Complete nucleotide sequence of the multidrug resistance IncA/C plasmid pR55 from Klebsiella pneumoniae isolated in 1969

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Objectives: To determine the complete nucleotide sequence of the multidrug resistance IncA/C plasmid pR55 from a clinical Klebsiella pneumoniae strain that was isolated from a urinary tract infection in 1969 in a French hospital and compare it with those of contemporary emerging IncA/C plasmids.

Methods: The plasmid was purified and sequenced using a 454 sequencing approach. After draft assembly, additional PCRs and walking reads were performed for gap closure. Sequence comparisons and multiple alignments with other IncA/C plasmids were done using the BLAST algorithm and CLUSTAL W, respectively.

Results: Plasmid pR55 (170810 bp) revealed a shared plasmid backbone (>99% nucleotide identity) with current members of the IncA/C2 multidrug resistance plasmid family that are widely disseminating antibiotic resistance genes. Nevertheless, two specific multidrug resistance gene arrays probably acquired from other genetic elements were identified inserted at conserved hotspot insertion sites in the IncA/C backbone. A novel transposon named Tn6187 showed an atypical mixed transposon configuration composed of two mercury resistance operons and two transposition modules that are related to Tn21 and Tn1696, respectively, and an In0-type integron.

Conclusions: IncA/C2 multidrug resistance plasmids have a broad host range and have been implicated in the dissemination of antibiotic resistance among Enterobacteriaceae from humans and animals. This typical IncA/C2 genetic scaffold appears to carry various multidrug resistance gene arrays and is now also a successful vehicle for spreading AmpC-like cephalosporinase and metallo-β-lactamase genes, such as blaCMY and blaNDM, respectively.

Keywords: antibiotic resistance spread, horizontal genetic transfer, transposons, IncA/C2 scaffold

Introduction

In recent years, there has been a growing interest in the characterization of plasmids belonging to the IncA/C incompatibility group, because of their widespread distribution among enteric bacteria of food-producing animals, their ability to confer multidrug resistance (MDR) and also their broad host range.1–3 Moreover, the A/C replicon type is mainly associated with plasmids harbouring and disseminating extended-spectrum β-lactamase (ESBL) genes in humans and animals.4 IncA/C MDR plasmids similar to pR55 were described in recent isolates of Salmonella enterica serovar Newport and Escherichia coli from animal sources in France (D. Meunier, HPA, personal communication).5 In addition, the emergence of the AmpC cephalosporinase blaCMY-2 carried by IncA/C MDR plasmids during the last decade in food animals, and the recent emergence of the blaNDM-1 carbapenemase gene in Klebsiella pneumoniae and E. coli in part associated with IncA/C plasmids in humans have caused considerable concern in the public health community.2,3,6

In the plasmid classification scheme based on incompatibility during conjugation, a subgroup of well-characterized IncA/C plasmids containing pRA1, pR55 and pIP40a was used as ‘reference/positive controls’ in the different approaches to determine Inc groups.7,8 Carattoli et al.4,8 using their PCR-based replicon typing method, reported that current IncA/C plasmids carrying ESBL genes represented a new replicon variant, named repA/C2 (IncA/C), compared with the IncA/C ‘reference’ plasmid pRA1 (renamed IncA/C1). Recently, plasmid restriction length polymorphism confirmed that pRA1 is more distantly related to other early-isolated IncA/C plasmids, i.e. pR55 and pIP40a.9
Figure 1. (a) Circular representation of IncA/C plasmid pR55. Nucleotide composition (GC plot) is represented on the distance scale (in bp) around the map. The two MDR loci, Tn6187 and the floR-ISC2 element, are indicated. Genes are colour coded, depending on functional annotations, as follows: blue, plasmid replication/maintenance/modification; green, transposition/recombination; yellow, conjugative plasmid transfer; red, antimicrobial resistance; and grey, other functions/hypothetical proteins. The sequence and annotation of pR55 are accessible in the GenBank database under
Phylogenetic comparisons of the conjugative transfer proteins of several IncA/C plasmids from different bacterial genera have revealed that their conjugative systems are the closest relatives of those identified in a group of integrative conjugative elements found in the chromosomes of different Gram-negative bacterial species. Moreover, the horizontal mobilization of the antibiotic resistance Salmonella genomic island 1 has recently been shown to be specifically mediated in trans by the IncA/C MDR plasmid family (i.e. pR55), suggesting a narrow interaction of their transfer functions.

In the present study, we report the complete nucleotide sequence of the early IncA/C MDR plasmid pR55 isolated in 1969. Plasmid pR55, also called R55 and pIP55, was isolated from a human pathogenic K. pneumoniae strain responsible for a urinary tract infection in November 1969 in Paris (Claude Bernard Hospital, France) and was characterized as a transferable MDR plasmid conferring high-level resistance to gentamicin and kanamycin, and also resistance to ampicillin, chloramphenicol and sulphonamides. The pR55 nucleotide sequence was compared with that of other fully sequenced IncA/C plasmids of different sources.

Materials and methods

Source of pR55

Plasmid pR55 was obtained from the Pasteur Institute (Paris, France) and historically used as a positive IncC control plasmid in incompatibility group determination.

Extraction, plasmid sequencing and plasmid restriction profile

Plasmid pR55 DNA was extracted from an E. coli K-12 strain J53 using the QIAGEN Plasmid Midi Kit (QIAGEN Inc., Toronto, Ontario, Canada). Sequencing was done using a GS-FLX sequencing kit and protocol (454 Life Sciences, Branford, CT, USA). Briefly, a single contiguous sequence with 18-fold coverage was obtained using draft assembly and PCR-based gap closure. The restriction profiles of pR55 using six different enzymes (EcoRV, EcoRI, HpaI, DraI, XmnI and SacI) were done to confirm the correct assembly of its complete sequence by comparison of the restriction profile determined from the sequence with the experimental restriction fragment length obtained.

Sequence comparison

Gene and protein sequences were compared and aligned with sequences in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The whole plasmid sequence of pR55 was also compared and aligned against sequenced IncA/C plasmids using the Artemis Comparison Tool (http://www.sanger.ac.uk/resources/software/act/) to determine the common genetic scaffold of this plasmid type.

Nucleotide sequence accession number

The complete nucleotide sequence of pR55 has been deposited in the GenBank database under accession no. JQ010984.

Results and discussion

Analysis of pR55 sequence

pR55 is a circular plasmid of 170810 bp containing 204 open reading frames, only 31 of which are transcribed in the opposite direction to the IncA/C replication initiation protein gene repA (Figure 1a). The correct assembly of its complete sequence was confirmed by restriction profiling using several enzymes (data not shown). pR55 and recent IncA/C plasmids show a high level of global synteny and share a highly conserved plasmid backbone, as previously described in the IncA/C MDR plasmid family. This common backbone is interrupted by two MDR loci, as shown by the deviating nucleotide composition and inserted at two conserved sites as in other IncA/C plasmids (Figure 1a).

The pR55 IncA/C scaffold of 131272 bp represents the sequence shared with different IncA/C plasmids (except the floR-ISCR2 (floresnecil resistance gene-insertion sequence common region) and Tn6187 elements) (Table 1). The pR55 IncA/C scaffold showed nucleotide identity ranging between 80% and 95% over about 86 kb in length with the IncA/C reference plasmid pRA1 isolated in 1971 from the fish pathogen Aeromonas hydrophila (Figure 1b). The phylogenetic analysis of the IncA/C pR55 repA gene revealed 100% nucleotide identity with the majority of current IncA/C2 plasmid repA genes, except those from plasmid pYR1 (seven nucleotide substitutions resulting in two amino acid variations) and all bla<sub>NDM-1</sub>-carrying IncA/C2 plasmids (nine nucleotide substitutions resulting in only one amino acid variation) (data not shown). The reference IncA/C1 repA gene of pRA1 exhibited 69 nucleotide substitutions in comparison with this large majority of IncA/C2 plasmids, which caused seven amino acid changes (data not shown). Thus, to the best of our knowledge, pR55 represents the oldest sequenced plasmid of the IncA/C<sub>2</sub> subtype including current IncA/C<sub>2</sub> ESBL-carrying plasmids.

Sequence comparisons of the IncA/C<sub>2</sub> core part of pR55 revealed a strong identity (>99% nucleotide sequence identity) to the plasmid backbone identified in recently described MDR IncA/C<sub>2</sub> plasmids of different animal and human sources (Figure 1b). This IncA/C<sub>2</sub> shared backbone encodes functions such as plasmid replication/partition, maintenance and type IV conjugative transfer. Depending on the compared IncA/C<sub>2</sub> plasmid, the common backbone extended from ~127 to 100 kb (see Table 1 from pIP1202 to pEH4H and Figure 1b). Plasmid pKPHS3 from a K. pneumoniae respiratory pathogen isolate is the smallest in size (~105 versus 160 kb on average) and thus
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ND, not determined.

<sup>a</sup>The pR55 scaffold corresponds to the whole sequence except the MDR regions: the ISCR2-floR element and Tn6187 from bp positions 31053 to 37983 and from bp positions 122700 to 155308, respectively. The grey coloured range in the eighth column represents the decrease in nucleotide identity from 99% to 88% and the width corresponds to the coverage (see the text for details).
showed a restricted shared backbone of 65 kb, but remaining highly similar (>99% identity) (Table 1). Like pR1A, the remaining sequenced IncA/C plasmid pXNC1 from the entomopathogenic endosymbiont Xenorhabdus nematophila appeared more distantly related to the MDR IncA/C2 plasmids from Enterobacteriaceae (Table 1). The main different hotspot integration sites previously described in other studies were also identified in pR55: (i) upstream of glmM-sul2 genes; (ii) at the 5′-end of the tcr gene; and (iii) between the rhs and ter genes (Figure 1a). Two specific MDR loci were found in pR55 at conserved integration hotspots (Figure 1a and b). These results together indicate that pR55 represents a historic IncA/C2 plasmid isolated in 1969 with a highly similar backbone with the current IncA/C2 ESBL-carrying plasmids.

**MDR loci in pR55**

The first MDR locus of pR55 consists of a specific floR-ISCR2 element and is inserted upstream of the glmM-sul2 genes, which represents the first integration hotspot in the IncA/C2 plasmid backbone. This hotspot, which is in the vicinity of the parAB genes, is where different MDR elements containing ISCR2 and/or IS26 are often found, such as the 16 kb floR-tetA-strAB-sul2 locus found in different blaCMY-2 plasmids from S. enterica and E. coli strains (Figure 1). The pR55 floR-ISCR2 element comprises two partial ISCR2 elements flanking the chloramphenicol and florfenicol efflux resistance gene floR. The two ISCR2 elements showed different deletions at their ter ends, i.e., deletion from the ter end extended to the 5′-end of transposase gene tnpA, resulting in a non-functional element regarding transposition. ISCR elements are recognized as powerful antibiotic resistance gene dissemination vehicles, able to drive MDR clusters and implicated in the evolution of the MDR IncA/C plasmid family.

The other MDR cluster of pR55 is located from position 122,700 to 155,308 between the rhs and ter genes, and represents a novel composite transposon (Tn) of 32.6 kb in size, named Tn6187 (Figures 1 and 2a). Tn6187 presents an unusual merged arrangement of a complete Tn21, a complete Tn1696, an In0-type class 1 integron and three IS5075 elements (for details, see Figure 2a). Tn6187 represents a unique mixed order of different Tn modules from Tn21 and Tn1696, i.e. both mercury resistance modules and both transposition modules. Few sequenced MDR regions described on plasmids from K. pneumoniae, such as the Tn2670-derived region in plasmid pRMH760, or hybrid transposons in IncA/C2 plasmid pRyc103T4 showed significant resemblance with Tn6187. Although built of similar transposons (Tn21 and Tn1696), IS and integrons, these mixed transposon structures consist of different genetic arrangements, suggesting different recombination events in their respective formation. Tn6187 is flanked by typical 38 bp terminal inverted repeats (TIR38) identical to those of Tn21 and Tn1696—the left one was intact, while the right one showed the insertion of an IS5075 element. The presence of a 5 bp duplication from each side of Tn6187 and the intact TIR38 flanking the mer1696 module suggested an initial transposition event of a Tn1696 element into the pR55 backbone (Figure 2b).

First, Tn6187 presents two 38 bp internal inverted repeats (IIR38) orientated face-to-face bracketing the chloramphenicol acetyltransferase gene catA1 and they are also flanked by 5 bp duplications (TATTAA) that are different from those found outside Tn6187 (Figure 2a). This region showed 99% identity to the rhs of Tn2670 found in plasmid R100. The two IIR38 were interrupted by the insertion of IS5075 elements. Second, Tn6187 harbours a mixed order of Tn modules related to Tn1696 or Tn21 from each side of the IS5075-inserted IIR38, as shown in Figure 2(a). Lastly, Tn6187 was also shown to contain an In0-type class 1 integron that harbours a resistance gene cassette array consisting of the aadB-bla<sub>OXA-21</sub> genes. To our knowledge, the gentamicin/kanamycin resistance gene cassette aadB has not yet been reported in association with the β-lactamase resistance gene cassette bla<sub>OXA-21</sub>. This In0-type integron is flanked by 25 bp imperfect inverted repeats, IRI and IRt, but not by 5 bp direct repeats (Figure 2a). IRI is flanked on the left by the ‘CATGG’ sequence that corresponded to the insertion site of In4 in Tn1696 and IRt is flanked by ‘TCCAT’ sequence that corresponded to the insertion site of In2 in Tn21. This result strongly suggested that the first steps of Tn6187 formation may be similar to that of the formation model of the pRMH760 MDR region previously proposed by Partridge and Hall. The Tn6187 configuration could have arisen in a first step by homologous recombination between two integrons with a single crossover within the conserved segment (5′-cs or 3′-cs), one in the same position as In4 in a Tn1696 integrated in the pR55 scaffold and the other with the same insertion as In2 in a Tn21 backbone found on an rhs of circle element derivative of the Tn2670 originally described from plasmid R100 (Figure 2b). This first recombination step would have resulted in a transitory hybrid transposon arrangement (Tn1696/Tn21) containing two integrons, the catA1 gene and the IS1 element from NR1 (Figure 2b). Then, the loss of one integron structure has probably occurred by recombination between the resolution site res<sub>I</sub> of Tn1696 and the resolution site of Tn21 (Figure 2b). This recombination would result in an excised fragment and a hybrid res<sub>I</sub> site in Tn6187 (Figure 2a). The hybrid Tn6186/Tn21 res<sub>I</sub> subsite suggested that the recombination has occurred at the recombination crossover point of the resolvase (GenBank accession no. JQ010984). Finally, a sequence inversion may have occurred between the second and third copy of IS5075, which could explain the mixed order of the different Tn1696 and Tn21 modules in Tn6187 (Figure 2a and b).

**Conclusions**

The IncA/C2 MDR plasmid family harbours a large arsenal of genetic weapons (integrons, transposons and ISCRs), giving it the ability to acquire and disseminate antibiotic resistance genes. Plasmid pR55 isolated in 1969 from K. pneumoniae represents the earliest sequenced IncA/C2 plasmid that shares a large common IncA/C2 backbone with current AmpC cephalosporinase-and metallo-β-lactamase-carrying plasmids. In all IncA/C2 members, antibiotic resistance genes are found clustered in a few integration hotspots in the IncA/C2 scaffold within complex genetic structures able to exchange and acquire novel antibiotic resistance genes via homologous recombination. As an alarming example, the current epidemic spread of the bla<sub>NDM-1</sub> gene acquired by IncA/C2 MDR plasmids has drastically reduced the therapeutic options available for physicians.
Figure 2. (a) Structure of the merged mercury resistance transposon Tn6187. Vertical thick black bars represent the 38 bp terminal or internal inverted repeat sequences of class II transposons; arrowheads below the bars indicate their orientations. The transposition or mercury resistance adjacent regions are indicated, i.e. mer1696 indicates the mercury resistance operon of Tn1696. The res sites are indicated by small open boxes. ISS075 and IS1326 are represented by open boxes; arrows in the boxes indicate the directions of the transposase genes. The In0-type class 1 integron is indicated; IRI and IRT are 25 bp imperfect inverted repeats defining the left and right ends of class 1 integrons, respectively. Antibiotic resistance gene cassettes are represented by horizontal arrows followed by a small circle (atC site). 5 bp target site duplications flanking Tn6187 (TTCTG) are indicated; the different 5 bp insertion sites from each side of the In0-type class 1 integron are also indicated. bp coordinates are from the complete pR55 sequence (GenBank accession no. JQ010984); for details see also Figure 1. (b) Three-step model for formation of Tn6187: (i) integration of an r-det circle of Tn2670 into Tn1696 of pR55 by homologous recombination between their integrons of In0-type and In4-type, respectively; (ii) recombination by crossing over between resI site results in the excision of the In4-type integron (not shown) and in the hybrid Tn1696/Tn21 resI site of Tn6187 shown in Figure 2(a); and (iii) sequence inversion between the second and third ISS075 element in opposite orientation would result in the Tn6187 structure with its specific order of transposon modules. Genetic features are as in Figure 2(a).
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Transparency declarations
None to declare.

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