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IncH-type plasmid harboring the *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{DHA-1}}$, and *qnrB4* genes recovered from animal isolates

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Running title: Complete sequence of an IncH-type plasmid bearing *bla*$_{\text{CTX-M-15}}$

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The whole sequence of plasmid pENVA carrying the extended-spectrum ß-lactamase gene *bla*$_{CTX-M-15}$ was determined. It has been identified from a series of clonally-related *Klebsiella pneumoniae* ST274 strains recovered from companion animals. This plasmid was 253,984-bp in-size and harbored, in addition to *bla*$_{CTX-M-15}$, a large array of genes encoding resistance to many antibiotic molecules including β-lactams (*bla*$_{TEM-1}$, *bla*$_{DHA-1}$), aminoglycosides (*aacA2*, *aadA1*), tetracycline (*tetA*), quinolones (*qnrB4*), trimethoprim *(dfrA15*), and sulfonamides (two copies of *sul1*). In addition, genes encoding resistance to mercury, tellurium, nickel, and quaternary compounds were identified. In addition, it carried genes encoding for DNA damage protection and mutagenesis repair, and also a CRISPR system locus corresponding to a immune system protecting against bacteriophages and plasmids. Comparative analysis of the plasmid scaffold showed that it possessed a similar structure with only a single plasmid, being pNDM-MAR encoding the carbapenemase NDM-1 and identified from human *K. pneumoniae* isolates. Both plasmids possessed two replicons, namely those of IncFIB-like and IncHIB-like plasmids, being significantly different from the previously characterized. The *bla*$_{CTX-M-15}$ gene, together with the other antibiotic resistance genes, was part of a large module likely acquired through a transposition process. We characterized here a new plasmid type encompassing the *bla*$_{CTX-M-15}$ gene identified in a *K. pneumoniae* of animal origin. It remains to determine to which extend this plasmid type may

spread efficiently, and possibly further enhance the dissemination of *bla*$_{\text{CTX-M-15}}$ among animal and human isolates.

Introduction

Antibiotic resistance within animal microbiomes (food-producing or companion animals) is now recognized as an emerging public health threat (1, 2). Resistance to broad-spectrum ß-lactams in *Enterobacteriaceae* is mainly caused by extended-spectrum β-lactamases (ESBL) and plasmid-encoded AmpC-type cephalosporinases (3). During the past decade, ESBLs of the CTX-M types have been recognized to be of growing importance worldwide, being frequently reported widely among enterobacterial isolates recovered from human specimens (4) either from the community (mainly *Escherichia coli*) or from hospitals (mainly *Klebsiella pneumoniae*). Furthermore, CTX-M-producing *E. coli* were identified among a wide range of animal species, including pets (5), poultry (6), cattle (7, 8), and wild animals (9, 10), and even from retail meat (11, 12), In addition, some plasmid-encoded AmpC-type β-lactamase genes, such as bla_{DHA} and bla_{CMY}, have been reported worldwide either from human and animal isolates (13, 14). Overall, these data raised some concerns about the transfer of ESBL and AmpC genes between human and animal bacterial strains.

Plasmids are often involved in dissemination of broad-spectrum ß-lactamase encoding genes. Six main plasmid families have been shown to mediate antimicrobial resistance dissemination among enterobacterial species, namely IncF, IncA/C, IncL/M, IncN, IncI and IncHI2

(14-17).

IncH-type plasmids are frequently involved in acquisition of antibiotic resistance both in human and animal bacterial isolates (5, 18). A large number of IncHI2 plasmids (usually with sizes larger than 250 kb) harboring $bla_{\text{CTX-M}}$ (with the exception of $bla_{\text{CTX-M-15}}$), bla_{SHV}, bla_{IMP}, bla_{VIM}, *armA*, *qnrA1*, *qnrS1* and *qnrB2* genes have been identified in many different enterobacterial species (5, 19).

CTX-M-15 is currently the most commonly identified CTX-M variant worldwide in enterobacterial species from human origin. By contrast, the most prevalent acquired ESBL among animal isolates is CTX-M-1, CTX-M-15 being rarely identified from animal isolates (4, 20, 21). The $bla_{\text{CTX-M-15}}$ gene has been identified onto plasmids belonging to unrelated incompatibility groups such as IncA/C, IncL/M, IncN, however the most frequently reported are IncI1 and IncF (14 22).

Recently, we identified phylogenetically-related CTX-M-15-producing *K. pneumoniae* isolates recovered from companion animals in France (23). In those isolates, the $bla_{\text{CTX-M-15}}$ gene was located onto a plasmid of ca. 250 kb in-size, that could not be classified in any of the known incompatibility groups, and that was self-transferable by conjugation to *E. coli* (23). The aim of this study was to determine the entire sequence of this plasmid in order to better evaluate its genetic relationship with those plasmids known to disseminate $bla_{\text{CTX-M-15}}$ among human isolates.

Material and Methods

Susceptibility testing. Susceptibility testing was performed by disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France), and minimal inhibitory concentrations (MICs) were determined by Etest (bioMérieux) on Mueller-Hinton agar plates at 37°C and interpretated according to the CLSI guidelines (24).

Sequencing, assembly and annotation of plasmid pENVA. A whole genome shotgun library was generated using 500 ng of plasmid DNA and sequenced on the Genome Sequencer FLX system (Roche Diagnostics, Mannheim, Germany) applying the Titanium chemistry in one quarter of a PicoTiterPlate. In the course of the Rapid Library preparation, according to the manufacturer's protocol, the DNA was tagged with a multiplex identifier (*GS Rapid Library MID Adaptors Kit*). Prior to emulsion PCR (emPCR), the pENVA plasmid library was combined with other MID-tagged libraries and subsequently sequenced. Sequencing reads were sorted based on their MID-tags and assembled by means of the GS *De novo* Assembler version 2.6 applying defaults settings (Roche Diagnostics). Ordering of contigs was done as described previously (25, 26), using the reference plasmid pNDM-MAR (Genbank accession no. JN420336) for mapping (27) by means of the computer program *r2cat* (28). Subsequently, 68 contigs showing homology to the reference plasmid were extracted, ordered according to the mapping results and stored in a new project. Reads protruding contig ends were used to identify contigs that flank a certain source contig. Sequence

gaps between contigs were closed by primer walking on plasmid template DNA. Moreover, contig graph information calculated by the GS *De Novo* Assembler was exploited to verify joining of contigs. Sequence finishing and polishing was accomplished using the CONSED software package (29). The complete sequence of plasmid pENVA was annotated by means of the GenDB genome annotation system version 2.0 (30). After automatic annotation, sequence information was refined manually as described previously (25, 26, 31). The plasmid genome plot was drawn and labeled as described (31, 32).

Phylogenetic analyses. For phylogenetic classification of plasmid pENVA, the replication initiation *repA* marker gene was used, since plasmids are commonly classified based on the amino acid sequence similarity of their replication initiator proteins (33). Nucleotide sequences of different related *repA* genes were extracted from corresponding database entries and aligned using the multiple sequence alignment program ClustalW (34). A phylogenetic tree was calculated by applying MEGA5 (35) applying the neighbor joining algorithm (36). A bootstrap analysis using 1,000 repetitions was carried out.

PCR-based replicon typing. PCR-based replicon typing aiming at identification of the main plasmid incompatibility groups reported for enterobacterial isolates was performed as described (37).

Comparative genome analyses. To analyze the similarity of the sequenced plasmid pENVA

and the reference plasmid pNDM-MAR, a comparative analysis was performed as described (31, 32, 38) by applying the tool M-GCAT (39).

Results and Discussion

Characterization of the plasmid bearing the *bla*$_{\text{CTX-M-15}}$ gene. Fourteen ST274-type, clonally-related, and CTX-M-15-producing *K. pneumoniae* isolates harbored a ca. 250-kb untypeable plasmid carrying the *bla*$_{\text{CTX-M-15}}$ gene. They had been recovered either from dogs, cats, sheep, and hedgehog (23). Once transferred by conjugation, this plasmid conferred resistance to broad-spectrum ß-lactams, tetracycline, gentamicin, sulfonamides, and trimethoprim, and reduced susceptibility to nalidixic acid to the *E. coli* recipient strain (23). Mating-out assays were performed with *K. pneumoniae* Kp15 recovered from urine of a cat, and the corresponding plasmid, named pENVA, was further studied.

Sequencing and general features of the antibiotic resistance plasmid pENVA. Plasmid pENVA was fully sequenced, yielding 43,358 sequence reads that were assembled into 1,258 large (> 500 bp) and 354 small (< 500 bp) contigs. Reads and contigs representing chromosomal contamination of the host bacterium *E. coli* were discarded. After filtering, plasmid pENVA consisted of 108 contigs, that were finally assembled after a PCR-based polishing approach. The complete plasmid pENVA has a size of 253,984 bp with an average GC content of 46.8%. The

plasmid sequence revealed 300 predicted coding sequences that could be assigned to 16 different functional modules (Fig. 1).

Backbone and accessory functions of plasmid pENVA backbone. Plasmid pENVA encoded two different replicon types, one belonging to the IncFIB family and the second corresponding to an IncHIB-type module. The first RepA protein (IncFIB-like) shared 40% with the canonical RepA of IncFIB (Genbank n°YP_788007.1). The second RepA (IncHIB-like) shared less than 60% amino acid identity with other reported IncHIB-type replication initiation proteins. However it shared a perfect identity with a recently identified replicase which gene was identified onto plasmid pNDM-MAR recovered from a clinical *K. pneumoniae* human isolate, harboring the $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$, and *qnrB1* antibiotic resistance genes (27). Hence, plasmid pENVA *repA* sequences are only distantly related to corresponding sequences from reference plasmids, explaining the lack of amplification when using the PBRT primers. Therefore, plasmid pENVA appeared to be an hybrid between two plasmid scaffolds, retaining their replication modules.

Plasmid pENVA harbored an *umuC-umuD* locus known to code for a mutagenesis repair system conferring resistance to UV light and related DNA damages (Fig. 2, panel A). A locus of five genes encoding a putative type III CRISPR-like system was also identified. Interestingly, CRISPR systems (clustered regularly interspaced short palindromic repeats) were shown to correspond to primitive immune systems involved in protection of host bacteria against bacteriophages and

plasmids (40). Plasmid pENVA harbored a functional partitioning system (*parA/parB*) and an higB-type toxin/antotoxin system. It also encoded a *flaC*-like gene, predicted to play a role in biosynthesis of flagella and therefore likely constituting a virulence factor.

Plasmid pENVA harbors a large multidrug resistance module comprising thirteen resistance genes encoding resistance to six different antibiotic classes. Nine antibiotic resistance genes were identified onto plasmid pENVA, namely *aacC2*, *aadA1* (resistance to aminoglycosides, including kanamycin, netilmicin, gentamicin, and tobramycin), $bla_{\text{TEM-1}}$ (resistance to penicillins), $bla_{\text{CTX-M-15}}$, $bla_{\text{DHA-1}}$ (resistance to broad-spectrum ß-lactams), *tetA* (resistance to tetracycline), *sul1* (two copies) (resistance to sulfonamides), *qnrB4* (resistance to quinolones), and *dfrA15* (resistance to trimethoprim). In addition, genes encoding resistance to mercury, tellurium, nickel, and quaternary ammonium compounds were identified. This array of genes encoding multidrug resistance was located in a large module, that was bracketed by two copies of insertion sequence IS*4321* (IS*110* family) inserted in opposite orientations to each other. Direct repeats representing possible transposition signatures were neither identified at the extremities of each of these IS*4321* elements nor at the extremities of the potential composite transposon formed by the two copies of IS*4321* flanking the multidrug resistance locus. This is in accordance with what has been observed for other members of the IS*110* family that do not generate target site duplications upon transposition (41). However, it may be speculated that the two copies of IS*4321* indeed form a

composite transposon carrying a large ca. 50-kb array of resistance genes. The latter putative composite transposon includes an internal resistance module bracketed by two IS*26* elements, and encompassing the IS*Ecp1*-made transposon (Tn*2012*) at the origin of $bla_{\text{CTX-M-15}}$ acquisition (Fig. 2A). As expected, a 5-bp target site duplication (TATGA) was identified at each extremity of Tn*2012*, as described (42, 43). The module flanked by IS*26* elements also harbored the *tetR* and *tetA* genes encoding inducible resistance to tetracycline (Fig. 2B), together with the AmpC ß-lactamase gene $bla_{\text{DHA-1}}$ that was associated to the LysR-type regulatory gene *ampR* (Fig. 2B). Moreover, the *qnrB4* gene was identified in association with genes encoding five putative phage-related shock proteins. This gene arrangement was similar to a gene cluster identified on plasmid pKP048 from China (44). Noteworthy, the promoter sequences (-35 [TTGGAC] and -10 [TACCAT]) upstream of the *qnrB4* gene were not part of the phage-related structures. The *lexA*-binding site, which was shown to be involved in regulation of a SOS response, was found in the vicinity of the *qnrB4* start codon, as previously described (45).

Plasmid pENVA harbors heavy metal resistance genes. Plasmid pENVA possessed two loci corresponding to putative heavy metal resistance genes. The first one was a complete mercuric resistance operon (*merD,A,C,P,T,R*) encoding a system known to transport mercuric-derivative compounds out of the bacterial cell (46). The second operon encoded resistance to tellurite-derivative compounds. The corresponding *ter*-type genes are marker characteristics of all IncHI2-

type plasmids (except for R476b). This operon has also been shown to be responsible for the control of resistance to infection by various bacteriophages (known as phage inhibition), and the resistance to pore-forming colicins (47).

Plasmid pENVA harbors additional insertion sequences. Among the different mobile elements identified within plasmid pENVA, insertion sequence IS*Kpn21* was identified. It belongs to the IS*NCY* family, and has recently been identified on different *K. pneumoniae* plasmids encoding the NDM-1 carbapenemase, including the IncH-type plasmid pNDM-MAR (27, 48). The recently-identified IS*Kpn20* element belonging to the IS*3* family was also identified. It was flanked by a 4-bp direct repeat (ACTT) being the likely signature of the insertion event. IS*Kpn20* was recently identified on a plasmid encoding the KPC-2 carbapenemase in Greece (49). A novel IS, that is related to IS*Raq1* (50) identified in *Rahnella aquatilis* (OrfB transposase sharing 90% amino acid identity) and therefore belongs to the IS*3* family was also identified with a target site duplication of 4 bp (GAAT) on each extremity. Considering that those IS were likely inserted as single elements, and therefore not associated to other mobilized DNA sequences, it is likely that they had minor impact on the evolution of plasmid pENVA.

Plasmid pENVA is related to the IncH-type *bla*$_{\text{NDM-1}}$-positive plasmid pNDM-MAR identified in *K. pneumoniae*. To estimate the genetic relationship of plasmid pENVA with other plasmids, a phylogenetic tree based on the *repA* marker gene was computed applying the tool

MEGA5 (35). The Neighbor-Joining algorithm (36, 51) was used comprising selected reference sequences (Table S1). This approach resulted in a phylogenetic tree which is divided into four different groups. Plasmid pENVA clusters together with the sequence originating from the IncH-type plasmid pNDM-MAR encoding NDM-1 from *K. pneumoniae* (27). Not only the *repA* gene products of both plasmids were identical, as mentionned above, but comparative analysis of both plasmids revealed that large regions of plasmid pENVA were nearly identical to those of pNDM-MAR (Fig. S1). Alignment of the homologous regions indicated that there were only 2.6% nucleotide mismatches over a length of 211,770 bp. In comparison to plasmid pNDM-MAR, pENVA harbored additional modules accounting for a total of 42,214 bp. Differences were mostly identified in the antibiotic resistance regions of both plasmids (Figure S1).

Regarding the resistance genes, the $bla_{\text{CTX-M-15}}$ gene was the sole gene identified on both plasmids, whereas all other antibiotic resistance genes were specific to each plasmid. Differences in the resistance modules of the plasmids resulted from insertions of different mobile genetic elements such as insertion sequences and transposons harboring antibiotic resistance genes. However, it is noteworthy that both plasmids harbored distinct resistance modules respectively flanked by IS*4321* and IS*26* elements, therefore highlighting the involvement of those IS elements in the genetic plasticity of that plasmid scaffold.

Apart from these resistance modules that contain different accessory genes, both plasmids

possessed very similar backbone modules and other accessory genes. However, one large segment encompassing the *tra* locus (conjugative transfer) and also the mercury and tellurite resistance loci was found to be in opposite orientations between the two plasmids, as highlighted in Figure 2B.

Concluding remarks. Plasmid pENVA encompassing two replicons and a large set of antibiotic resistance genes was identified from an animal isolate. Interestingly, a similar plasmid backbone has been recently identified from a *K. pneumoniae* clinical isolate recovered in Morocco (27, 52). In that latter case, the plasmid also harbored a series of clinically-relevant resistance genes, and in particular the $bla_{\text{NDM-1}}$ carbapenemase gene. It remains to evaluate whether this plasmid type might therefore play a significant role in dissemination of the $bla_{\text{CTX-M-15}}$ gene. Primers FIB-M FW and FIB-M RV which sequences have been reported by Villa et al. (27) are adequate for such screening approaches.

Plasmid pENVA additionally harbored genes encoding resistance to other clinically-relevant antibiotics, which are prescribed either in human and verterinary medicines. These antibiotics are quinolones, trimethoprim, aminoglycosides, sulfonamides, and tetracycline. It is noteworthy that this plasmid identified in animal isolates might have been selected from the environment taking in account the high number of heavy metal resistance genes. This hypothesis is reinforced by the fact that the plasmid harbors genes encoding resistance to heavy-metal derivatives such as mercury or tellurite compounds, but also to UV radiation.

Nucleotide sequence accession number. The annotated nucleotide sequence of plasmid pENVA was submitted to the GenBank database and is accessible under the accession no HG918041.

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Conflict of interest

None

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Figure 1. Genetic map of the fully sequenced plasmid pENVA. The circles represent (from inner to outer most): (i) GC skew; (ii) GC content; (iii) annotated coding sequences as arrows; (iv) plasmid modules. These modules are colored depending on their functional assignments as shown by labelling.

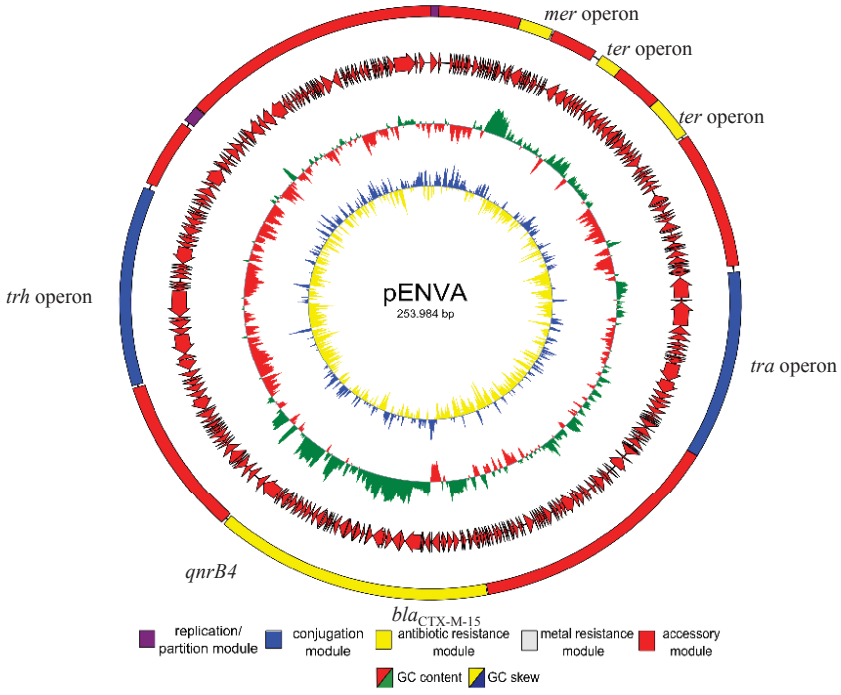

Figure 2.

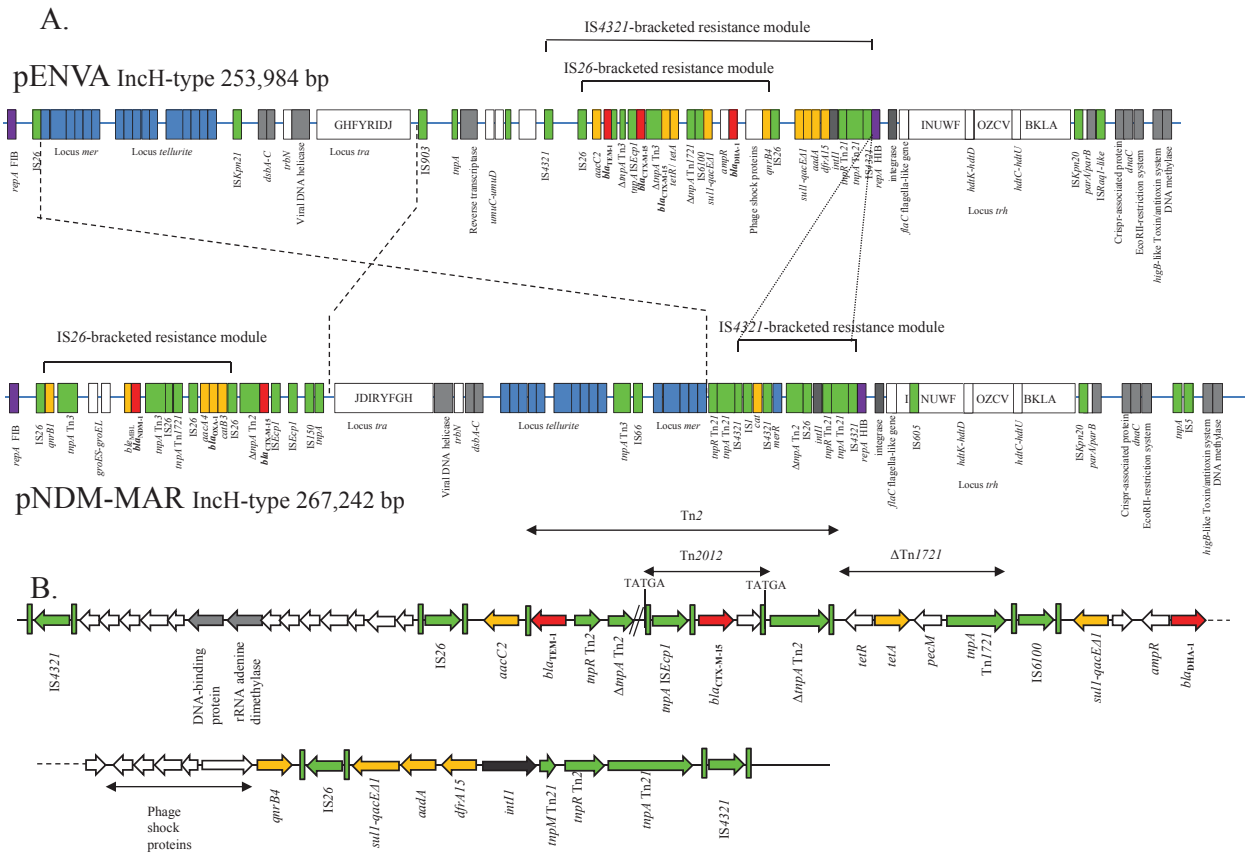

Figure 2. A. Major structural features of pENVA and comparison with the reference plasmid pNDM-MAR. White boxes indicate plasmid backbone regions that commonly occur in plasmids. The Tra locus (conjugative transfer) is indicated by white boxes with capital letters indicating the respective tra genes. Resistance genes are indicated by orange boxes, except for the β-lactamase genes, which are indicated by red boxes. Transposon-specific genes (*tnpA*, *tnpR*, *tnpM*), insertion sequences and class 1 integrase genes are indicated by green and dark grey boxes, respectively. Other genes are indicated by coloured boxes as follows: violet, replicase genes; light grey, partitioning systems and DNA methylase genes; blue, heavy metal resistance clusters.

B. Schematic representation of the multidrug resistance module of plasmid pENVA. The genes and their annotations are indicated by arrows and colored according to their functions.