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mcr-1-Mediated In Vitro Inhibition of Plasmid Transfer Is Reversed by the Intestinal Environment

Xiaoman Yang 1,2,†, Rundong Shu 1,†, Leqi Hou 1, Panpan Ren 1, Xin Lu 3, Zhi Huang 1,2, Zengtao Zhong 1,2 and Hui Wang 1,3,*

1 Department of Microbiology, College of Life Sciences, Nanjing Agricultural University, 210095 Nanjing, China; dx201980533@hust.edu.cn (X.Y.); 2018116064@njau.edu.cn (R.S.); 2020116063@stu.njau.edu.cn (L.H.); 2020116062@stu.njau.edu.cn (P.R.); zhuang@njau.edu.cn (Z.H.); zttzhong@njau.edu.cn (Z.Z.)
2 Department of Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, 430074 Wuhan, China
3 State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 102206 Beijing, China; luxin@icdc.cn
* Correspondence: wanghui@njau.edu.cn; Tel.: +86-25-84396645
† These authors contributed equally to this work.

Abstract: Colistin is regarded as an antibiotic of last resort against multidrug-resistant Gram-negative bacteria, including Klebsiella pneumoniae and Escherichia coli. Colistin resistance is acquired by microorganisms via chromosome-mediated mutations or plasmid-mediated mobile colistin resistance (mcr) gene, in which the transfer of mcr is the predominant factor underlying the spread of colistin resistance. However, the factors that are responsible for the spread of the mcr gene are still unclear. In this study, we observed that mcr-1 inhibited the transfer of the pHNSHP45 backbone in liquid mating. Similar inhibitory effect of mcr-1.6 and chromosomal mutant ∆mgrB suggested that colistin resistance, acquired from either plasmid or chromosomal mutation, hindered the transfer of colistin resistance-related plasmid in vitro. Dual plasmid system further proved that co-existing plasmid transfer was reduced too. However, this inhibitory effect was reversed in vivo. Some factors in the gut, including bile salt and anaerobic conditions, could increase the transfer frequency of the mcr-1-containing plasmid. Our results demonstrated the potential risk for the spread of colistin resistance in the intestine, provide a scientific basis against the transmission of colistin resistance threat.

Keywords: mcr-1; colistin resistance; conjugation; bile salt; Klebsiella pneumoniae; Escherichia coli

1. Introduction

Increasing antibiotic resistance in multidrug-resistant (MDR) Gram-negative bacteria poses a severe threat to public health and safety [1,2]. Due to the lack of development of new antimicrobial agents, an old cationic antimicrobial peptide, colistin, has regained the spotlight in the mid-1990s as the last resort against multidrug-resistant Enterobacteriaceae, including Klebsiella pneumoniae and Escherichia coli. Unfortunately, a rapid global resistance towards colistin has subsequently emerged, which markedly reduces the efficiency of colistin-related antibiotics and makes treatment more difficult.

Colistin resistance is acquired mainly via two methods. One of the mechanisms is chromosome-mediated mutations. The mutation of pmrAB [3], phoPQ [4], or mgrB [5] is reported to interfere with the synthesis of lipopolysaccharide (LPS) by increasing the modification of lipid A. However, the rate of genomic mutation is low (approximately 10⁻⁹ to 10⁻⁶) and easy to reverse [6,7]. The other mechanism is the acquisition of the plasmid-mediated mobile colistin resistance (mcr) gene, which encodes a phosphoethanolamine transferase resulting in the addition of phosphoethanolamine to lipid A [8]. Compared with genomic
mutations, horizontal gene transfer (HGT) with higher frequency is the predominant mode of acquiring colistin resistance among bacterial cells [9,10].

The first report of mobilizable colistin resistance in 2015 was mediated by pHNSHP45, whose strong transfer frequency is up to $10^{-1}$ under laboratory conditions [8]. The mcr-1 gene on pHNSHP45 encodes phosphoethanolamine (pEtN) transferase that alters the cell surface charge by catalyzing the addition of a pEtN to lipid A [11]. To date, various mcr variants were widely spread over 60 countries [12–14] which raises concerns about the advent of the post-antibiotic era.

However, the acquisition of colistin resistance presumably comes with fitness costs. Our previous study indicated that colistin resistance conferred by either chromosomal mgrB deletion or mcr-1 expression on a plasmid renders carbapenem-resistant K. pneumoniae more sensitive to phage infection [15]. It implied that the chemical modifications of the lipopolysaccharide molecules of the outer membrane might change some behavior of recipient bacteria. Plasmid HGT is the predominant method to introduce colistin resistance, and bacterial conjugation is the most efficient strategy for HGT [16,17]. In this study, we examined the transfer frequency of an mcr-1-related plasmid in vitro and in vivo using various host strains to evaluate the potential effect of mcr-1 on gene spreading and the related factors in the process.

2. Results

2.1. Liquid Conjugation was Established for Quantitative Measurement of Plasmid Transfer Rate

Conjugation is the process of exchanging plasmids between two bacteria. To establish a suitable mating system to observe the variation in the transfer frequency of plasmids, the equal density of donor strain K. pneumoniae A2312NM(pHNSHP45) and recipient strain K. pneumoniae D20-2 were mixed and incubated on a filter (filter mating) or in a liquid medium (liquid mating). The transfer frequency of the plasmid was determined after conjugation. The pHNSHP45 displayed a high level of transfer (from $10^{-3}$ to $10^{-2}$) by filter mating (Figure 1). However, liquid mating exhibited a relatively lower plasmid transfer frequency (from $10^{-5}$ to $10^{-4}$), which is suitable to quantify the transfer efficiency variation in this research. Thus, liquid mating was applied to explore the spread of colistin resistance via HGT.

![Figure 1. Different conjugation systems for quantitative measurement of plasmid transfer rate. The pHNSHP45 plasmid was transferred from K. pneumoniae A2312NM to K. pneumoniae D20-2 through a filter or liquid mating. Cultures were incubated till the log phase. Donor and recipient strains were mixed in equal proportions and concentrated 50-fold. The mixture was incubated on a filter or suspended in a liquid medium for 4 h at 37 °C, after which the plasmid transfer frequency was measured. Data are the mean and SD of three independent experiments. Significance was determined using t-test; ** $p < 0.01$.](image-url)
2.2. mcr-1 Inhibits pHNSHP45 Transfer through Conjugation

mcr-1 is carried by the plasmid pHNSHP45, hereafter referred to as the mcr-1 plasmid. To examine whether the mcr-1 gene contributes to the transfer of pHNSHP45, we replaced the mcr-1 gene with a kanamycin-resistant cassette (Km\textsuperscript{R}) on pHNSHP45 to obtain the pAC22 plasmid, hereafter named the \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid. We also restored the mcr-1 at an intergenic region on the pAC22 to obtain the pAC23 plasmid, hereafter named the \(\text{mcr-1}^c\) plasmid.

*K. pneumoniae* A2312NM with these different mcr-1-related plasmids (mcr-1, \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) and \(\text{mcr-1}^c\)) were verified using PCR to amplify three evenly distributed genes on pHNSHP45 (mcr-1, parA and virB) and antimicrobial susceptibility testing (Supplementary Figure S1).

*K. pneumoniae* A2312NM harboring a plasmid with or without the mcr-1 gene exhibited no difference in terms of growth (Figure 2A), suggesting that mcr-1 expression had no significant effect on the growth of A2312NM. Moreover, no competition was observed between A2312NM strains carrying the mcr-1 plasmid and the \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid when they were cultured together (Figure 2B). The transfer frequency of the mcr-1 plasmid, \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid, and \(\text{mcr-1}^c\) plasmid from *K. pneumoniae* A2312NM to *K. pneumoniae* D20-2 were firstly measured to evaluate the influence of the mcr-1 gene. The spontaneous mutation rate was approximately \(10^{-8}\) (data not shown), while the conjugation frequency of the mcr-1 plasmid ranged from \(10^{-5}\) to \(10^{-4}\) (Figure 2C, red). The \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid exhibited a transfer frequency of \(10^{-3}\) to \(10^{-2}\), which was approximately 100-fold higher than that of the mcr-1 plasmid. In addition, the \(\text{mcr-1}^c\) plasmid restored the plasmid transfer frequency (Figure 2C, red), suggesting that the mcr-1 gene had a negative effect on the conjugal transfer of the plasmid backbone. To explore whether this phenotype is strain-specific, we repeated this experiment with *K. pneumoniae* A1502 as the recipient strain. Similar results demonstrated that the plasmid transfer with the mcr-1 plasmid (the mcr-1 plasmid ranged from \(10^{-5}\) to \(10^{-4}\)) was lower than the plasmid without the mcr-1 gene (the \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid ranged from \(10^{-3}\) to \(10^{-2}\))(Figure 2C, black). Further, the transfer frequency of the \(\text{mcr-1}^c\) plasmid was reduced from \(10^{-4}\) to \(10^{-3}\). These results suggested that the mcr-1 gene inhibited the conjugal transfer of its plasmid backbone within *K. pneumoniae*.

We further extend the assay to *E. coli*. The related plasmids were introduced into the conjugal donor strain *E. coli* MG1655. Compared to the transfer frequency of the mcr-1 plasmid, the deletion of the mcr-1 gene had an approximately 100-fold increase (Figure 2D), indicating that the mcr-1 gene inhibited the conjugal transfer rate when the plasmid was transferred from *E. coli* MG1655 to *E. coli* Nissle 1917.

To assess the effect of the mcr-1 gene on the plasmid transfer between different genera, conjugation was performed between the donor *K. pneumoniae* A2312NM and the recipient *E. coli* MP13. Interestingly, consistent with the aforementioned results, the mcr-1 plasmid and the \(\text{mcr-1}^c\) plasmid exhibited a lower transfer frequency than the \(\Delta\text{mcr-1}::\text{Km}\textsuperscript{R}\) plasmid (Figure 2E). Collectively, mcr-1 inhibited the transfer of the plasmid backbone through conjugation, and this negative effect is common and not limited to any specific bacteria.
A2312NM carrying different plasmids. (K. pneumoniae plasmid. It indicated that mcr-1 phosphoethanolamine transferases. An mcr-1 pHNSHP45. We further repeated it with an IncP Plasmid pMCR1.6 _ P053 [23]. An some-mediated colistin resistance [15]. Then 2.3. Colistin Resistance has Inhibitory Effect on Plasmid Transfer 2.51 × 10² frequency of the pMCR1.6_P053 plasmid (carrying mcr-1.6 contains two single-nucleotide polymorphisms that do not impact the activity of phos- 2.3. Colistin Resistance has Inhibitory Effect on Plasmid Transfer Since the first report of IncI2-type plasmid pHNSHP45, the mcr-1 gene was found to be carried by diverse plasmid replicon types, such as IncI2, IncH12, IncP, IncFIP, and IncX4 [18–22]. The previous data implied an inhibitory effect of mcr-1 on the IncI2 plasmid pHNSHP45. We further repeated it with an IncP Plasmid pMCR1.6 _ P053 [23]. An mcr-1 variant, named mcr-1.6, was carried by pMCR1.6_P053. Compared with mcr-1, mcr-1.6 contains two single-nucleotide polymorphisms that do not impact the activity of phosphoethanolamine transferases. An mcr-1.6-deletion plasmid, hereafter named the Δmcr-1.6::ApraR plasmid, was constructed by replacing the mcr-1.6 gene fragment with an apramycin-resistant cassette in pMCR1.6_P053. Further, we compared the transfer frequency of the pMCR1.6_P053 plasmid (carrying mcr-1.6 gene) and Δmcr-1.6::ApraR from K. pneumoniae A2312NM to E. coli MP13. The transfer frequency of pMCR1.6_P053 was 2.51 × 10⁻⁷ (Figure 3A), which was significantly lower than that of the Δmcr-1.6::ApraR plasmid. It indicated that mcr-1.6 inhibited the conjugal transfer of the plasmid.
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100-fold less than in the A2312NM WT donor (a colistin-sensitive strain; Figure 3B). These K. pneumoniae with the same donor strain (Δmcr-1::ApraR plasmid) was 100-fold lower than that of the deletion of the mcr-1 gene plasmid (Δmcr-1::KmR plasmid) (Figure 3B). However, the transfer frequency of the Δmcr-1::KmR plasmid was 10−5 in the A2312NM ΔmgrB donor (a chromosomally mediated colistin-resistant strain), which was approximately 100-fold less than in the A2312NM WT donor (a colistin-sensitive strain; Figure 3B). These data indicated that colistin resistance, either from the acquisition of plasmid-mediated mcr or chromosomal mutation, can inhibit the transfer of the mcr-1-related plasmid.

2.4. The Impact of Colistin Resistance on Helper Plasmid Transfer

As previously shown, the colistin-resistant gene expression on a plasmid had a negative effect on its backbone transfer. Further study was performed to explore the effect of colistin resistance on plasmids that are not related to colistin resistance. We developed a dual plasmid system where the helper plasmid pRK2013 and the mcr-1-related plasmids (mcr-1 plasmid or Δmcr-1::ApraR plasmid which was also named as pAC24) co-existed in the donor strain K. pneumoniae A2312NM (Figure 4A). Liquid mating was performed with the recipient strain K. pneumoniae D20-2. As Figure 4B shows, the conjugation frequency of the mcr-1 plasmid was lower than that of the Δmcr-1::ApraR plasmid (Figure 4B, left). Meanwhile, the conjugation frequency of pRK2013 was at approximately 10−3 when it co-existed with the mcr-1 plasmid, which was approximately 52 times less than that of pRK2013 co-existed with the Δmcr-1::ApraR plasmid (Figure 4B, right), the trend is similar to that of the mcr-1-related plasmids.

Figure 3. The impact of plasmid or chromosome mediated colistin resistance on plasmid transfer. (A) Transfer frequency of mcr-1.6 plasmid and Δmcr-1.6::ApraR plasmid from K. pneumoniae A2312NM to E. coli MP13. (B) mcr-1-related plasmid transfer frequency. Donor strains were A2312NM ΔmgrB containing Δmcr-1::KmR plasmid and A2312NM containing mcr-1 plasmid or Δmcr-1::KmR plasmid. They were conjugated individually with the recipient strain K. pneumoniae D20-2. Data are mean and SD of three independent experiments. Significance was determined using t-test; * p < 0.05, ** p < 0.01.
Figure 4. The impact of colistin resistance on the transfer frequency of co-existing plasmid. (A) A dual plasmid system was constructed with pRK2013 combined with an mcr-1 plasmid (orange) or Δmcr-1::ApraR plasmid (purple) in the donor strain K. pneumoniae A2312NM. (B) Conjugation frequency of mcr-1 or Δmcr-1::ApraR plasmid (left) and pRK2013 in the dual plasmid system was measured separately. (C) Conjugation frequency of pRK2013 with donor K. pneumoniae A2312NM WT or A2312NM ΔmgrB and recipient K. pneumoniae D20-2. Data are the mean and SD of more than three independent experiments. The significance was determined using t-test; ** p < 0.01.

Transfer of pRK2013 under chromosomal-mediated colistin resistance was also tested. A2312NM(pRK2013) (colistin-sensitive) or A2312NM ΔmgrB(pRK2013) (colistin-resistant) were set as the donor strain, and K. pneumoniae D20-2 acted as the recipient strain. A consistent trend was shown in Figure 4C that the frequency for pRK2013 transfer without colistin resistance was 3.85 folds higher than that with mgrB-mutation-mediated colistin
resistance. Collectively, these data indicated that the co-existing plasmid transfer was affected by colistin resistance.

2.5. mcr-1 Plasmid Transfers In Vivo

The intestinal tract is regarded as a ‘melting pot’ for gene exchange which provides several ideal conditions, such as high density and diversity of microbiota, stable temperature, biofilm formation, and so on [25–27]. To explore the transfer of mcr-1 in vivo, we selected the indigenous bacteria E. coli MP13 as the recipient strain while K. pneumoniae A2312NM carrying the mcr-1 plasmid or the Δmcr-1::KmR plasmid as the donor strain. Adult mice were pretreated with streptomycin to clean the intestinal flora [28,29]. An equal volume of the donor and recipient strain was mixed and immediately administered to the mice intragastrically. Fecal samples were collected after 3 days. The transfer frequency of the mcr-1 plasmid (1.19 × 10^{-3}) was higher than that of the Δmcr-1::KmR plasmid (6.93 × 10^{-4}), which was opposite to the previous trends in vitro (Figure 5A and 2E), and suggested that colistin resistance support the plasmid transfer in vivo.

**Figure 5.** Influence of oxygen and bile salt on plasmid transfer. (A) Plasmid transfer in vivo. Five-week-old CD-1 mice were pretreated with streptomycin. Approximately 10^8 cells of different donor strains, namely, K. pneumoniae A2312NM harboring mcr-1 plasmid, A2312NM containing the Δmcr-1::KmR plasmid, were mixed with recipient strain K. pneumoniae MP13 separately and immediately administered to each mouse intragastrically. Fecal samples were collected after 3 days, and the transfer frequency was calculated. (B) The effect of mucin and bile salt on the transfer of mcr-1 plasmid. Conjugation was performed with or without additional mucin or bile salt. (C) Conjugation was performed with or without bile salt under different oxygen concentrations. Data are the mean and SD of three and more independent experiments. The significance was determined using t-test; ns, no significance; ** p < 0.01, *** p < 0.001.
The intestine is a complex mini-ecosystem with lots of specific environmental factors which might influence the HGT [30]. Several key factors (mucins, bile salt, and anaerobic conditions) were introduced in the liquid mating system in vitro with the same donor and recipient strains in vivo to test the potential impact. First, we determined the transfer frequency of the \( mcr-1 \) plasmid in the presence of mucin or bile salt. Mucins are the major macromolecular constituent of mucus which act as a barrier in the intestinal epithelium [31,32], and bile salt are an important component of bile that provide not only antibacterial protection in the intestine but also signaling molecules for virulence expression of multiple pathogens [33]. No significant difference was observed in terms of conjugation frequency with an additional 0.01% of mucin in the LB medium, whereas it decreased by 3-fold in the presence of 0.1% mucin (Figure 5B). Interestingly, the transfer frequency of the \( mcr-1 \) plasmid increased by 3–11 fold with bile salt, which was consistent with the results of conjugation in vivo (Figure 2E and 5A,B). This suggested that bile salt facilitates the conjugation-mediated spread of colistin resistance among bacterial cells.

Anaerobic condition is an important signal that is involved in a variety of physiological activities in the gut flora [30]. To evaluate whether oxygen concentration has an impact on bile salt-facilitated plasmid transfer, we performed conjugation under aerobic (shown as “O\(^2\)”) and anaerobic conditions (shown as “O\(^2\)–”). The transfer frequency of the \( mcr-1 \) plasmid under aerobic conditions was \( 10^{-5} \) (Figure 5C, blue). After the addition of 0.5% bile salt, the frequency notably increased by 21-fold (Figure 5C, red). It kept increasing to approximately \( 10^{-3} \) plus anaerobic treatment (Figure 5C, green), suggesting that both bile salt and anaerobic condition play an important role in the process.

Subsequently, we measured the transfer frequency of the \( \Delta mcr-1::Km^R \) plasmid under \( O_2^+ \) conditions. The transfer frequency of the \( \Delta mcr-1::Km^R \) plasmid was \( 4.21 \times 10^{-4} \), which was 50-fold higher than that of the \( mcr-1 \) plasmid (Figure 5C, blue and gray). It was consistent with previous results that \( mcr-1 \) inhibits the conjugal transfer of the pHNSHP45 plasmid (Figure 2C–E). Conjugation under \( O_2^+ \) conditions or \( O_2^- \) conditions combined with bile salt exhibited similar transfer frequencies around \( 10^{-3} \) (Figure 5C, gray and dark gray). We stopped testing more factors because the transfer frequency in this situation is too high to observe additional promotion. In summary, intestinal environmental factors such as bile salt and anaerobic conditions may increase plasmid transfer frequency and thus accelerate the spread of colistin resistance. The intestine is a potential niche for the spread of conjugative plasmids.

3. Discussion

HGT via conjugation is considered one of the major contributors to the spread of antibiotic resistance. Many factors are included in affecting the transfer of plasmids.

One of the factors is the nature of the strain. It was reported that the recipient bacteria can influence the yield of transconjugants when the pVCM29188_146 plasmid was transferred to *Salmonella Kentucky* CVM29188, *S. Newport* SL317, and *E. coli* DH10B [34,35]. Moreover, the transfer frequency of RP1/RP4 was \( 2.05 \times 10^{-1} \), with *E. coli* HB101 as the donor and *E. coli* X7 as the recipient. The transfer frequency was \( 2.56 \times 10^{-2} \) when the RP1/RP4 plasmid was transferred within *E. coli* BJ4 itself [36]. Liu reported that the transfer frequency of pHNSHP45 can reach up to \( 10^{-1} \) with *E. coli* SHP45 as the donor and *E. coli* C600 as the recipient using the filter mating technique [8]. Our results revealed that pHNSHP45 had a high rate of transfer frequency (\( 10^{-2} \)) in filter mating when it was transferred from *K. pneumoniae* A2312NM to *K. pneumoniae* D20-2 (Figure 1). However, when pHNSHP45 was transferred from *K. pneumoniae* A2312NM to *E. Coli* MP13 instead of *K. pneumoniae* D20-2, the transfer frequency was decreased by approximately 10-fold in liquid mating (Figure 2C,E). These results indicated that the nature of the donor and recipient is important for plasmid transfer.

The structural integrity of bacteria, especially LPS, is also important for plasmid transfer [37]. For example, Ishiwa [38] revealed that PilV adhesin located at the top of thin pili determines the specificity of the recipient by recognizing its LPS. Duke [39] reported
that the plasmids Flac and R1drd19 are easier to transfer to the S218 wild-type strain than its polysaccharide core-related LPS mutant. To acquire colistin resistance, the major mechanism is to alter the structure of cell surface LPS, which results in interference with the electrostatic binding of colistin [5,11]. Our results demonstrated that colistin resistance, acquired either from the IncI2-type plasmid containing the mcr-1 gene or chromosomal mgrB mutation, reduced the transfer frequency of mcr-1-related plasmids and co-existed helper plasmid in vitro (Figure 2C–E and 4B,C). The influence was repeatable with the IncP-type plasmid pMCR1.6_P053 harboring mcr-1.6. Although various transfer frequencies occur in different types of plasmids, mcr-1.6 still displayed a significant inhibitory effect on its backbone (Figure 3A). The altering of the donor strain (colistin R) in LPS may interfere with the contact with the recipient strain.

Another factor that affects the plasmid transfer is the conjugation conditions. The stabilization of cell-to-cell contact determines the frequency of plasmid transfer. In liquid mating, the conjugation process benefits from mating-pair stabilization either provided by F-pili or type IVb pili that hold cells together and maintain close contact during plasmid transfer [40]. Filter mating fulfills high cell density and close proximity for donor and recipient cells, facilitating the formation of mating pairs for plasmid transfer [30,41,42]. Kosuke reported that the transfer frequency of pCAR1 or pDK1 was significantly different between liquid mating and filter mating [43]. Our results were consistent with this observation (Figure 1).

Environmental factors, like antibiotics, temperature and chemical compounds, are thought to be involved in mediating the plasmid transfer. Hastings [40] reported that ROS response induced by several antibiotics can promote genetic change and the evolution of antibiotic resistance. Aviv [41] reported that the conjugation frequency of pES1 was significantly higher at 37 °C than at 27 °C. Besides, the transfer frequency gradually increased with the increasing salt concentration in the conjugation mixture. Additionally, plasmid transfer can be influenced by intestinal tract factors [42,43]. Garcia and Aviv [41,44] reported that the transfer of pES1 and pSLT plasmids were affected by lower oxygen level and bile salt. In this study, we observed that the intestinal environment plays an important role in spreading colistin resistance among bacteria by conjugation (Figure 5A). We observed that additional mucin is ineffective in the conjugation (Figure 5B). Mucin is reported as the barrier in the intestinal epithelium, which is associated with bacterial colonization [31,32], and this barrier may interfere with mating-pair formation. Meanwhile, bile salt and anaerobic conditions facilitated the transfer of the plasmid and, in turn, colistin resistance. The conjugation process is dependent on the cell membrane, as increasing the cell membrane permeability will promote HGT. Xiao found that exposure to a subinhibitory concentration of colistin resulted in a break in the membrane barrier and significantly stimulated the conjugation frequency of mcr-1- and blaNDM-5-positive [45]. As a digestive secretion, bile can destabilize membranes and disrupt bacterial cellular homeostasis via its detergent-like properties [46] and, presumably, may help promote plasmid transfer. In addition, bile salt has been reported to activate virulence gene expression of Vibrio cholerae in anaerobic conditions such as in the small intestine [47]. We suspected that bile salt and limited oxygen concentration might participate in the spread of colistin resistance in vivo. Further studies are required to understand the underlying mechanism.

Polymyxin drugs used in clinics and colistin-containing feeds in animal husbandry could accelerate the spread of colistin resistance. After the discovery of plasmid-mediated mobile colistin resistance in China in 2015, several variants of mcr genes have been isolated from fecal samples of humans and livestock [48]. For example, up to 31% of mcr-resistant strains were isolated directly from the feces of patients [49]. mcr-1 [50], mcr-1.1 [51], mcr-1.4 [51], mcr-5 [52], mcr-10 [53] were isolated from hospital wastewater. Additionally, mcr-1 [54], mcr-5.3 [55], and mcr-8 [56] were isolated from animal waste. Moreover, the presence of mcr has been reported in cases from farms, slaughterhouses, and municipal sewage, where human beings and animals live [57–59]. Here, we reported that the intestinal tract may be an ideal niche for the plasmid-mediated spread of colistin resistance (and
potentially resistance to other antibiotics) among bacteria. This also warns us of the danger of antibiotics overuse.

4. Materials and Methods

4.1. Bacteria and Growth Condition

The strains and plasmids used in this study are listed in Table 1. All bacteria strains were propagated by Luria-Bertani (LB) broth with the appropriate antibiotic at 37 °C unless otherwise stated.

Table 1. Strains and plasmids used in this study.

| Strain and Plasmid       | Description                                      | Reference or Source |
|--------------------------|--------------------------------------------------|---------------------|
| **Strains**              |                                                  |                     |
| E. coli                  |                                                  |                     |
| MG1655                   |                                                  | [60]                |
| MP13                     |                                                  | [61]                |
| Nissle 1917              |                                                  | [62]                |
| **K. pneumoniae**        |                                                  |                     |
| A2312NM                  | Clinical isolate, Str<sup>R</sup>, Tc<sup>R</sup> | [15]                |
| D20-2                    | Clinical isolate, Gen<sup>R</sup>                | This study          |
| A1502                    | Clinical isolate, Gen<sup>R</sup>                | This study          |
| **Plasmids**             |                                                  |                     |
| pHNSHP45                 | IncI2 type plasmid, harboring mcr-1 gene, colistin<sup>R</sup> | [8]                 |
| pAC22                    | mcr-1 gene in pHNSHP45 is replaced by kanamycin resistance, Km<sup>R</sup> | [15]                |
| pAC23                    | Recombine mcr-1 gene on pAC22, colistin<sup>R</sup> | [15]                |
| pAC24                    | mcr-1 gene in pHNSHP45 is replaced by apramycin resistance, Apra<sup>R</sup> | This study          |
| pMCR1.6_P053             | IncP type plasmid, harboring mcr-1.6, colistin<sup>R</sup> | [23]                |
| pRK2013                  | Auxiliary plasmid for mating, Km<sup>R</sup>     | [63]                |

4.2. Growth Curve Assay

Saturated cultures of K. pneumoniae A2312NM harboring different plasmids were washed with fresh LB, then diluted 1:100 into 3 mL of LB medium containing the appropriate antibiotic at 37 °C. OD<sub>600</sub> was measured at a dedicated time.

4.3. Competition Assay

A2312NM with the mcr-1 plasmid or Δmcr-1::Km<sup>R</sup> plasmid were cultured overnight at 37 °C. Saturated cultures were equally inoculated in fresh LB at 37 °C and transferred to LB medium every day at 1:100. Daily samples were counted for viable bacteria on plates with appropriate antibiotics.

4.4. Conjugation In Vitro

The liquid conjugation in vitro was performed with modifications [64]. Briefly, log-phase cultures were concentrated 10-fold in LB medium. Equal volumes (100 µL) of donor and recipient strain were mixed and concentrated 5-fold with LB medium containing different concentration of bile salt (0, 0.5%, 1%) or mucin (0, 0.01%, 0.1%). Similarly, treated non-mixed donors or recipients were used as controls. Conjugation proceeded aerobically (standing) or anaerobically (chamber, standing) for 4 h at 37 °C. Samples were serially diluted and plated on selected plates containing the appropriate antibiotics (gentamicin 20 µg/mL, colistin 5 µg/mL, kanamycin 100 µg/mL, chloramphenicol 20 µg/mL, streptomycin 100 µg/mL, apramycin 50 µg/mL, nalidixic acid 10 µg/mL). Representative colonies of conjugants harboring different mcr-1-related plasmid were confirmed using PCR to amplify three evenly distributed genes on pHNSHP45 (mcr-1 primer F-5′TTGCGGATTTACCTCCACCAG3′/R-5′TGGAGTGTCCGGGTTTGG3′; parA primer F-5′GCTGCTCTGCATTGTTTGTG3′/R-5′AGCTACGGCGCAACAACAC3′; vibB primer F-5′CCAGACGGCAAGATTGGAG3′/R-5′ATCTGCCAGAAGGAAGACTAAG3′) and antimicrobial susceptibility testing. Conjugation frequency was calculated as a ratio of the total number of transconjugants to the total number of recipient cells, and spontaneous mutation
rates were calculated for the same treated recipient bacteria. For filter mating, the only difference is that the mixed culture is placed on a filter for conjugation.

4.5. Conjugation Frequency In Vivo

A streptomycin-pretreated adult mice model was performed as previously described [65] with some modifications. Briefly, a five-week-old CD-1 mouse was provided with drinking water supplied with 5 g/L of streptomycin and 0.05 g/L of aspartame throughout the experiment. Approximately 10^8 cells of the strains *K. pneumoniae* A2312NM harboring the *mcr-1* plasmid, A2312NM containing the Δ*mcr-1::KmR* plasmid, and recipient strain MP13, were mixed equally and intragastrically administered to mice following the treatment with 50 µL of 10% (wt/vol) NaHCO₃. Fecal samples were collected and homogenized, serially diluted, and then plated on LB medium with the appropriate antibiotics. Conjugants were confirmed using PCR (to amplify *mcr-1*, *parA* and *virB*) and antimicrobial susceptibility testing. The plasmid transfer frequency was determined as a ratio of the total number of transconjugants to the total number of recipient cells.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11070875/s1, Figure S1: Confirmation of the presence of plasmids.

Author Contributions: Conceptualization, X.Y. and H.W.; Formal analysis, X.Y. and R.S.; Z.H. and Z.Z.; Investigation, X.Y., R.S., L.H. and P.R.; Methodology, R.S., L.H. and P.R.; Resources, X.L.; Visualization, X.Y. and R.S.; Writing—original draft, X.Y.; Writing—review and editing, H.W., R.S., L.H. and P.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Natural Science Foundation of China (32070131, HW) and the Natural Science Foundation of Jiangsu Province (BK20191314, HW).

Institutional Review Board Statement: All animal experiments were carried out in strict accordance with animal protocols that were approved by the Ethical Committee of Animal Experiments of Nanjing Agricultural University [SYXK (Su) 2017-0007]. All efforts were made to minimize animal suffering. Euthanasia was performed by CO2 inhalation.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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