RESEARCH ARTICLE

REVISED Understanding the genetic basis of the incompatibility of IncK1 and IncK2 plasmids [version 2; peer review: 2 approved]

Marta Rozwandowicz[1], Arie Kant[2], Jaap Wagenaar[3], Dik Mevius[2+], Joost Hordijk[1], Michael Brouwer[2]

[1]National Institute for Public Health and the Environment, Bilthoven, 3721 MA, The Netherlands
[2]Wageningen Bioveterinary Research, Lelystd, 8221 RA, The Netherlands
[3]Utrecht University, Utrecht, 3584 CS, The Netherlands

[+] Deceased author

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Abstract

Antimicrobial resistance is a persistent challenge in human and veterinary medicine, which is often encoded on plasmids which are transmissible between bacterial cells. Incompatibility is the inability of two plasmids to be stably maintained in one cell which is caused by the presence of identical or closely related shared determinants between two plasmids originating from partition or replication mechanisms. For I-complex plasmids in *Enterobacteriacae*, replication-based incompatibility is caused by the small antisense RNA stem-loop structure called RNAI. The I-complex plasmid group IncK consists of two compatible subgroups, IncK1 and IncK2, for which the RNAI differs only by five nucleotides. In this study we focussed on the interaction of the IncK1 and IncK2 RNAI structures by constructing minireplicons containing the replication region of IncK1 or IncK2 plasmids coupled with a kanamycin resistance marker. Using minireplicons excludes involvement of incompatibility mechanisms other than RNAI. Additionally, we performed single nucleotide mutagenesis targeting the five nucleotides that differ between the IncK1 and IncK2 RNAI sequences of these minireplicons. The obtained results show that a single nucleotide change in the RNAI structure is responsible for the compatible phenotype of IncK1 with IncK2 plasmids. Only nucleotides in the RNAI top loop and interior loop have an effect on minireplicon incompatibility with wild type plasmids, while mutations in the stem of the RNAI structure had no significant

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1. **Manuel Ares-Arroyo**, Institut Pasteur, Paris, France

2. **Lu Yang**, Shanghai University of Sport, Shanghai, China
China Agricultural University, Beijing, China

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effect on incompatibility. Understanding the molecular basis of incompatibility is relevant for future *in silico* predictions of plasmid incompatibility.

Keywords
plasmid, incompatibility, IncK, minireplicon

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Corresponding author: Michael Brouwer (mike.brouwer@wur.nl)

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REVISED **Amendments from Version 1**

The main difference in the new version of this manuscript is additional background information about stability and incompatibility of wt IncK1 and IncK2 plasmids. Moreover, the authors elaborated further on the design choices and their meaning for the obtained results.

Any further responses from the reviewers can be found at the end of the article

Introduction

Antimicrobial resistance is a health threat that is emerging globally and threatens human and veterinary medicine. One of the factors that has facilitated this spread is the transfer of mobile genetic elements between bacteria, for which plasmids are mostly responsible in Gram negative bacteria such as *Escherichia coli*[1]. In *Enterobacteriaceae* there are 40 plasmid types described, according to their incompatibility[1,2]. IncK1 plasmids are often associated with $bla_{CTX\text{-}M\text{-}14}$ and IncK2 plasmids predominantly carry $bla_{CMY\text{-}2}$[3]. IncK1 plasmids are found in various sources and IncK2 plasmids are predominantly associated with poultry, which makes it an adapted and specialized vector spreading antimicrobial resistance among poultry[4].

Incompatibility is the inability of two plasmids to be stably maintained in one cell[5]. This phenotypic trait was long used as a plasmid typing tool for epidemiological studies but this has now mostly been replaced by molecular diagnostic tools[6]. Incompatibility is caused by the presence of identical or closely related shared determinants between two plasmids, originating from partition or replication mechanisms[7].

Replication and the copy number of plasmids can be regulated by antisense RNA. For IncF, IncL, IncM, IncQ, I-complex plasmids (containing IncI, IncK, IncB/O and IncZ plasmids), ColE1 and plasmid pT181 (rep7a) incompatibility is mediated by an antisense stem-loop RNA structure (RNAI) that inhibits translation of the *rep* mRNA[8–14].

Involvement of the antisense RNAI in replication control and incompatibility, was extensively studied for I-complex plasmids[15–20]. RNAI controls replication through interaction with stem-loop I (SLI)[21,22]. The most important step is the formation of the stable kissing complex by pairing between the single-stranded RNAI and SLI[22]. An excellent graphical representation of I-complex plasmid replication, including IncK1 and IncK2, was previously published[23].

Minireplicons can be used to determine if incompatibility of IncK plasmids is determined by the replication control region, and not the partitioning region. Minireplicons contain a minimal part of a plasmid that can replicate at the same copy number as the original plasmid and that maintains the same incompatibility behaviour of the original plasmid[24]. The role of nucleotides in the top loop domain and upper stem of the RNAI structure in the interaction with the stem-loop I (SLI) was studied in detail for IncB/O plasmids[15]. IncB/O and IncK plasmids share a high degree of similarity in their RNAI structures. Using systematic mutagenesis of nucleotides in the RNAI structure, it was concluded that for IncB/O plasmids, three nucleotides on the top of the top loop (C37, C38 and C39) are crucial for the initial kissing interaction of RNAI with SLI. The interior loop in the upper stem is involved in the intra-strand melting and inter-strand pairing of RNAI with SLI[23]. Mutations disrupting the structure of the interior loop have a significant effect on plasmid compatibility[15]. Mutations at other positions only had significant effects if the mutation caused a base mismatch and therefore altered the structure.

Two compatible lineages of IncK plasmids were described in literature[3,25]. Four SNPs and one indel were identified that differ in the RNAI sequence of IncK1 and IncK2 plasmids, which may contribute to the compatibility and copy number of these plasmids. RNAI is a target allowing distinction between IncK1 and IncK2 plasmids. In this paper we examined the influence of these five polymorphisms in the RNAI structure for the compatibility of IncK1 and IncK2 plasmids. These results provide insights into the basis of incompatibility of IncK plasmids and support previous results for IncB/O plasmids.

Methods

Plasmids and vectors used

IncK2 plasmid pT.1.09 described in this study was recovered from *E. coli* from a poultry faeces sample and IncK1 plasmid p754 was recovered from a dog faeces sample (Table 1)[3]. Vector pMW2 is a 4,4kb pBlueScript-derivative carrying the kanamycin resistance gene *aph(3')-III*[26].

Table 1. Plasmids used and constructed in this study.

Plasmid name	Resistance gene	Source	Reference
p754 (IncK1)	$bla_{CTX\text{-}M\text{-}14}$	dog	3
pT.1.09 (IncK2)	$bla_{CMY\text{-}2}$	poultry	3
pIncK1 mini	*aph(3')-III*	-	this study
pIncK2 mini	*aph(3')-III*	-	this study
pIncK1 mini RNAI_delA2	*aph(3')-III*	-	this study
pIncK1 mini RNAI_T10C	*aph(3')-III*	-	this study
pIncK1mini RNAI_G25T	*aph(3')-III*	-	this study
pIncK1 mini RNAI_G41C	*aph(3')-III*	-	this study
pIncK1 mini RNAI_G41C	*aph(3')-III*	-	this study
pIncK2 mini RNAI_insA2	*aph(3')-III*	-	this study
pIncK2 mini RNAI_G3C	*aph(3')-III*	-	this study
pIncK2 mini RNAI_C9T	*aph(3')-III*	-	this study
pIncK2 mini RNAI_T24G	*aph(3')-III*	-	this study
pIncK2 mini RNAI_C40G	*aph(3')-III*	-	this study

Minireplicon construction

We examined the effect of point mutations in the RNAI structure on the compatibility of IncK plasmids. To determine the effect of the RNAI structure only and exclude involvement of any other plasmid structures, we designed minireplicons that contain the replication region of the IncK plasmid, and a kanamycin resistance cassette, which were ligated in a MW2 vector.

Minireplicons were constructed by cloning the replication region of the IncK1 or IncK2 plasmid into the vector pMW2. This vector was chosen because of the presence of kanamycin resistance gene *aph(3')-III*. The replication region, corresponding to the one previously used for a minireplicon construction, contained repA, repB and RNAI[27]. Amplifying the replication region was performed using the rep754 mini fw and rv primers for the IncK1 plasmid and repT1.09 mini fw and rv primers for IncK2 (Table 2). The PCR reaction was performed according to the protocol: 95°C 2 min, 95°C 30 sec, 57°C 30 sec, 72°C 2 min, 72°C 5min for 30 cycles. The PCR product was purified using the Gene Clean Turbo Kit (MoBio), digested with BamHI and KpnI for IncK1 and SacI and KpnI (Thermo Fisher Scientific) for IncK2. The digested replication region was ligated into vector MW2 after digestion with the respective enzymes, using T4 Ligase with standard manufacturers protocol (Thermo Fisher Scientific). The ligated product was electroporated into *E. coli* DH10B Electro MAX competent cells (Thermo Fisher Scientific) according to the manufacturer's protocol. These cells were chosen because of the high transformation efficiency. Transformants were grown on agar plates containing 25 µg/mL of kanamycin (Sigma). The presence of the minireplicon was confirmed by PCR using IncK replicon targeting primers (K1 fw and rv and K2 fw and rv) and Sanger sequencing. All created minireplicons are listed in Table 1. The size of the IncK1 minireplicon is 3789 bp and IncK2 3922 bp.

Single nucleotide mutagenesis was performed with the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific) according to the manufacturer's protocol, using primers with the designated mutation (Table 2). The presence of the minireplicon was confirmed with PCR using primers targeting the replication region (Table 2). The presence of the mutation was confirmed by Sanger sequencing.

The RNAI structure of the wild type (wt) and mutated RNAI genes was predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

Stability

The stability of minireplicons was determined in triplicate by independent overnight culturing in LB broth without selection. Serial dilutions of the culture were plated on LB agar plates without selection and incubated overnight at 37°C. 40 colonies were picked and PCR was performed to amplify the resistance gene present on the minireplicon. Stability was determined as a percentage of colonies containing the minireplicon. All results were statistically analysed using the Mann–Whitney U test. Stability of wild type IncK1 and IncK2 plasmids has been tested in a prior study[3].

Plasmid copy number

To determine the plasmid copy number, three independent DNA extractions using DNeasy UltraClean Microbial Kit (Qiagen) were performed from overnight culturing in LB broth with selection for each strain, and qPCRs using iQ SYBR

Table 2. Primers used in this study. "Mini" in the primer name means that the primer targets the minireplicon. Each primer name additionally depicts the mutation that is introduced in the RNAI structure, using this primer.

Primer name	Primer sequence	Reverse primer used	Reference
Creating minireplicons			
rep754 mini fw	CATGGTACCGGCCTGCAGTTCTGACAGAC		This study
rep754 mini rv	ATGTGATCATAGGCACGGTGCTGCGTTTG		This study
repT1.09 mini fw	CAGGGTACCACTGAGCCAGATACCAGTT		This study
repT1.09 mini rv	CAGGAGCTCTACGAGCGTGTACTGAGGAC		This study
IncK1/IncK2 plasmids identification			
K1 fw	ATCGTCAGGATCCGGGAAGTC		3
K1 rv	GAGCGATTGTGCCGTGTATT		3
K2 fw	ATGCTCGCGGTCCGGAAAGCC		3
K2 rv	GTGCCGTGCGTTAATGCACTGCAA		3

Primer name	Primer sequence	Reverse primer used	Reference
Single nucleotide mutagenesis			
Phu-754-R1	GGGATAAGTATATATGAAACCGTACCAGAG		This study
Phu-754-R2	TAGTAGGGGCGTTCACAGAATACGGGATAA		This study
Phu-T1.09-R1	GGGATAAGTATATATGAAACCGTGTCAGAG		This study
Phu-T1.09-R2	TAGTGGGGGCCTCACAGAATACGGGATAAG		This study
Phu-754-A2-Del-Fw	GTATTCTGTGACGCCCCTACTATCTTTCACG	Phu-754-R1	This study
Phu-754-C4G-Fw	GTATTCTGTGAAGGCCCCTACTATCTTTCACG	Phu-754-R1	This study
Phu-754-T10C-Fw2	GTATTCTGTGAACGCCCCCACTATCTTTCACG	Phu-754-R1	This study
Phu-754-G25T-Fw	TCTTTCACGATTCCCGCCAAAGTTCGAGGAAAGAT	Phu-754-R2	This study
Phu-754-G41C-Fw	TCTTTCACGAGCCCGCCAAAGTTCGACGAAAGAT	Phu-754-R2	This study
Phu-T1.09-A2-Ins-Fw	GTATTCTGTGAAGGCCCCCACTATCTTTCACG	Phu-T1.09-R1	This study
Phu-T1.09-G3C-Fw	GTATTCTGTGACGCCCCCACTATCTTTCACG	Phu-T1.09-R1	This study
Phu-T1.09-C9T-Fw	GTATTCTGTGAGGCCCCTACTATCTTTCACG	Phu-T1.09-R1	This study
Phu-T1.09-T24G-Fw	TCTTTCACGAGCCCGCCAAAGTTCGACGAAAGAT	Phu-T1.09-R2	This study
Phu-T1.09-C40G-Fw	TCTTTCACGATCCCGCCAAAGTTCGAGGAAAGAT	Phu-T1.09-R2	This study
Resistance genes detection			
Kan fw	ATGATGCTATGGCTGGAAGG		This study
Kan rv	CGCAGAAGGCAATGTCATAC		This study
CTX-M-14 fw	CTATTTTACCCAGCCGCAGC		28
CTX-M-14 rv	GTTATGGAGCCACGGTTGAT		28
CMY fw	ATGATGAAAAAATCGTTGCTGC		29
CMY rv	GCTTTTCAAGAATGCGCCAGG		29
aph(3′)-III fw	GGCTAAAATGAGAATATCACCGG		30
aph(3′)-III rv	CTTTAAAAAATCATACAGCTCGCG		30

Green Supermix (BioRad), targeting the *aph(3'')-III* replicon and *uidA* gene, were carried out in triplicate for each extraction. Plasmid copy number per chromosome was calculated using the formula described by San Millan *et al.*[31] cn = $[(1 + Ec)^{Ctc}/(1 + Ep)^{Ctp}]$ x (Sc/Sp), where cn is the plasmid copy number per chromosome, Sc and Sp are the sizes of the chromosomal and plasmid amplicons (in bp), respectively, Ec and Ep are the efficiencies of the chromosomal and plasmid qPCRs (relative to 1), respectively, and Ctc and Ctp are the threshold cycles of the chromosomal and plasmid reactions, respectively. Plasmid copy number was determined using aph(3'')-III fw and aph(3'')-III rv primers for IncK1 minireplicons and uidA fw and uidA rv for the chromosomal target (Table 2). Obtained data were analysed using the Mann–Whitney U test.

Incompatibility testing

IncK minireplicons are non-conjugative, therefore electroporation was chosen as a method to deliver the minireplicons into the bacterial cell. Although IncK1 or IncK2 wt plasmids are conjugative, in order to standardize the methods, they were electroporated into *E. coli* DH10B according to the manufacturer's protocol. *E. coli* cells carrying either the IncK1 or IncK2 wt plasmid were made electrocompetent from 250 mL liquid culture of OD_{600} 0.5 in LB media. Cultures were spun down for 10 min at 3560×*g* at 4°C. Pellets were washed twice with 250 and 125 mL ice-cold water, washed with 10 mL ice-cold 10% glycerol and finally resuspended in 0.5 mL ice-cold 10% glycerol and frozen at −80°C. For electroporation, minireplicons were isolated using the Wizard Plus SV Miniprep kit (Promega) and transformed as described above. Transformants

were subsequently selected on LB plates supplemented with 25 μg/mL kanamycin (Sigma-Aldrich) to select for the minireplicon and 2 μg/mL cefotaxime (Sigma-Aldrich) to select for the IncK wt plasmid.

To test the incompatibility of the IncK plasmid and the minireplicon combinations, the heteroplasmid population was grown overnight and plated on non-selective LB agar plates. For a detailed description see the "stability" paragraph.

Results

RNAI structure comparison

The RNAI sequence of IncK1 and IncK2 sequences differ by four SNPs and one indel. The RNAI structures of the wt plasmids were predicted as well as the structures of variants where one polymorphism of IncK2 is introduced in IncK1 and vice versa (Figure 1). The RNAI structures of wt IncK1 and IncK2, which consist of 61 and 60 nucleotides respectively, mainly differ in the top loop region. The top loop of the IncK2 RNAI structure is substantially bigger (12 nucleotides) compared to the one from IncK1 (8 nucleotides) based on *in silico* RNA structure predictions.

All of the nucleotide positions that are different between IncK1 and IncK2 plasmids were subjected to site-directed mutagenesis. Each mutated nucleotide was substituted with the corresponding nucleotide from the opposite plasmid group. The predicted size of the loop is controlled by a single polymorphism at the base of the loop while all other polymorphisms affect the stem or interior loop of the molecule. All of the mutations have an effect on the predicted structure, either creating or dissolving the second interior loop at the base of the stem or creating an overhang of several 'free' bases. However, mutation G25T/T24G is the only mutation that affects the top loop and therefore has the biggest impact on the conformation of the predicted structure (Figure 1).

Minireplicon stability

The stability of the minireplicons as well as the vector pMW2 was examined in triplicate after 24 hours of culture without antibiotic selection and was defined as the percentage of colonies that contained the minireplicon at the end of experiment. Stability of the vector pMW2 was 40%, which is lower compared to IncK1 minireplicons (Figure 2). However, the difference between stability of pMW2 and any minireplicon was not statistically significant, probably due to high standard deviation. The stability of the minireplicons was much lower compared to their parental plasmids[3]. The IncK1 wt minireplicon had a higher stability compared to the IncK2 wt minireplicon (p=0.034). All IncK1 mutated minireplicons have a higher stability than the corresponding IncK2 mutated minireplicons ($p \leq 0.05$). For IncK1 plasmids there were no statistically significant differences between the wt minireplicon and mutated variants (Figure 2). For IncK2 minireplicons,

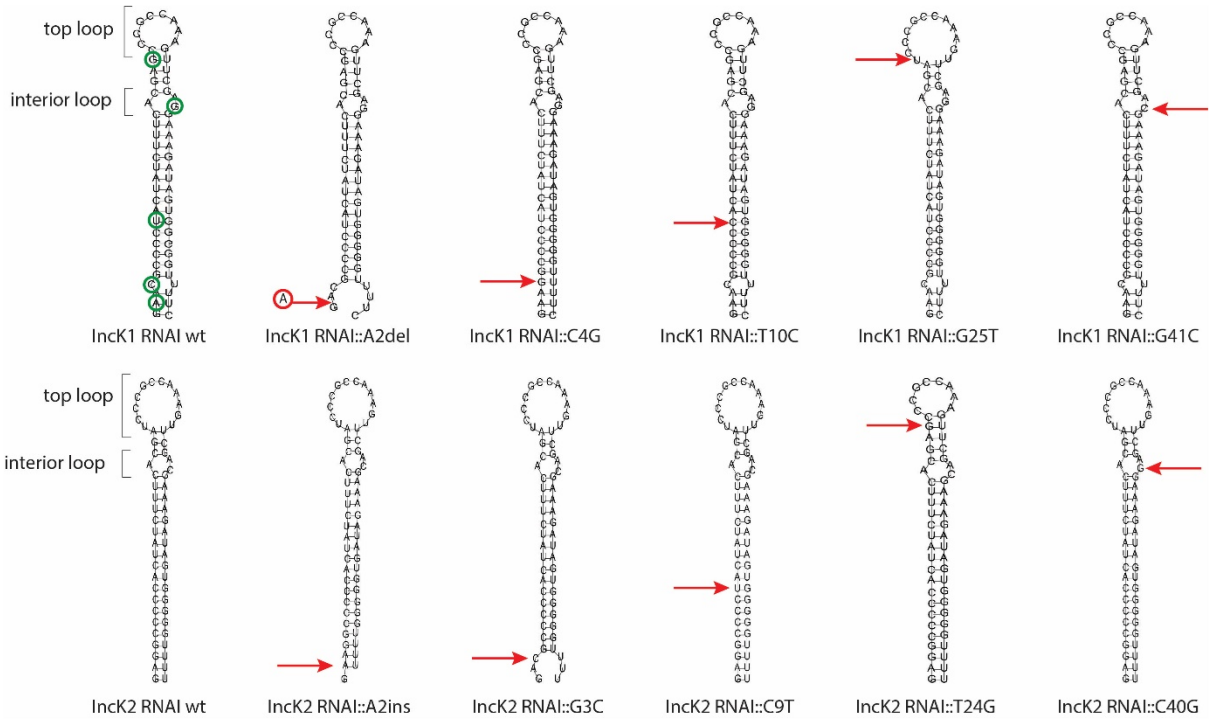

Figure 1. RNA structure prediction of all mutated RNAI variants. Red arrows point out the different mutation sites of the various minireplicons that were tested. Green circles indicate all SNPs between IncK1 and IncK2 RNAI.

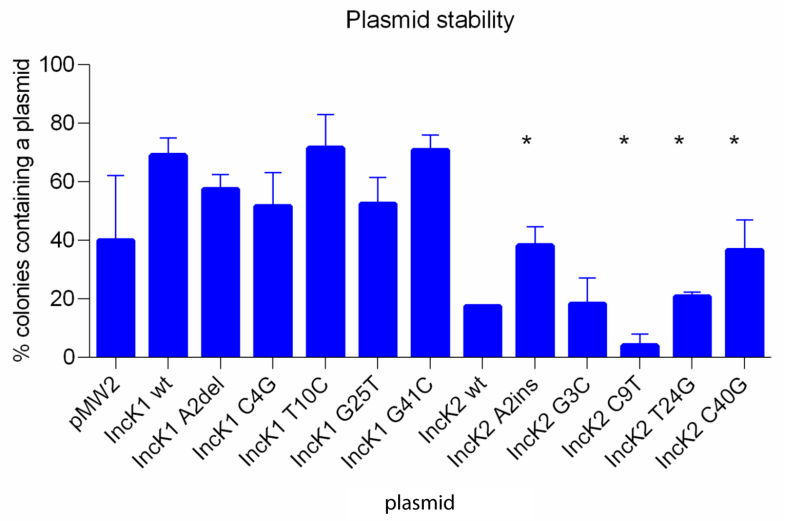

Figure 2. Stability of wt and mutant minireplicons after 24 hours of non-selective growth. Asterisks indicate the mutant minireplicons for which stability was significantly different compared to the minireplicons containing the wt RNAI ($p \leq 0.05$).

mutations A2ins, T24T and C40G resulted in a statistically significant increase in stability of the minireplicon compared to wt (p=0.037 for A2ins and C40G and p=0.034 for T24T). On the other hand, mutation C9T caused a statistically significant decrease in stability of the minireplicon compared to the IncK2 wt (p=0.037). This low stability and high variability of the IncK2-derived minireplicons may affect the results and IncK2-derived minireplicons were therefore excluded from plasmid copy number and incompatibility testing.

Plasmid copy number

Plasmid copy number was determined using qPCR targeting *aph(3")-III* as a plasmid target gene and *uidA* as a genomic target gene. Vector pBlueScript, which is a backbone for the vector pMW2, is a high-copy number plasmid which was confirmed in our experiment. pMW2 has an average copy number of 125. The IncK1-based minireplicons have an average copy number of 2. This result corresponds to the previous reports showing that copy number of IncK1 wt plasmids is 1–2 copies per cell[4]. For all of the minireplicons with mutated RNAI there is no significant difference in copy number compared to the wt minireplicon (Figure 3).

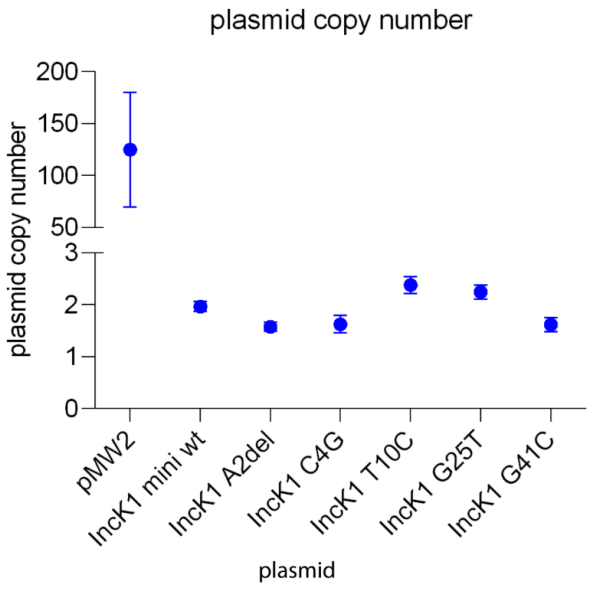

Figure 3. Plasmid copy number.

Minireplicon incompatibility

Incompatibility of IncK1 wt-derived mutated minireplicons was checked in triplicate against the parental and non-parental IncK wt plasmids. We performed overnight incompatibility tests to assess if the tested minireplicons and wt plasmids could be stably present together in one cell without antibiotic selection. The compatibility of IncK1 wt and IncK2 wt plasmids was determined previously[3].

All heteroplasmid strains were created by electroporating a mutated minireplicon with wt IncK1 or IncK2 plasmid into one cell. The compatibility of plasmids from all obtained heteroplasmid cells are shown in Figure 4. We determined the percentage of cells carrying either both a minireplicon and IncK wt plasmid, only one of these or none. All minireplicons were compatible with their non-parental IncK wt plasmid (Figure 4, Extended data[32], Table S1). Mutations G25T and G41C have a critical effect on compatibility of the IncK1 minireplicon with the IncK1 wt parental plasmid. Mutations A2del and T10C also cause some degree of compatibility with the IncK1 parental plasmid.

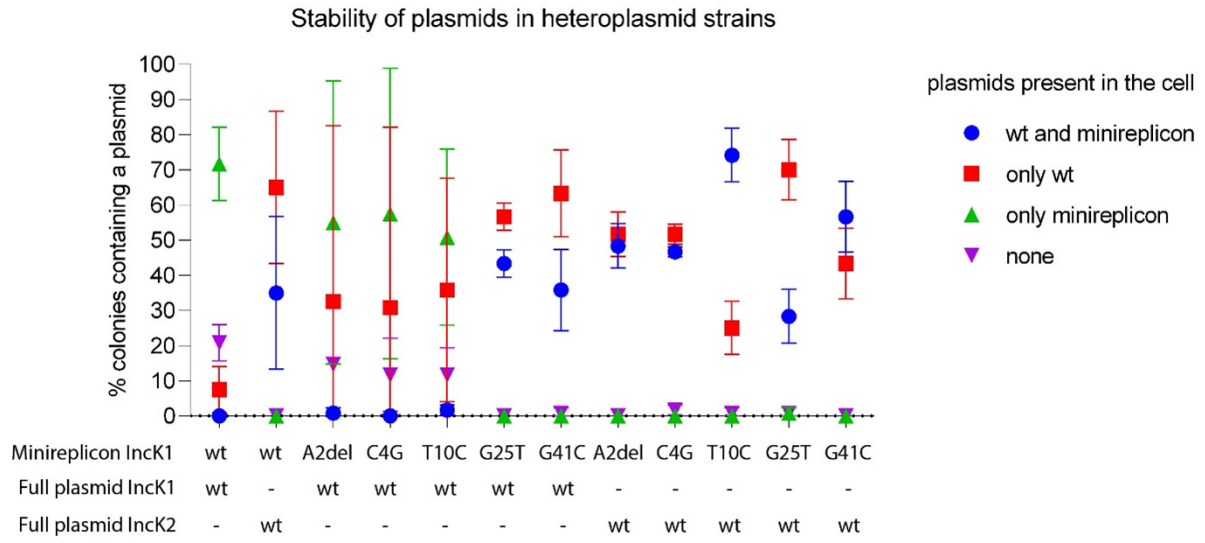

Figure 4. Compatibility of wt and mutated minireplicons tested in triplicate against parental and non-parental IncK plasmids. Raw data can be found in Supplementary Table 1.

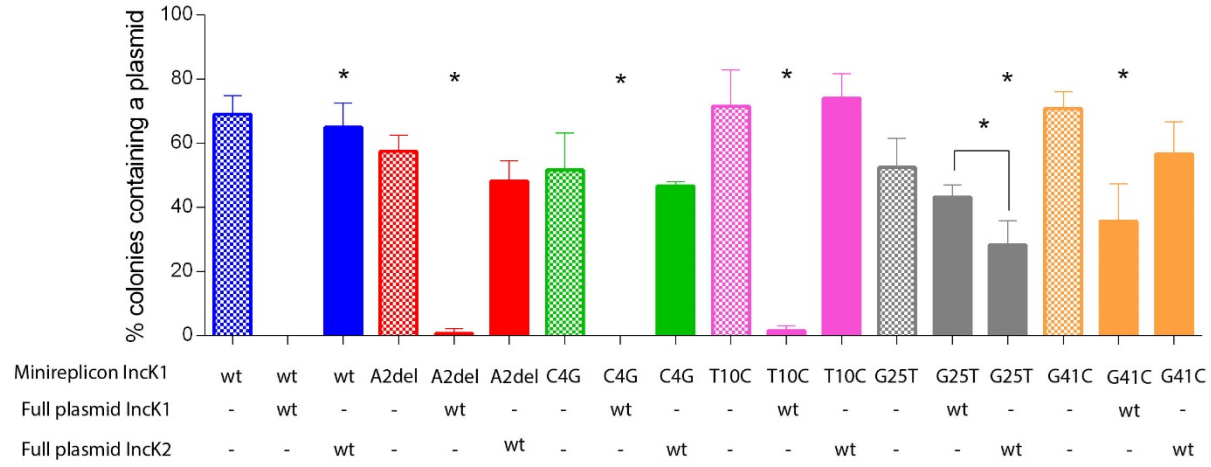

Figure 5. Comparison of single minireplicon stability with its stability in the heteroplasmid strain (co-existing with parental or non-parental plasmids). Dotted bars represent stability of the minireplicon alone, full bars represent stability of minireplicons with the IncK1 wt plasmid. * Means that the minireplicon stability in the heteroplasmid sample is statistically significantly different from the stability of a single minireplicon.

Based on our incompatibility experiments, we compared the stability of single minireplicons to the plasmids from heteroplasmid strains containing both the minireplicon and their (non-) parental IncK wt plasmids (Figure 5). All minireplicons that co-existed with their parental plasmid had a statistically significant lower stability in comparison with plasmids from cells containing only the minireplicon (Figure 4). The only exception is mutation G25T on the IncK1 minireplicon, where there was no statistical difference in stability between the heteroplasmid or single plasmid strains. This is also the only mutation that restored full compatibility of the minireplicon and its parental plasmid. Mutation G25T on the IncK1 minireplicon resulted in a decreased compatibility with its non-parental plasmid.

Discussion

In this study we examined the molecular basis of incompatibility of IncK plasmids by determining the effect of point mutations in the RNAI structure on incompatibility. We created minireplicons carrying the replication region of either the IncK1 or IncK2 plasmid, in which we subsequently introduced point mutations in the RNAI structure.

The minireplicons constructed in this study consisted of the replication region of IncK1 or IncK2 plasmids and an *aph(3")-III* gene. Vector pMW2 used in this study has a relatively low stability itself. This low stability combined with a very high copy number is in line with the nature of an expression vector. No stability determinants were introduced in the design as they could possibly affect incompatibility relationships. The low stability of vector pMW2 had an effect on the results of the study, which should be taken into account. Moreover, stability of IncK2 minireplicons was too low to be included in the study.

The copy number of the plasmid is regulated by the replication region. IncK wt plasmids have been shown to have a copy number of 1–2 per cell[4]. Lack of the partitioning system on the low-copy minireplicons leads to random segregation during cell division and therefore rapid plasmid loss. Additionally, the IncK wt plasmid has a toxin-antitoxin system stabilizing it in a cell, which is missing on the minireplicons. This could explain the instability of the minireplicons created in this study. High instability of minireplicons was previously reported for IncL/M plasmids[33]. Additionally, the fact that the minireplicons constructed in this study lack the partitioning system, may also contribute to the relatively weak observed incompatibility. Moreover, IncK2 plasmids were shown to be adapted to poultry body temperature (42°C)[4]. It is possible that testing at a higher temperature would result in higher stability of IncK2 plasmids. However, plasmid incompatibility was initially defined as a displacement of a resident plasmid (in this case wild type IncK plasmid), which might explain low stability of wt plasmid in the incompatibility experiments[5]. The obtained results indicate that replication-based incompatibility may not be a straight-forward system based on plasmids being present or not in one cell. It may be similar to partitioning-based incompatibility of IncF plasmids, where both strong and weak incompatibility was reported[34].

The results obtained in this study showed that mutations in the top loop and interior loop in the upper stem of the RNAI structure have a critical effect on the compatibility of IncK plasmids. Similar findings were previously demonstrated for IncB/O plasmids[15]. Three nucleotides in the top loop of RNAI of IncB/O plasmid structure, G37, C38, C39, have the most significant effect on the compatibility of IncB/O plasmids, because they are responsible for the formation of the initial kissing complex between RNAI and SLI[15]. Mutations in the bottom part of the top loop of IncB/O plasmid have less significant effect on compatibility, which would explain the results that heteroplasmid strains containing IncK wt plasmid and IncK minireplicon with the top loop mutation, G25T for IncK1 were compatible. The interior loop in the upper stem of RNAI is involved in inter-strand pairing between RNAI and SLI[23]. Preserving the structure of the interior loop is crucial for the interaction with SLI. Mutation G41C, that was introduced into RNAI in this study, does not disrupt the interior loop and therefore has a limited effect on the incompatibility of IncK plasmids. Additionally, different mutations cause a different degree of incompatibility for IncK1 and IncK2 plasmids. For IncK1 minireplicons, mutations G25T and G41C have a critical effect on their compatibility with the IncK1 wt parental plasmid. Mutations A2del and T10C only have a small effect on the compatibility. These changes in compatibility of IncK1 minireplicons could be observed regardless its low stability. These results implicate that mutation T10C does not affect compatibility of IncK1 and IncK2 plasmids, but has an effect on replication and copy number control of IncK1 plasmid. It is possible that exchanging thymine to cytosine results in stronger bonding with guanine on the opposite strands which interferes with the formation of the extended kissing complex. These findings are in line with previous research, which showed that different mutations in the RNAI structure can cause a different degree of *rep* inhibition[15]. Incompatibility of IncK plasmids can be caused by the cumulative effect of the mutations in the RNAI structure. However, low stability of the minireplicons may be the sole reason why not all initially heteroplasmid strains contain both plasmids. More research is required to be able to fully understand the role of mutations in RNAI and the interplay of partitioning system in the compatibility of these plasmids.

In recent years, compatibility of many plasmids was re-examined[3,25,35,36]. The most used and well established method to determine compatibility of two plasmids is conjugation of both into one cell, followed by selective plating[37]. However, the present study has indicated the added value of examining plasmid incompatibility over time in comparison with the classical method where incompatibility is measured as an ability to form a heteroplasmid strain. This greatly affected the interpretation of results. Incompatible plasmids could be found in one cell with selective pressure and were unstable together after removal of the selective pressure.

Based on previous sequence alignments, RNAI-structure predictions and minireplicon incompatibility experiments, this manuscript has confirmed the predicted effect of certain bases in the RNAI structure of IncK1 and IncK2 plasmid incompatibility. It shows that a single mutation in the RNAI structure of the IncK1 plasmid can change its compatibility. The RNAI structures used in this study were predicted *in silico*. However, using crystallographic RNAI structures could provide a better resolution and insights about the exact mechanism underlying the compatibility changes caused by the introduced mutations. Further research in this field, including more plasmid types, can possibly lead to a design of a sequence-based tool predicting plasmid compatibility. Such a tool would eliminate the necessity to perform laborious compatibility experiments and allow faster and easier plasmid compatibility predictions. Knowledge about plasmids compatibility would allow to improve existing plasmid classification, which would further help understanding epidemiology of these plasmids.

Data availability

Underlying and extended data

Biostudies: Underlying and extended data for "Understanding the genetic basis of the incompatibility of IncK1 and IncK2 plasmids." https://www.ebi.ac.uk/biostudies/studies/S-BSST977

This project contains the following underlying data:

Data file 1. Raw data underlying figures 2, 3, 4, and 5 and extended data.

ENA: Sequencing data of the strains used in this study

https://www.ebi.ac.uk/ena/browser/view/ERR1551799

https://www.ebi.ac.uk/ena/browser/view/ERR1607717

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Authors contribution

MR Conceptualization, Formal Analysis, Investigation, Methodology, Writing

AK Investigation, Methodology

JAW Conceptualization, Funding Acquisition, Methodology, Writing

DJM Conceptualization, Methodology, Writing

JH Conceptualization, Methodology, Writing

MSMB Conceptualization, Funding Acquisition, Methodology, Writing

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 15 December 2023

https://doi.org/10.21956/openreseurope.18124.r35951

✔ **Lu Yang**
[1] Shanghai University of Sport, Shanghai, China
[2] China Agricultural University, Beijing, Beijing, China

I do not have further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, Antimicrobial resistance, Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 16 November 2023

https://doi.org/10.21956/openreseurope.18124.r35952

✔ **Manuel Ares-Arroyo**
Department of Genomes and Genetics, Institut Pasteur, Paris, Île-de-France, France

I do not have further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmid biologist. Mobile Genetic Elements. Bacterial evolution.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 02 August 2023

https://doi.org/10.21956/openreseurope.16358.r33419

?

Lu Yang
[1] Shanghai University of Sport, Shanghai, China
[2] China Agricultural University, Beijing, Beijing, China

The manuscript titled "Understanding the genetic basis of the incompatibility of IncK1 and IncK2 plasmids" by Rozwandowicz et al. aims to investigate the genetic basis of incompatibility between IncK1 and IncK2 plasmids in bacteria. Overall, the study is well-conducted and holds valuable insights into the dynamics of plasmid interactions within bacterial cells. However, I have some concerns and suggestions that should be addressed before the manuscript can be considered for indexing. Please find my specific comments below:

1. The stability of the vector pMW2 is reported to be 40% and widely dispersed, but its potential impact on the study's results is not clearly addressed. I recommend the author to provide a more detailed description of the experimental design and explain why to choose this vector and competent cells in this study. I also suggest the author include a brief discussion in the manuscript's conclusion about how the low stability of the vector might affect the experimental outcomes. Addressing this issue would enhance the scientific rigor and credibility of the study, ensuring a more cautious interpretation of the results.

2. In the manuscript, there appears to be no direct comparison of the incompatibility of IncK2 mutated minireplicons with the IncK1 wild-type plasmids. This omission limits the comprehensive understanding of the plasmid's behavior and compatibility between these two types. It would greatly benefit the readers and the significance of the study if you could address this issue.

3. To enhance its significance, I recommend explicitly discussing the relationship between this type of plasmid and antibiotic resistance. This will strengthen the manuscript's impact on the field.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, Antimicrobial resistance, Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Oct 2023

Marta Rozwandowicz

Lu Yang The manuscript titled "Understanding the genetic basis of the incompatibility of IncK1 and IncK2 plasmids" by Rozwandowicz et al. aims to investigate the genetic basis of incompatibility between IncK1 and IncK2 plasmids in bacteria. Overall, the study is well-conducted and holds valuable insights into the dynamics of plasmid interactions within bacterial cells. However, I have some concerns and suggestions that should be addressed before the manuscript can be considered for indexing. Please find my specific comments below:

1. The stability of the vector pMW2 is reported to be 40% and widely dispersed, but its potential impact on the study's results is not clearly addressed. I recommend the author to provide a more detailed description of the experimental design and explain why to choose this vector and competent cells in this study. I also suggest the author include a brief discussion in the manuscript's conclusion about how the low stability of the vector might affect the experimental outcomes. Addressing this issue would enhance the scientific rigor and credibility of the study, ensuring a more cautious interpretation of the results.

- The reason for choosing pMW2 vector was the presence of kanamycin resistance gene and *E. coli* DH10B Electro MAX were the authors' choice because of their high transformation efficiency. Both arguments are now included in the methods under the "Minireplicon construction" paragraph. The discussion about the impact of pMW2 vector's low stability is now included in the discussion part of the manuscript.

1. In the manuscript, there appears to be no direct comparison of the incompatibility of

IncK2 mutated minireplicons with the IncK1 wild-type plasmids. This omission limits the comprehensive understanding of the plasmid's behavior and compatibility between these two types. It would greatly benefit the readers and the significance of the study if you could address this issue.

- The authors believe that due to the very low stability of IncK2 minireplicons such an experiment would not yield reliable results. An appropriate comment is included at the end of the "Minireplicon stability" paragraph of the results section.

1. To enhance its significance, I recommend explicitly discussing the relationship between this type of plasmid and antibiotic resistance. This will strengthen the manuscript's impact on the field.

- Resistance genes most commonly associated with IncK1 and IncK2 plasmids, as well as the sources where these plasmids are found are now discussed in the introduction part of the manuscript. Additionally, a strong association of IncK2 plasmids to poultry sources is mentioned. **Is the work clearly and accurately presented and does it cite the current literature?** Yes **Is the study design appropriate and does the work have academic merit?** Yes **Are sufficient details of methods and analysis provided to allow replication by others?** Yes **If applicable, is the statistical analysis and its interpretation appropriate?** Yes **Are all the source data underlying the results available to ensure full reproducibility?** Yes **Are the conclusions drawn adequately supported by the results?** Yes ***Competing Interests:*** No competing interests were disclosed. ***Reviewer Expertise:*** Microbiology, Antimicrobial resistance, Genomics **I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Competing Interests: No competing interests were disclosed.

Reviewer Report 20 July 2023

https://doi.org/10.21956/openreseurope.16358.r33413

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Manuel Ares-Arroyo
Department of Genomes and Genetics, Institut Pasteur, Paris, Île-de-France, France

The aim of the work is to characterize the molecular basis of incompatibility within and between two related subgroups of plasmids from the I-complex, the IncK1 and IncK2. Combining an *in silico* analysis and a molecular biology approach, the authors show how just one SNP in the RNAI of these plasmids turn them into compatible replicons. Despite other features of the plasmids are not tested (e.g., the partitioning system), this represents an interesting piece of work for plasmid biologists in particular, which shows how replication-mediated incompatibility works in two related lineages of plasmids.

In general terms, there are not big concerns regarding the bibliography, reproducibility, statistical analysis, etc. Notwithstanding, there are few concerns I would like to comment regarding the present manuscript that make me consider the work Approved With Reservations:

General concerns:

Stability of pT.109 and p754. It seems that the authors did not test the stability of the wt IncK1 (pT.109) and IncK2 (p754), or data is not shown. I find a bit disturbing the result in Figure 4, in which the incompatibility between the minireplicon IncK1 and the full plasmid IncK1 results in the loss of the full wt plasmid: 70% of the colonies carry the minireplicon, whereas less than 10% carry the full wt plasmid. According to the authors, these plasmids encode for a partitioning system in addition to a toxin/antitoxin system. Given the expected stability of this plasmid, isn't it unexpected that the minireplicon is the one that is mostly maintained in the population after an overnight? How stable are these wt plasmids in E. coli DH10B? Any comments about this result?

Incompatibility between pT.109 and p754. The authors tested the incompatibility between the minireplicons with the full wt plasmids. However, at no point the authors tested the incompatibility between the two full wt plasmids. Did the authors calculate it? Does it show similar results to the minireplicon IncK1 with the full plasmid IncK2? Likewise, it could help to understand to which extent other factors could be implicated in the plasmid incompatibility, commented at some points within the text.

Temperature (37ºC and 42ºC): IncK1 and IncK2 usually inhabit niches with different temperatures, being IncK2 plasmids specially adapted to temperatures of 42ºC (poultry temperature). It was shown by authors of this work that both IncK1 and Inck2 lineages show different plasmid copy number, conjugation rates and could have different fitness cost depending on this temperature (Rozwandowicz et al., 2019, Frontiers Microbiology)[1]. Since all the experiments were done at 37ºC, it is not unexpected to find that the stability of IncK2-derivatives is lower than that of IncK1-derivatives. Did the authors test if the IncK2 minireplicons at 42ºC have a stability comparable to the IncK1 at 37ºC, so that they are not discarded from the work? Did the authors tested different temperatures?

Specific concerns:

Introduction.

1st Paragraph. It states that "there are 40 plasmid types described, mostly based on their incompatibility". There are 40 types according to the incompatibility, but additional classification methods are being used (MOB, MPF, PTUs,...) and they not included in these 40 aforementioned types. I would change 'mostly based on' by 'according to'.

2nd Paragraph. It could be convenient a reference to the definition of incompatibility.

3rd Paragraph. The authors mention that plasmid replication regulated by antisense RNA occurs in IncF, IncL, IncM and I-complex plasmids. However, within the references cited (5-11), there is also the plasmid R1162 (reference 9, which is an incQ) and the gram positive plasmid pT181 (reference 5, typed as rep7a). Additionally, ColE1 replication could be added to the list.

4th Paragraph. A schematic figure representing the replication mechanism of I-complex plasmids (with the role of RNAI) might be useful for readers not used to this plasmid complex. The second half of the **5th paragraph** could also benefit of the figure. Additionally, it could include the general genetic map of the minireplicons used (present in the extended data).

Methods.

Plasmids and vectors used. Either in the text and/or in the table could be indicated which wt plasmid is IncK1 or Inck2 (pT.109 or p754).

Incompatibility testing. The minireplicons were electroporated because they are non-conjugative, the authors wrote. But, the full wt plasmids were electroporated as well (next sentence). Are they also non-conjugative? What is their size? No information on the manuscript.

Results.

RNAI structure comparison. The last sentence of the 1st paragraph ("The predicted size… …interior loop of the molecule") could fit better in the second paragraph, with the result of G25T/T24G, or at least after mentioning that some substitutions were tested.

Figure 1. The RNAfold server has the option to color the nucleotides according to the base-pair probability. Have the authors considered the option to include the probabilities in the secondary structure inference? Do the wt RNAI's secondary structures show high probabilities? And the mutated ones?

Minireplicon stability. Does the minireplicons' stability correlate with the one of the wt original plasmids? Is pT.109 more stable than p754 in these conditions?

Minireplicon stability. Some SNPs in the RNAI of IncK2 increase/decrease the stability, but the secondary structure (in Figure 1) is really conserved. Do the base-pair probabilities of these structures change?

Minireplicon incompatibility. '…against the parental and non-parental IncK wt plasmids' means against pT.109 and p754?

Figure 4. For the representation of this Figure, might the authors benefit from a stacked histogram.

Discussion.

1st Paragraph. In addition to the RNAI differences, how similar are the rep genes? Could these rep genes also provide some incompatibility? Additionally, how conserved are these RNAI in nature? All IncK1 and IncK2 plasmids have the exact same RNAIs or are there differences dissimilarities in between wt plasmids?

2nd Paragraph. If full wt plasmids should be more stable (toxin/antitoxin, partitioning), one would expect them to be more stable when assessing the compatibility, not being more loss than the

minireplicon (as it is in Figure 4). Any explanation? Are these full wt plasmids more stable than the minireplicons? No data on this aspect in the text.

2nd Paragraph. "The observed incompatibility is weaker than expected". What were the expectation of the authors?

2nd Paragraph. The authors state that "the results indicate that replication-based incompatibility may not be a straight-forward system based on plasmids being present in one cell." But indeed, this is exactly what is occurring in the experiment. Among the bacteria carrying both the full IncK1 and the minireplicon IncK1, 0% of the colonies tested carry both plasmids after just one overnight. Despite other plasmid characteristics may be involved, it does suggest that incompatibility is infeluncing the compatibility via replication-based mechanisms.

3rd Paragraph. Full stop (".") missing between reference 21 and the next sentence ("Preserving the structure...").

3rd Paragraph. "T10C causes the highest increase in plasmid copy number". In results, it was shown that there are no statistical differences in copy number between the IncK1 minireplicons.

4th Paragraph. Do the authors have references for 'the most used method to determine compatibility of two plasmids is conjugation?'

References

1. Rozwandowicz M, Brouwer MSM, Mughini-Gras L, Wagenaar JA, et al.: Successful Host Adaptation of IncK2 Plasmids.*Front Microbiol*. 2019; **10**: 2384 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmid biologist. Mobile Genetic Elements. Bacterial evolution.

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Author Response 23 Oct 2023

Marta Rozwandowicz

Manuel Ares-Arroyo *Institut Pasteur Departement de Virologie, Paris, Île-de-France, France The aim of the work is to characterize the molecular basis of incompatibility within and between two related subgroups of plasmids from the I-complex, the IncK1 and IncK2. Combining an in silico analysis and a molecular biology approach, the authors show how just one SNP in the RNAI of these plasmids turn them into compatible replicons. Despite other features of the plasmids are not tested (e.g., the partitioning system), this represents an interesting piece of work for plasmid biologists in particular, which shows how replication-mediated incompatibility works in two related lineages of plasmids. In general terms, there are not big concerns regarding the bibliography, reproducibility, statistical analysis, etc. Notwithstanding, there are few concerns I would like to comment regarding the present manuscript that make me consider the work Approved With Reservations:* ***General concerns: Stability of pT.109 and p754****. It seems that the authors did not test the stability of the wt IncK1 (pT.109) and IncK2 (p754), or data is not shown. I find a bit disturbing the result in Figure 4, in which the incompatibility between the minireplicon IncK1 and the full plasmid IncK1 results in the loss of the full wt plasmid: 70% of the colonies carry the minireplicon, whereas less than 10% carry the full wt plasmid. According to the authors, these plasmids encode for a partitioning system in addition to a toxin/antitoxin system. Given the expected stability of this plasmid, isn't it unexpected that the minireplicon is the one that is mostly maintained in the population after an overnight? How stable are these wt plasmids in E. coli DH10B? Any comments about this result?*

Stability of IncK1 and IncK2 plasmids was not performed in the present study, however, It was checked in previous study describing compatibility of these plasmids (Rozwandowicz et al. 2017). In Rozwandowicz et al. 2017 stability of IncK1 or IncK2 plasmid was determined in the cell carrying both IncK1 and IncK2 plasmids. After 72 h, 98% of the IncK1 plasmids were still present using selective agar and 100% using nonselective agar. The IncK2 plasmid showed 100% stability on both selective and nonselective agar. An appropriate sentence was added to the "Minireplicon stability" part of the results.Obtained results are actually not that surprising considering that plasmid incompatibility was initially defined as a displacement of a resident plasmid (in this case wild type IncK plasmid). Moreover, in a direct competition, the authors predict that the minireplicons have a lower fitness cost compared to the wt plasmid.

Incompatibility between pT.109 and p754*. The authors tested the incompatibility between the minireplicons with the full wt plasmids. However, at no point the authors tested the incompatibility between the two full wt plasmids. Did the authors calculate it? Does it show similar results to the minireplicon IncK1 with the full plasmid IncK2? Likewise, it could help to understand to which extent other factors could be implicated in the plasmid incompatibility, commented at some points within the text.*

The incompatibility of two wt plasmids is not presented in this manuscript, but it was demonstrated in previous work from the authors (Rozwandowicz et al. 2017). Although the exact pair used in this study was not tested previously, authors believe that the results can be extrapolated. The results presented in the previous study correlate nicely with the current study. In the pair of IncK2 wt and IncK1 minireplicon, the InK2 plasmid is nearly 100% stable. An appropriate sentence was added to the "Minireplicon incompatibility" part of the results.

Temperature (37ºC and 42ºC)*: IncK1 and IncK2 usually inhabit niches with different temperatures, being IncK2 plasmids specially adapted to temperatures of 42ºC (poultry temperature). It was shown by authors of this work that both IncK1 and Inck2 lineages show different plasmid copy number, conjugation rates and could have different fitness cost depending on this temperature (Rozwandowicz et al., 2019, Frontiers Microbiology)1. Since all the experiments were done at 37ºC, it is not unexpected to find that the stability of IncK2-derivatives is lower than that of IncK1-derivatives. Did the authors test if the IncK2 minireplicons at 42ºC have a stability comparable to the IncK1 at 37ºC, so that they are not discarded from the work? Did the authors tested different temperatures?*

The authors did not test stability of these plasmids at different temperatures. It is possible that replication and stability of IncK2 plasmid is dependent on the temperature. An appropriate comment is now added in the 3rd paragraph of the discussion of the manuscript.

Specific concerns: Introduction*.* ***1st Paragraph****. It states that "there are 40 plasmid types described, mostly based on their incompatibility". There are 40 types according to the incompatibility, but additional classification methods are being used (MOB, MPF, PTUs,…) and they not included in these 40 aforementioned types. I would change 'mostly based on' by 'according to'.*

The change was made according to the reviewer's suggestion.

2nd Paragraph. *It could be convenient a reference to the definition of incompatibility.*

The appropriate reference for a definition of incompatibility was added.

3rd Paragraph*. The authors mention that plasmid replication regulated by antisense RNA occurs in IncF, IncL, IncM and I-complex plasmids. However, within the references cited (5-11), there is also the plasmid R1162 (reference 9, which is an incQ) and the gram positive plasmid pT181 (reference 5, typed as rep7a). Additionally, ColE1 replication could be added to the list.*

The change was made according to the reviewer's suggestion

4th Paragraph*. A schematic figure representing the replication mechanism of I-complex plasmids (with the role of RNAI) might be useful for readers not used to this plasmid complex. The second half of the* ***5th paragraph*** *could also benefit of the figure. Additionally, it could include the general genetic map of the minireplicons used (present in the extended data)*

The authors agree that a schematic figure would be useful. However, since the I-complex replication mechanism in general is not the main focus of the paper and would take up a relatively large space, a relevant reference with an excellent description has been included.

***Methods**. **Plasmids and vectors used.** Either in the text and/or in the table could be indicated which wt plasmid is IncK1 or Inck2 (pT.109 or p754).*

An appropriate addition was made both to the text and the table.

***Incompatibility testing.** The minireplicons were electroporated because they are nonconjugative, the authors wrote. But, the full wt plasmids were electroporated as well (next sentence). Are they also non-conjugative? What is their size? No information on the manuscript.*

The wt full plasmids are conjugative, but they were electroporated in order to standardize the methods. An appropriate comment is made in the text.

***Results**. **RNAI structure comparison**. The last sentence of the 1st paragraph ("The predicted size… …interior loop of the molecule") could fit better in the second paragraph, with the result of G25T/T24G, or at least after mentioning that some substitutions were tested.*

The change was made according to the reviewer's suggestion.

***Figure 1.** The RNAfold server has the option to color the nucleotides according to the base-pair probability. Have the authors considered the option to include the probabilities in the secondary structure inference? Do the wt RNAI's secondary structures show high probabilities? And the mutated ones?*

The colored version of the graph generated by the RNAfold was not used because in the colored version single nucleotides were not visible and it would be difficult to show where the actual point mutations were made. Both wt IncK1 and IncK2 RNAI structures show overall very high base-pair probabilities. Introduced point mutations might slightly reduce base-pair probabilities, but don't drastically change the whole structure integrity.

***Minireplicon stability.** Does the minireplicons' stability correlate with the one of the wt original plasmids? Is pT.109 more stable than p754 in these conditions?*

Stability of wt IncK1 and IncK2 plasmids was shown to be very high (98-100%) therefore observed stability of the minireplicons is much lower. As suggested by the reviewer, a possible explanation as to why the IncK2 minireplicon is much more unstable compared to the IncK1 minireplicon could be that the IncK2 plasmid replication is adapted to 42^{o}C and performs semi-optimal at 37^{o}C. A remark has been added in the discussion (3rd paragraph).

***Minireplicon stability.** Some SNPs in the RNAI of IncK2 increase/decrease the stability, but the secondary structure (in Figure 1) is really conserved. Do the base-pair probabilities of these structures change?*

The base-pair probabilities of these structures don't seem to change drastically. Although the structure is conserved, if these mutations occur, they might indeed affect the interactions of RNAI with its target and therefore altering stability of the whole minireplicon.

***Minireplicon incompatibility**. '...against the parental and non-parental IncK wt plasmids' means against pT.109 and p754?*

By "parental and non-parental IncK wt" the authors mean wt IncK plasmids (pT.109 and p754). Parental plasmid for IncK1 minireplicon will be wt IncK1 and non-parental wt IncK2 plasmid.

***Figure 4.** For the representation of this Figure, might the authors benefit from a stacked histogram.*

The authors chose for this data representation to be able to show standard deviation between three replicates of the experiment, which would be difficult in a stacked histogram.

***Discussion. 1st Paragraph.** In addition to the RNAI differences, how similar are the rep genes? Could these rep genes also provide some incompatibility? Additionally, how conserved are these RNAI in nature? All IncK1 and IncK2 plasmids have the exact same RNAIs or are there differences dissimilarities in between wt plasmids?*

The rep genes of pT.109 and p754 share little DNA identity. RNAI has a complementary region in *repBA* mRNA. Besides that, the authors are not aware of any involvement of the *repA* gene in the incompatibility. To our best knowledge there is no variation in IncK1 and IncK2 RNAI sequence, which is very conserved.

***2nd Paragraph.** If full wt plasmids should be more stable (toxin/antitoxin, partitioning), one would expect them to be more stable when assessing the compatibility, not being more loss than the minireplicon (as it is in Figure 4). Any explanation? Are these full wt plasmids more stable than the minireplicons? No data on this aspect in the text.*

Wt IncK1 and IncK2 plasmids are more stable than their minireplicons. However in a direct competition between them it might play a role which plasmid is resident (wt IncK) and which one is incoming (minireplicon). Moreover, the minireplicon will probably have smaller fitness cost compared to the wt plasmid, based on its size and the number of encoded genes.

***2nd Paragraph.** "The observed incompatibility is weaker than expected". What were the expectation of the authors?*

Additionally, the fact that the minireplicons constructed in this study lack the partitioning system, may also contribute to the relatively weak observed incompatibility

***2nd Paragraph.** The authors state that "the results indicate that replication-based incompatibility may not be a straight-forward system based on plasmids being present in one cell." But indeed, this is exactly what is occurring in the experiment. Among the bacteria carrying*

both the full IncK1 and the minireplicon IncK1, 0% of the colonies tested carry both plasmids after just one overnight. Despite other plasmid characteristics may be involved, it does suggest that incompatibility is infeluncing the compatibility via replication-based mechanisms.

What the authors meant by this statement was that almost none of shown mutations result in full incompatibility. In the pairs of IncK1 minireplicon and IncK2 plasmid, heteroplasmid cells were detected, but no cells harbored only IncK1 minireplicon. These pairs show lower level of incompatibility then IncK1 wt with an IncK1 minireplicon.

***3rd Paragraph**. Full stop (".") missing between reference 21 and the next sentence ("Preserving the structure...").*

The change was made according to the reviewer's suggestion.

***3rd Paragraph**. "T10C causes the highest increase in plasmid copy number". In results, it was shown that there are no statistical differences in copy number between the IncK1 minireplicons.*

The incorrect statement was deleted from the manuscript.

***4th Paragraph**. Do the authors have references for 'the most used method to determine compatibility of two plasmids is conjugation?'*

The following reference were added to that statement. References: Rozwandowicz M, Brouwer MS, Zomer AL, Bossers A, Harders F, Mevius DJ, Wagenaar JA, Hordijk J. Plasmids of Distinct IncK Lineages Show Compatible Phenotypes. Antimicrob Agents Chemother. 2017 Feb 23;61(3):e01954-16.

References** 1. Rozwandowicz M, Brouwer MSM, Mughini-Gras L, Wagenaar JA, et al.: Successful Host Adaptation of IncK2 Plasmids.Front Microbiol. 2019; **10**: 2384 PubMed Abstract | Publisher Full Text **Is the work clearly and accurately presented and does it cite the current literature?** Yes **Is the study design appropriate and does the work have academic merit?** Yes **Are sufficient details of methods and analysis provided to allow replication by others?** Yes **If applicable, is the statistical analysis and its interpretation appropriate?** Yes **Are all the source data underlying the results available to ensure full reproducibility?** Yes **Are the conclusions drawn adequately supported by the results?** Yes **Competing Interests:** No competing interests were disclosed. **Reviewer Expertise:** Plasmid biologist. Mobile Genetic Elements. Bacterial evolution. **I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.