

University of Groningen

Next-generation sequencing to investigate antimicrobial resistance

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DOI:
[10.33612/diss.250884559](https://doi.org/10.33612/diss.250884559)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Fleres, G. (2022). *Next-generation sequencing to investigate antimicrobial resistance: a one-health perspective*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.
<https://doi.org/10.33612/diss.250884559>

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Chapter 7

Detection of a Small IncX₄ Plasmid Carrying the *mcr-1.1* Gene in a Pig Oral Fluid Sample by Shotgun Metagenomic Sequencing

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Journal of Global Antimicrobial Resistance (2020)

<https://doi.org/10.1016/j.jgar.2020.12.020>

Antimicrobial resistance driven by antibiotic overuse and bacterial evolution is a global threat, not only in healthcare settings but also in agriculture. Considering that only a few antimicrobial drugs are in development (1), limiting the use of last-resort antibiotics is of particular importance. Colistin is an example of such an antibiotic for severe infections caused by carbapenemase-producing multidrug-resistant Gram-negative bacteria (2). However, plasmid-mediated mobile colistin resistance (*mcr*) has become a public health concern (2). Here, we report the characterization of a small IncX4 plasmid carrying the *mcr-1.1* gene, found in a pig oral fluid (OF) sample, using short- (SRS) and long-read sequencing (LRS).

The OF sample was obtained from a German farm in October 2017. Nucleic acids were isolated using the MagMAX CORE Nucleic Acid Purification Kit (Applied BioScience). A SRS library was generated using the KAPA HyperPlus Kit (Roche) and sequenced on a NextSeq 500 (2 × 150bp) (Illumina). Sequence data was trimmed and assembled with CLC Genomics Workbench v12.0.3 (Qiagen). A LRS library was generated with the Rapid PCR Barcoding Kit (SQK-RPB004) (Oxford Nanopore Technologies [ONT]) and sequenced on a MinION (ONT). LRS data was basecalled with Albacore v2.5.11 (<https://github.com/Albacore/albacore>), demultiplexed with Porechop (<https://github.com/rrwick/Porechop>), assembled using MetaFlye v2.5 (<https://github.com/fenderglass/Flye>) and polished using Racon v1.4.3 (<https://github.com/isovic/racon>). Finally, the assembly was corrected with Medaka v.0.10.0 (<https://github.com/nanoporetech/medaka>) and polished twice with SRS data using Racon. Metagenomic binning was performed using MaxBin2 v2.2.4-1 (<https://sourceforge.net/projects/maxbin2/>) followed by taxonomic classification with OneCodex (<https://www.onecodex.com/>). For characterization of the plasmid replicon sequences, ABRicate v0.8 (<https://github.com/tseemann/abricate>) with the PlasmidFinder v2.1 database was used. Finally, online tools ISFinder (<https://isfinder.biotoul.fr/>) and OriTfinder v1.1 (<https://bioinformml.sjtu.edu.cn/oriTfinder/>) were used to screen for Insertion Sequences (IS) and the OriT, respectively. Circlator v1.5.5 (<https://github.com/sanger-pathogens/circlator>)

was used to confirm plasmid circularity. Annotation was performed manually using the best BLASTx hits. A comparison with the best reference (CP042970.1) was illustrated with EasyFig v2.2.2 (<https://mjsull.github.io/Easyfig/>).

The *mcr* gene was located on a 18,156 bp long circular contig named pLJN-*mcr1.1_IncX4* (GenBank accession: MT106093) with an average read coverage of 17.56 (SRS) and 31.06 (LRS). It was classified as an IncX4 plasmid consisting of 34 coding sequences and one copy of an IS26 with no additional antibiotic resistance genes. A BLASTn search showed that pLJN-*mcr1.1_IncX4* was almost identical (99.86% identity) but significantly smaller than eight IncX4-type *mcr-1*-harboring plasmids ($\geq 33,303$ bp) (GenBank accession: CP042970.1, MK869758.1, MK869757.1, MK172815.1, CP018773.2, CP016550.1, MF449287.1 and KY689634.1). This was due to a large IS26-mediated deletion of part of the type IV secretion system (T4SS) and an additional 6.3 kb which contained the *tbrM* gene (also named *kikA*) (Fig. 1). The deletion of the T4SS and its adjacent region mediated by IS26 has already been described in evolutionary in vitro studies in *Escherichia coli* (3). T4SS, the conjugational transfer machinery, imposes a burden on the bacterial host cell that occurs at the expense of reduced vertical transfer (3). Therefore, a knockout of the main transfer machinery is likely beneficial in terms of growth rate (3). While a plasmid missing part of the T4SS is not expected to conjugate (4), the transfer of non-self-transmissible plasmids through mobilization and co-integration have been described previously with the help of co-resident conjugative plasmids (5). The relaxase of the non-self-transmissible plasmid can bind its *oriT* and can then be recognized and transferred by the type IV coupling protein and T4SS of a co-resident conjugative plasmid (5). The pLJN-*mcr1.1_IncX4* plasmid subsequently contained a relaxase and an *oriT*-like region (coordinate 1737...1814) containing a pair of 14-bp inverted repeats (IRs) (GCAGGTGAGCAAAG...CTTTGTTACCTGC).

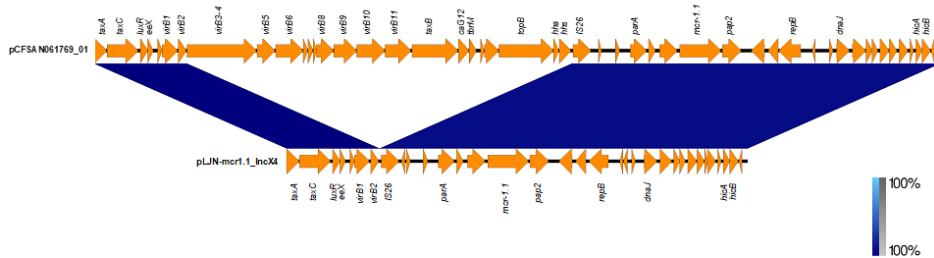


Figure 1. Comparison of the reference plasmid pCFSAN061769_01 (33,304 bp) (GenBank accession no. CP042970.1) and our plasmid pLJN-*mcr1.1*_IncX4 (18,156 bp) (GenBank accession no. MT106093). The missing region corresponds to part of the T4SS. The arrows indicate the direction and position of the genes. Areas with high homology between sequences are represented in blue.

The culture-independent nature of shotgun metagenomics does not allow for in vitro conjugation experiments or host determination, which can be a limitation of the methodology. However, binning yielded 48 bins, with *Acinetobacter* spp. (83.69%), *E. coli* (6.98%), and *Salmonella enterica* (2.33%) found in the bin containing the *mcr-1.1* gene. We believe plasmid pLJN-*mcr1.1*_IncX4 is probably hosted by an *E. coli* for several reasons: i) IncX4 is the most prevalent plasmid type in *E. coli* (5); ii) 75/95 of the BLASTn hits of pLJN-*mcr1.1*_IncX4 were attributed to *E. coli*; iii) *E. coli* was highly abundant in the sample and present in the *mcr* gene bin.

In summary, a small IncX4 plasmid harbouring the *mcr-1.1* gene was recovered directly from a porcine sample. It shares high homology with previously characterized plasmids in Enterobacterales from human, animal, food, and environmental specimens from several continents (2). However, the plasmid differed significantly in size due to the absence of part of the T4SS. This may ultimately limit the dissemination potential of these plasmids in these hosts. However, it is likely a trade-off between horizontal and vertical transmission (5). Metagenomic binning did not determine a specific plasmid host, but evidence suggests that *E. coli* is the probable host. Bacteria have an ever-changing nature and can adapt to highly competitive environments. It is essential to monitor these continual modifications in the event human or animal health is threatened. Here, we show the potential of applying SMg for the broad surveillance of antimicrobial resistance genes using OF samples.

Ethical approval

The animal samples used for this study were collected within the Food Pro-tec-ts project, which has been classified as an animal study and was approved on the 22.09.2017 by the respective state office for nature, environment, and consumer protection (file reference: 84.02.05.40.17.079).

Acknowledgements

We want to thank Christopher Erdmann, Erley Lizarazo and Hayley Cassidy for expert technical assistance and the farms for their participation.

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