Spreading Advantages of Coresident Plasmids $bla_{CTX-M}$-Bearing IncFII and $mcr-1$-Bearing IncI2 in Escherichia coli

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ABSTRACT Two diverse conjugative plasmids can interact within bacterial cells. However, to the best of our knowledge, the interaction between $bla_{CTX-M}$-bearing IncFII plasmid and $mcr-1$-carrying IncI2 plasmid colocated on the same bacterial host has not been reported. This study was initiated to explore the interaction and to analyze the reasons that these two plasmids are often coresident in multidrug-resistant Escherichia coli. To assess the interactions on plasmid stabilities, fitness costs, and transfer rates, we constructed two groups of isogenic derivatives, C600FII, C600I2, and C600FII₁I2 of E. coli C600 and J53FII, J53I2, and J53FII₁I2 of E. coli J53, respectively. We found that carriage of FII and I2 plasmids, independently and together, had not impaired the growth of the bacterial host. It was difficult for the single plasmid FII or I2 in E. coli C600 to reach stable persistence for a long time in an antibiotic-free environment, while the stability would be striking improved when they coresided. Meanwhile, plasmids FII and I2, whether together or apart, could notably enhance the fitness advantage of the host; moreover, E. coli coharboring plasmids FII and I2 presented more obvious fitness advantage than that carrying single plasmid FII. Coresident plasmids FII and I2 could accelerate horizontal cotransfer by conjugation. The transfer rates from a strain carrying coresident FII and I2 plasmids increased significantly when it mated with a recipient cell carrying one of them. Our findings highlight the advantages of coinhabitant FII and I2 plasmids in E. coli to drive the persistence and spread of plasmid-carried $bla_{CTX-M}$ and $mcr-1$ genes, although the molecular mechanisms of their coresidence warrant further study.

IMPORTANCE More and more Enterobacteriaceae carry both $bla_{CTX-M}$ and $mcr-1$, which are usually located on IncFII-type and IncI2-type plasmids in the same bacterial host, respectively. However, the study on advantages of coresident plasmids in bacterial host is still sparse. Here, we investigated the stability, fitness cost, and cotransfer traits associated with coresident IncFII-type and IncI2-type plasmids in E. coli. Our results show that coinhabitant plasmids in E. coli are more stable, confer more fitness advantages, and are easier to transfer and cotransfer than a single plasmid IncFII or IncI2. Our findings confirm the advantages of coresident plasmids of $bla_{CTX-M}$-bearing IncFII and $mcr-1$-bearing IncI2 in clinical E. coli, which will pose a serious threat to clinical therapy and public health.

KEYWORDS $bla_{CTX-M}$, $mcr-1$, IncFII, IncI2, coresident
bla<sub>CTX-M</sub>-type ESBLs (4–6). Similarly, the IncI2-type conjugative plasmid is the most epidemiological successful vector for horizontal spread of mcr-1 (1, 7–9).

Recently, resistant genes bla<sub>CTX-M</sub> and mcr-1 have been simultaneously detected in Enterobacteriaceae species isolated from humans and animals (8, 10, 11). Although they sometimes coexist on the same plasmid (8, 12), the bla<sub>CTX-M</sub> and mcr-1 genes are usually located on diverse plasmids of the same bacterial host (13–15). In a previous survey on antimicrobial-resistant bacterial isolates in China, Escherichia coli LWY24 was isolated from healthy chicken feces in Henan Province (15). The isolate LWY24 harbored bla<sub>CTX-M-55</sub> and mcr-1, which were located on an IncFII replicon pLWY24J-3 (bla<sub>CTX-M-55</sub>-bearing, MN702385) and an IncI2 replicon pLWY24Jmcr-1 (mcr-1-carrying, MN689940), respectively, and could carry out horizontal transfer by plasmid conjugation, independently or simultaneously.

Previous studies confirmed that two distinct conjugative plasmids could interact within bacterial cells (16–20). To the best of our knowledge, the interaction between bla<sub>CTX-M</sub>-bearing IncFII plasmid and mcr-1-carrying IncI2 plasmid colocated on the same cell has not been reported. This study was initiated to explore the characteristics of coresident IncFII-type and IncI2-type plasmids in E. coli.

RESULTS AND DISCUSSION

The plasmids FII and I2 had no effect on bacteria growth. Growth kinetics of the four isogenic strains were plotted respectively based on optical density at 600 nm (OD<sub>600</sub>) values and the log<sub>10</sub> CFU values after 14-h assessment (Fig. 1). No obvious difference in growth was observed among bacteria, which indicated that carriage of FII and I2 plasmids, independently and together, had not impaired the growth of the bacterial host. The results coincided with previous studies on bla<sub>CTX-M</sub>-carrying IncFII plasmid (9) and mcr-1-harboring IncI2 plasmid (7), which further explained why IncFII-replicon and IncI2-replicon plasmids were dominant in bla<sub>CTX-M</sub>-carrying and mcr-1-harboring E. coli strains, respectively (6, 9).

Coresident plasmids FII and I2 improved stabilities. In order to analyze the stability of acquired plasmids over time in the absence of selection, we propagated the isogenic strains, C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>FII+I2</sub> in antibiotic-free culture medium for 15 days (i.e., ~150 generations) (Fig. 2). The results demonstrated that the single plasmid FII in the host could not be stably maintained. It was partially lost from day 2.5, and only about 73.5% remained at day 15. Meanwhile, the plasmid I2 loss occurred from day 12.5, with a total loss of about 8.1% at the end. From this, the stability of two plasmids was decreased in different degrees in the absence of antibiotic selective pressure, especially that of plasmid FII.

Intriguingly, when plasmids FII and I2 coresided in the host, almost no plasmid loss was detected over the 15 days of the experiment, which implied that the stability of bla<sub>CTX-M</sub>-carrying FII plasmid and mcr-1-harboring I2 plasmid could significantly improve by coresidence. The results were consistent with previous studies that the stability of coexistent
plasmids increased (17), which contributed to elucidating why \textit{bla}_{CTX-M} and \textit{mcr-1} are easier to locate on diverse plasmids in the same bacterial host worldwide (13–15).

Cells coharboring plasmids FII and I2 presented fitness advantages. To determine the relative carriage costs of plasmids FII and I2, pairwise competitions were carried out between the plasmid-free strain \textit{E. coli} DH5\textalpha{} and three isogenic derivatives, C600FII, C600I2, and C600FII\_1I2. The outcome competition revealed that there were no fitness costs between plasmid-free strains \textit{E. coli} DH5\textalpha{} and \textit{E. coli} C600 (Fig. 3a). However, the plasmid-harboring strains presented high fitness advantages in comparison with \textit{E. coli} DH5\textalpha{}, which obviously increased over time (Fig. 3a). The isogenic strains, C600I2, C600FII\_1I2, and C600FII, significantly outcompeted \textit{E. coli} DH5\textalpha{} from day 3 (relative fitness \([RF] = 1.28 \pm 0.0085, P = 0.0443\)), 3 (RF = 1.29 \pm 0.021, P = 0.0166), and 4 (RF = 1.56 \pm 0.05, P = 0.0465), respectively. Thus, plasmids FII and I2, whether together or apart, bestowed the fitness advantages on the host bacteria, which were consistent with some previous reports (7, 21) and contributed to the plasmids gradually becoming the capital vehicles for \textit{bla}_{CTX-M} and \textit{mcr-1} horizontal disseminations (6, 22).

Thereafter, the results of plasmid-plasmid competition assays described that no fitness costs were observed between C600I2 and C600FII\_1I2 after 144-h assessments (Fig. 3b), indicating that plasmid FII would not incur any additional fitness costs on its host cell when transferred to strain C600I2. In contrast, strain C600FII\_1I2 showed significant competition advantages over strain C600I2 from 48 h (RF = 1.22 \pm 0.09, P = 0.0317) to 144 h (RF = 1.67 \pm 0.09, P = 0.0008), demonstrating that plasmid I2 could further decrease fitness costs on its host when entering strain C600I2 (Fig. 3b). Together, \textit{E. coli} coharboring plasmids FII and I2 will not confer more fitness costs than bacteria carrying one plasmid FII or I2, which helps to promote the coexistence of plasmid-carried \textit{bla}_{CTX-M} and \textit{mcr-1} in \textit{E. coli} and to clarify why more and more \textit{Enterobacteriaceae} carry both \textit{bla}_{CTX-M} and \textit{mcr-1} (11, 15).

Coinhabitant plasmids FII and I2 contributed to cotransfer. The transfer speeds of plasmids FII and I2 were analyzed by serial transfer experiments (Table 1). The plasmid FII exhibited higher transfer speeds than plasmid I2. Furthermore, whether the plasmids FII and I2 coexisted in the same cell or in different cells, the transconjugants, cohaboring plasmids FII and I2, could be obtained, but this was easier and faster in the former situation, which partially explained why isolates more often harbored multiple plasmids. These results proved that coresident plasmids FII and I2 in \textit{E. coli} could accelerate horizontal cotransfer.

We measured the conjugation rates of eight pairs of plasmids (Fig. 4). First, we compared the mating rates of each plasmid when in the presence of a coresident plasmid with its own mating rates to the mating rates of each plasmid when alone in the donor cell (Fig. 4b and d). The conjugation rates of I2 plasmid in the donor C600FII\_1I2 were severely decreased, approximately 141-fold lower than those in the donor C600I2, while there was no significant difference in the conjugation rates of FII plasmid between the donors C600FII\_1I2 and C600I2. We also examined the conjugation frequencies of single plasmids in matings to recipient cells carrying another plasmid (Fig. 4b and d). Similar
to the above results, the conjugation rates of plasmid I2 were also significantly decreased, by 31.1-fold, when recipient cells harbored plasmid FII compared with those when the recipient was plasmid-free. These results implied that plasmid FII could inhibit the transfer of plasmid I2 whether plasmid FII presented in the donor or in the recipient, while plasmid I2 had no effect on the transfer of plasmid FII in the similar case.

Further, we explored the conjugation frequencies of each plasmid in the presence of a coresident plasmid in donor cells mating with recipient cells carrying another plasmid (Fig. 4c and e). Surprisingly, the conjugation rates of I2 and FII plasmids in this situation all increased significantly compared with those of the other situations. We speculate that transfer of the other plasmid will possibly increase significantly when the donor contains two plasmids and the recipient contains one of the plasmids. A previous study reported that negative interactions were significantly more frequent when plasmids occupied the same cell (18). Meanwhile, our above results also demonstrated that plasmid FII could inhibit the transfer of plasmid I2 when the plasmids were coresident in the donor, although further studies are needed to verify the reason this happens.

TABLE 1 The transfer speeds of plasmids pLWY24J-3 and pLWY24Jmcr-1, independently and together, in diverse settings

| Donor          | Recipient | Transconjugant | Conjugation time (min) |
|----------------|-----------|----------------|------------------------|
|                |           |                | 0  | 5  | 10 | 20 | 60 |
| C600<sub>FII</sub> | E. coli J53 | T<sub>J1</sub> | +   | +  | +  | +  | +  |
| C600<sub>I2</sub>  | E. coli J53 | T<sub>I2</sub> | +   | +  | +  | +  | +  |
| C600<sub>1,FII</sub> | E. coli J53 | T<sub>J1</sub> | +++ | +++| +++| +++| +++|
|                | E. coli J53 | T<sub>J2</sub> | 0   | +  | +  | +  | +  |
|                | E. coli J53 | T<sub>J1FII</sub> | 0   | 0  | +  | +  | +  |
| C600<sub>1,FII</sub>/C600<sub>I2</sub> (1:1) | E. coli J53 | T<sub>J1</sub> | +++ | +++| +++| +++| +++|
|                | E. coli J53 | T<sub>J2</sub> | 0   | +  | +  | +  | +  |
|                | E. coli J53 | T<sub>J1FII</sub> | 0   | 0  | 0  | 0  | +  |

*The plasmids pLWY24J-3 and pLWY24Jmcr-1 are abbreviated FII and I2, respectively. The strains C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>1,FII</sub> were isogenic derivatives of E. coli C600, which harbored the plasmids FII and/or I2. “+” represents that there were 1 to 10 transconjugants on all LB agar plates supplemented with colistin and/or cefotaxime, “+++” represents 11 to 99 transconjugants, “++++” represents ≥100 transconjugants, and “0” represents no transconjugants. The naming of transconjugants is as follows: capital “T” stands for the transconjugant, capital “J” represents the recipient, and subscript of transconjugants represents the plasmid which was transferred from the donor to the transconjugant.

FIG 3 Pairwise competition in vitro between E. coli C600, E. coli DH5α, C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>1,FII</sub> normalized to a 50/50 starting ratio. (a) Relative fitness of E. coli C600, C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>1,FII</sub> against E. coli DH5α, respectively. (b) Pairwise competition of C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>1,FII</sub>. The strains C600<sub>FII</sub>, C600<sub>I2</sub>, and C600<sub>1,FII</sub> were isogenic derivatives of E. coli C600, which harbored the plasmids FII and/or I2 (abbreviations of plasmids pLWY24J-3 and pLWY24Jmcr-1, respectively). The pairwise strains were competed in LB broth medium for 6 days, with six passages. Each boxplot represents the distribution of relative fitness values for each time point: the horizontal line in the box is the median, and the bottom and top of the box are the lowest and the highest values. Asterisks denote significant differences using unpaired Student’s t test (*, P < 0.05; **, P < 0.01; ****, P < 0.001).
Many factors could affect plasmid stability, fitness cost, and conjugation, such as sizes, copy numbers, and replications (17, 23, 24). In this study, the plasmids FII and I2 were of comparable size (Table 2) and conferred similar effects on the host cell. It is worth noting that the plasmids FII and I2 present positive interactions, which appears to be different from the transfer interactions between plasmids pLWY24J-3 and pLWY24Jmcr-1. The plasmids pLWY24J-3 and pLWY24Jmcr-1 are abbreviated FII and I2, respectively. (a) A diagram of distinct donors, recipients, and transconjugants in the dual conjugation assays. Orange ellipse represents E. coli C600, green ellipse represents E. coli J53, and small circles in ellipses represent plasmids FII and/or I2. (b and c) The transfer rates of plasmid I2. (d and e) The transfer rates of plasmid FII. Control groups are indicated in the title of each plot. Each boxplot represents the distribution of transfer rate values: the horizontal line in the box is the median, and the bottom and top of the box are the lowest and the highest values. Asterisks denote significant differences using unpaired Student's t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant).

**FIG 4** The transfer interactions between plasmids pLWY24J-3 and pLWY24Jmcr-1. The plasmids pLWY24J-3 and pLWY24Jmcr-1 are abbreviated FII and I2, respectively. (a) A diagram of distinct donors, recipients, and transconjugants in the dual conjugation assays. Orange ellipse represents E. coli C600, green ellipse represents E. coli J53, and small circles in ellipses represent plasmids FII and/or I2. (b and c) The transfer rates of plasmid I2. (d and e) The transfer rates of plasmid FII. Control groups are indicated in the title of each plot. Each boxplot represents the distribution of transfer rate values: the horizontal line in the box is the median, and the bottom and top of the box are the lowest and the highest values. Asterisks denote significant differences using unpaired Student’s t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant).
positive epistasis between small and large plasmids described by Millan et al. (17), indicating that positive interactions may be related to the plasmid nature but not to the plasmid size. Meanwhile, we conducted the plasmid copy numbers by quantitative real-time PCR according to the previous study with some modifications (17, 25, 26). The results demonstrated that the copy numbers of plasmid FII and I2 per chromosome were $1.01 \pm 0.11$ and $2.0 \pm 0.15$, respectively, while those of coinhabitant plasmids FII and I2 significantly declined to $0.84 \pm 0.31$ copies per cell (FII, $P = 0.002$) and $1.69 \pm 0.20$ (I2, $P = 0.001$) copies per cell. Although IncFII and IncI2 plasmids are low-copy-number conjugative plasmids (4), their copy numbers further decrease when they coexist in the same host, which partially explains improved persistence, better adaptability, and easier transfer of coinhabitant plasmids FII and I2. Further studies are needed to analyze other factors.

**Conclusion.** In conclusion, compared with single plasmids carried in *E. coli*, coinhabitant IncFII-type and IncI2-type plasmids in *E. coli* were stably persistent, conferred more fitness advantages, and were easier to transfer and cotransfer. Our findings highlight the advantages of coresident IncFII-type and IncI2-type plasmids in *E. coli* to drive the persistence and spread of plasmid-carried *bla*$_{CTX-M}$ and *mcr-1*, which could pose a serious threat to clinical therapy and public health.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** We used the following bacterial strains: azide-resistant *E. coli* JS3, rifampin-resistant *E. coli* C600, and *E. coli* DH5α. The conjugative plasmids, pLWY24J-3 (*bla*$_{CTX-M,55}$-bearing, 68.72 kb, IncFII; F33:A−B−, abbreviated FII) and pLWY24Jmcr-1 (*mcr-1*-carrying, 62.01 kb, IncI2 replicon, abbreviated I2), were obtained from one multidrug-resistant isolate, *E. coli* LSY24 O3:H25 ST93, from chicken in China (15) and were used to transform *E. coli* C600 or *E. coli* JS3 by electroporation, independently and together, and to generate two groups of isogenic derivatives, designated C600FII, C600I2, and C600FII:1 and J53FII, J53I2, and J53FII:1, respectively. The strains and conjugative plasmids used in the study are detailed in Table 2 and 3.

**Growth kinetics and plasmid stability.** Growth curves for *E. coli* C600 and its isogenic strains, C600FII, C600I2, and C600FII:1, were established. After overnight incubation at 37°C, the cultures were inoculated (1:1000 dilution) into four tubes containing 5 mL of fresh LB broth and inoculated at 37°C and 180 rpm. The OD$_{600}$ values were measured at intervals (0 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 14 h) using a UV spectrophotometer. Meanwhile, the culture broths were serially diluted with 0.9% saline and plated onto LB agar plates. CFU were counted after 18 h of incubation at 37°C, and the log$_{10}$ CFU values were calculated. Three independent biological replicates were performed.

To investigate plasmid stability, we propagated the isogenic strains, C600FII, C600I2, and C600FII:1, in antibiotic-free LB broth for 15 days, diluting the cultures (1:1,000) every 12 h, as described previously.

**TABLE 2 Conjugative plasmids used in this study**

| Plasmids       | Group         | Size (kb) | Abbreviation | Resistance genes       | Origin         | Yr  | Reference | Accession no |
|----------------|---------------|-----------|--------------|------------------------|----------------|-----|-----------|--------------|
| pLWY24J-3      | IncFII, F33:A−B− | 68.72     | FII          | *bla*$_{CTX-M,55}$, *bla*$_{TEM,18}$, *rmtB* | *E. coli* LSY24 | 2016 | 15        | MN702385     |
| pLWY24Jmcr-1   | IncI2         | 62.01     | I2           | *mcr-1*                | *E. coli* LSY24 | 2016 | 15        | MN689940     |

**TABLE 3 Strains used in this study**

| Strains       | Description and characteristics                                                                 | Reference | Accession no |
|---------------|-------------------------------------------------------------------------------------------------|-----------|--------------|
| *E. coli* LSY24 | O3:H25-ST93, isolated from chicken, conferred resistance to cefotaxime, gentamicin, amikacin, oxytetracycline, doxycycline, florfenicol, colistin, enrofloxacine, fosfomycin, and sulfamonomethoxine/trimethoprim. | 15        | CP054556     |
| *E. coli* DH5α | Used as a reference strain for fitness assays *in vitro*.                                       | 7         |              |
| *E. coli* C600 | Rifampin resistance, plasmid-free, used as a recipient to construct isogenic derivatives, C600FII, C600I2, and C600FII:1, and used as a reference strain for growth kinetics assays. | This study |              |
| *E. coli* JS3  | Azide resistance, plasmid-free, used as a recipient to construct isogenic derivatives, JS3FII, JS3I2, and JS3FII:1, and used as a recipient for transfer experiments. | This study |              |
| C600FII, C600I2, and C600FII:1 | Isogenic derivatives of *E. coli* C600, which harbored the plasmids FII and/or I2. | This study |              |
| JS3FII, JS3I2, and JS3FII:1 | Isogenic derivatives of *E. coli* JS3, which harbored the plasmids FII and/or I2. | This study |              |
(7, 27, 28). Periodically, the culture broths were serially diluted in 0.9% saline and plated onto LB agar. Colonies from each viable count were replica plated onto LB agar plates containing 4 mg/L of cefotaxime and/or colistin. Cultures from each viable count were replica plated onto LB agar plates containing 4 mg/L of cefotaxime and/or colistin and were randomly selected for confirmation of the presence of either CTX-M and/or mcr-1 and the corresponding replicon typed by PCR.

**In vitro fitness assays.** To determine the fitness costs associated with bearing the plasmids FII and I2, both together and apart, four strains, *E. coli* DH5α, C600φ, and C600φ, were competed in fresh LB broths according to the method described in previous studies with some modifications (16, 25). The experiment was set up with a full-factorial design so that each strain was competed against every other strain. Meanwhile, we also compared the fitness advantages of plasmid-free *E. coli* DH5α and *E. coli* C600. To initiate growth competition, each overnight culture was inoculated in fresh LB medium and grown to an OD600 of 0.5, mixed in pairs at a ratio of 1:1, 10−2 diluted into LB broth, and grown for 24 h. Then, the mixture was again diluted 10−2-fold into fresh LB broth. This procedure was repeated until the competition experiment had lasted for 144 h (6 cycles). The total number of bacteria was determined by spreading properly diluted samples of each competition mixture on LB agar containing 4 mg/L of cefotaxime and/or 2 mg/L colistin at 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h. Ten colonies per plate were randomly selected for confirmation according to the above description.

The relative fitness (RF) was calculated as follows according to the methods described previously (7, 17), using the formula RF = \( \frac{\log_{10}S_{1d0} - \log_{10}S_{1d}}{\log_{10}S_{2d0} - \log_{10}S_{2d}} \), where \( S_{1d0}, S_{1d}, S_{2d0}, \) and \( S_{2d} \) are the respective CFU densities of the strains and \( t \) is time in days. If RF is not equal to 1, there exists a fitness difference between the competitors, that is, RF > 1 indicates that there exists a fitness advantage, whereas RF < 1 represents a fitness cost. Statistical analysis was carried out via the software GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA).

**Serial transfer experiments.** To compare the relative transfer speeds of the plasmids FII and I2, the retransfer experiments were carried out using isogenic derivatives of *E. coli* C600 as the donor and *E. coli* J53 as the recipient. The donors were three settings as follows: (i) the donor cell was C600φ or C600φ, (ii) the donor cell was C600φ, or (iii) the donor cell was composed of a 1:1 mixture of C600φ and C600φ. Overnight cultures were dilated 1:1,000 in fresh LB broth and incubated with shaking at 37°C to an OD600 of 0.5 and then mixed in pairs in a 4.0 mL total volume at a ratio of 1:1 and mated for 0 min, 5 min, 10 min, 20 min, and 60 min at 37°C. Matings were stopped by cooling the samples on ice for 1 min. Next, the samples were centrifuged at 4°C and 1,000 rpm for 10 min, 3.5 mL of supernatants was discarded, and then the residues were vigorously vortexed for 1 min to remix evenly. The mixtures (100 µL per plate) were plated on 5 agar plates supplemented with colistin and/or cefotaxime. The plates were subsequently incubated overnight at 37°C to allow colony formation and to count. Ten colonies per plate were randomly chosen for confirmation as described above. Experiments were repeated in three separate assays.

To compare intracellular and intercellular interactions between the plasmids FII and I2 on transfer efficiencies, the dual conjugation assays were further performed using isogenic derivatives of *E. coli* C600 as the donor and *E. coli* J53 as the recipient. The donors were five settings as follows: (i) the donor cell was C600φ or C600φ, (ii) the donor cell was C600φ, or (iii) the donor cell was composed of a 1:1 mixture of C600φ and C600φ. Overnight cultures were dilated 1:1,000 in fresh LB broth and incubated with shaking at 37°C to an OD600 of 0.5 and then mixed in pairs in a 4.0 mL total volume at a ratio of 1:1 and mated for 0 min, 5 min, 10 min, 20 min, and 60 min at 37°C. Matings were stopped by cooling the samples on ice for 1 min. The samples were centrifuged at 4°C and 1,000 rpm for 10 min, 3.5 mL of supernatants was discarded, and then the residues were vigorously vortexed for 1 min to remix evenly. The mixtures (100 µL per plate) were plated on 5 agar plates supplemented with colistin and/or cefotaxime. The plates were subsequently incubated overnight at 37°C to allow colony formation and to count. Ten colonies per plate were randomly chosen for confirmation as described above. Experiments were repeated at least three independent biological replicates were included for each sample. Finally, mating efficiencies were calculated and evaluated by statistical analyses.

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**REFERENCES**

1. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168. https://doi.org/10.1016/S1473-3099(15)00424-7.

2. Bevan ER, Jones AM, Hawkey PM. 2017. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. J Antimicrob Chemother 72:2145–2155. https://doi.org/10.1093/jac/dkx146.

3. Mahéraud AC, Kembel H, Magnan M, Gachet B, Roche D, Le NH, Tenaillon O, Denamur E, Branger C, Landraud L. 2019. Advantages of the F2A1:B-IncFII pandemc plasmid over IncC plasmids in vitro acquisition and evolution of blaCTX-M gene bearing plasmids in *Escherichia coli*. Antimicrob Agents Chemother 63:e01130-19. https://doi.org/10.1128/AAC.01130-19.

4. Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez ZB, Guerra B, Mevis DJ, Hordijk J. 2018. Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. J Antimicrob Chemother 73:1121–1137. https://doi.org/10.1093/jac/dkx488.

5. Wang J, Zeng ZL, Huang XY, Ma ZB, Guo ZW, Lv LC, Xia YB, Zeng L, Song QH, Liu JH. 2017. Evolution and comparative genomics of F33: A1–B–plasmids carrying blaCTX-M-55 or blaCTX-M-65 in *Escherichia coli* and *Klebsiella pneumoniae* isolated from animals, food products, and humans in China. mSphere 3:e00137-18. https://doi.org/10.1128/mSphere.00137-18.

6. Furlan JP, Lopes R, Gonzalez IH, Ramos PL, Stehling EG. 2020. Comparative analysis of multidrug resistance plasmids and genetic background of CTX-M-producing *Escherichia coli* recovered from captive wild animals. Appl Microbiol Biotechnol 104:6707–6717. https://doi.org/10.1007/s00253-020-10670-4.
16. Morton ER, Platt TG, Fuqua C, Bever JD. 2014. Non-additive costs and interactions alter the competitive dynamics of co-occurring ecologically distinct plasmids. Proc Biol Sci 281:20132173. https://doi.org/10.1098/rspb.2013.2173.

17. Millan AS, Heilbron K, MacLean RC. 2014. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. ISME J 8:601–612. https://doi.org/10.1038/ismej.2013.182.

18. Gama JA, Zilhão R, Dioníssio F. 2017. Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. Plasmid 93:6–16. https://doi.org/10.1016/j.plasmid.2017.08.003.

19. Gama JA, Zilhão R, Dioníssio F. 2017. Multiple plasmid interference – pledging allegiance to my enemy’s enemy. Plasmid 93:17–23. https://doi.org/10.1016/j.plasmid.2017.08.002.

20. Gama JA, Zilhão R, Dioníssio F. 2017. Co-resident plasmids travel together. Plasmid 93:24–29. https://doi.org/10.1016/j.plasmid.2017.08.004.

21. Choi Y, Lee JY, Lee H, Park M, Kang K, Lim SK, Shin D, Ko KS. 2020. Comparison of fitness cost and virulence in chromosome and plasmid-mediated colistin-resistant Escherichia coli. Front Microbiol 11:798. https://doi.org/10.3389/fmicb.2020.00798.

22. Anyanwu MU, Jaya IF, Nwobi OC. 2020. Occurrence and characteristics of mobile colistin resistance (mcr) gene-containing isolates from the environment: a review. JEBPH 17:1028. https://doi.org/10.3390/jjerp17031028.

23. Virolle C, Goldlust K, Djermoun S, Bigot S, Lesterlin C. 2020. Plasmid transfer by conjugation in Gram-negative bacteria: from the cellular to the community level. Genes 11:1239. https://doi.org/10.3390/genes11111239.

24. Pinto UM, Pappas KM, Winans SC. 2012. The ABCs of plasmid replication and segregation. Rev Nat Microbiol 10:75–76. https://doi.org/10.1038/nmico2882.

25. Yang J, Wang HH, Lu YY, Yi LX, Deng YY, Lv LC, Burrus V, Liu JH. 2021. A ProQ/FinO family protein involved in plasmid copy number control favours fitness of bacteria carrying mcr-1-bearing IncI2 plasmids. Nucleic Acids Res 49:3981–3996. https://doi.org/10.1093/nar/gkab149.

26. Lee C, Kim J, Shin SG, Hwang S. 2006. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. J Biotechnol 123:273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014.

27. Wang J, Guo ZW, Zhi CP, Yang T, Zhao JJ, Chen XJ, Zeng L, Lv LC, Zeng ZL, Liu JH. 2017. Impact of plasmid-borne qnrAB on the development of fluoroquinolone resistance and bacterial fitness in Escherichia coli. J Antimicrob Chemother 72:1293–1302. https://doi.org/10.1093/jac/dkw576.

28. Dorado MP, Garcillán BMP, Lasa I, Solano C. 2021. Fitness cost evolution of natural plasmids of Staphylococcus aureus. mBio 12:e03094-20. https://doi.org/10.1128/mBio.03094-20.