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Novel *mcr-3.40* variant co-located with *mcr-2.3* and $bla_{CTX\text{-}M\text{-}63}$ on an IncHI1B/IncFIB plasmid found in *Klebsiella pneumoniae* from a healthy carrier in Thailand

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Sir,

The emergence of the mobilizable colistin resistance (*mcr*) genes raises concerns for public health, as colistin is considered one of the last-line therapeutics for treatment of infections with MDR bacteria, including carbapenem-resistant Enterobacteriaceae.[1] Since its first description from China in 2015, 10 *mcr* families have been reported worldwide, identified in several bacterial species originating from various animal, human and environmental samples.[2] The event of co-occurrence of two *mcr* variants on the same plasmid is uncommon.[3] Here, we report a novel *mcr* variant, *mcr-3.40*, co-located with *mcr-2.3* and $bla_{CTX\text{-}M\text{-}63}$ on an IncHI1B/IncFIB plasmid found in *Klebsiella pneumoniae*.

Colistin-resistant *K. pneumoniae* strain 90CM2 was isolated from a stool sample of a healthy farmer in North-Eastern Thailand as a part of a cross-sectional study.[4] The study was conducted according to the Helsinki declaration and the protocol involving human participants was approved by the Khon Kaen University Ethics Committe (Project ID: HE612268 and 0514.1.75/66, respectively). Informed consent was obtained after the experimental procedures had been completely explained. MIC determination using the EUVSEC Plate (Trek diagnostics, Thermo Scientific, USA), interpreted using EUCAST guidelines (www.eucast.org), showed that strain 90CM2 was resistant to colistin (MIC >16 mg/L), third-generation cephalosporins (MIC >8 mg/L for ceftazidime and MIC >4 mg/L for cefotaxime) and other antimicrobial agents (Table S1, available as Supplementary data at *JAC* Online).

Hybrid assembly was performed using Unicycler[5] based on short reads obtained using Illumina Novoseq 6000 (Novogene, en.novogene.com) and long reads obtained using a MinION device (Rapid Sequencing Kit SQK-RBK004 and Flow Cell R9.4; Oxford Nanopore Technologies, UK). Annotation was performed using Prokka v1.14.6 and the BLAST tool. Antibiotic resistance and virulence genes were identified using the online CGE platform (http://www.genomicepidemiology.org/) and the Kleborate tool,[6] respectively. ISs were explored using ISfinder (https://isfinder.biotoul.fr/).

WGS demonstrated that *K. pneumoniae* strain 90CM2 (BioProject no. PRJNA669428) belonged to ST17 with a chromosome size of 5 285 993 bp and five plasmids, of which four were complete and circularized during assembly (Figure S1). Antibiotic resistance and virulence determinants are listed in Table S2. Genes encoding aerobactin and yersiniabactin were found, rendering this strain a hypervirulent *K. pneumoniae*.[7] Interestingly, plasmid p90CM2-MCR (253 649 bp) harboured both *mcr-2.3* and *mcr-3*-like genes. The MCR-3 allele differed by one amino acid compared with MCR-3 (Q486T, KY924928.1), MCR-3.11 (V373G, MG489958.1) and MCR-3.24 (N468T, NG060580.1). The new allele variant was designated as *mcr-3.40* (MT872722) by NCBI. Additionally, p90CM2-MCR also harboured several other antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}63}$ (β-lactams), *aphA-2* (aminoglycosides), *strA* and *strB* (streptomycin), *qnrS1* (quinolones), *mph*(A) (macrolides) and *bleO* (bleomycin)]. Further investigation of the plasmid structure revealed that p90CM2-MCR had an average GC content of 46.2% and encoded 263 predicted ORFs, including three replicons (IncHI1B-like, IncFIB-like and IncN). Furthermore, self-transferability of p90CM2-MCR into *Escherichia coli* J53 (Azi^R) during liquid mating was confirmed by S1-PFGE (Figure S2). The average plasmid transfer frequency was $1.12 \times 10^{-3} \pm 5.7 \times 10^{-4}$ transconjugants per recipient (Supplementary Materials and methods).

Plasmid p90CM2-MCR exhibited a high backbone similarity (>78% coverage and >98% identity) to plasmid pNDM-MAR, plasmid pENVA and plasmid pUNNAMED1, including IncHI1B/IncFIB replicons, a plasmid transfer (*tra* and *trh*) locus, tellurium resistance (*ter*) genes and a type IV CRISPR[8] (Figure 1a). Thus, p90CM2-MCR was considered a member of the novel IncH plasmid family, sharing a common ancestry with compared plasmids (pNDM-MAR, pENVA and pUNNAMED1). By contrast, p90CM2-MCR contained an IS*Kpn25*-like insertion (7.9 kb) and a unique MDR region (37 kb) not identified on the reference plasmids. To the best of our knowledge, an association between this plasmid type and *mcr* genes has never been reported before.

Both *mcr* genes were embedded within an MDR region surrounded by the remnants of Tn*5393* (IRL-*strAB* and Δtransposase-IRR) (Figure 1b). Each identified module of

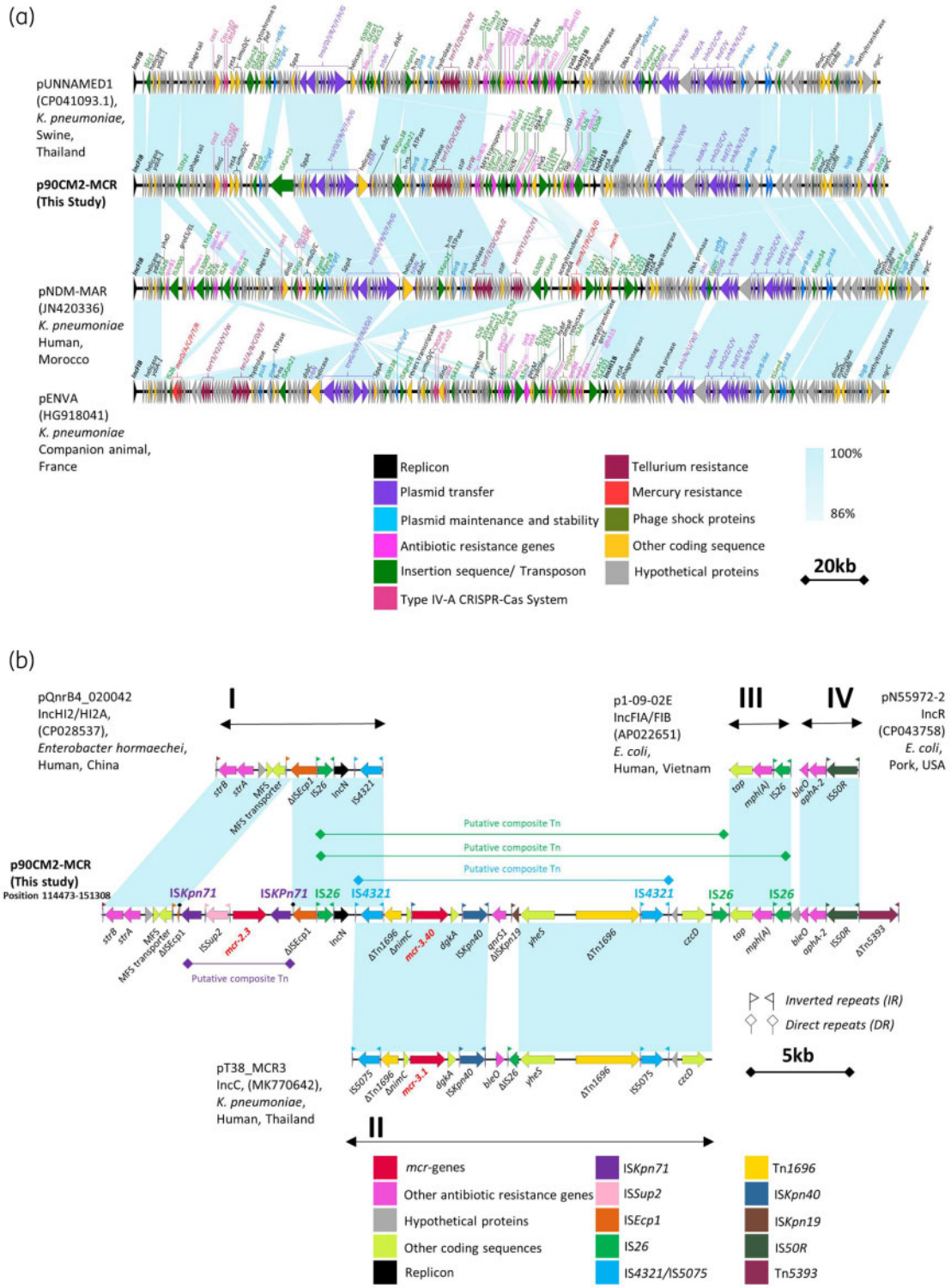

Figure 1. (a) Linear comparison of p90CM2 and three reference plasmids (pNDM-MAR, pENVA and pUNNAMED1). (b) Linear comparison of the MDR region of p90CM2-MCR with segments from different plasmids.

the MDR region demonstrated a high similarity to several different plasmid segments. The two largest modules (module I containing *mcr-2.3* and module II containing *mcr-3.40*) were highly similar (~99%) to regions located on plasmid pQnrB4_020042 (accession number CP028537) and plasmid pT38_MCR3 (accession number MK770642), respectively. Within module I, a putative composite transposon (~5 kb) harbouring *mcr-2.3* was flanked by duplicates of novel IS elements, designated as IS*Kpn71* (IS*256* family) by ISfinder. A pair of direct repeats, ATTATTTTT, flanking the putative composite transposon, indicated its previous insertion into this region and, thus, possible mobilization. Additionally, the putative transposon contained IS*Spu2*, located right upstream of *mcr-2.3*. To the best of our knowledge, this is the first report of an *mcr* gene associated with IS*Sup2*.

Conversely, *mcr-3.40* was detected in a known *mcr-3* genetic context, *mcr-3.40-dgkA*-IS*Kpn40*, within a region flanked by complete copies of IS*4321*. A similar segment was discovered on plasmid pT38-MCR3 (Figure 1b, module II) within antibiotic resistance island ('ARI')-A.[9] Moreover, this region was bracketed by three copies of IS*26*; one identified upstream and the other two identified downstream from the IS*4321*-flanked region. Due to the opposite directions of IS*26*,[10] a segment encompassing modules I-II-III could potentially be moved by a new composite transposon. However, it is inconclusive whether the identified putative transposons were involved in the events that tailored the highly mosaic structure of the MDR region of p90CM2-MCR.

Self-transferability of p90CM2-MCR and possible mobilization of individual MDR segments could ultimately pose a public health threat. Moreover, coexistence of resistance and virulence plasmids within a single *K. pneumoniae* clone recovered from an asymptomatic carrier is also of concern.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2, Figures S1 and S2 and Supplementary Materials and methods are available as Supplementary data at *JAC* Online.

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