for partitioning/replication (yellow), conjugative transfer (green), antibiotic resistance (red), and mobility (gray) are depicted. Prime symbols indicate truncated genes.

The assembly of the *K. pneumoniae*, KLPN57, reads resulted in a genome of 5.23 Mb with ST-1 and three plasmids (321 kb, 175 kb and 77 kb). The $bla_{OXA-427}$ gene was identified on the 321,455 bp plasmid pKLPN57_01 plasmid that carried 417 predicted coding sequences(Figure 2). The other 2 plasmids are of type IncFIb with resistance genes $bla_{CTX-M-15,}$ $bla_{TEM-1B,}$ aph(3')-Ia, *aadA2*, *sul1*, and of type IncFII with *qnrS1*.

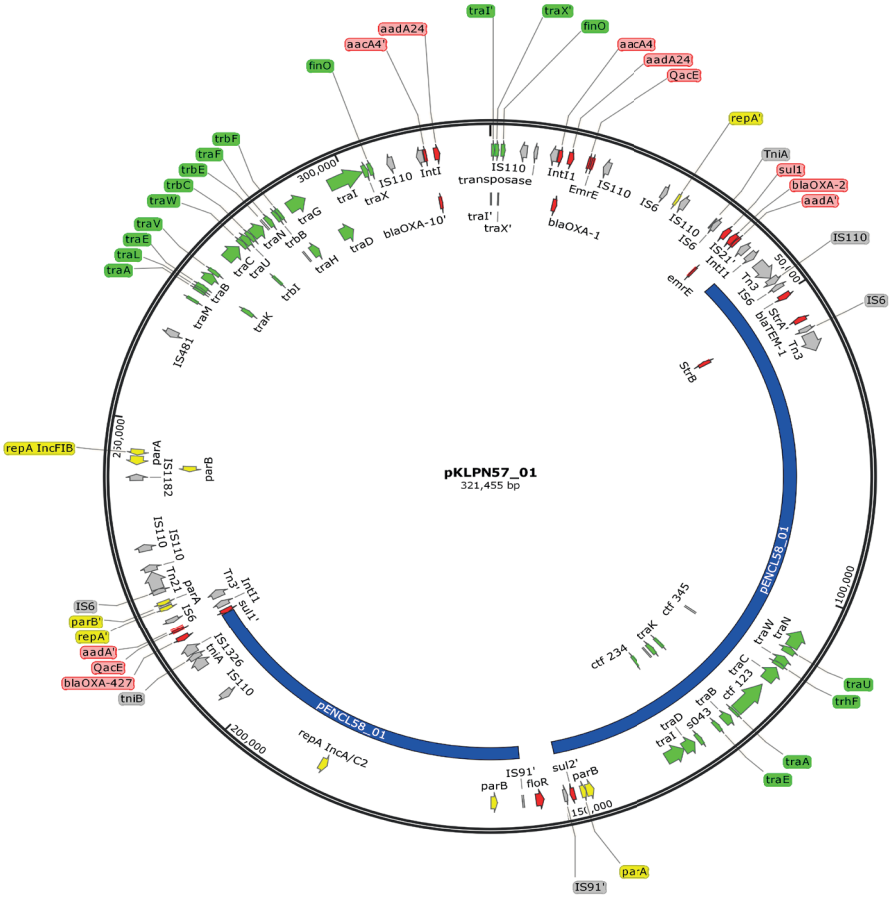

Figure 2. Circular representation of pKLPN57_01, harbouring *bla*$_{OXA-427}$. The plasmid parts identical to those in pENCL58_01 (>99% similarity) are indicated in blue. Only genes for partitioning/replication (yellow), conjugative transfer (green), antibiotic resistance (red), and mobility (gray) are depicted. Prime symbols indicate truncated genes.

The plasmid pKLPN57_01 contained seven resistance genes for aminoglycoside resistance (*aadA24*, *aacA4*, *strB*), β-lactam resistance (*bla*$_{OXA-10}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-427}$), chloramphenicol resistance (*floR*) and sulphonamide resistance (*sul1*). Five additional resistance genes (*strA*, *bla*$_{OXA-2}$, *sul2*, *bla*$_{OXA-10}$ and *aacA4*) were truncated and were potentially dysfunctional. Interestingly, two types of *repA* genes coding for plasmid replication proteins were detected on that plasmid. These *repA* genes belong to two distinct incompatibility plasmid groups namely, IncA/C2 and IncFIb. Further analysis confirmed that the pKLPN57_01 is composed

of a novel plasmid belonging to IncFIb group, and an IncA/C2 plasmid highly similar to pENCL58_01 identified in E. cloacae ENCL58 (Figure 3). Additionally, the plasmid carries 2 parts of a repA gene related to colW separated by the IncA/C2 region, together comprising the complete replication gene. Based on the presence of different repA genes and the large number of mobile genetic elements, the pKLPN57_01 plasmid is in fact a megaplasmid of 321 kb and could be a result of a multiple fusions of different plasmids and/or mobile elements. The IncFIb region showed highest homology with K. pneumoniae plasmid CP021541.1_(99% identity, 68% coverage), and the ColW region with Achromobacter xylosoxidans subsp. denitrificans plasmid HF679279.1 (100% identity, 37% coverage). Unfortunately, we were not able to identify the sources of these plasmid elements.

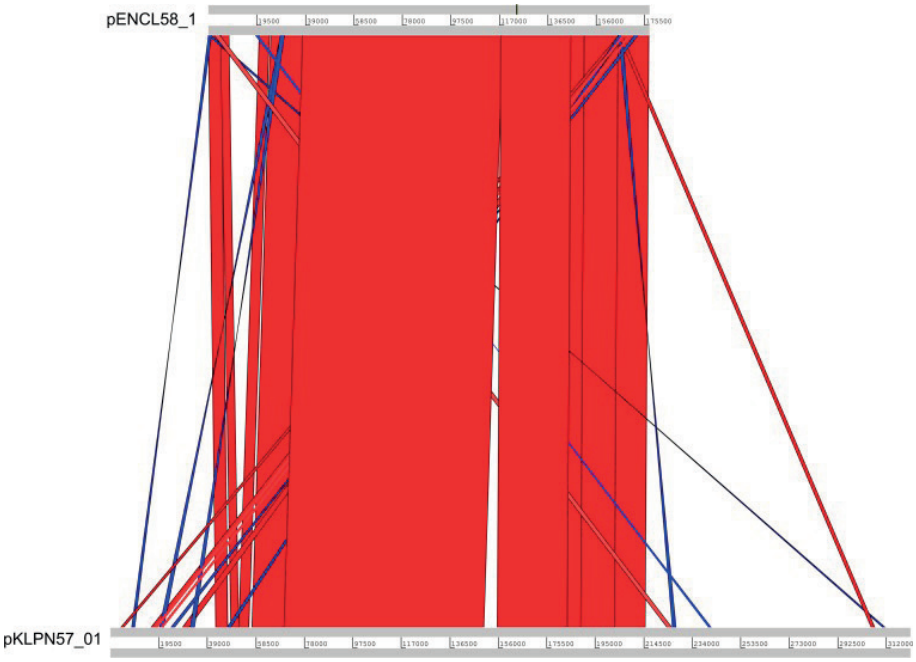

Figure 3. Pairwise comparison of pENCL58_01 (upper sequence) and pKLPN57_01 (lower sequence). The *intI1* gene of pENCL58_01 is used as a starting point. At the starting position, the homologous plasmid is disrupted and integrated into the pKLPN57_01 megaplasmid. Connecting bars represent regions with BLASTN matches of >99.9% in the same (red) and inverted (blue) orientations, visualized with the Artemis Comparison Tool [8].

The genetic environment downstream of the *bla*$_{OXA-427}$ gene, characterised by IS1326 and a Tn3 family transposon, is identical in the two plasmids. Upstream of the *bla*$_{OXA-427}$ gene, however, in pKLPN57_01, compared to pENCL58_01, additional resistance genes are present (Figure 1 and Figure 2).

In Figure 3, the alignment of pENCL58 and pKLPN57 is shown. In the pKLPN57_01 megaplasmid, the IncA/C2 region is flanked by identical class I integron integrase genes, *IntI1*, at both sides. At the 5' side, the *IntI1* gene is fused with a resistance integron. At the 3' side, the *IntI1* gene of IncA/C2 is fused with a partial *colW*-like *repA* gene by a transposase gene of the Tn3-family transposases and an IS6 insertion sequence. In both regions of fusion, there is an abundance of Tn3 family transposases, IS6, and IS110 insertion sequences. The region homologous to pENCL58 is divided in two parts by a transposon flanked on both sides by IS91, carrying the resistance gene *floR*.

We conclude that the megaplasmid in the *K. pneumoniae* isolate is a result of cointegration of an IncA/C2-type plasmid harbouring *bla*$_{OXA-427}$ with an IncF1b-type plasmid. The ability of Tn3 and insertion sequences to mediate in cointegration of plasmids is well known [9]. Plasmid cointegrates have been reported previously in carbapenemase producing *Enterobacteriaceae* strains[4,10]; they showed IncFIa-type plasmid backgrounds, and IS26 insertion sequences flanking the regions of fusion. To our knowledge, however, cointegrates formed from large resistance plasmids with sizes of > 300kb, have not been reported previously. The plasmid cointegration seems to represent an important molecular pathway for inter-species transfer of plasmids and their resistance genes. Cointegration with disruption of replicons, as observed in pKLPN57_01, may allow plasmid incompatibility to be overcome [11]. Next, cointegration with a plasmid that has co-evolved in *K. pneumoniae* may provide the transferred plasmid with specific conjugation and restriction modification machinery [11]. The cointegration of plasmids into pKLPN57_01 has probably taken place in recent years. Future studies will need to clarify whether megaplasmids of the size presented in our study are persistent, or that they represent a temporary initial stage in antibiotic resistance plasmid evolution.

Accession numbers

The annotated plasmid sequences of pKLPN57_01 and pENCL58_01 were deposited at DDBJ/EMBL/GenBank under the accession numbers LT882698 and LT882699, respectively.

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