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Muhammad Kamruzzaman

muhammad.kamruzzaman@sydney.edu.au

Westmead Institute for Medical Research, The University of Sydney <https://orcid.org/0000-0003-2005-9846>

Janani Jeyachandran

Jonathan Iredell

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Leading region anti-defense genes drive conjugation success of IncM and IncL plasmids

Muhammad Kamruzzaman^{1, 2, 3*}, Janani Jeyachandran¹, Jonathan R. Iredell^{1, 2, 3, 4*}

¹Centre for Infectious Diseases and Microbiology, The Westmead Institute for Medical Research,
Westmead, New South Wales, Australia;

²Faculty of Medicine and Health, University of Sydney, Sydney, New South Wales, Australia

³Sydney Infectious Diseases Institute, The University of Sydney, New South Wales, Australia

⁴Westmead Hospital, Westmead, New South Wales, Australia;

***Corresponding authors:** muhammad.kamruzzaman@sydney.edu.au (MK), and
jonathan.iredell@sydney.edu.au (JI)

Abstract

Conjugative plasmids are major drivers of horizontal gene transfer and play a central role in the dissemination of antibiotic resistance (AbR) genes among bacteria. To establish successfully in new hosts, these plasmids must overcome recipient defenses, often through anti-defense proteins expressed from the leading DNA strand immediately upon transfer. IncM and IncL plasmids are globally significant vectors for dissemination of important AbR genes, yet the key leading-strand determinants underlying their success have remained unclear. Here, we systematically analysed the leading regions of these plasmids and identified a network of previously uncharacterised anti-defense genes driven by single-strand-specific promoters. Experimental deletion of this region resulted in a marked reduction in conjugation efficiency across diverse bacteria. This reduction was linked to the presence of recipient restriction enzymes targeting incoming plasmid DNA. Together, our findings reveal the leading region as a critical anti-defense hub that promotes efficient conjugation and establishment of high-risk AbR plasmids.

Introduction

Bacterial conjugation is a key process of horizontal gene transfer, enabling the direct transfer of genetic material, such as plasmids, from a donor to a recipient cell (1, 2). This mechanism significantly contributes to bacterial evolution and dissemination of adaptive traits, including antibiotic resistance and pathogenicity (3, 4).

During conjugation, plasmid DNA is typically transferred via a specialised mating apparatus as a single-stranded molecule (ssDNA), beginning at the origin of transfer (*oriT*) (1, 5). Successful establishment of a plasmid in the recipient bacterium requires not only the physical transfer of DNA but also its protection and integration into the new cellular environment. Recipient bacteria deploy a variety of defense mechanisms, such as restriction-modification (R-M) systems, CRISPR-Cas immunity and other innate nucleic acid-targeting mechanisms that act to degrade or inactivate foreign genetic elements (6-8).

Successful conjugative plasmids commonly encode powerful anti-defense strategies (4, 9-12), several of which are located on the first (leading) strand of the plasmid DNA to enter the recipient cell during conjugation (13, 14). This strategic positioning ensures gene expression immediately upon entry and increases the likelihood of successful establishment and replication in the new host. These include the genes encoding anti-restriction proteins, CRISPR inhibitors/anti-CRISPR proteins, DNA methyl transferases, SOS inhibitors, TA anti-toxins, ssDNA binding proteins and nucleoid-associated proteins that modulate host responses (14).

IncM and IncL plasmids have emerged as important vectors in the spread of critical resistant genes, including carbapenemase and extended-spectrum β -lactamase (ESBL) genes such as *bla*_{IMP-4}, *bla*_{NDM}, *bla*_{KPC} and *bla*_{CTX-M} (15). Initially, IncL and IncM plasmids were grouped in a single

incompatibility group (16) but have distinct incompatibility determinant regions (*inc*), relaxase (*traX*), and entry exclusion genes (*traY*, *excA*) (17). The important role of IncM plasmids in the spread of antibiotic resistance can be attributed, at least in part, to their broad host range, stable maintenance and efficient conjugative transfer (18, 19).

In this study, we analysed previously uncharacterised genes in the leading regions of IncM and IncL plasmids to identify candidate anti-defense systems and experimentally assessed their role in the conjugative transfer of IncM plasmids across diverse bacterial hosts. We also identified defense systems encoded by recipient bacterial chromosomes.

Results

The leading region of the IncM plasmid is enriched with diverse anti-defense genes.

Anti-defense genes are frequently enriched in the first ~30 open reading frames (ORFs) to enter the host upon conjugative transfer (14). We analysed the ~12.5 kb leading region of the IncM plasmid pJIBE401 (20), which differs by only a single nucleotide polymorphism from the reference IncM plasmid pEl1573 (21). This region comprises 22 protein-coding ORFs, of which only two are currently annotated as anti-defense genes: encoding a single-strand binding protein (Ssb) and an anti-restriction protein (KlcA). One additional gene encodes the transcriptional regulator KorC, while the 17 ORFs are annotated as hypothetical proteins or proteins of unknown function.

To refine these annotations, we performed comprehensive reannotation, NCBI BLASTp search and conserved domain analysis. This approach identified several additional candidate anti-defense and regulatory genes, including three putative methyltransferases, one additional putative anti-

restriction protein, a DNA repair protein (RadC), two putative antitoxins (HicB-like and MqsA-like) of type II toxin–antitoxin system, and two transcriptional regulators (KorB-like and Hha-like). Overall, this ~12.5 kb region encodes nine putative anti-defense genes (three methyltransferases, two anti-restriction proteins, two type II antitoxins, one DNA repair protein, and Ssb) alongside three transcriptional regulators (KorC, KorB-like, and Hha-like) (Fig.1A). These findings indicate that the IncM leading region serves as a concentrated reservoir of diverse anti-defense functions, details of which are provided in Table 1.

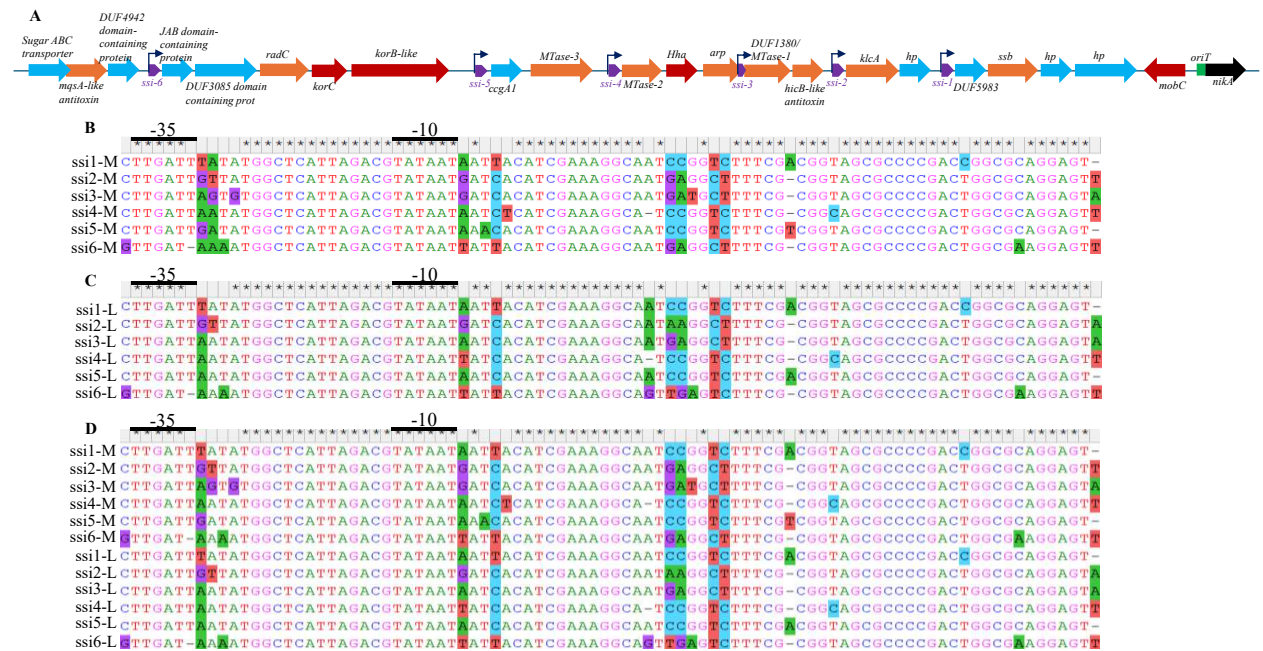


Fig. 1. Genetic organisation of IncM plasmid leading region with newly annotated putative anti-defense candidates. Plasmid putative anti-defense genes, regulatory genes, and single-strand-specific promoter regions present in the ~12.5 kb leading region of IncM plasmid pJIBE401 are shown (A). The green bar represents the plasmid origin of transfer (oriT) region, and the orange arrows indicate putative candidates of different anti-defense genes. The red arrows represent regulatory genes, and the blue arrows denote genes with unknown functions. Six single-strand

specific promoter regions are shown by small purple arrows (*ssiI-6*). **(B-D)** Nucleotide sequence alignments of the putative single-strand-specific promoter sequences identified in the leading region of the IncM and IncL plasmids. Alignments of all six promoter sequences (*ssiI-6*) of IncM **(B)**, IncL **(C)** and IncM and IncL together **(D)** are shown. Asterisks on top of the alignment are for identical nucleotides, and colour shadings are for non-identical nucleotides. The -10 and -35 promoter regions are marked with a thick black line over the aligned nucleotides.

Table 1. List of putative anti-defense genes on the leading region of IncM plasmids and their relative position from the oriT

Gene	Description	Distance from the oriT
<i>ssb</i>	Single-strand binding protein	~2.1 kb
<i>klcA</i>	Anti-restriction protein	~3.3 kb
<i>hicB</i> -family antitoxin	Endoribonuclease anti-toxin of type II TA	~3.9 kb
<i>DUF1380/MTase-1</i>	Putative methyltransferase	~4.3 kb
<i>arp</i>	Putative anti-restriction protein	~4.8 kb
<i>Hha-like</i>	Putative co-regulator of H-NS	~5.3 kb
<i>MTase-2</i>	Methyltransferase	~5.6 kb
<i>MTase-3</i>	Methyltransferase	~6.2 kb
<i>KorB-like</i>	Transcriptional repressor	~7.9 kb
<i>korC</i>	Transcriptional regulator	~9.3 kb
<i>radC</i>	DNA repair protein	~9.6 kb
<i>mqsA</i> -like	Endoribonuclease anti-toxin of type II TA	~11.4 kb

Multiple single-stranded specific promoter sequences in the IncM plasmid leading region.

Plasmid leading region anti-defense genes are usually located in the T-strand in such an orientation that the transcription can be initiated immediately in recipient bacteria, well before completion of plasmid transfer and strand pairing as dsDNA. The leading region also carries single-strand-specific promoter sequences, also known as *Frpo* or *ssi*, with secondary structures that facilitate binding of RNA polymerase to start transcription of these anti-defense genes from ssDNA (22-24). We identified six *ssi*-like sequences (*ssi*-1 to *ssi*-6) carrying conserved -35 and -10 elements (Fig. 1A, B) in the leading region of IncM plasmids, suggesting that these *ssi*-like sequences may work as promoters for the early transcription of diverse genes from the single-stranded leading region of IncM plasmids immediately after the transfer into the recipient bacteria. The length of the *ssi*-like sequences identified in the IncM plasmid is 89-90 bp, and all six *ssi*-like sequences are composed of closely identical nucleotide sequences.

Five out of six *ssi*-like promoter sequences are identified immediate upstream of the putative anti-defense genes and another one is located upstream of the DUF3085 and downstream genes are *radC* and *korC* (Fig. 1A). One *ssi*-like sequence was identified upstream of the *ssb* (single strand binding protein) gene, and it was reported that transcription from *ssi* is highly stimulated by the Ssb protein (22). The localisation of the *ssb* gene in the very beginning of the IncM leading strand and the presence of a single-strand-specific promoter immediately upstream of *ssb* is consistent with the known role of Ssb in stabilising ssDNA, promoting interactions early in translocation and facilitating transcription from other *ssi*-like promoter regions (22, 25).

IncM and IncL plasmids share a very similar leading region.

IncL plasmids are closely related to IncM plasmids and both are within the same plasmid MOB (relaxase) subtype MOB_{P13} (26) and are therefore expected to share a highly similar leading-region architecture. Consistent with this, alignment of the ~12.5 kb leading regions of IncM and IncL plasmids revealed 98.4% nucleotide identity, with even higher conservation at the amino acid level (Fig. S1). Both regions encode an identical set of open reading frames (ORFs) and a close match of the six leading region *ssi*-like sequences (Fig. 1C, D). We therefore selected the IncM plasmid as a representative model for subsequent experimental analyses.

Leading-region genes of IncM plasmids are expressed early in recipient cells during conjugation.

Previous studies have experimentally demonstrated the early expression of genes located in the leading regions of conjugative plasmids, such as IncI and IncP, in recipient bacteria during plasmid transfer (9, 22, 27). We performed quantitative real-time PCR (qRT-PCR) during time-course mating experiments. Filter mating conjugation assays were conducted using *Escherichia coli* J53Az carrying an IncM plasmid as the donor and *E. coli* UB5201Rf as the recipient, as previously described (20). Samples were collected at 0, 15, 30, 45, and 60 minutes post-mating, and transcript levels of selected genes were quantified (S1 Table), which revealed elevated expression of all tested leading-region genes during conjugation (Fig. 2A), indicating active transcription shortly after transfer into recipient cells. We examined 13 genes, including eight candidate anti-defense genes, two transcriptional regulators, a DUF3085 domain-containing protein, the *pemI* antitoxin (located outside the leading region) and the bacterial chromosomal *recA* gene. Notably, expression of key anti-defense candidates, such as the *arp* anti-restriction gene, *hicB*-like antitoxin and *mqsA*-like antitoxin gene, was detectable as early as 15 minutes after mating. Most other genes were expressed from 30 minutes onward, excepting one putative methyltransferase (*MTase-I*), which

showed delayed expression beginning at 45 minutes. The *pemI* antitoxin gene, located outside the leading region, showed no detectable expression during the mating time course and served as a negative control for early transcriptional activation. Chromosomal *recA* gene expression was detected during conjugation, suggesting the accumulation of single-stranded DNA molecules in the recipient bacteria after conjugation transfer of the plasmid DNA, associated with the ssDNA specific bacterial SOS response (28).

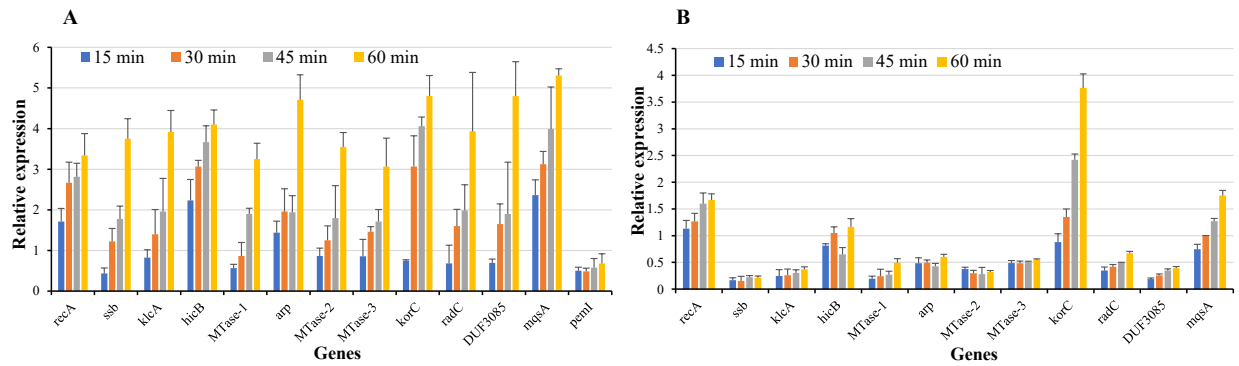


Fig. 2. Relative expression of wild-type conjugation-efficient (A) and conjugation-deficient (B) IncM plasmid leading region genes during solid mating conjugation experiments.

Conjugation experiments were performed between donors J53Az (pJIMK45) or J53Az (pJIMK45 Δ traY-excA) and recipient UB5201Rf strains. Data are from the three independent solid mating experiments with standard errors.

traY and *excA* genes are essential for conjugative transfer, and deletion completely abolishes the transfer capability of IncM plasmids (18). We therefore constructed a frameshift deletion mutant of the *traY-excA* genes on the IncM plasmid pJIMK45 (Fig. S2 and Table S1) to confirm that the elevated expression of leading-region genes observed during conjugation originates from recipient cells after plasmid transfer rather than from donor cells.

Conjugation assays between *E. coli* J53Az (pJIMK45 Δ *traY-excA*) as the donor and *E. coli* UB5201Rf as the recipient revealed markedly reduced expression of all those leading-region genes that were shown to be upregulated in the wild-type plasmid (Fig. 2B). No significant *recA* induction was observed, consistent with the lack of transfer of single-stranded plasmid DNA. This result indicates that elevated transcription of these genes depends on successful DNA transfer into recipient cells.

Interestingly, *korC* and the *mqsA*-like gene retained elevated expression even under non-conjugative conditions. KorC is a DNA-binding transcriptional regulator that represses operons involved in stable maintenance functions and is typically upregulated during an SOS response (29). In contrast, the *mqsA*-like gene was previously annotated as a hypothetical protein; however, our reannotation identified homology to the MqsA antitoxin of a type II toxin–antitoxin system, and domain analysis revealed the presence of a predicted DNA-binding domain. MqsA is an *rpoS* repressor, with more direct effects on dampening the host bacterial response to incoming ssDNA (30). These features suggest possible roles for the MqsA-like protein as: (i) an antitoxin capable of neutralising endogenous toxins, particularly endoribonucleases, and/or (ii) a transcriptional regulator, possibly suppressing SOS responses to incoming DNA. The sustained expression of both *korC* and *mqsA*-like in donor cells suggests functions in maintaining plasmid stability and/or regulating transcriptional networks prior to transfer, as well as important roles in adaptation to the new host environment by coordinating the expression of anti-defense genes.

Leading-region genes directly contribute to the successful conjugative transfer of IncM plasmids.

To determine the functional contribution of leading-region anti-defense genes to IncM plasmid transfer, we next compared conjugation efficiencies between a wild-type IncM plasmid (pJIMK45,

a derivative of pJIBE401, S2 Table) and derivatives carrying targeted deletions within the leading region.

We first constructed a partial deletion mutant, pJIMK45 Δ 2.2LR, in which ~2.2 kb at the beginning of the leading region was removed (Fig. S3A, Table S2). This deleted segment includes two putative anti-defense genes, *ssb* and *klcA*, alongside two other genes of unknown function. Conjugation assays into three *E. coli* recipient strains of the wild-type IncM plasmid pJIMK45 from *E. coli* J53Az as donor revealed no significant differences in transfer frequency between the wild-type plasmid (pJIMK45) and the Δ 2.2 kb mutant (Fig. 3A). The deleted region is a small part of the leading region, which has only two putative anti-defense genes, one anti-restriction *klcA* and one *ssb*. The anti-restriction protein KlcA was found to be very weak and unable to block normal restriction endonuclease activity (31). Maybe the other anti-defense gene products in the leading region, or their combined effects, are necessary to observe a significant difference in the conjugation transfer of the IncM plasmid.

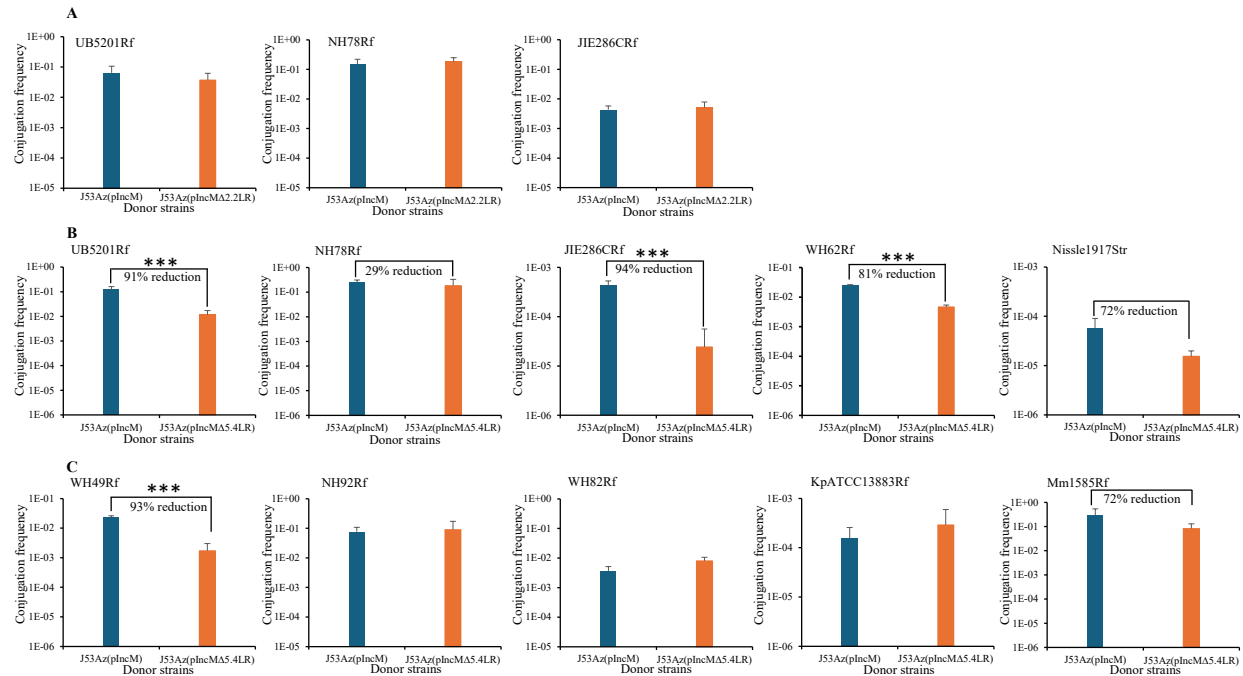


Fig. 3. Conjugation frequency comparison between the IncM plasmid and the leading region-deleted versions of the IncM plasmid (panel A, ~2.2 kb deletion; panels B and C, ~5.4 kb deletion) in different Enterobacteriaceae species. Donor strain is J53Az carrying IncM plasmid pJIMK45 or pJIMK45Δ2.2LR or pJIMK45Δ5.4LR, and the recipient is different *E. coli*, *Klebsiella pneumoniae* and *Morganella morganii* strains, all resistant to rifampicin, except Nissle1917, which is resistant to streptomycin. The recipient strain's name is at the top of each graph. Panels A and B: all *E. coli*; panel C: the first four are *K. pneumoniae*, and the last one is an *M. morganii* strain. Data are the mean of three independent solid mating experiments with standard errors. *P* value <0.05 are considered statistically significant and denoted by asterisks.

A second mutant, pJIMK45Δ5.4LR, has a larger ~5.4-kb segment of the leading region deleted. This includes the ~2.2 kb segment described above plus an additional ~3.2 kb encompassing one additional anti-restriction gene, three putative methyltransferase (MTase) genes, one HicB-like

241 anti-toxin gene, and an Hha-like regulatory protein (Fig. S3B, Table S2). Importantly, this deletion
 242 removes seven of the nine predicted anti-defense genes located within the ~12.5 kb leading region.
 243 We then measured the conjugation transfer frequency of the IncM plasmids pJIMK45 (intact
 244 leading region) and pJIMK45 Δ 5.4LR from *E. coli* J53AZ to recipients of different
 245 Enterobacteriaceae species, including laboratory and clinical strains of *E. coli*, *K. pneumoniae*, and
 246 *Morganella morganii* (S3 Table). Conjugation assays using this mutant revealed a marked
 247 reduction in transfer efficiency across a diverse panel of recipient bacteria. In five *E. coli* strains,
 248 including both laboratory and clinical isolates, as well as the probiotic strain *E. coli* Nissle1917,
 249 the Δ 5.4 kb mutant exhibited a 29–94% decrease in conjugation frequency compared to the wild-
 250 type plasmid (Fig. 3B). The conjugation frequency of pJIMK45 Δ 5.4LR was significantly reduced
 251 in three *E. coli* strains (81%, 91%, and 94%). This strongly suggests that the leading region of the
 252 IncM plasmid plays a pivotal role in the successful transfer and subsequent establishment of IncM
 253 and similar plasmids (e.g., IncL) in recipient bacteria.
 254 We next extended our analysis to other Enterobacteriaceae. Conjugation assays were performed
 255 using four *Klebsiella pneumoniae* strains (one reference strain and three clinical isolates) and one
 256 *Morganella morganii* strain as recipients. The Δ 5.4 kb mutant exhibited a significant reduction in
 257 transfer frequency in one clinical *K. pneumoniae* isolate (93% decrease), but no measurable effect
 258 in the remaining *K. pneumoniae* strains (Fig. 3C). In contrast, transfer into *M. morganii* was
 259 substantially impaired, with a 72% reduction in conjugation efficiency.
 260 These species- and strain-dependent differences likely reflect variation in host defense systems. *K.*
 261 *pneumoniae* is a major reservoir for IncM and IncL plasmids in clinical settings. The relatively
 262 modest impact of leading-region deletion in most *K. pneumoniae* strains may indicate that these
 263 plasmids are well adapted to this species or that *K. pneumoniae* have weaker defense mechanisms

against IncM plasmids. In contrast, the stronger effects observed in *E. coli* and *M. morganii* suggest that these hosts impose more stringent barriers to plasmid establishment, necessitating a broader array of anti-defense mechanisms. This is consistent with epidemiological observations that IncM and IncL plasmids are more frequently associated with *K. pneumoniae* than *E. coli*, despite both being common members of the Enterobacteriaceae (17, 32-34). Also, these plasmids have numerous anti-defense weapons in their leading regions against defense mechanisms of *E. coli* and other Enterobacteriaceae, and those anti-defense genes may contribute significantly to the success of these plasmids in disseminating into broad-host-range bacterial species.

Host defense systems shape the impact of leading-region anti-defense genes on conjugation efficiency.

The strain-specific variation in conjugation efficiency observed for the leading-region deletion mutant (pJIMK45Δ5.4LR) across Enterobacteriaceae prompted us to investigate whether these differences correlate with the presence of host defense systems, particularly restriction-modification (RM) systems. Notably, the absence of any detectable reduction in conjugation frequency in most *Klebsiella pneumoniae* isolates and in one *E. coli* ST131 isolate suggested that host-encoded restriction barriers may play a key role in determining the contribution of plasmid-encoded anti-defense functions. Whole-genome sequencing of all recipient strains using Oxford Nanopore long-read sequencing, with annotation of chromosomal assemblies using Bakta and Restriction-ModificationFinder1.1 (CGE Server) revealed that four of the *E. coli* isolates encoded multiple restriction enzymes, including EcoKI, Eco31I, EcoAI/EcoEI, and EcoMII, whereas one isolate lacked detectable restriction enzyme genes (Table 2). IncM plasmid also carries a variable number of cutting sites for most of those enzymes (Table 2). Importantly, the presence of these RM systems strongly correlated with the observed conjugation phenotype:

strains harbouring restriction enzymes showed a significant reduction in conjugation efficiency of the leading-region-deleted plasmid, whereas no difference was observed in the restriction enzyme negative strains (Fig. 3 and Table 2).

Table 2. Restriction enzyme present in the recipient bacterial chromosome and their cutting sites on the plasmid tested

Recipient strain	Species	Restriction enzyme	Number of RE cutting sites on pJIMK45
UB5201Rf	<i>E. coli</i>	EcoKI, EcoKMcrA, EcoKMrr, EcoKMcrBC(EcoKMcrB), EcoKMcrBC(EcoKMcrC)	EcoKI= 5; others cut methylated DNA only
JIE286Rf	<i>E. coli</i>	Eco31I, EcoMII, Eco13461III	Eco31I=0, EcoMII=7/8 sites, Eco13461III=N/A
Nissle1917-str	<i>E. coli</i>	Eco31I, EcoMII	Eco31I=0, EcoMII=7/8
WH62Rf	<i>E. coli</i>	EcoAI/EcoEI, Type II Restriction endonuclease	EcoAI=5, EcoEI=9
NH78Rf	<i>E. coli</i>	None	N/A
WH82Rf	<i>K. pneumoniae</i>	None	N/A
NH92Rf	<i>K. pneumoniae</i>	None	N/A
WH49Rf	<i>K. pneumoniae</i>	None	N/A
KpATCC13883Rf	<i>K. pneumoniae</i>	None	N/A
Mm1585Rf	<i>M. morganii</i>	EcoAI/EcoEI, EcoKI	EcoAI=5, EcoEI=9; EcoKI=5

Although most *K. pneumoniae* isolates showed no difference, one clinical isolate (WH49) displayed a significant reduction in transfer efficiency between wild-type and mutant plasmids despite lacking any RM systems recognised by Restriction-ModificationFinder1.1. This suggests the presence of uncharacterised defense mechanisms, such as novel restriction systems or other defense modules that remain undetected by current annotation tools. In *Morganella morganii*, the presence of EcoAI/EcoEI and EcoKI restriction enzyme genes was associated with a marked reduction in conjugation efficiency (~72%) of the Δ 5.4 kb mutant plasmid compared to the wild-type (Fig. 3C).

Discussion

The early events that occur in the recipient cell immediately after plasmid transfer are increasingly recognised as critical determinants of horizontal gene transfer success (9, 14, 22). In this study, we demonstrate that the leading region of IncM plasmids constitutes a highly specialised genetic module that is enriched in anti-defense genes, equipped with multiple single-stranded DNA (ssDNA)-responsive promoter sequences, and rapidly expressed upon entry into recipient cells. These features collectively promote plasmid establishment by overcoming host defense systems during the vulnerable period between DNA transfer and plasmid replication. Our findings reveal that the leading region is not merely a conserved structural component of IncM plasmids but functions as an adaptive regulatory and anti-defense platform that directly enhances conjugation efficiency.

The enrichment of multiple classes of anti-defense genes within this region suggests a co-ordinated strategy to counteract several layers of host defense, and particularly highlight the importance of restriction enzymes: putative methyltransferases to protect incoming plasmid DNA from endonuclease restriction by mimicking host methylation patterns (35), anti-restriction proteins to directly inhibit restriction enzymes (31), putative HicB-like and MqsA-like type II anti-toxins to directly neutralise bacterial endonucleases (36) and RadC-like proteins for DNA repair.

Conjugative DNA transfer is known to induce a bacterial SOS response in response to single-stranded DNA intermediates (28). To counteract this, many conjugative plasmids, including members of the IncF and IncI groups, encode PsiB, which inhibits RecA activation and thus SOS induction (9, 37, 38). Transfer of PsiB and the ssDNA-binding protein Ssb by the plasmid conjugation apparatus type 4 secretion system (T4SS) directly suppresses the SOS response to the incoming ssDNA transfer intermediate (the T strand covalently bound at the 5' end with relaxase)

(39). This enhances plasmid stability and transfer efficiency by limiting host stress responses and tends to be associated with narrow host-range plasmids of higher G-C content (40). However, the G-C content of the IncM plasmid we used in this experiment is 52.2%, and the G-C content of IncM and IncL plasmids generally ranges from 52-55%.

Nucleoid-associated heat-stable nucleotide structuring (H-NS) proteins have a silencing role on expression of plasmid *traJ* and chromosomal *arcA*, providing a strategy in IncF type (MOB_{F12A}) ‘narrow-host range’ plasmids, such as R1, to control or suppress the development of transformation competence (41). IncM plasmid lacks both *psiAB* and *hns* but retains high conjugation efficiency in its usual hosts (18, 20, 42).

In our experiments, induction of *recA* expression in recipient cells was observed early during conjugation, consistent with the expected SOS response to incoming single-stranded DNA. The absence of PsiB-like activity suggests that IncM plasmids tolerate, rather than suppress, the SOS response during transfer. This observation highlights a potentially distinct strategy of defense evasion, in which protection against restriction enzymes and other host barriers may be sufficient to ensure successful establishment without directly modulating SOS signalling. Alternatively, other uncharacterised genes in the leading region may partially mitigate SOS-associated stress through indirect mechanisms. Early and sustained expression of the transcriptional regulator KorC, the HicB-like protein, and the MqsA-like protein (Fig. 2A) suggests important roles of these proteins in the early establishment of the plasmid. HicB is an antitoxin of the type II TA system, HicBA, and neutralises the RNA endoribonuclease toxin, HicA. HicA domain-containing proteins are widely distributed in bacteria and play a major role as a bacterial defense against bacteriophages, plasmids, and other mobile genetic elements (43). The presence of a HicB-like gene within the leading region of IncM and IncL plasmids, together with its rapid and sustained

349 expression in recipient cells during conjugation, suggests that it functions as an anti-defense factor.
350 Specifically, the HicB-like protein may counteract host HicA-domain toxins and other mRNA-
351 targeting endoribonucleases, thereby promoting the efficient expression of incoming plasmid
352 genes and facilitating successful plasmid establishment following transfer.

353 The DUF3085 domain-containing protein identified in the leading region is preceded by a putative
354 single-strand-specific promoter. DUF3085 has been highlighted as an indicative marker of
355 successful broad host range plasmids and the highly conserved DUF3085 RNA secondary
356 structure is thought to act as an important cis-regulatory element in DUF3085 domain-containing
357 proteins found in conjugative plasmids and integrative conjugative elements (ICEs) (40). The
358 IncM MOB_{P13} plasmid pJIBE401 has been previously identified as a broad-host-range plasmid
359 (44), and the presence of DUF3085 domain-containing protein further confirms its broad-host-
360 range nature.

361 Functional studies further demonstrated that leading-region genes actively promote successful
362 plasmid transmission, as disruption of the leading region reduced transfer efficiency. These
363 findings indicate that conjugation depends not only on DNA transfer machinery but also on post-
364 transfer establishment functions that help incoming DNA survive, replicate, and persist in recipient
365 cells. Importantly, the contribution of anti-defense genes depended on the recipient's defense
366 repertoire, indicating that their fitness benefits are context-dependent.

367 Together, these findings establish a clear relationship between host defense capacity and the
368 requirement for plasmid-encoded anti-defense systems. Specifically, the presence of active RM
369 systems in the recipient bacterium increases reliance on leading-region anti-defense genes for
370 successful plasmid transfer and establishment. Even when plasmids contain multiple restriction
371 sites targeted by host enzymes, the presence of a full complement of anti-restriction and

372 methyltransferase genes enables efficient transfer. In contrast, removal of these genes renders the
373 plasmid highly susceptible to host restriction, resulting in a substantial decrease in conjugation
374 efficiency (Fig. 3B, C; Table 2).

375 RM systems are among the most widespread and effective bacterial defense mechanisms and are
376 known to play a central role in limiting horizontal gene transfer, including plasmid conjugation
377 and bacteriophage infection (10, 45-47). Classic studies have demonstrated that Type I restriction
378 enzymes such as EcoKI and EcoAI can significantly reduce plasmid transfer efficiency by
379 degrading unmethylated incoming DNA (10, 48). The strength of this barrier often correlates with
380 the number and distribution of restriction enzyme recognition sites within the plasmid sequence.
381 However, plasmids have evolved multiple strategies to counteract these defenses. Anti-restriction
382 proteins can directly inhibit restriction enzymes, while plasmid-encoded methyltransferases can
383 pre-modify DNA to mimic host methylation patterns, thereby avoiding cleavage (10). Our findings
384 are consistent with this model and demonstrate that IncM plasmids rely on a combination of these
385 mechanisms to overcome host barriers. The cumulative loss of multiple such genes in the $\Delta 5.4$ kb
386 mutant substantially compromises this defense, highlighting the importance of redundancy and
387 functional diversity in plasmid survival strategies.

388
389 At the mechanistic level, the localisation of these anti-defense genes within the leading region is
390 likely to be critical. During conjugation, plasmid DNA is transferred as a single-stranded molecule,
391 which is initially less susceptible to restriction enzymes that predominantly target double-stranded
392 DNA. However, once complementary strand synthesis begins, the plasmid becomes vulnerable to
393 restriction. The presence of single-strand-specific promoters in the leading region enables rapid
394 transcription of anti-defense genes immediately upon entry into the recipient cell. This early

expression likely allows the plasmid to pre-emptively neutralise restriction enzymes before the formation of double-stranded DNA, thereby enhancing survival and establishment.

Moreover, the presence of multiple transcriptional regulators, including KorC-, KorB- and Hha-like proteins, suggests a tightly controlled regulatory network governing gene expression in the leading region. Such regulation may be critical to balance early expression of anti-defense functions with the later establishment of plasmid maintenance and replication systems.

Despite these insights, the precise mechanisms by which individual anti-defense genes contribute to defense evasion remain incompletely understood. In particular, the roles of newly identified or poorly characterised proteins, such as DUF3085-containing proteins or atypical regulators, require further investigation. Dissecting the specific interactions between plasmid-encoded anti-defense systems and host defense pathways will be essential to fully understand the molecular basis of plasmid dissemination.

From a broader perspective, these findings have important implications for antimicrobial resistance spread. IncM and IncL plasmids are major vectors of clinically relevant resistance genes, and their ability to efficiently transfer across diverse bacterial hosts is a key factor in their global dissemination. Targeting anti-defense systems in the leading region may represent a promising strategy to limit plasmid transfer and curb the spread of resistance genes.

Methods and Materials

Primers, plasmids, bacterial isolates and media

Primers, plasmids, and bacterial strains used in this study are described in the supplementary tables S1, S2, and S3, respectively. Bacteria were grown in LB Lennox broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and or agar (1.5% agar), supplemented with the following where necessary: sodium azide (AZ; 100 mg/L); rifampicin (RIF; 30, 60 or 90 mg/L); cefotaxime (CTX; 8 mg/L), tetracycline (TET; 10 mg/L), streptomycin (STR, 100 mg/L) and/or kanamycin (KAN; 50 mg/L). A varying concentration of RIF (30, 60 or 90 mg/L) was used to generate RIF-resistant mutant strains, while RIF 90 mg/L was used throughout the work to grow RIF-resistant strains and select transconjugants in combination with TET. AZ was used to grow and select J53Az.

Bioinformatic analysis of the leading region

The FASTA format sequence of the IncM plasmid pJIBE401 (one SNP variant of pE11573; Accession no. JX101693) was annotated using the online tool Bakta ([Bakta](#)) and tested for the presence of restriction modification systems using RestrictionModificationFinder1.1 ([CGE Server](#)). Each of the open reading frames in the ~12.5 kb leading region of pJIBE401 was analysed using NCBI BLASTp search to determine their identity with known proteins in GenBank. We also performed domain analysis of each ORF in the ~12.5 kb leading region using the NCBI Conserved Domain Search Tool ([CD-Search: New Query](#)).

Gene expression analysis using quantitative real-time PCR

The relative expression of plasmid leading region putative anti-defense genes from the IncM plasmid pJIMK45 was measured using quantitative real-time PCR (qRT-PCR) as described previously (49). Briefly, a solid filter mating experiment was set with the donor *E. coli* J53Az carrying IncM plasmid pJIMK45 and the recipient *E. coli* UB5201Rf strain. Another mating experiment was performed using donor J53Az carrying a *traY-excA* deletion version of the pJIMK45 (pJIMK45Δ*traY-excA*) with the recipient UB5201Rf. The mating was terminated at

different time intervals (15, 30, 45, and 60 min) by mixing the membrane filter into 5 mL saline solution and vortexing. One mL of mating mixtures from each time point was centrifuged, and cells were collected for RNA isolation and subsequent qPCR analysis using the protocol described previously (49, 50). All cDNA samples were amplified using gene-specific qRT-PCR primers (S1Table) and SYBR Green PCR reagent (Qiagen, Germany) on a Rotor-Gene 6000 real-time thermocycler. The qPCR program includes initial denaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 10 sec.

The CT values of all reactions were obtained using Rotor-Gene Q Series Software. The internal reference gene *rpoB* was used. Using the $2^{-\Delta\Delta CT}$ method, the relative expression levels of the target gene were normalised to the internal reference gene. The procedure was repeated to obtain three biological replicates of total RNA.

Construction of gene deletion mutants

A homologous recombination-based allelic exchange method was used to construct gene deletion mutants, using the lambda-red recombinase system (51, 52). An FRT-kanamycin cassette was amplified from the plasmid pKD4 with the primers P1 and P2 carrying 40-bp homologous region from the upstream and downstream of the desired deletion region on the plasmid pJIMK45. The primers used for generating three different deletion mutants (~2.2-kb, ~5.4-kb leading region and the *traY-excA* deletion) were described in the S1 Table. PCR was performed using high-fidelity pfu polymerase (NEB, USA) according to the manufacturer's instructions, and the PCR product was purified using a PCR purification kit (Invitrogen, USA). 1.0 -2.0 µg of the purified PCR product was electroporated into the electrocompetent *E. coli* UB5201Rf bacteria carrying plasmid pJIMK45 and lambda-red recombinase plasmid pMK200. The transformants carrying the desired gene-deletion plasmid were selected on agar plates containing kanamycin (KAN). A few colonies

were tested for gene-specific PCR to confirm the deletion. The temperature-sensitive lambda-red plasmid will be lost at 37-42°C. The sequences of the final constructs were examined by whole-genome sequencing.

Generation of rifampicin resistant host strains

To construct RIF resistant strains, WH62, WH49, WH82, and NH92 were each grown overnight at 37°C in LB, diluted 1:10⁶ in fresh LB and 200 mL of culture transferred into each well of a 96-well plate with RIF (30 mg/mL). After overnight incubation at 37°C, cultures with bacterial growth were spotted onto LB agar plates containing RIF (60 mg/mL) and incubated at 37°C overnight. These colonies were then spotted onto LB agar plates containing 90 mg/mL RIF. A RIF resistant phenotype is often due to a mutation in *rpoB* (53), so *rpoB* was amplified from two colonies by PCR (S1 Table) and Sanger sequenced to identify such mutations (Australian Genome Research Facility, Australia).

***In vitro* conjugation**

Donor and recipient strains were each grown overnight at 37°C in LB with appropriate antibiotics. Cultures were pelleted by centrifugation and washed once with an equivalent volume of sterile saline solution (0.85% NaCl), and the cell pellets were resuspended in saline. The OD₆₀₀ of each culture was adjusted to 1.9-2.0, and 1 mL of each of the donor and recipient cultures were mixed, centrifuged and the pellets resuspended in 50 mL saline. The culture was spotted onto nitrocellulose membranes on LB agar plates. Mating mixtures were incubated at 37°C for 20 h without shaking. Mating was ended by placing the filters in 5 mL saline and vortexing. Dilutions of the mating mixtures were plated on LB agar with appropriate antibiotics to select for transconjugants.

To determine conjugation frequencies, the number of transconjugants on selective plates were counted. Donor cell numbers were also determined by plating dilutions of the donor cultures used for mating onto LB agar plates. Conjugation frequencies were calculated by dividing the number of transconjugants by the number of original donor cells. Conjugation frequency was measured from three independent mating experiments, and the data were presented with the mean and standard errors (Fig. 3).

Whole genome sequencing and analysis

E. coli, *K. pneumoniae* and *Morganella morganii* isolates were long-read sequenced using Oxford Nanopore Technologies (ONT). Colonies (¼-½ of a 10 µl inoculating loop) were taken from an overnight LB plate supplemented with appropriate antibiotics and pellets stored at -30°C. Genomic DNA was extracted from pellets thawed at room temperature using the Qiagen DNeasy UltraClean Microbial Kit (Qiagen, Germany) following the manufacturer's instructions, except that bead-beating was limited to 2 min and all subsequent mixing steps were performed by gentle inversion or flicking, not vortexing, to preserve DNA fragment length. DNA was eluted in 50 µL of elution buffer and assessed for purity (NanoDrop spectrophotometer) and concentration (Qubit fluorometer). Sequencing libraries were prepared using the ONT Rapid DNA Barcoding Kit 24 v14 (SQK-RBK114.24 or SQK-RBK114.96, Oxford Nanopore Technologies, United Kingdom) and sequenced with R10.4.1 flow cells, according to the manufacturer's instructions. Approximately 200 ng of input DNA was used per reaction, and the final pooled library was loaded at 1610 ng and run on a GridION sequencer. Output was automatically demultiplexed and basecalled during the run using "super-accurate basecalling" in MinKNOW 24.06.15/Dorado 7.4.14 with default settings. Sequence data was processed using an in-house quality-control pipeline, using kraken 2.1.3 (<https://github.com/DerrickWood/kraken2/wiki>) in quick mode (db

k2_standard_16gb_20240112) to check for contamination and confirm species, and NanoPlot 1.43.0 (<https://github.com/wdecoster/NanoPlot>) to check read length and depth statistics. Data was assembled using dragonflye_autocycler 1.2.0 (<https://github.com/rpetit3/dragonflye>) with -depth 100 and gsize 5150000. Assemblies were assessed for quality and contamination with CheckM 1.2.2 (<https://github.com/Ecogenomics/CheckM>). Annotation of bacterial sequences was verified by Bakta-web (54). Restriction modification (RM) systems in bacterial chromosomes were identified using Restriction-ModificationFinder 1.1 (<https://cge.food.dtu.dk/services/Restriction-ModificationFinder/>) (55) with default parameters. RestrictionMapper version 3 (<https://restrictionmapper.org/index.html>) was used to identify restriction sites on plasmids.

Statistical analysis

A one-way ANOVA was performed in Microsoft Excel (16.54) to compare conjugation transfer frequency. A p -value < 0.05 indicates a significant difference between two plasmids.

Data availability.

Data generated and presented in the manuscript has been found in the manuscript and in the supplementary information file.

Sequence data: The whole-genome sequences of bacterial isolates have been submitted to GenBank, and the accession numbers have not been assigned yet. The bioproject and biosample numbers of the bacterial sequences are as follows, and the sequences can be accessed by clicking the isolate hyperlink.

1. [WH62.fsa](#) (Bioproject no. PRJNA1491500, Biosample no. SAMN61463020)
2. [WH82_contig00001.fsa](#) (Bioproject no. PRJNA1491501, Biosample no. SAMN61463091)

3. [WH49_contig00001.fsa](#) (Bioproject no. PRJNA1491503, Biosample no. SAMN61463093)
4. [NH92_contig00001.fsa](#) (Bioproject no. PRJNA1491507, Biosample no. SAMN61463147)
5. [Mm1585Rf_contig00001.fsa](#) (Bioproject no. PRJNA1491510, Biosample no. SAMN61463376)
6. [UB5201Rf_chr.fsa](#) (Bioproject no. PRJNA1491545, Biosample no. SAMN61463927)
7. [NH78Rf_chr.fsa](#) (Bioproject no. PRJNA1491577, Biosample no. SAMN61465146)
8. [JIE286CRf_contig00001.fsa](#) (Bioproject no. PRJNA1491580, Biosample no. SAMN61465259)

Supporting Information:

Supplementary Figure S1. Nucleotide sequence alignment between IncM and IncL plasmids leading regions. Black bars represent non-identical nucleotide sequences.

Supplementary Figure S2. IncM plasmid derivative pJIMK45 Δ traY-excA has been constructed by deleting the part of *traY-excA* region from the pJIMK45 plasmid.

Supplementary Figure S3. IncM plasmid leading region deletion mutants. (A) a ~2.2 kb leading region was deleted from the IncM plasmid pJIMK45 by inserting a neomycin resistance gene. (B) a ~5.4 kb leading region (contains previous ~2.2 kb and additional ~3.2 kb) of IncM plasmid pJIMK45. The orange arrows are for putative anti-defense genes, the red arrows represent regulatory genes, and the blue arrows denote genes with unknown functions. Six single-strand specific promoter regions are shown by small purple arrows (ssi1-6).

Supplementary Table S1. Primers used in this study

Supplementary Table S2. Plasmids used in this study

Supplementary Table S3. Bacterial strains used in this study

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Author contributions

M.K. conceived the study, performed the experiments, conducted data analysis, provided supervision and overall guidance, secured funding and was responsible for writing the original draft, review & editing.

J.J. performed experiments, analysed data and contributed to manuscript revision.

J.R.I. provided study resources, secured funding, performed project administration, supervised and contributed to review & editing.

Competing Interest

The authors declare no competing interests.

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