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Silent transmission of an IS1294b-deactivated mcr-1 gene with inducible colistin resistance
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ABSTRACT
Global dissemination of the mobile colistin resistance mcr-1 is of particular concern as colistin is one of the last-resort antibiotics for the treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria. In this study, an inactive form of mcr-1 in a fluoroquinolone-resistant and colistin-susceptible uropathogenic Escherichia coli isolate (ECO3347) was characterised. The mcr-1 gene was deactivated by insertion of a 1.7-kb IS1294b element flanked by two tetramers (GTTC) and located on a 62-kb pHNSHP45-like plasmid (p3347-mcr-1). Single-step and multistep selections were used to induce colistin resistance in vitro in ECO3347. ECO3347 acquired colistin resistance (MIC = 16–32 mg/L) only after a serial passage selection with increasing concentrations of colistin (2–8 mg/L). Deactivated mcr-1 was re-activated by loss of IS1294b without any remnants in most colistin-resistant mutants. In addition, a novel amino acid variant (Leu105Pro) in the CheY homologous receiver domain of PmrA was detected in one colistin-resistant mutant. Plasmid p3347-mcr-1+ carrying the re-activated mcr-1 gene is transferable to E. coli J53 recipient with a high conjugation rate (ca. 10−1 cells per recipient cell). Conjugants showed an identical growth status to J53, suggesting lack of a fitness cost after acquiring p3347-mcr-1+. These results highlight that the disrupted mcr-1 gene has the potential for wide silent dissemination with the help of pHNSHP45-like epidemic plasmids. Inducible colistin resistance may likely compromise the success of clinical treatment and infection control. Continuous monitoring of mcr-1 is imperative for understanding and tackling its dissemination in different forms.

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1. Introduction
Polymyxins (colistin and polymyxin B) are the last-resort antibiotics for treating infections caused by carbapenem-resistant Enterobacteriaceae, thus the emergence of colistin resistance has received a great deal of attention. Most reported mechanisms of polymyxin resistance are chromosomally mediated, especially via specific mutations in two-component regulatory systems (e.g. pmrAB as well as phoPQ) and its negative regulator mrgB in the case of Klebsiella pneumoniae), resulting in modifications of the lipid A component of lipopolysaccharide (LPS) by the addition of phosphoethanolamine or 4-amino-4-deoxy-L-arabinose, or in unusual cases the total loss of the LPS [1]. Other strategies are also occasionally employed to resist the activities of polymyxins, including efflux pumps, the formation of capsules, and overexpression of the outer membrane protein OprH [1].

Recently, plasmid-encoded colistin resistance mediated by the mobile colistin resistance-1 (mcr-1) gene has been identified in Escherichia coli and K. pneumoniae in China [2]. MCR-1 is a phosphoethanolamine transferase that catalyses the addition of a phosphoethanolamine group to lipid A, leading to decreased affinity of colistin for LPS [2]. The great concern of this finding is that the plasmid-borne colistin resistance trait leads to its interspecies transferability, and transfer of the resistance to multidrug-resistant, especially carbapenem-resistant, Enterobacteriaceae would seriously limit current treatment options [3]. Available evidence suggests that the reservoir of the mcr-1 gene is in animals [4]. Subsequent studies have reported that the mcr-1 gene has been detected in isolates from humans, food and environmental samples worldwide, mostly in E. coli [5–7]. In addition, the gene has been detected in numerous species of Enterobacteriaceae, e.g. Enterobacter cloacae, Enterobacter aerogenes, K. pneumoniae, Salmonella enterica and

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Citrobacter spp., harboured by plasmids of various incompatibility (Inc) groups including IncI2, IncX4, IncFI, IncFII, IncY, IncHI2 and IncP [8–15]. Identification of the mcr-1 gene on different plasmid backbones suggests that its spread corresponds to multiple genetic events that have occurred independently in distantly related geographic areas [6].

Intriguingly, two different inactive forms of mcr-1 were recently identified. One was due to an intragenic 22-bp duplication in a Shigella sonnei isolate from Vietnam [16] and the other was caused by insertion of IS10R in an E. coli isolate from the Netherlands [17]. Of note, the inactive form of mcr-1 caused by duplication is able to be reactivated by deletions under colistin selection and can be mobilised via horizontal plasmid transfer. Such silent transmission of the mcr-1 gene raises a concern that inactive colistin resistance may compromise last-line treatment choices, thus threatening successful treatment outcomes. In addition, the inactive form of the mcr-1 gene can easily escape antimicrobial resistance surveillance based on routine phenotypic data. Since inactive mcr-1 genes have been rarely reported, an understanding of how these genes disseminate and how phase switching (from inactive to active) might be triggered remains elusive. In this study, we report the finding of another inactive form of the mcr-1 gene in an E. coli isolate and its selective re-activation, resulting in high-level colistin resistance. The transferability and fitness cost of the mcr-1-harbouring plasmid were further evaluated.

2. Materials and methods

2.1. Bacterial strains

A series of E. coli isolates (n = 1270) collected in 2011–2012 were screened for the presence of mcr-1 by PCR using the primers CLR5-F (5′-CGGTGCTTGGTTGCT-3′) and CLR5-R (5′-CTTGGCTGCTTGTCT GTAGG-3′) as described previously [2]. Escherichia coli strain ECO3347 was identified with a positive PCR result. Species identification was performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Diagnostics, Bremen, Germany).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using a VITEK®2 with AST-GN13 test cards (bioMérieux, Marcy-l’Étoile, France), except for colistin whose susceptibility was evaluated by broth microdilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) reference method (2016). Results were interpreted according to the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (v.6.0, 2016) (http://www.euCAST.org/clinical_breakpoints/).

2.3. Plasmid analysis

Plasmid size was estimated by S1 nuclease pulsed-field gel electrophoresis (PFGE). The mcr-1 gene location was determined by Southern blotting. Conjugative transfer of plasmids was carried out with the recipient strain sodium azide-resistant E. coli J53 using a 2:1 ratio of donor and recipient at 37°C overnight. Transconjugants were selected on Mueller–Hinton (MH) medium with sodium azide (100 mg/L) and colistin (2 mg/mL). Selected transconjugants were later confirmed by amplification of the mcr-1 gene using the primers MCR-1-IS-FW (5′-GTGGATGTAATCTGTCTTCAGG-3′) and MCR-1-IS-REV (5′-AAATTCGTGGTTGGTAC-3′). Plasmid replicon typing was performed by submitting the genomic sequences to PlasmidFinder (https://cge. cbs.dtu.dk/services/plasmidFinder). Comparison of plasmid sequences was done using BLASTn.

2.4. In vitro induction of colistin resistance

Single-step and multistep selections were used to induce colistin resistance in ECO3347: (i) single-step selection: 100 μL of an overnight culture (ca. 2 × 10⁹ CFU) was plated on MH medium with the addition of 2, 4 and 8 mg/L colistin, respectively; and (ii) multistep selection: a serial passage selection process was performed for the induction of colistin resistance. Briefly, 20 μL of an overnight culture of ECO3347 was re-inoculated into 2 mL of fresh MH broth (Oxoid Ltd., Basingstoke, UK) containing 2 mg/L colistin and was incubated at 37°C overnight [optical density at 600 nm (OD₆₀₀) of ca. 0.6–0.8]. The obtained colistin-adapted cultures were sequentially inoculated into MH broth containing 4 mg/L and 8 mg/L colistin with a 1:100 ratio of inoculum. Cultures recovered at different colistin concentrations were selected on MH agar with 2 mg/L colistin. The mcr-1 gene of mutants was checked by PCR using primers MCR-1-IS-FW and MCR-1-IS-REV to identify the genetic changes. To determine the stability of induced colistin resistance, colistin-resistant mutants were inoculated in MH broth without colistin once per day and the passage was continued for 5 days. The resulting cultures were selected on MH agar plates without colistin. The colistin minimum inhibitory concentration (MIC) of the obtained colonies (n = 10) was determined by the broth microdilution method, and the existence of the mcr-1-harbouring plasmid was tested by replicon typing.

2.5. Whole-genome sequencing, bioinformatic analysis and gap closure of the mcr-1-harbouring plasmid

Genomic DNA was prepared using a Puregene Kit (QiAGEN, Hilden, Germany). Genomes were sequenced using a HiSeq 2500 instrument (Illumina Inc., San Diego, CA) using 2 × 125-bp paired-end libraries. De novo assembly was performed using CLC Genomics Workbench v.10.0 (QiAGEN) after quality trimming (Qs ≥ 20); annotation was performed by uploading data to the Rapid Annotation using Subsystem Technology (RAST) server (rast.nmpdr.org); multilocus sequence typing (MLST), serotyping, and resistome and toxome analysis were done using the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/); and detection of single nucleotide polymorphisms (SNPs) was performed as previously described [18]. The phylogenetic group of E. coli was determined in silico by searching the sequences of chuA and yjaA genes and DNA fragment TSPE4.C2 in genomes as previously described [19].

Two pairs of primers were used to close the plasmid encoding the mcr-1 gene: (i) MCR-1-IS-FW and MCR-1-IS-REV were used to fill in the gap between the broken ends of the mcr-1 gene; and (ii) pmcr-1-FW (5′-TGCTGGATGTTACGTTACG-3′) and pmcr-1-REV (5′-TAAATTCGTGGTTGGTGTCAC-3′) were used to extend the two ends of the scaffold acquired from step (i). PCR was performed using Taq DNA Polymerase (TaKaRa, Tokyo, Japan) with the recommended concentrations of reagents under the following conditions: initial denaturation at 95°C for 15 min; 30 cycles of 95°C for 30 s (denaturation), 56°C for 30 s (annealing) and 72°C for 1 min (extension); and a final extension at 72°C for 5 min. The PCR products were sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.6. Growth assay

Fitness costs were determined by growth assays. Briefly, overnight cultures were re-inoculated in fresh MH medium by dilution of 1:100 and the growth curve was determined by recording the OD₆₀₀ of cultures.
3. Results

3.1. Characterisation of isolate ECO3347

Isolate ECO3347 was obtained from a urine sample of a 67-year-old female patient with a urinary tract infection who was admitted to a secondary hospital in Hebei Province, China, in March 2012. Uropathogenic E. coli (UPEC) isolate ECO3347 was resistant to fluoroquinolones (ciprofloxacin and levofloxacin MICs of ≥4 mg/L and ≥8 mg/L, respectively), non-susceptible to ampicillin (MIC = 16 mg/L) and susceptible to colistin (MIC = 0.125 mg/L). The isolate was classified as phylogenetic group B1 and its serotype was identified as 029:H51. ECO3347 was assigned to ST359 (43–41–15–90–11–8–6) using the Warwick MLST scheme [20].

Analysis of the acquired resistome identified four drug resistance genes in ECO3347: floR; sul2; a novel blacMY variant (sharing ca. 75.8% amino acid identity with CMY-47); and an interrupted mcr-1 gene resulting in two moieties. This is in accordance with the MIC of colistin described above. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties. Two mutations in GyrA (Ser83Leu) and ParC (Ser80Ile) responsible for fluoroquinolone resistance as well as an interrupted mcr-1 gene resulting in two moieties.

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3.2. Structure of the mcr-1-harboring plasmid

A pair of primers (MCR-1-IS-FW and MCR-1-IS-REV) was designed to identify the insertion interrupting the mcr-1 gene, and a 1707-bp PCR product was obtained. DNA sequencing determined that the mcr-1 gene was interrupted by an IS1294b insertion, resulting in two moieties (521 bp and 1105 bp). IS1294b, an element showing 95% identity to much of IS circle replication [21]. IS1294b was framed to oris (the origin of replication) and terIS (the replication terminator) ends by tetramers GTTC and GCTT. Blasting the contig encoding the mcr-1 gene with GenBank suggests that the mcr-1 gene was carried by a plasmid. This is further supported by the results of Southern blot that the mcr-1 gene was detected on an ca. 60-kb plasmid (Supplementary Fig. S1).

The mcr-1-harboring plasmid was cloned using primers pmcr-1-FW and pmcr-1-REV, resulting in a 62343-bp circular plasmid, named p3347-mcr-1 (Fig. 1A). p3347-mcr-1 was typed as an IncI2 plasmid and showed high homology with two E. coli plasmids (>99% identity, >90% coverage): pBA76-MCR-1 (GenBank accession no. KX013540) from the United Arab Emirates; and pSLv21 (GenBank accession no. CP016405) from the USA. Different from the prototypic pHNSHP45, pHNSHP45 includes transposons and three regions major differences between p3347-mcr-1, pBA76-MCR-1 and pHNSHP45 include the existence of transposons and three regions encoding various hypothetical proteins (Fig. 1B).

3.3. In vitro induction of colistin resistance

Single-step and multistep selections were performed to induce colistin resistance in ECO3347. In the single-step selection, mutants with a frequency of ca. 10–6 were obtained by plating on MH medium with addition of 2 mg/L or 4 mg/L colistin. No mutants appeared on the medium with the addition of 8 mg/L colistin. Only one mutant recovered from 4 mg/L colistin (named ECO3347-4R) showed stable colistin resistance with an MIC of 32 mg/L. Mutants able to retain the colistin resistance after passage on MH plates with 2 mg/mL colistin were defined as stable colistin-resistant mutants, otherwise they were defined as unstable mutants. No mutants with stable resistance were obtained from 2 mg/L colistin. The colistin MIC of unstable mutants was 0.25 mg/L. No changes were detected by PCR using primers MCR-1-IS-FW and MCR-1-IS-REV in the disrupted mcr-1 gene of ECO3347-4R or in the mutants with unstable colistin resistance.

In addition, a serial passage selection process started with 2 mg/L colistin was performed for inducing colistin resistance. Numerous colonies were recovered from cultures with 2 mg/L and 4 mg/L colistin. Twenty mutants were randomly selected from each concentration to test their susceptibility to colistin, all of which showed unstable colistin resistance with MICs of 0.25 mg/L. Hundreds of colonies were recovered from cultures with 8 mg/L colistin, of which ten were randomly selected to test their susceptibility to colistin. Intriguingly, the ten mutants all showed stable colistin resistance with high colistin MICs of 16–32 mg/L. An ca. 1.7-kb deletion was detected in mcr-1::IS1294b by PCR in the ten mutants (Fig. 2).

3.4. Genetic changes in colistin-resistant mutants

To identify the genetic determinants of inducible colistin resistance, seven mutants derived from the multistep induction were sequenced, including the colistin-resistant mutant ECO3347-4R recovered from 4 mg/L colistin, two colistin-resistant mutants with a 1.7-kb deletion in mcr-1::IS1294b recovered from 8 mg/L colistin (ECO3347-8R-1 and ECO3347-8R-2), and four susceptible/unstable mutants recovered from 2 mg/L (ECO3347-2S-1 and ECO3347-2S-2) or 4 mg/L colistin (ECO3347-4S-1 and ECO3347-4S-2). Genomic comparisons revealed that IS1294b was completely lost in ECO3347-8R-1 and ECO3347-8R-2, resulting in an intact mcr-1 gene without any remnants (Fig. 2). Further SNP analysis identified eight SNPs among the seven mutants, including six non-synonymous (NS) SNPs and one synonymous SNP (Table 1). The seven mutants shared three SNPs, independent of the selection strategy. A NS-SNP located within the asmA gene (encoding an outer membrane assembly protein) was exclusively found in ECO3347-8R-1 and ECO3347-8R-2, resulting in

Table 1

<table>
<thead>
<tr>
<th>Reference nucleotide</th>
<th>Nucleotide in mutants a</th>
<th>Gene</th>
<th>Annotation</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>A</td>
<td>aaeA</td>
<td>p-Hydroxybenzoic acid efflux pump subunit</td>
<td>Pro2Ser</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
<td>glfD</td>
<td>Glycolate dehydrogenase, iron-sulfur subunit</td>
<td>His744Arg</td>
</tr>
<tr>
<td>T</td>
<td>C</td>
<td>glcF</td>
<td>Maltose-6'-phosphate glucosidase</td>
<td>Thr391Ala</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>glvA</td>
<td>A/G-specific adenine glycosylase</td>
<td>Ala60Ser</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>purS</td>
<td>Phosphoribosylformylglycinamidine synthase, synthetase subunit</td>
<td>Gln160Stop</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>AsmA</td>
<td>Transcriptional regulatory protein BasR/PmrA</td>
<td>Leu105Pro</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>N/A</td>
<td>-</td>
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<tr>
<td>G</td>
<td>T</td>
<td>N/A</td>
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<td>G</td>
<td>G</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a SNPs are indicated in bold.
Fig. 1. Schematic representation of plasmid p3347-mcr-1 harbouring the deactivated mcr-1 gene. (A) Genetic map of p3347-mcr-1. A circular representation of p3347-mcr-1 was generated using DNAPlotter. Genes are classified by different colours and the names of some genes are shown. (B) Comparison of novel mcr-1 plasmid p3347-mcr-1 with mcr-1 plasmids pBA76-MCR-1 (GenBank accession no. KX013540) and pHNSHP45 (GenBank accession no. KP347127). Grey blocks indicate regions of sequence homology between two plasmids. Genes are classified by different colours as shown in (A).
and the other was caused by insertion of IS
sonnei reported. One was due to an intragenic 22-bp duplication in an
E. coli isolate, which was also recently identified in the Netherlands
by transient alterations of certain genes at transcription level may play
a role in the colistin adaptation, whilst direct chromosomal muta-
tions (except in pmrB in K. pneumoniae) often confer a high
fitness cost [27]. All selected susceptible and resistant mutants shared
three SNPs, suggesting sequential acquisition of colistin resistance
during the induction procedure. We suppose that the mutants with
re-activated mcr-1 are derived from the mutants pre-adapting to
the pressure of low colistin concentrations (2–4 mg/L). The role of
the three SNPs in the pre-reversal stage at low colistin concentra-
tions would be interesting to be studied in the future.

Currently, the intrinsic (mcr-1-independent) resistance mecha-
nism remains unclear, especially for E. coli. Here a novel amino acid
variant in the N-terminal receiver domain of PmrA, resulting in a high amino acid change (Leu105Pro) in the CheY-like response regulatory receiver domain.

3.5. Transferability of p3347-mcr-1

Dependent on the re-activated mcr-1 gene, conjugation assays showed that p3347-mcr-1
was able to be transferred into E. coli J53 at a high frequency of ca. 10^{-1} cells per recipient cell. The
coliSTIN MIC of p3347-mcr-1
was almost identical and they reached the same
density at stationary phase (OD600 ≈0.8) (Supplementary Fig. S2). WT, wild-type.

3.6. Fitness cost

The fitness cost of p3347-mcr-1
was determined by growth curve assays. The growth curve of the transconjugant J53-p3347-mcr-1
and recipient J53 was almost identical and they reached the same
density at stationary phase (OD600 = 0.8) (Supplementary Fig. S2).

4. Discussion

Global dissemination of colistin resistance has received great at-
tention, especially since the first report of plasmid-encoded colistin
resistance mediated by the mcr-1 gene identified in China [2]. To
date, MCR-1-producing isolates are mostly E. coli strains, which have
been reported in many different countries throughout Europe, Asia
and North America. The population of MCR-1-producing E. coli is
highly diverse and no prevalent clones have been detected as yet. In this study, we obtained an mcr-1
harbouring UPEC isolate belonging to ST359. This isolate was resistant to quinolones with
inducible colistin resistance, largely limiting clinical treatment stra-
tegies for UPEC. Interestingly, an mcr-1-containing E. coli ST359 isolate
was also recently identified in the Netherlands [17]. ST359 is known
as one of the prevalent extended-spectrum \( \beta \)-lactamase (ESBL)
-producing clones of phylogroup B1 increasingly associated with
urinary tract infections in multiple countries and is thus sug-
gested as an emerging UPEC clone [24]. In addition, this clone is
frequently isolated from animals and chicken retail meat, showing
a high PFGE similarity (70\%) with isolates of human origin [25].
This suggests that ST359 with zoonotic potential may be acting as
a reservoir mediating dissemination of the mcr-1 gene between
animals and humans in the future.

Prior to this study, two different inactive forms of mcr-1 had been
reported. One was due to an intragenic 22-bp duplication in an S.
onnei isolate [16] and the other was caused by insertion of IS10R
in an E. coli isolate [17]. Intriguingly, the ORF of the duplication-
inactivation mcr-1 gene can be restored by deleting one copy of the tandem repeat under the selection of high colistin concentrations (8–32 mg/L) [16]. This is similar to the current results that the IS1294b-interrupted mcr-1 can be reactivated by loss of IS1294b under the selection of 8 mg/L colistin. No remnants were detected in the mcr-1 gene after the loss of IS1294b. This is because IS1294b is an IS91-like element using one-ended, rolling-circle replication. IS1294b inserts specifically at the 3' end of the sequences 5'-CTTG or 5'-
GTTT of the target DNA, without duplication of the target sequence after transposition [23]. Of note, an IS1294 element was previously detected adjacent to the mcr-1 gene in an E. coli ST354 strain isolated from a child in Cambodia in 2012 [26]. This suggests that the mcr-1 gene and its surrounding region could be a hotspot of IS1294-like elements. In addition, blast analysis against the
sequence of IS1294b in GenBank showed that most matches belong
to the plasmids of E. coli and Shigella spp., indicating that the plas-
mids of E. coli are one of the major reservoirs of IS1294-like sequences.

The IS10R-deactivated mcr-1 gene cannot be reactivated by
colistin induction [17]. Of note, the current study showed that
reactivation of the IS-interrupted mcr-1 gene is strategy-dependent. The disrupted mcr-1
can only be reactivated by a serial passage sele-
tion process but not by single-step selection, suggesting that
reactivation of the disrupted mcr-1 is dependent on a stepwise
process. At low colistin concentrations (2–4 mg/L), almost all re-
covered mutants acquired unstable colistin resistance, implying that
transient alterations of certain genes at transcription level may play
a role in the colistin adaptation, whilst direct chromosomal muta-
tions (except in pmrB and mgrB in K. pneumoniae) often confer a high
fitness cost [27]. All selected susceptible and resistant mutants shared
three SNPs, suggesting sequential acquisition of colistin resistance
during the induction procedure. We suppose that the mutants with
re-activated mcr-1 are derived from the mutants pre-adapting to
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the three SNPs in the pre-reversal stage at low colistin concentra-
tions would be interesting to be studied in the future.
induction in *E. coli*. In fact, numerous studies support that mutations occurring in *pmrB* are more frequent than those in *pmrA* [29], but the underlying mechanism is yet unknown.

Of more concern, the three ‘silent’ mcr-1 genes reported previously and here are carried by an IncI2 plasmid (pEG430-1 and p3347-mcr-1) and an IncX4 plasmid (pmcr-1), respectively, and are mobilisable via horizontal plasmid transfer. In particular, the backbone of p3347-mcr-1 and pEG430-1 is highly similar to that of the prototypic mcr-1-encoding plasmid pHN5H4P5, indicating that the inducible ‘silent’ mcr-1 genes are generated by epidemic plasmids. Moreover, p3347-mcr-1 showed a very high conjugation rate, comparable with that of pHN5H4P5. It is suggested that successful dissemination of resistance plasmids largely depends on the fitness cost imposed on hosts [30]. However, previous studies have reached inconsistent conclusions about the fitness costs of mcr-1–harboring plasmids. The initial study of the mcr-1 gene showed that acquisition of plasmid pHN5H4P5 does not impose a fitness cost [2]. Likewise, here we found that acquisition of p3347-mcr-1 is not associated with reduced fitness as the transconjugants showed a comparable growth status to the recipient J53. In contrast, an mcr-1-carrying IncX4 plasmid pECMCR-1101 (GenBank accession no. KX570748) shows a low fitness cost [31]. We suppose that the fitness cost may be plasmid-dependent. Lack of a fitness cost imposed on hosts by acquiring pHN5H4P5-like plasmids greatly facilitates their spread, thus contributing to propagation of the mcr-1 gene. Taken together, this study raises a concern that the inducible ‘silent’ mcr-1 genes have the potential for wide dissemination mediated by pHN5H4P5-like epidemic plasmids and may likely compromise the success of clinical treatment and infection control.

In conclusion, we have identified the third inactivate form of the colistin resistance gene mcr-1 carried on a pHN5H4P5-like plasmid in a UPEC isolate. Re-activation of the disrupted gene is dependent on the sequential acquisition of mutations for colistin resistance. Acquisition of the plasmid with mcr-1 does not impose a fitness cost, suggesting a widespread potential for the ‘silent’ mcr-1 gene in the future.

GenBank accession nos.

The nucleotide sequences of ECO3347, ECO3347-2S-1, ECO3347-2S-2, ECO3347-4S-1, ECO3347-4S-2, ECO3347-4R, ECO3347-8R-1 and ECO3347-8R-2 have been deposited in GenBank under accession nos. NSBS000000000–NSBL000000000.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jantimicag.2018.01.004.


