Characterization of a Clinical *Enterobacter hormaechei* Strain Belonging to Epidemic Clone ST418 Co-carrying bla_{NDM-1}, bla_{IMP-4} and *mcr*-9.1

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Short report

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Abstract

An *Enterobacter hormaechei* isolate (ECL-90) simultaneously harboring bla_{NDM-1}, bla_{IMP-4} and *mcr-9.1* was recovered from the secretion specimen of a 24-year-old male patient in a tertiary hospital in China. The whole genome sequencing of this isolate was complete, and 4 circular plasmids with variable sizes were detected. Multi-locus sequence typing (MLST) analysis assigned the isolate to ST418, known as a carbapenemase-producing epidemic clone in China. bla_{IMP-4} and *mcr-9.1* genes were co-carried on an IncHI2/2A plasmid (pECL-90-2) and bla_{NDM-1} was harbored by an IncX3 plasmid (pECL-90-3). The genetic context of *mcr-9.1* was identified as a prevalent structure, "*rcnR-rcnA-pcoE-pcoS*-IS*903-mcr-9-wbuC*", which is a relatively unitary model involved in the mobilization of *mcr-9*. Meanwhile, bla_{NDM-1} gene was detected within a globally widespread structure known as NDM-GE-U.S ("IS*Aba125*– bla_{NDM-1} – bla_{MBL}"). Our study warrants that the convergence of genes mediating resistance to last-resort antibiotics in epidemic clones would largely facilitate their widespread in clinical settings, thus representing a potential challenge to clinical treatment and public health.

Introduction

The worldwide prevalence of carbapenem-resistant *Enterobacteraceae* (CRE) has been well known (1), with *Klebsiella pneumoniae* carbapenemases (KPCs), New Delhi metallo-lactamases (NDMs), imipenemases (IMPs) and OXA-48-like enzymes being the most prevalent carbapenemase (2). Moreover, co-occurrence of these genes in a single strain has been frequently identified (3, 4). Currently, colistin is one of the last-resort antibiotics for the CRE infections (5). However, the emergence of plasmid-borne colistin resistance gene *mcr* frustrates colistin's efficiency. MCR encodes a phosphoethanolamine transferase which is involved in the modification of lipopolysaccharide, the target of colistin. At present, the prevalence of *mcr* is the most widespread mechanism of colistin resistance (6). As of now, ten different *mcr* variants noted from *mcr-1* to *mcr-10* have been described (6–8). Of more concern, co-existence of *mcr* and carbapenemase genes has been sporadically reported (9). For instance, *mcr-1*, *mcr-3.5*, and bla_{NDM-5} are found in an *Escherichia coli* isolate (10), *mcr-4.3* and bla_{NDM-1} are co-identified in a clinical *E. cloacae* isolate from China (11), and bla_{VIM-4} and *mcr*-9 are found in an *E. hormaechei* isolate in USA (12). The rapid emergence of such new resistance phenotypes has broken through the last defense line and severely limited therapeutic options. In this study, we characterized a clinical *E. hormaechei* isolate simultaneously harboring bla_{NDM-1}, bla_{IMP-4} and *mcr*-9.1. The structures of plasmids carrying three resistance genes were fully dissected to understand their dissemination and accumulation pattern.

Materials And Methods

Bacterial isolate

This *E. hormaechei* isolate (ECL-90) was recovered from the secretion specimen of a 24-year-old male patient in October, 2017, who was admitted into a large tertiary hospital in Nanjing because of an acute community-acquired pneumonia. The *E. hormaechei* strain ECL-90 was identified by matrix-associated laser desorption ionization–time of flight mass spectrometry BioMerieux, Craponne, France) as *E. cloacae* complex, and further confirmed by whole-genome sequencing (WGS).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed toward ertapenem, imipenem, meropenem, cefepime, ceftazidime, cefotaxime, cefuroxime, cefazolin, cefmetazole, piperacillin/tazobactam, amikacin, gentamicin, funantuoyin, trimethoprim and sulphame-thoxazole, aztreonam, piperacillin, ciprofloxacin, levofloxacin aztreonam/avibactam, tigecycline, polymyxin, fosfomycin and ceftazidime/avibactam by microbroth dilution method. The results were interpretated according to the CLSI 2019(13). The cutoff values for tigecycline and polymyxin was referring to Eucast (www.eucast.org).

Resistance gene detection

Carbapenemase genes were detected by PCR according to the protocol described previously(14). PCR products were sent to Qingke Biotechnology Co., Ltd for DNA sequencing when targeted DNA bands appeared on the 1.5% gel after Ethidium bromide staining. Exact gene variants were assigned by blasting resulted DNA sequences into the NCBI database.

Conjugation assay

Broth conjugation experiments using a sodium azide-resistant *E. coli* J53 isolate as a recipient strain were performed to determine the transferability of carbapenemases encoding genes. LB agar plates containing 150 μg/mL sodium azide plus 1 μg/mL meropenem were prepared to select transconjugants. bla_{NDM-1} or *imp-4* within transconjugants was confirmed by PCR and DNA sequencing.

S1-pulsed-field gel electrophoresis (S1-PFGE)

In order to analyze the plasmids within this strain, the strain was embedded in agarose and made into plugs, after plugs were digested by S1 nuclease (Takara, Japan) (15), electrophoresis was carried out at 6.0 V/cm, The switch time was increased from 3 to 36 s at a gradient of 6 V/cm for 18.5 h, with an angle of 120º at 14℃ by using the CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA, USA). Gel was visualized with the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

DNA extraction

The genomic DNA of this isolate was extracted and whole-genome sequencing was performed on the Hiseq (Illumina, San Diego, CA, USA) as described previously (16).

Whole genome sequencing

In order to further analyze the chromosome and plasmid characterization, this isolate was firstly sequenced for whole genome by Hiseq 4000 instrument (Illumina, San Diego, CA, USA). The genome data were submitted to the NCBI database after the data were dealt for *de novo* Assembly, Scaffolding, and Annotation. To obtain full sequences of the plasmids, this isolate was further sequenced by Nanopore platform (Nanopore, Oxford, UK), and hybrid assembly was performed with Illumina sequencing data by using Unicycler version 0.4.8 (17).

Analysis of genome

Multi-locus sequence typing (MLST) analysis using MLST 2.0; antibiotic resistance genes were identified by using Resfinder v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1/). The completeness of these plasmids was further verified by PCR loop experiment.

Results

The antimicrobial susceptibility

Antimicrobial susceptibility test using microbroth dilution method showed that this strain was resistant to ertapenem (16 μg/ml), imipenem (8 μg/ml), meropenem (> 16 μg/ml), cefepime (> 32 μg/ml), ceftazidime (> 32 μg/ml), cefotaxime (> 32 μg/ml), cefuroxime (> 64 μg/ml), cefazolin (> 32 μg/ml), cefmetazole (> 64 μg/ml), piperacillin/tazobactam (> 256 μg/ml), amikacin (8 μg/ml), gentamicin (64 μg/ml), funantuoyin (128 μg/ml), trimethoprim and sulphame-thoxazole (> 32 μg/ml), aztreonam (> 128 μg/ml), piperacillin (> 256 μg/ml), ciprofloxacin (8 μg/ml), levofloxacin (4 μg/ml), and ceftazidime/avibactam (> 32 μg/ml), while remained susceptible to aztreonam/avibactam (< 0.25 μg/ml), tigecycline (1 μg/ml), colistin B (0.25 μg/ml) and fosfomycin (32 μg/ml).

Genome content

We found a chromosome with size of 4,584,517 bp, and 4 plasmids (pECL-90-1, pECL-90-2, pECL-90-3, and pECL-90-4) ranging in sizes from 6,364 bp to 348,891 bp (Table 1). The sizes of 4 plasmids were was in accordance with the results of S1-PFGE analysis, and the completeness of these plasmids were further verified by PCR loop experiment (Primers and the sizes of products were shown in Table S1). *fosA* and *bla*ACT-16 were identified in the chromosome, and the other resistance determinants were carried by pECL-90-2 and pECL-90-3.

Table 1
The key features of the *Enterobacter hormaeche* isolate

	Size (bp)	G + C(%)	Resistant determinants	plasmid	Accession No
Chromosome	4584517	55.58	*fosA* and bla_{ACT-16}	ST418	CP061744
pECL-90-1	106756	51.02	NA	IncFIB	CP061745
pECL-90-2	348891	48.59	bla_{IMP-4}, *mcr-9.1*, a*ac(3)-IId*, *aac(6')-IIc*, *aac(6')-Ib3*, *aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id*, bla_{SFO-1}, bla_{SHV-12}, bla_{TEM-1B}, *ere(A)*, *mph(A)*, *catA2*, *aac(6')-Ib-cr*, *sul1*, tet(D), *dfrA19*	IncHI2/2A	CP061746
pECL-90-3	44961	46.49	*blaNDM-1*	IncX3	CP061747
pECL-90-4	6364	51.21	NA	Untypeable	CP061748
NA: not applicable					

MLST analysis assigned ECL-90 to sequence type (ST) 418 (allelic profile 53-35-154-44-45-4-6) (18), which is known as one of the predominant epidemic clones of carbapenemase-producing *E. cloacae* in China (19, 20).

Antimicrobial Resistant Genes

Antibiotic resistance genes including genes conferring beta-lactam resistance (bla_{NDM-1}, bla_{IMP-4}, bla_{SFO-1}, bla_{SHV-12}, bla_{TEM-1B}, bla_{ACT-16}), colistin resistance (*mcr-9.1*), aminoglycoside resistance [*aac*(3)-*IId*, *aac(6')-IIc*, *aac(6')-Ib3*, *aph(3")-Ib*, *aph(3')-Ia*, *aph*(6)-*Id*, *aph*(6)-*Id*], fluoroquinolone and aminoglycoside resistance [*aac(6')-Ib-cr*], macrolide resistance (*ereA*, *mphA*), phenicol resistance *(catA2*), sulphonamide resistance (*sul1*), tetracycline resistance (*tetD*), and trimethoprim (*dfrA19*) were identified. Overall, the genotypes identified were consistent with the resistance phenotypes.

PCR detection of resistance genes also identified bla_{NDM-1}, bla_{IMP-4} and *mcr-9*. Broth conjugation assays using a sodium azide-resistant *E. coli* J53 isolate as a recipient showed that bla_{NDM-1} was transferable, but not for *mcr-9.1* and bla_{IMP-4}.

Characterization of *mcr*-9.1

ECL-90 was susceptible to colistin although *mcr-9.1* was detected. The IPTG-induced expression of *mcr*-9.1 in *E. coli* BL21(DE3) containing pET28a-*mcr*-9.1 did not confer resistance to colistin, and the resistance to colistin could not be induced by using sub-MIC concentration of colistin. This is consistent with the previous report that *mcr* − 9.1 is inactive in colistin resistance (21). A recent study with global data revealed that *Enterobacter spp.*, was the predominant host of *mcr-9* (37%) (22).

The genetic environment of IMP-4 and mcr-9.1

bla $_{IMP-4}$ and *mcr*-9.1 were co-identified on an IncHI2/2A-type plasmid pECL-90-2, which was 348,891 bp in length with an average GC content of 48.59% (Fig. 1). Consistently, a recent report identified that IncHI2-type plasmids may serve as a critical reservoir of *mcr-9* (22). Blasting the sequence of pECL-90-2 in GenBank database showed the best matches were plasmid pGW1 carried by a *Cronobacter sakazakii* strain GZcsf-1 (CP028975, 86.8% query coverage and 99.99% sequence identity) and plasmid p17277A_477 carried by a *Klebsiella quasipneumoniae subsp. quasipneumoniae* strain M17277 (CP043927, 79.54% query coverage and 99.99% sequence identity). This suggests that the plasmid could widely disseminate among *Enterobacteriaceae*. The downstream regulatory genes (*qseC* and *qseB*) detected in p17277A_477 (CP043927) were replaced by an IS*26* here (Fig. 1).

The *bla*$_{IMP-4}$ gene was carried by a class I integron designated as In*823b*, which located in an IS*6100*-IS*26* transposon-like structure (23, 24). The structure has previously been identified as being prevalent mediating the dissemination of *bla*$_{IMP-4}$ gene in China (24). The *intI1* gene of In*823b* was disrupted by the insertion of IS*26*, and a single resistance gene cassette *bla*$_{IMP-4}$-attC*bla*$_{IMP-4}$ adjacent to a group IIc intron Kl.pn.I3 was identified (Fig. 1). The typical 3'-conserved segment of In*823b* was absent.

NDM-1 genetic environment

The *bla*$_{NDM-1}$ gene was carried on a 44,961-bp IncX3 plasmid (pECL-90-3) (Fig. 2). IncX3-type plasmids mediating the dissemination of *bla*$_{NDM-1}$ among these homologous strains have been previously evidenced intensively (25). Query against GenBank showed that pECL-90-3 shared the highest similarity with pNDM5-L725 carried by *E. coli* strain L725 (CP036205, 99.73% query coverage and 99.99% sequence identity) and pBM527-2 carried by *Citrobacter sp*. strain CF971 (CP041048, 99.73% query coverage and 99.99% sequence identity).

Discussion

Carbapenemase-producing *Enterobacteriaceae* (CPE) spread at a high rate and colistin is the last-resort therapeutic for the infection caused by CPE. However, the emergence of plasmid-borne *mcr* genes highly facilitates the wide dissemination of colistin resistance, thus largely threatens the clinical use of colistin. Here, we for the first time characterized a clinical *Enterobacter hormaechei* strain co-producing *bla*$_{NDM-1}$, *bla*$_{IMP-4}$ and *mcr*-9.1 belonging to an epidemic clone (ST418). The accumulation of genes mediating resistance to last-resort antibiotics in epidemic clones would largely facilitate their widespread in clinical

settings, which may cause disastrous consequence with respect to antimicrobial resistance. Understanding how resistance genes were accumulated in a single strain could help us to track the evolutionary trajectory of drug resistance. Our finding highlights the importance of surveillance on the epidemic potential of colistin-resistant CPE, and effective infection control measures to prevent the resistance dissemination.

Genomic analysis showed that, the genetic context of *mcr-9*.1 gene "*rcnR-rcnA-pcoE-pcoS*-IS*903-mcr-9-wbuC*" is known as a prevalent structure for *mcr-9* (22). And a "IS*903-mcr-9-wbuC*-IS*26*" genetic structure has been found in 71% sequences harboring *mcr-9* in the NCBI Nucleotide Collection database (26), indicating the importance of IS*903B* in the spread of *mcr-9* gene. In addition, the genetic context of bla_{NDM-1} gene "IS*Aba125*– bla_{NDM-1} – ble_{MBL}" has recently been named NDM-GE-U.S. and has been found to be widespread globally (27). The wider context of bla_{NDM-1} gene IS*Aba125*– bla_{NDM-1} – ble_{MBL}-*ΔtrpF-dsbC* was flanked by IS*3000* and IS*26*, which was identical to that detected in *E. coli* strain BJ01 (JX296013) isolated in China (28). The bla_{IMP-4} gene carried by In*823b*, has been found to be located in an IS*6100*-IS*26* transposon-like prevalent structure mediating the dissemination of bla_{IMP-4} gene in China (23, 24). Altogether, numerous mobile genetic elements flanking *mcr-9.1*, bla_{IMP-4} and bla_{NDM-1} gene indicate the transferable potential independent of the plasmid mobilization.

Noteworthily, the backbone structures of plasmids identified in our study shared a high similarity with plasmids harbored in multiple *Enterobacteriaceae* family such as *E. coli*, and *K. pneumoniae*. Under the selective pressure of antimicrobial agents, these resistance-encoding determinants might be recruited into a variable genetic locus flanked by mobile elements such transposons and insertion sequences, leading to a successful transmission among various *Enterobacteriaceae* species. Especially, the convergence of genes mediating the resistance to last-resort antibiotics (e.g. carbapenems and colistin) in epidemic clones would largely facilitate their widespread in clinical settings, thus represents a potential challenge to clinical treatment and public health.

In summary, we here for the first time characterized a clinical epidemic *E. hormaechei* clone ST418 co-harboring bla_{NDM-1}, bla_{IMP-4} and *mcr*-9.1. The accumulation of genes conferring resistance to last-resort antibiotics via various mobile genetic elements highlights that stricter infection control measurements should be conducted to prevent the dissemination of such "chimera superbug".

Accession number (s): The chromosome of ECL-990 has been deposited in GenBank nucleotide sequence database under accession number of CP061744; four plasmids have been deposited in the GenBank nucleotide sequence database under accession number of CP061745 (pECL-90-1), CP061746 (pECL-90-2), CP061747 (pECL-90-3), and CP061748 (pECL-90-4).

Abbreviations

MLST: multi-locus sequence typing

CRE: carbapenem-resistant Enterobacteriaceae

KPC: *Klebsiella pneumoniae* carbapenemases

NDM: New Delhi metallo-lactamases

IMP: imipenemases

WGS: whole-genome sequencing

CLSI: Clinical and Laboratory Standards Institute

PCR: Polymerase Chain Reaction

S1-PFGE: S1-pulsed-field gel electrophoresis

CPE: Carbapenemase-producing *Enterobacteriaceae*

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

WC and ZLH performed the susceptibility testing and whole genome sequencing; SHW, DDH XWW interpreted the data regarding antimicrobial susceptibility testing and whole genome sequencing; XLC

and KZ designed the work and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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None

Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the related Department in China.

Conflict of interest statement

None declared

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Figures

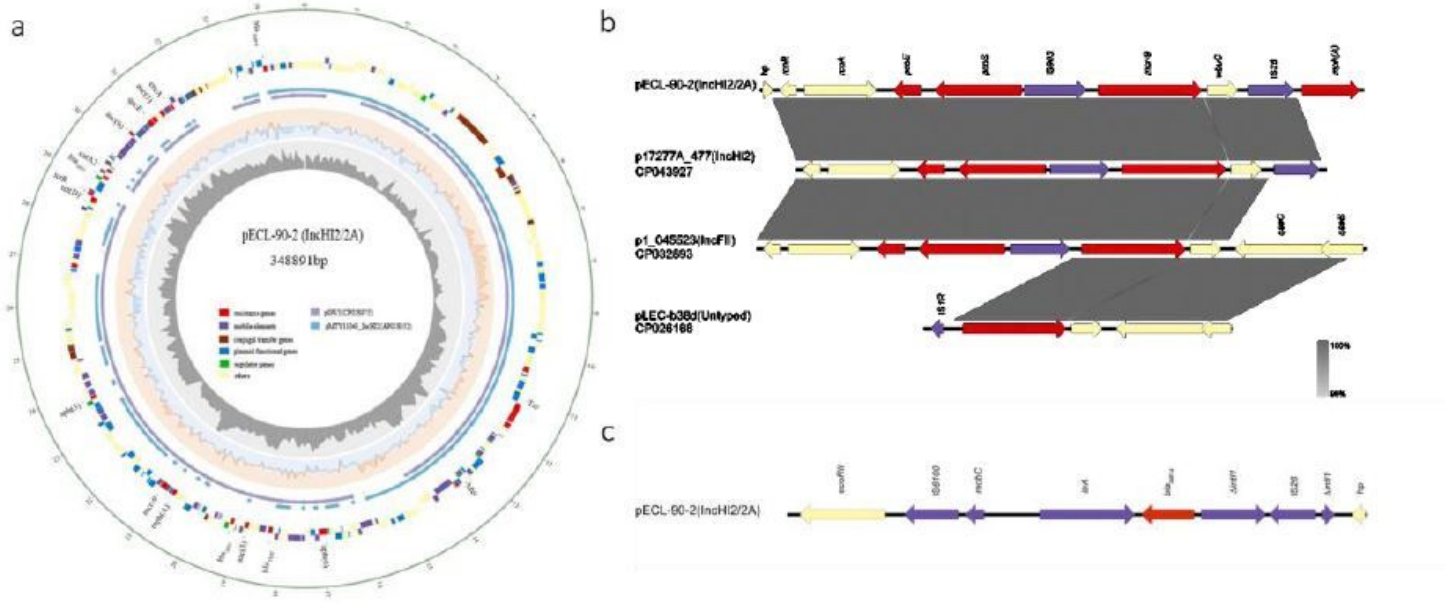


Figure 1

Analysis of mcr-9.1-harboring IncHI2/2A-type plasmid pECL-90-2 and the genetic context of mcr-9.1 and blaIMP-4. (a) Plasmid structure of pECL-90-2 compared to pGW1 (GenBank accession number CP028975) and pMTY11043_IncHI2 (GenBank accession number AP018352) is shown. Open reading frames are indicated by colored columns based on predicted gene function. Dark gray, GC content; light blue, GC skew (+); orange, GC skew (-); (b) Comparison of mcr-9.1 genetic context harbored by pECL-90-2, p17277A_477 (IncHI2) (GenBank accession number CP043927), P1_045523 (IncFII) (GenBank accession number CP032893), and pLEC-b38d (Untypeable) (GenBank accession number CP026168). Dark fray shading denotes regions of shared homology among different plasmids; (c) Genetic context of blaIMP-4 carried by pECL-90-2.

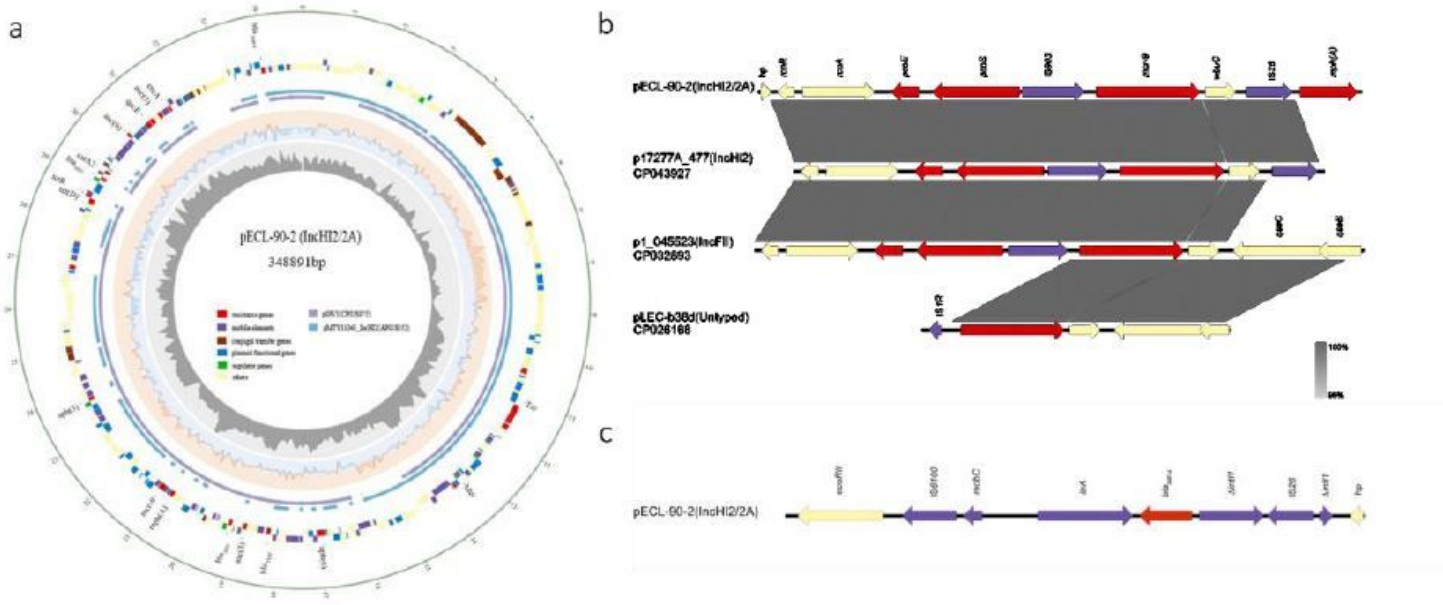


Figure 1

Analysis of mcr-9.1-harboring IncHI2/2A-type plasmid pECL-90-2 and the genetic context of mcr-9.1 and blaIMP-4. (a) Plasmid structure of pECL-90-2 compared to pGW1 (GenBank accession number CP028975) and pMTY11043_IncHI2 (GenBank accession number AP018352) is shown. Open reading frames are indicated by colored columns based on predicted gene function. Dark gray, GC content; light blue, GC skew (+); orange, GC skew (-); (b) Comparison of mcr-9.1 genetic context harbored by pECL-90-2, p17277A_477 (IncHI2) (GenBank accession number CP043927), P1_045523 (IncFII) (GenBank accession number CP032893), and pLEC-b38d (Untypeable) (GenBank accession number CP026168). Dark fray shading denotes regions of shared homology among different plasmids; (c) Genetic context of blaIMP-4 carried by pECL-90-2.

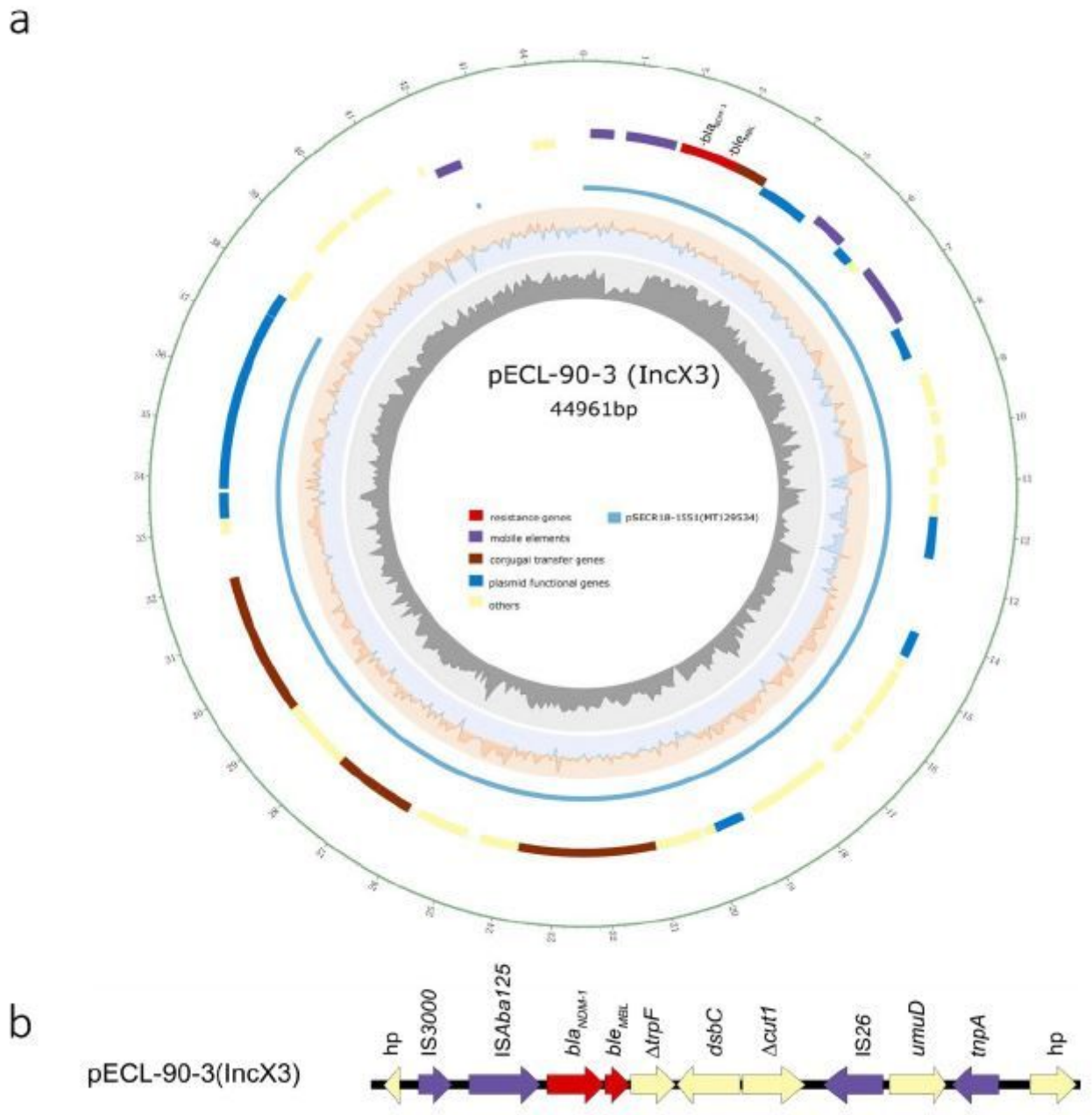
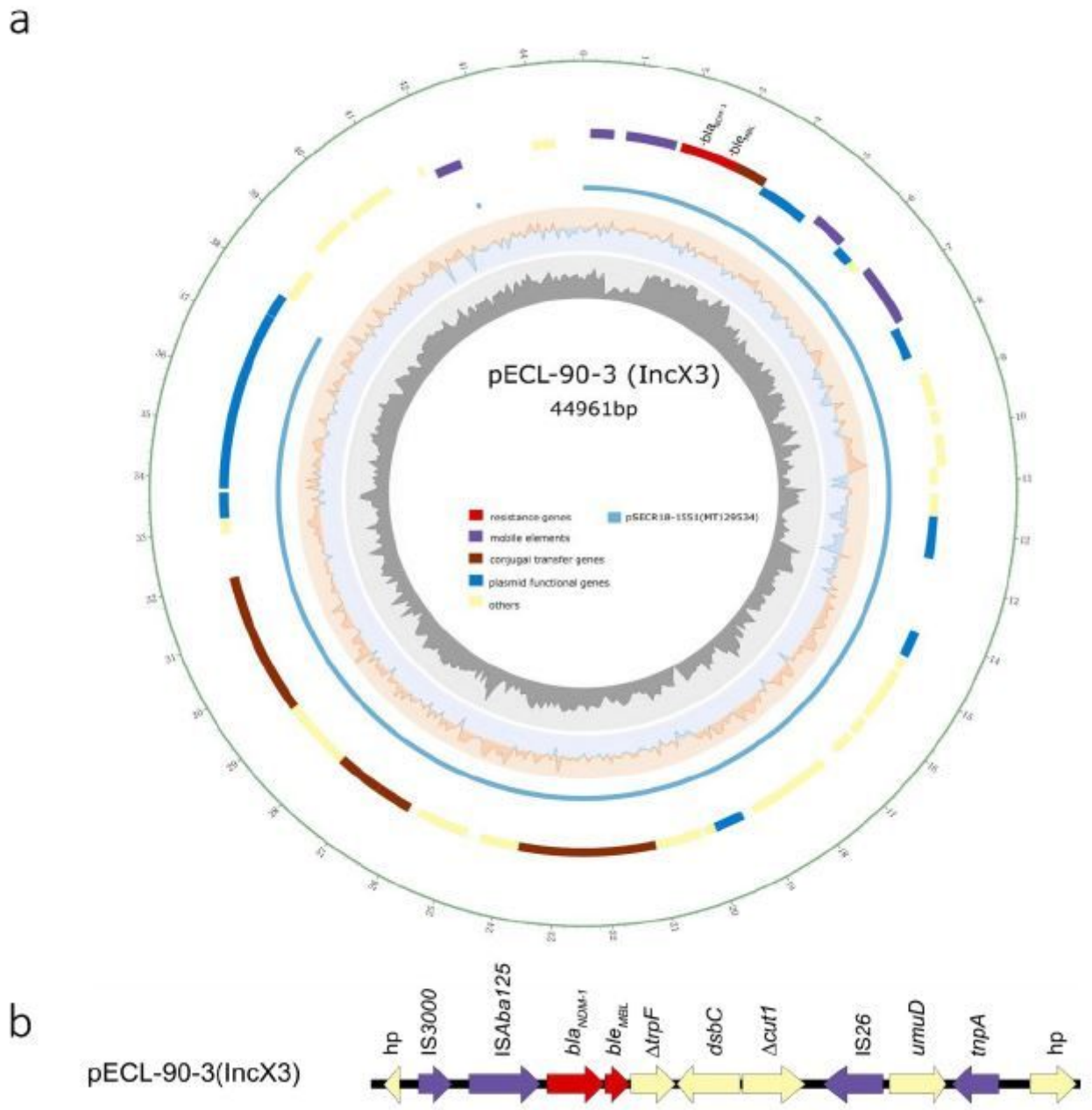


Figure 2

Analysis of blaNDM-1-harboring IncX3-type plasmid pECL-90-3 and the genetic context of blaNDM-1. (a) Plasmid structure of pECL-90-3 compared to pSECR18-1551 (GenBank accession number MT129534). Open reading frames are indicated by colored column based on predicted gene function. Dark gray, GC content; light blue, GC skew (+); orange, GC skew (-); (b) Genetic context of blaNDM-1 detected on pECL-90-3.



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Supplementary Files

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- TableS1.primersforringformationexperimentofPlasmid.docx
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