A P7 Phage-Like Plasmid Carrying mcr-1 in an ST15 *Klebsiella pneumoniae* Clinical Isolate

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A *Klebsiella pneumoniae* clinical strain, named SCKP83, was isolated and found to be resistant to colistin thanks to the presence plasmid-borne colistin resistant gene \(mcr-1\). The strain was subjected to whole genome sequencing and conjugation experiments. The subsequent analysis indicated that the strain belongs to ST15 and the capsular type K41. In SCKP83, \(mcr-1\) was carried by a 97.4-kb non-self-transmissible plasmid, a 90.9-kb region of which was predicted as an intact phage. This phage was 47.79% GC content, encoded 105 proteins and contained three tRNAs. \(mcr-1\) was located downstream of two copies of the insertion sequence \(IS_{Ap}l1\) (one complete and one truncated) and was inserted in the \(ant1\) gene, which encodes a putative antirepressor for antagonizing C1 repression, in this phage. The phage is highly similar to phage P7 (77% coverage and 98% identity) from *Escherichia coli*. Several similar \(mcr-1\)-carrying plasmids have been found in *E. coli* at various locations in China, suggesting that these phage-like plasmids have circulated in China. The findings in this study suggest that the P7 phage-like plasmids are not restricted to *E. coli* and may represent new vehicles to mediate the inter-species spread of \(mcr-1\).

**Keywords:** colistin, resistance, phagemid, plasmids, *Klebsiella pneumoniae*

**INTRODUCTION**

*Klebsiella pneumoniae* is a major pathogen causing a variety of infections in humans. Colistin is the last resort antimicrobial agent to treat infections caused by *K. pneumoniae* including those with resistance to carbapenems. However, colistin-resistant *K. pneumoniae* have emerged worldwide (Olaitan et al., 2014a). A few mechanisms including both chromosomal and plasmid-borne ones have been identified to be responsible for resistance to colistin in *K. pneumoniae* (Olaitan et al., 2014b). Plasmid-borne colistin resistance genes including \(mcr-1\) (Liu et al., 2016), \(mcr-2\) (Xavier et al., 2016), and \(mcr-3\) (Yin et al., 2017) have been found recently. In particular, \(mcr-1\) has been identified in various species of the Enterobacteriaceae in many countries (Poirol et al., 2017).

Bacteriophages (phages) are viruses able to infect and replicate within bacteria. Phages mediate the transfer of genetic components between bacteria via transduction. Phages may have a lytic cycle or a lysogenic cycle or both. In the lytic cycle, phage genomes are replicated and are assembled into particles, which cause cell lysis and are then released. In the lysogenic cycle, phage genomes integrate into the chromosome of host bacterial cells to exist in a latent or dormant state without causing cell lysis (Feiner et al., 2015). The structure of phages typically consists of a protein head.

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that encapsulates a DNA or RNA genome and a tail that attacks the bacterial host (Wurtz, 1992). Phage genomes vary remarkably in form and size but usually encode products for host takeover, replication, virion assembly, or lysis (Black and Thomas, 2012). Some phages may integrate into plasmids and can therefore be transferred by the host plasmid (Oliver et al., 2005; Shin and Ko, 2015).

mcr-1 is commonly carried by plasmids of the IncI2 or IncX4 replicon type and has also been found on IncF, IncHI2, or IncP plasmids (Poirel et al., 2017). We have found a plasmid carrying mcr-1 and phage P7-like sequences, which is reported here.

METHODS

Bacterial Strain

*K. pneumoniae* strain SCKP83 was recovered from a sputum sample of a 90-year-old male patient with severe pneumonia in February 2017 in China, who did not receive colistin before. Species identification was performed using Vitek II (bioMérieux, Marcy-l’Étoile, France) and MALTI-TOF (Bruker, Billerica, MA, USA). In vitro susceptibility of colistin was performed using the broth dilution method of the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2017) and breakpoints of colistin defined by EUCAST (http://www.eucast.org/) were applied. The presence of plasmid-borne colistin resistant genes *mcr-1, mcr-2,* and *mcr-3* was screened by PCR as described previously (Xavier et al., 2016; Zhao and Zong, 2016; Yin et al., 2017).

Whole Genome Sequencing and Analysis

The strain was subjected to whole genome sequencing. Genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and whole genome sequencing was performed using the HiSeq X10 Sequencer (Illumina, San Diego, CA). The coverage was approximately 300 × coverage, which was calculated based on the estimated genome size and the average output of the sequencer. Reads were trimmed using Trimmomatic (version 0.36) (Bolger et al., 2014) and were then assembled to contigs using SPAdes (version 3.11) (Bankevich et al., 2012) with careful mode turned on. Sequence type and capsular type were determined using the genomic sequence to query the multi-locus sequence typing and wzi allele databases of *K. pneumoniae* available at http://bigdb.pasteur.fr/klebsiella/klebsiella.html. Antimicrobial resistance genes were identified from genome sequences using the ABRicate program (https://github.com/tseemann/abricate) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/). The plasmid carrying *mcr-1,* designated pMCR_SCKP-LL83, was circularized using PCR and Sanger sequencing to fill in gaps between contigs. Plasmid replication was determined using the PlasmidFinder tool at http://genomic epidemiology.org/. Similar plasmids were retrieved from the GenBank and pairwise comparisons were performed using BLASTn alignment (Altschul et al., 1990) and BRIG (Alikhan et al., 2011). The presence of phages was screened using PHASTER (http://phaster.ca/) (Arndt et al., 2016). tRNAs were screened using tRNA-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe and Chan, 2016).

Nucleotide Sequence Accession Numbers

Draft whole-genome sequence of strain SCKP83 has been deposited into GenBank under the accession number NOKM0000000. Short reads of the whole-genome sequence of strain SCKP83 has been deposited into Short Reads Achieve under the accession number SRP099296. The complete sequences of pMCR_SCKP-LL83 has been deposited into GenBank under the accession number MF510496.

Conjugation and Transformation Experiments

Conjugation experiments were performed using both broth- and filter-based methods as described previously (Coque et al., 2002; Novais et al., 2006; Valenzuela et al., 2007). The azide-resistant *Escherichia coli* strain J53 was used as the recipient and 2 µg/ml colistin plus 150 µg/ml sodium azide were used for selecting transconjugants. Plasmids were prepared from strain SCKP83 using alkaline lysis (Sambrook and Russell, 2001) and were used for electroporation. Electroporation was conducted using a Gene Pulser (Bio-Rad, Hercules, CA, USA) with an electrical pulse of 25 µF capacitance, 2.5 kV and 200 Ω sample resistance. *E. coli* strain DH5α and a colistin-susceptible *K. pneumoniae* strain 020018 were used as recipient strains. Potential transformants were selected on agar plates containing 2 µg/ml colistin.

Induction of Bacteriophage

To determine the nature of pMCR_SCKP-LL83, we performed the induction assay using ultraviolet ray and mitomycin C as described previously (Mitsui et al., 1973; Raya and H’bert, 2009). Briefly, for UV induction, 1 ml culture of strain SCKP-LL83 in the exponential phase was harvested and resuspended in 0.05 M phosphate buffer (pH 6.8). The suspension was adjusted to the

![FIGURE 1](Image 313x149 to 542x322) | The genetic context of mcr-1 on pMCR_SCKP-LL83. The ISAp1Δ-ISAp1-mcr-1-phoΔ structure is inserted into ant1 but without the 2-bp DR characteristic of the insertion of ISAp1. The two ISAp1 are at contrary oppositions. Δ refers to truncated genes or elements. ant1 encodes a putative antirepressor. The phage genes surrounding ant1 include repL (encoding replication protein), kA (encoding a putative host killing protein), simB and simC (both encoding proteins for host immunity).
TABLE 1 | Features of pMCR_SCKP-LL83.

| Featurea | Position (start–end) | Function |
|----------|----------------------|----------|
| 0001     | 356–1912             | Type I restriction-modification system subunit M |
| 0002     | 1909–3114            | Restriction endonuclease subunit S |
| 0003     | 3235–6351            | Type I restriction enzyme EcoRI24II R protein |
| 0004     | 6616–7122            | 3’-Phosphatase, 5’-polynucleotide kinase |
| 0005/pmgS| 7195–8457            | Putative morphogenetic protein |
| 0006     | 8459–8677            | Hypothetical protein |
| 0007     | 8759–9480            | Hypothetical protein |
| 0008/pphA| 9457–10134           | Serine/Threonine protein phosphatase |
| 0009/pmgP| 10131–10757          | Putative morphogenetic protein |
| 0010     | 11259–11414          | Hypothetical protein |
| 0011/pmgM| 11481–12059          | Putative morphogenetic function protein |
| 0012     | 12062–12307          | Putative morphogenetic protein |
| 0013     | 12571–12831          | Baseplate protein |
| 0014     | 12841–14058          | Tail protein |
| 0015     | 14062–14790          | Tail protein |
| 0016     | 14777–15562          | Hypothetical protein |
| 0017     | 15564–16580          | Tail length tape measure protein |
| 0018     | 16573–17205          | Putative baseplate protein |
| 0019     | 17252–18250          | Hypothetical protein |
| 0020/a| 18250–19614          | Replicative DNA helicase |
| 0021     | 19900–19975          | tRNA-Met |
| 0024/locA| 20250–20675          | Putative tellurite or colicin resistance protein |
| 0025     | 21187–21360          | Hypothetical protein |
| 0026     | 21603–21678          | tRNA-Thr |
| 0027     | 21681–21756          | tRNA-Asn |
| 0028/dmtG| 22429–24693          | DNA adenine methylase family protein |
| 0029/dgC| 24690–25595          | Recombination-associated protein RdgC |
| 0030     | 25588–25872          | Hypothetical protein |
| 0031     | 25857–26096          | Hypothetical protein |
| 0032     | 26335–27123          | Hypothetical protein |
| 0033     | 27163–27595          | Outer membrane lytic protein |
| 0034/upB| 27763–28155          | Hypothetical protein |
| 0035     | 28048–28311          | Hypothetical protein |
| 0036/repA| 28491–29375          | Initiator replication family protein of p011-like replicon |
| 0037     | 29668–30477          | Helicase |
| IS1294   | 32106–32205          | Insertion sequence |
| 0040/parA| 32334–33530          | Plasmid partition protein A |
| 0041/parB| 33547–34548          | Plasmid partition protein B |
| 0042     | 34774–36480          | Putative baseplate protein |
| 0043     | 36541–38130          | Hypothetical protein |
| 0044     | 38140–38965          | Tail tube protein |
| 0045/pmgG| 38991–39572          | Putative morphogenetic protein |
| 0046/pilB| 39584–40093          | Putative baseplate structural protein |
| 0047     | 40217–40423          | Hypothetical protein |
| 0048     | 40547–40792          | Hypothetical protein |
| 0049/repL| 40843–41682          | Replication protein |
| 0050/kIA| 41718–42518          | Putative host killing protein |

(Continued)
**TABLE 1 | Continued**

| Feature<sup>a</sup> | Position (start–end) | Function |
|---------------------|----------------------|----------|
| 0009                | 91208–91417          | C1 repressor inactivator |
| 0100                | 91528–92379          | Primary repressor of lytic function |
| 0101                | 92405–93889          | Putative large terminase protein |
| 102/pacA            | 93889–95082          | Terminase A protein |
| 0103/paI            | 95169–95621          | Late promoter activating protein |
| 0104                | 95710–96753          | Hypothetical protein |
| 0105                | 96781–96960          | Hypothetical protein |
| 0106/doc            | 96965–97345          | Toxin Doc |
| 0001                | 356–1912             | Type I restriction-modification system subunit M |
| 0002                | 1909–3114            | Restriction endonuclease subunit S |
| 0003                | 3235–6351            | Type I restriction enzyme EcoRI24II R protein |
| 0004                | 6616–7122            | 3′-Phosphatase, 5′-polynucleotide kinase |
| 0005/pmgS           | 7195–8457            | Putative morphogenetic protein |
| 0006                | 8459–8677            | Hypothetical protein |
| 0007                | 8799–9480            | Hypothetical protein |
| 0008/pphA           | 9457–10134           | Serine/Threonine protein phosphatase |
| 0009/pmgP           | 10131–10757          | Putative morphogenetic protein |
| 0010                | 11259–11414          | Hypothetical protein |
| 0011/pmgM           | 11481–12059          | Putative morphogenetic protein |
| 0012                | 12062–12307          | Putative morphogenetic protein |
| 0013                | 12571–12831          | Baseplate protein |
| 0014                | 12841–14058          | Tail protein |
| 0015                | 14062–14790          | Tail protein |
| 0016                | 14777–15562          | Hypothetical protein |
| 0017                | 15564–16580          | Tail length tape measure protein |
| 0018                | 16573–17205          | Putative baseplate protein |
| 0019                | 17252–18250          | Hypothetical protein |
| 0018/dnaB           | 18250–19614          | Replicative DNA helicase |
| 0021                | 19900–19975          | tRNA-Met |
| 0024/locA           | 20250–20675          | Putative tellurite or colicin resistance protein |
| 0025                | 21187–21360          | Hypothetical protein |
| 0026                | 21603–21678          | tRNA-Thr |
| 0027                | 21681–21756          | tRNA-Asn |
| 0028/dnt            | 22429–24893          | DNA adenine methylase family protein |
| 0029/rdgC           | 24890–25595          | Recombination-associated protein RdgC |
| 0030                | 25588–25872          | Hypothetical protein |
| 0031                | 25857–26096          | Hypothetical protein |
| 0032                | 26335–27123          | Hypothetical protein |
| 0033                | 27163–27585          | Outer membrane lytic protein |
| 0034/upfB           | 27763–28155          | Hypothetical protein |
| 0035                | 28048–28311          | Hypothetical protein |
| 0036/reptA          | 28491–29375          | Initiator replication family protein of pO111-like replicon |
| 0037                | 29688–30477          | Helicase |
| IS1724              | 32106–32205          | Insertion sequence |
| 0040/paA            | 32334–33530          | Plasmid partition protein A |
| 0041/paB            | 33547–34548          | Plasmid partition protein B |
| 0042                | 34774–36480          | Putative baseplate protein |

(Continued)
TABLE 1 | Continued

| Featurea | Position (start–end) | Function |
|----------|---------------------|----------|
| 0093     | 85982–87286         | Hypothetical protein |
| 0094     | 87343–87984         | Maturation control protein |
| 0095/ref | 88173–88733         | Recombination enhancement function protein |
| 0096     | 88981–89190         | Putative lysozyme establishment protein |
| 0097/cre | 89343–90374         | GST-ixP-cre recombinase fusion protein |
| 0098/cra | 90382–90603         | Putative Cre-associated regulatory protein |
| 0099     | 91208–91417         | C1 repressor inactivator |
| 0100     | 91528–92379         | Primary repressor of lytic function |
| 0101     | 92405–93889         | Putative large terminase protein |
| 102/pacA | 93889–95082         | Terminase A protein |
| 0103/paa | 95169–95621         | Late promoter activating protein |
| 0104     | 95710–96753         | Hypothetical protein |
| 0105     | 96781–96980         | Hypothetical protein |
| 0106/doc | 96965–97345         | Toxin Doc |

aFeatures: genes, mobile genetic elements or C-segments. The allele numbers of genes present on pMCR_SCKP-LL83 are shown.

0.5 McFarland turbidity. Six aliquots of 150 μl were spotted on a 9 cm Petri dish and irradiated by a germicidal UV lamp at a distance of 100 cm. The drops were collected at 10, 20, 30, 60, 90, and 120 s serially, each of which was then incubated with 1 ml LB broth under 37°C in dark for 3–4 h. Lysis was observed by naked eyes. For mitomycin C induction, 100 ml cultures of strain SCKP-LL83 were added with mitomycin C to a final concentration of 0.1, 1, 10, 20, and 40 μg/ml and were incubated under 37°C with shaking. Aliquots (1 ml) were sampled at 2, 4, 12, and 24 h. The cultures were filtrated through 0.22 μm polycethersulfone membranes (Merck Millipore, Billerica, MA, USA) and the membranes were used for the plaque formation test, which was carried out via the agar overlay method (Kropinski et al., 2009). All of the tests were performed in triplicate.

Assay for Replication Module

The replication initiation protein-encoding gene repB and its replication origin sequence (ori) of pMCR_SCKP-LL83 were amplified with self-designed primers OriF (GGGAATTGAAATGGATCAACATTGACTATACG) and OriR (GGGAATTGACATACACCAGTGGATGAGA; EcoRI sites are underlined). The amplicons were cloned onto the vector pKC1139, which has a temperature sensitive origin of replication and cannot replicate at temperatures higher than 30°C. The ligated vectors were transformed into E. coli DH5α and the transformants were screened by apramycin (100 μg/ml) at 37°C. The presence of repB and ori in transformants were confirmed by PCR with M13(-21) Forward and M13-R primers binding to the clone vector and Sanger sequencing.

RESULTS AND DISCUSSION

Strain SCKP83 was resistant to colistin (MIC, 8 μg/ml) and had mcr-1 but not mcr-2 and mcr-3 genes. Whole genome sequencing of strain SCKP83 generated 5,247,124 clean reads, which were then assembled to 119 contigs (89 > 1,000 bp) with a 50.38% GC content. Strain SCKP83 belonged to ST15, which is a relative common type of K. pneumoniae seen in China (Zhang et al., 2017b). The capsular type of strain SCKP83 was K41.

mcr-1 was carried by a 97.4-kb plasmid, pMCR_SCKP-LL83, which did not carry any additional known antimicrobial resistance genes. Despite repeated attempts, no colistin resistant transconjugants were obtained, suggesting that pMCR_SCKP-LL83 is not self-transmissible. In addition, the transformation of this plasmid into E. coli strain DH5α and a colistin-susceptible K. pneumoniae strain was unsuccessful. This suggests that this plasmid may be strain-specific or its transformation occurs at a low frequency, which could not be detected in our experiments. pMCR_SCKP-LL83 had a single pO111I plasmid replicon. Transformants containing repB and its ori were obtained. The presence of repB and ori allows the temperature sensitive vector pKC1139 to replicate at 37°C, suggesting that the replication module of pMCR_SCKP-LL83 indeed leads to the replication of this plasmid.

On pMCR_SCKP-LL83, mcr-1 was located downstream of a complete insertion sequence ISAp1. The phosphoesterase-encoding pho gene that is always located downstream of mcr-1 was truncated at its 3'-end with only 38 bp out of the 747-bp gene remaining. Surprisingly, immediate upstream of the complete ISAp1 (1,070 bp in length) lies another ISAp1 that is truncated at its 5'-end with the presence of 223 bp including an intact right-hand inverted repeat (IRR) (Figure 1). When we artificially subtract the ISAp1Δ-ISAp1-mcr-1-phoΔ region from pMCR_SCKP-LL83, the remaining artificially-joining sequence perfectly matched the ant1 gene, which encodes a putative antirepressor for antagonizing C1 repression by formation of Anti1/Ant2/C1 complex. It therefore becomes evident that the ISAp1Δ-ISAp1-mcr-1-phoΔ structure is inserted into ant1. It has been found that a single copy of ISAp1 is able to mobilize mcr-1 and pho together with itself (Li et al., 2017; Zhao et al., 2017). The insertion of ISAp1 would generate 2-bp direct target repeats (DR). However, no 2-bp DRs were present flanking the ISAp1Δ-ISAp1-mcr-1-phoΔ structure, suggesting that the formation of such a complex structure was not directly due to the insertion mediated by ISAp1. The mechanism responsible for generating the ISAp1Δ-ISAp1-mcr-1-phoΔ structure remains unclear but might have involved recombination.

A 90.9-kb region of the 97.4-kb pMCR_SCKP-LL83 was predicted as an intact phage. Neither the appearance of lysis nor the formation of plaques was observed in the UV induction. In mitomycin C induction, no plaques were formed at the tested concentrations and intervals. These results suggest that pMCR_SCKP-LL83 was indeed a plasmid. Nonetheless, the phage region on pMCR_SCKP-LL83 had 47.79% GC content, encoding 105 proteins and contained three tRNAs, i.e., tRNA-Asn, tRNA-Thr, and tRNA-Met (Table 1). pMCR_SCKP-LL83 is highly similar (72% coverage and 98% identity) to the 101.7-kb Enterobacteria phage P7 (GenBank accession no. AF503408). Phage P7 (previously called as φamp) was isolated from E. coli of human fecal flora (Smith, 1972) and exists as a...
FIGURE 2 | Comparison of pMCR_SCKP-LL83 with phage P7 (GenBank accession no. AF503408). Similar regions are indicated with the degree of nucleotide identity being shown in gray scales. Mobile genetic elements, type I restriction-modification (RM) systems and C-segments are shown in green, yellow, and blue, respectively.

FIGURE 3 | Comparison of phage P7 and similar mcr-1-carrying plasmids. The comparison is a pairwise BLASTn alignment performed using BRIG (Alikhan et al., 2011). Plasmids are pMCR_SCKP-LL83 (this study), pHYEC7-mcr1 (GenBank accession no. KX518745) and pSLK172-1 (GenBank accession no. CP017632) and pMCR-1-P3 (GenBank accession no. KX880944). Coding sequences (CDS) of phage P7 (GenBank accession no. AF503408) are indicated. CDS of phage P7 absent from pMCR_SCKP-LL83 or vice-versa are listed in Table S1.
nonintegrated autonomous circular plasmid that constitutes a unique compatibility group (Hedges et al., 1975). Compared with P7, pMCR_SCKP-LL83 did not have the \( \beta \)突如其来－lactam carrying transposon Tn3, the type I restriction-modification system EcoP7, a 4-kb invertible C-segment and a few genes, most of which encode proteins of unknown function (Table S1 in the Supplementary file and Figure 2). C-segment contains several genes encoding phage tail fibers and also determines the host specificity of the phage (Iida, 1984). In contrast, pMCR_SCKP-LL83 had a few extra genes including an unnamed type I restriction-modification system, mcr-1 and a 5-kb putative invertible C-segment (Table S1), which is highly similar (92% coverage and 99% identity) to the multiple DNA inversion region min on plasmid p15B of \( \textit{E. coli} \) 15T (Sandmeier et al., 1991).

It is well known that plasmids can transfer genetic components between bacterial isolates, but the role of plages in disseminating antimicrobial resistance genes is still a matter of debate (Colavecchio et al., 2017; Enault et al., 2017). Nonetheless, some studies have found that plages are able to transfer genes conferring resistance to aminoglycosides (\( \text{aadA}, \text{aphA1}, \text{strA}, \text{strB} \)), \( \beta \)‐lactams (\( \text{blaCMY}-2, \text{blaTX}-M\), \( \text{blaOXA}-2, \text{blaOXA}-20, \text{blaPER}-1, \text{blaTEM} \)), chloramphenicol (\( \text{floR} \)), or tetracycline (\( \text{tetA}, \text{tetB}, \text{tetG}, \text{tetO}, \text{tetW} \)) via transduction (Zhang and LeJeune, 2008; Colomer-Lluch et al., 2014; Bearson and Brunelle, 2015; Ross and Topp, 2015; Shousha et al., 2015; Anand et al., 2016). In addition, a recent study has identified that two \( \textit{E. coli} \) plages could promote the transformation of plasmids carrying antimicrobial resistance genes (Keen et al., 2017).

During the process of this work, mcr-1 in either complete or interrupted version has been found on plasmids containing similar phage sequences including pHYEC7-mcr1 (GenBank accession no. KX518745), pSLK172-1 (GenBank accession no. CP017632) (Bai et al., 2017), and pMCR-1-P3 (GenBank accession no. KX880944) (Zhang et al., 2017a). All of these plasmids have been recovered from \( \textit{E. coli} \) at various locations of China and are highly similar (75–79% coverage, 97–99% identity, identified by BLAST; Figure 3) to pMCR_SCKP-LL83. This suggests that the phage sequence-containing plasmids represent new vehicles, which may have circulated in China, to mediate the spread of mcr-1 in addition to plasmids of IncI2, X4, F, H12, and P types. The identification of pMCR_SCKP-LL83 from a

Escherichia coli (aEPEC) recovered in China. \( \textit{J. Antimicrob. Chemother.} \) 72, 1531–1533. doi: 10.1093/jac/dkw564

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