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Original Research Article

Incompatibility and phylogenetic relationship of I-complex plasmids

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ABSTRACT

Plasmid incompatibility is the inability of two plasmids to be stably maintained in one cell, resulting in loss of one of the plasmids in daughter cells. Dislodgement is a phenotypically distinct form of incompatibility, described as an imperfect reproduction, manifesting in rapid exclusion of a resident plasmid after superinfection. The relationship between plasmids of the phenotypic incompatibility groups IncB/O and IncZ is unclear. Their inability to co-exist was initially referred to as dislodgement while other research reached the conclusion that IncB/O and IncZ plasmids are incompatible. In this manuscript we re-evaluated the relationship between IncB/O and IncZ plasmids to settle these conflicting conclusions. We performed dislodgement testing of R16Δ (IncB/O) and pSFE-059 (IncZ) plasmids by electroporation in a bacterial cell and checked their stability. Stability tests of the obtained plasmid pair showed that the IncB/O plasmid was exclusively and almost completely lost from the heteroplasmid *Escherichia coli* population. Other IncB/O – IncZ pairs could not form a heteroplasmid population, using conjugation or electroporation. Our data supports the previous suggestion that IncB/O and IncZ plasmids may be considered phenotypically incompatible.

1. Introduction

The first widely adopted plasmid typing scheme was based on phenotypically screened incompatibility, which is defined as an inability of plasmids to be stably maintained in a single cell (Datta and Hedges, 1971; Hedges and Datta, 1971). The I-complex plasmid group, designated due to the morphological and serological properties of their pili (Bradley, 1984; Falkow et al., 1974), consists of phenotypically incompatible plasmids including IncI1α and IncI1γ, IncI2, IncK, IncB/O and IncZ. Incompatibility of I-complex plasmids is caused by a small stem-loop structure called RNAI (Praszkier et al., 1989). Interaction of RNAI with *repB* (or *repY*, which is an equivalent gene to *repB* in I-type plasmids) is required for translation of replication initiation protein RepA (Praszkier and Pittard, 2005). The presence of two almost identical RNAI structures coming from two different plasmids leads to copy number destabilization and plasmid loss. Replication-based incompatibility results in a roughly even spread of loss of the plasmids upon superinfection, regardless of which plasmid was resident and which plasmid was coming into the recipient cell. The phenomenon can easily be confused with partition-based incompatibility and its phenotypically distinct form – dislodgement (Coetzee et al., 1972). Partition-based incompatibility describes the phenomenon where superinfection leads to the stable maintenance of a specific plasmid type over another, regardless of whether this plasmid was the resident or the invading plasmid (Novick 1987). Plasmid dislodgement refers to the phenomenon where, upon superinfection, the resident plasmid is always lost and (nearly) all recipient cells will only contain the invading plasmid. Plasmid dislodgement was described for several plasmid groups including IncI, IncH and IncT (Grindley et al., 1973; Hedges and Datta, 1973; Taylor and Grant, 1977). For some plasmid groups dislodgement was later disproven. It was shown that the IncT plasmid R394, which showed dislodgement with IncN plasmids, was in fact a co-integrate of these two replicons (Hauman et al., 1982). Another plasmid feature that can be confused with plasmid incompatibility is entry exclusion. It is a process where transfer of plasmid between bacterial cells is inhibited due to identical or closely related exclusion proteins, often encoded on related plasmids (Sakuma et al., 2013).

Tschäpe and Tietze first described the IncZ plasmid group and reported an unusual relationship of IncB (R16) and IncZ (pIE545) plasmids (Tschäpe and Tietze, 1983). In a heteroplasmid bacterial population carrying both IncB/O and IncZ plasmids, the resident plasmid was rapidly and exclusively lost. This observation was designated plasmid dislodgement. To test the incompatibility relationships, they used wild-type plasmids. Later, Praszkier et al. cloned minireplicon

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versions of IncB (pMU707) and IncZ (pIE545) into pBR322 and introduced them into IncB or IncZ minireplicon-containing cells (Praszkier et al., 1991). They obtained the same results of loss of the resident plasmid, but this was interpreted as incompatibility of IncB and IncZ plasmids, followed by the suggestion that these groups are effectively the same group of plasmids.

After the introduction of PCR-Based Replicon Typing (PBRT) and the introduction of a reliable and commercially available kit, this molecular method has become a universal standard to identify plasmids in Enterobacteriaceae (Carattoli et al., 2005). The grouping in this scheme has also become the basis for in silico replicon typing in whole-genome sequencing (WGS) data. Nonetheless, discrimination between replicon types based on phenotypical data is still useful for more in-depth studies of plasmids (Carattoli et al., 2014; Moran et al., 2015). In this study we describe the DNA sequence of a plasmid R16Δ, a derivative of the archetype IncB/O plasmid R16 (Evans et al., 1968), using both short and long read sequencing techniques. We compared the RNAI sequences of R16Δ to other plasmids that represent either IncB/O or IncZ. We tested incompatibility of IncB/O and IncZ plasmids using conjugation and electroporation. Additionally, we have performed stability testing between IncB/O and IncZ plasmids to determine their ability to stably persist in bacterial cells together.

2. Materials and methods

2.1. Plasmid and bacterial stains used

The strain carrying R16Δ was obtained from the Belgian Nuclear Research Centre (Evans et al., 1968). All other plasmids described in this study were recovered from human and animal samples that were collected and analysed in the Dutch national AMR monitoring program as reported in MARAN-report (www.wur.nl/en/Research-Results/Projects-and-programmes/MARAN-Antibiotic-usage.htm) and the EU-SAFEFOODERA project08176 (Wu et al., 2013) (Table 1). *E. coli* OT-ESBL-0589 was isolated from cattle in 2011, *E. coli* SFE-059 was isolated from a human urine sample in 2009 and *E. coli* SFE-199 was isolated from poultry meat in 2009. As a recipient in conjugation experiments, we used *E. coli* MG1655-YFP (Handel et al., 2015).

2.2. Sequencing

Illumina sequencing was performed on a Miseq platform using 2 × 250 bp reads and a 300 bp insert size. Long-read sequencing libraries were prepared according to the manufacturer's protocols using sequencing kit SQK-LSK108 and native barcoding kit EXP-NBD103 (Oxford Nanopore Technologies). Sequencing was performed on the MinION MK1B with flowcell type R9.4 (Oxford Nanopore Technologies). The hybrid assembly was performed using Unicycler v0.4.6 with default settings (Wick et al., 2017). Annotation was performed using prokka (Seemann, 2014). The resistance region of plasmid R16Δ was analysed using Galileo AMR (ARC BIO) (Partridge and Tsafnat, 2018). Toxin-antitoxin systems were predicted using TA finder based on TADB (Shao et al., 2010). Virulence genes were predicted using VirulenceFinder 2.0 (Joensen et al., 2014). RNAI structure prediction was performed using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) with default settings. Levenshtein distance of IncB/O and IncZ RNAI sequences was calculated using the online tool "Levenshtein Distance" (https://planetcalc.com/1721/).

2.3. Phylogenetic analysis

Phylogenetic trees were constructed from both newly sequenced plasmids and downloaded sequences. Alignment was performed with MAFFT (Katoh et al., 2002). For the RNAI-based phylogenetic tree we used Q-INS-I strategy and the gap opening penalty was set to 1.00. For the *repA*-based phylogenetic tree, default settings were used. Phylogenetic trees were built using IQ-TREE with the default settings (Trifinopoulos et al., 2016). Phylogenetic trees were visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Dislodgement testing

2.4.1. Electroporation

Plasmid R16Δ was isolated using the Wizard Plus SV Miniprep kit (Promega) and transferred to *E. coli* DH10B Electro MAX cells (Thermo Fisher Scientific) by electroporation. *E. coli* DH10B Electro MAX were transformed using: 2.0 kV, 200 Ω, 25 μF conditions with plasmid R16Δ miniprep DNA and transformants were selected on LB plates supplemented with 3 μg/ml tetracycline (Sigma-Aldrich). Electrocompetent *E. coli* cells carrying IncB/O or IncZ plasmids were prepared from a liquid culture (OD_{600} = 0.5) in LB with appropriate antibiotics. Cultures were centrifuged for 10 min with 3560 x*g* at 4 °C. Pellets were washed twice with ice-cold water, resuspended in ice-cold 10% glycerol and frozen at −80 °C. *E. coli* DH10B with R16Δ (DH10B-R16Δ) was transformed with plasmid pSFE-059 (IncZ) and transformants were selected on LB plates supplemented with 3 μg/ml tetracycline and 2 μg/ml cefotaxime (Sigma-Aldrich). The resultant strain was designated DH10B-R16Δ/pSFE-059. The presence of both replicons in the cell after electroporation was confirmed with replicon-specific PCRs using primers listed in Table 2. Additionally, we performed the same experiment with the reverse order of plasmids, namely plasmid pSFE-059 was transferred first to *E. coli* DH10B and later plasmid R16Δ was added as a superinfecting plasmid. That strain was designated DH10B-pSFE-059/R16Δ. These strains were used for stability experiments.

2.4.2. Liquid conjugation

Overnight cultures of *E. coli* DH10B, carrying an IncB/O or IncZ plasmid, and *E. coli* MG1655-YFP were renewed in fresh LB broth (Tritium Microbiologie) and grown to OD_{600} = 0.5, then mixed in 1:1 ratio and grown for 16 h at 37 °C. Transconjugants were recovered on LB plates supplemented with appropriate antibiotics. Five colonies were recovered from each plate and subjected to PCR using primers targeting the IncB/O or IncZ replicons (Table 2). Positive controls were included in each experiment, in which either pSFE-059 or pR3521 were conjugated into an empty *E. coli* MG1655-YFP. All experiments were performed in triplicate. As an additional control, compatibility of IncB/O

Table 1
Plasmids or plasmid sequences used in this study.

Plasmid name	Incompatibility group	Reference	Accession number
R16Δ	IncB/O	this manuscript	PRJEB30795
R16	IncB/O	(Evans et al., 1968); (Moran and Hall, 2019)	MK758104
SFE-059	IncZ	this manuscript	MN335639
SFE-199	IncZ	this manuscript	MN335638
OT-ESBL-0589	IncZ	this manuscript	MN335640
pIE545	IncZ	(Praszkier et al., 1991)	M93064.1 (only replication region)
pMU707	IncB/O	(Praszkier et al., 1991)	M93062.1 (only replication region)
p3521	IncB/O	(Papagiannitsis et al., 2011)	GU256641.1

Table 2
PCR primers used in the study.

Primer name	Primer sequence	Incompatibility
R16 fv	5′ CACAATCGCTCCGCCATAAG 3′	B/O
R16 rv	5′ GCAGGCAGTTTCTTCAGACG 3′	B/O
IncZ fv	5′ ACCGTCAGGTAAAGAACCCG 3′	Z
IncZ rv	5′ CCGCGACATTATCATGCGTT 3′	Z
3521 fv	5′ CGCTTCCGGAGATACTCAGC 3′	Z
3521 rv	5′ CCAGAGATTCAACCCTGTGC 3′	Z

Table 3
Pairs of plasmids used for the dislodgement and incompatibility testing.

donor \ recipient	pSFE-059	pR3521
pSFE-059	np	x
pOT-ESBL-0589	np	x
pSFE-199	x	x
pR3521	x	np

np = experiment was not performed, x = experiment was performed but no transconjugants or transformants were obtained.

and IncZ plasmid pairs was examined using both electroporation and conjugation as described. Plasmids pSFE059 and pR3521 were used as resident plasmids and plasmids pSFE199, OT-ESBL-0589, pSFE059 and pR3521 were used as an incoming plasmid (Table 3).

2.4.3. Stability

Stability of IncB/O and IncZ plasmids was examined in LB broth without antibiotic selection. *E. coli* DH10B-R16Δ/pSFE-059 and DH10B-pSFE-059/R16Δ were each grown overnight in LB broth without antibiotics and plated on non-selective LB plates. After overnight incubation, 100 colonies were replicated onto LB plates supplemented with 3 μg/ml tetracycline, 2 μg/ml cefotaxime or combinations of both antibiotics. 10 μl of the overnight culture was used to inoculate 10 ml of LB broth and grown overnight again. Culturing was repeated once more resulting in 72 h of growth. Plating was repeated after each sampling point. Stability tests were performed in triplicate. Ten colonies from each sampling time point that grew on double selection plates were tested with PCR for presence of IncB/O and IncZ plasmids.

3. Results

3.1. Sequence analysis

All plasmids in this study were sequenced using both Illumina and Nanopore sequencing platforms followed by hybrid assembly of the data. Overall, IncB/O and IncZ plasmids share a high degree of homology (Fig. 1). Besides plasmid R16Δ, which had lost some conjugal transfer genes, the conjugation regions of IncB/O and IncZ plasmids were highly similar. The region encoding genes responsible for the pili formation seems to be more diverse. IncZ plasmids share only about 80% homology compared to the 11,727 bp pili region of p3521, 87% homology to the same regions of pSFE-059 and pOT-ESBL-0589, and 75% for pSFE-199. The entry exclusion system inhibits the transfer of closely related plasmids and in I-complex plasmids it is mediated by two proteins ExcA and TraY (Smith et al., 2015). The *excA* gene of plasmid p3521 shares 93% identity with plasmids pSFE-199 and pSFE-059 and 96% with plasmid OT-ESBL-0589. The *traY* gene of plasmid p3521 shares 89% identity with plasmids pSFE-199 and pSFE-059 and 98% with plasmid OT-ESBL-0589. The gene encoding the RepA protein does not share homology between IncB/O and IncZ plasmids, which is in line with previous reports (Praszkier et al., 1991). A recently published manuscript discusses extensive mosaicism in IncB/O plasmids backbones (Moran et al., 2019). Plasmids pOT-ESBL-0589, pSFE-0569 and pSFE-199 described in this manuscript are also characterized by such mosaicism. The *oriT/nikA* and *nikB* region of plasmid pOT-ESBL-0589 is most closely related to the same region in plasmid p805a (accession number: MK088173) whereas its *traY-excA* region is identical to pEB1 (accession number: CP005999). The *oriT/nikA* and *nikB* regions of plasmids pSFE-0569 and pSFE-199 are most closely related to plasmid pCERC6 (accession number: MH287044), whereas the *traY-excA* region shares high and comparable degree of similarity to described *traY-excA* regions. It is identical to *traY-excA* region of plasmid pEc631_1 (accession number CP040264.1).

3.1.1. R16Δ

R16Δ is a 73,376 bp IncB/O non-conjugative derivative of plasmid R16. R16Δ shares 94% identity with the replication region of pMU707 (2167 bp), the reference IncB/O plasmid. It contains RelE/ParE and PndAC family plasmid stabilization systems. The resistance region of plasmid R16Δ is identical to the resistance region of previously published plasmid R16 (Moran and Hall, 2019).

Although plasmid R16 was previously described as capable of conjugative transfer (Tschäpe and Tietze, 1983), all three conjugation attempts of R16Δ in the present study failed. We compared the conjugation and pili encoding regions of R16Δ with R16. This analysis showed that the transfer region of plasmid R16Δ was incomplete (Fig. 2). Some essential genes for conjugation were absent, including *traC*, *pilO*, *pilQ*, *sogL*, *traM*, *traO*, *traQ* (Lawley et al., 2003; Yoshida et al., 1999; Narahara et al., 1997).

3.1.2. IncZ plasmids pSFE-059, pSFE-199, pOT-ESBL-0589

The three IncZ plasmids that were sequenced contained regions of high similarity, but the region that is known to contain accessory functions, like antibiotic resistance genes and associated mobilizing elements, differed significantly (Fig. 3). As a result of these differences, pOT-ESBL-0589 was 93,189 bp, pSFE-059 was 118,539 bp and pSFE-199 was 117,058 bp. A comparison of the replication regions of these plasmids to the reference plasmid pIE545 show that they had 98%, 100% and 98,37% sequence identity respectively. All three of the plasmids contained the RelBE or ParDE toxin antitoxin systems, which act in plasmid stabilization.

In plasmid pOT-ESBL-0589, the ESBL gene $bla_{\text{CTX-M-1}}$ is associated with IS*Ecp1* which is likely to be responsible for its insertion and is identical to the region described in the IncI1 plasmid pCTX1261 (HF549090)(Schink et al., 2013). Of note is that the element has not inserted in the region that is usually associated with accessory functions, but within the conjugation region.

In pSFE-199 and pSFE-059 incorporation of various integrative elements into the accessory regions of the plasmids have led to mosaic regions in which multiple antimicrobial resistance genes are present (Fig. 3). In pSFE-199 the tetracycline resistance gene *tetA*(A) and its regulator *tetR*(A) were incorporated by insertion of Tn*1721*. A partial copy of Tn*21* is present, which is interrupted by insertions of IS*26* and a partial copy of IS*Ecp1*. This region is similar in sequence and structure to an integration previously described in IncN and IncI plasmids and encodes for the ESBL gene $bla_{\text{CTX-M-1}}$ and the macrolide resistance gene *mphA* (Cullik et al., 2010). In the accessory region of pSFE-059, an aminoglycoside resistance gene *aadA22* is part of a class 1 integron, as previously described in *Salmonella* Chailey (Kim et al., 2011). The ESBL gene $bla_{\text{CTX-M-32}}$ is flanked by a copy of IS*Ecp1* which was interrupted by an insertion of IS*Kpn26* and *orf477*, as previously described in an *E. coli* strain isolated in Portugal (Tacao et al., 2012). This complete cassette is flanked by two copies of IS*26*.

Plasmids pSFE-059 and pSFE-199 carry a Per-activated serine protease autotransporter, which is part of a type V secretion system protein. An identical autotransporter protein is encoded on another IncZ plasmid - pH2332-107 (accession number: KJ484627.1). The Type V secretion system is involved in secretion to the cell surface of some proteases and lipases, as well as proteins involved in bacterial adhesion to surfaces and biofilm formation (Meuskens et al., 2019).

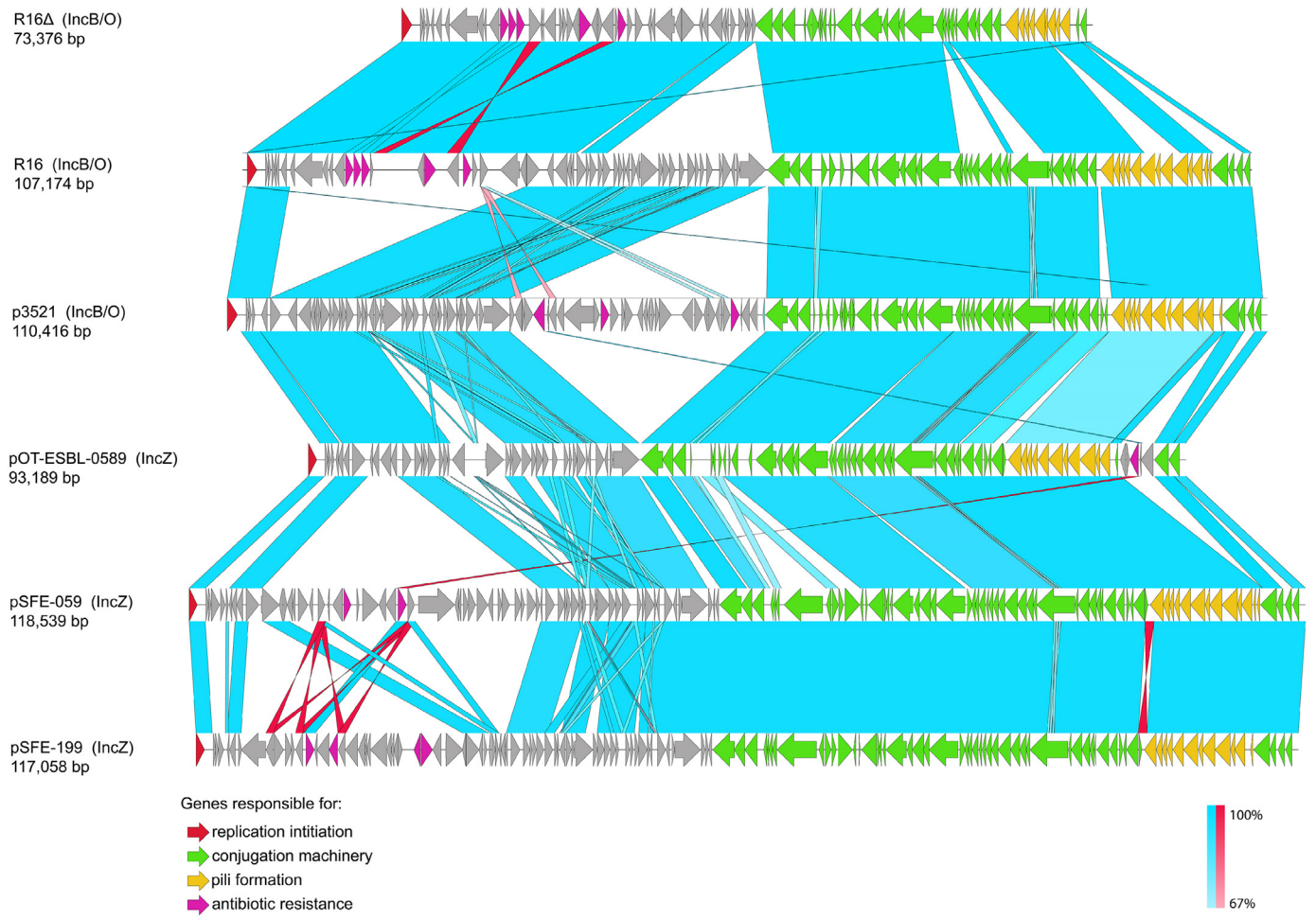

Fig. 1. Comparison of the four plasmids sequenced in this study with previously sequenced IncB/O plasmid p3521. These are linearized representations of the plasmid sequences. Open reading frames are indicated by coloured arrows to match the gene's functions as indicated at the bottom of the figure. Regions of homology are connected by blue boxes, inversions are indicated by red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Comparison of the incompatibility and replication regions

An alignment of the DNA sequence of the RNAI structure of available sequences of IncB/O and IncZ plasmids was performed (Fig. 4). The nucleotide sequence and the resulting stem loop structure of the predicted RNAI molecule differed between plasmids from different groups while they are strongly conserved within plasmid groups, which is in line with previous findings (Zhang et al., 2019; Moran and Hall, 2019; Moran et al., 2015; Praszkier et al., 1991) There is 83% identity between the IncB/O and IncZ RNAI DNA sequence, and they have a Levenshtein distance of 9 mutations/indels (Levenshtein, 1966).

Two phylogenetic trees, based on the *repA* gene and RNAI regions,

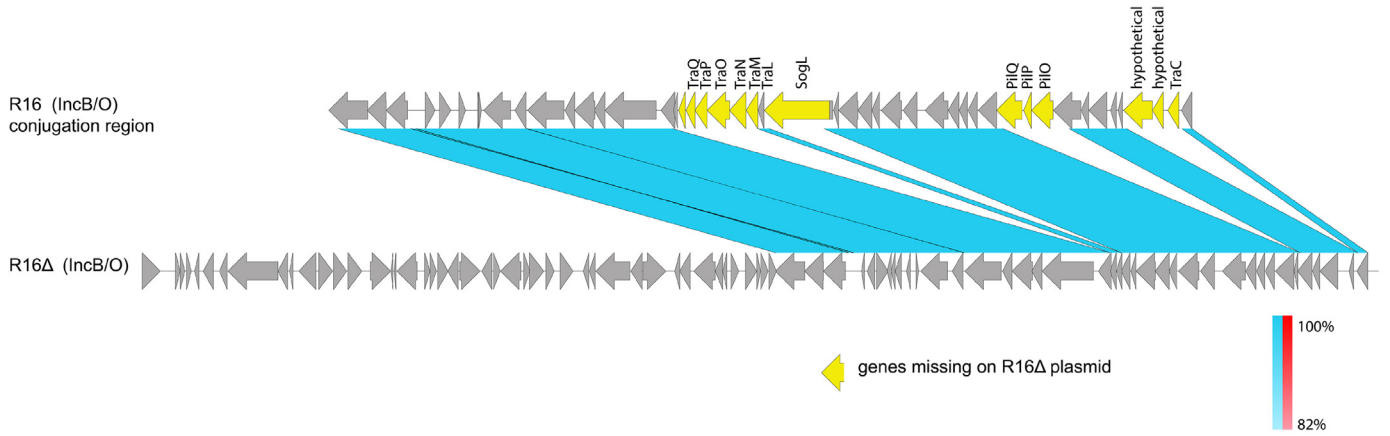

Fig. 2. Comparison of the conjugation region of R16 plasmid (top) with whole sequence of plasmid R16Δ (bottom). Yellow arrows indicate genes missing on plasmid R16Δ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

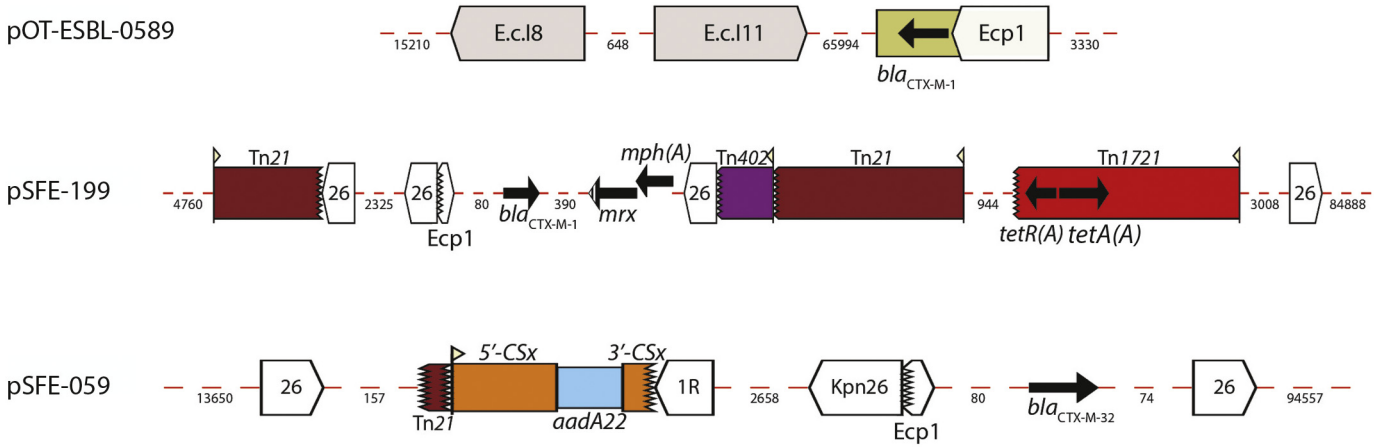

Fig. 3. Resistance region of plasmids pOT-ESBL-0589, pSFE-199 and pSFE-059. Copies of previously described mobile elements are shown as blocks, partial mobile elements show jagged lines at the edges. Complete resistance genes are depicted as arrows in the orientation of transcription. Inverted and direct repeats are shown at the edge of a mobile element by stemloop structures.

were constructed independently in order to examine the relatedness of plasmids from the I-complex based on these regions. The *repA*-based phylogenetic tree shows two main clusters. One is formed by IncK1, IncB/O and IncI plasmids and the other from IncK2 and IncZ plasmids (Fig. 5). A representative IncI2 plasmid (R721) is phylogenetically further apart from IncB/O and other IncI plasmids. The RNAI-based phylogenetic tree shows a different relationship between these plasmid groups (Fig. 6). IncK1 and IncK2 plasmids are phylogenetically distant from IncB/O, IncZ and IncI plasmids. We were unable to identify homologs of RNAI on the archetype IncI2 plasmid R721.

3.3. Testing for incompatibility and dislodgement

To determine the biological relationship between the IncZ and IncB/O plasmids, we performed a series of electroporation and conjugation experiments with *E. coli* strains carrying one IncB/O or IncZ plasmid (Table 3). R16Δ and pSFE-059 were chosen to represent IncB/O and IncZ in an experiment where both plasmids were introduced into a single cell by two independent rounds of electroporation, resulting in cells containing both plasmids when cultured on double selective agar plates. When both plasmids were in the same bacterial cell, we observed a rapid loss of the plasmid R16Δ when cultured in non-selective media, while plasmid pSFE-059 remained 100% stable in *E. coli* cells, as determined by subculturing individual colonies on single selective or double selective agar plates (Fig. 7). This occurred regardless of the order in which the plasmids entered the bacterial cell. Part of the population, which was growing on double selection plates, contained both IncB/O and IncZ plasmids, which means that for at least three consecutive rounds of O/N culture, plasmid R16Δ was not lost completely. Presence of both plasmids in the cell was confirmed by PCR in ten colonies. *E. coli* DH10B containing either plasmid R16Δ or pSFE-059 were tested separately in order to confirm their stability and both were stable for three consecutive rounds of O/N culture without selection (data not shown).

Two additional IncZ and one IncB/O plasmids were chosen to test the compatibility of these plasmid groups via conjugation (Table 3). All plasmids were used in donor and recipient cells in triplicate. None of the tested plasmid pairs resulted in a heteroplasmid population, which was confirmed by PCRs targeting both plasmids. Compatibility of the same plasmid combinations was additionally tested via electroporation. All tested plasmids were used both as a resident or superinfecting plasmid. Heteroplasmid populations were also not obtained via electroporation, which was confirmed by PCRs targeting both plasmids. Positive control experiments were performed to demonstrate that these plasmids could be transformed into *E. coli* DH10 and conjugated into *E. coli* MG1655-YFP. These experiments all resulted in positive results (data not shown).

4. Discussion

In this study we sequenced the archetype IncB/O plasmid R16Δ and three IncZ plasmids – pSFE-059, pSFE-199, pOT-ESBL-0589 using Illumina and Nanopore sequencing techniques. Having the full plasmid sequence, rather than a collection of contigs, is essential for detailed plasmid sequence analysis (George et al., 2017).

Plasmid R16 was originally reported to be conjugative, but our experiments, supported by the DNA sequence analysis, showed that the plasmid we were working with had lost the ability to conjugate, probably due to several deletions in the tra- and pil-regions. These genes are responsible for pilin maturation, DNA processing and entry exclusion (Klimke et al., 2005). These deletions may have occurred after long-term storage, but we could not locate any other source for

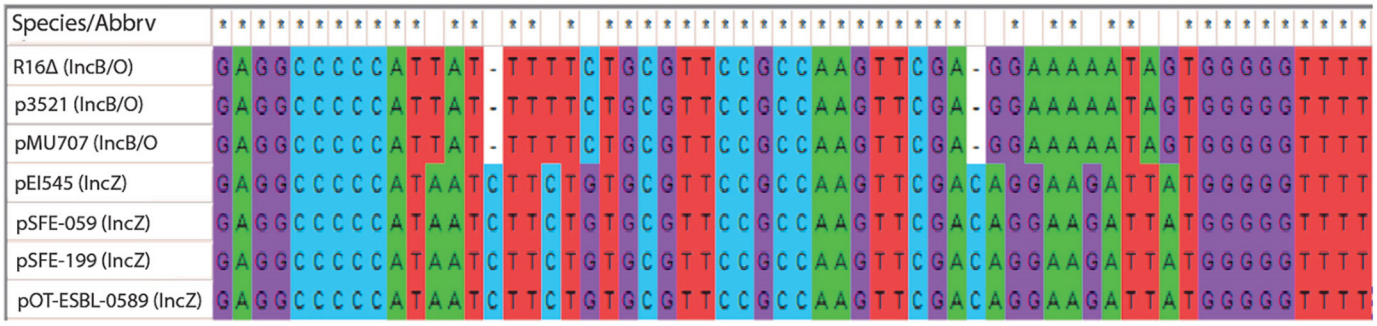

Fig. 4. Comparison of RNAI sequences of IncB/O and IncZ plasmids. Asterisks on top of the sequence indicate conserved nucleotides.

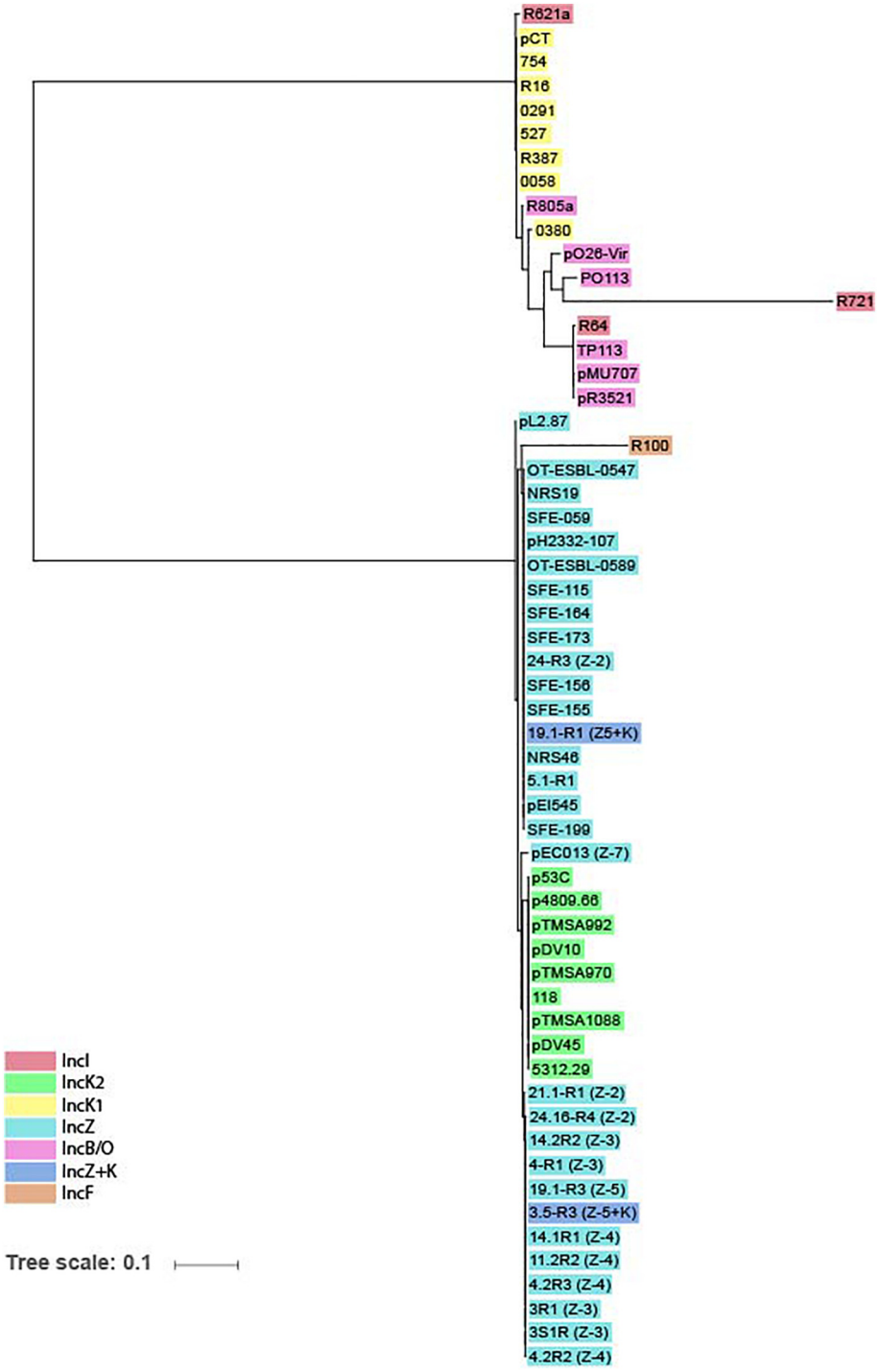

Fig. 5. RepA-based phylogenetic tree of I-complex plasmids. For visualization purposes, branches intersected by // are displayed as 10% of the actual length. Plasmid names are coloured based on the incompatibility group in which they were organised in the original studies, based phenotypic incompatibility typing, PBRT or in silico PBRT. See supplementary table (S1) for accession numbers.

this specific plasmid. As such, this R16 derived plasmid is referred to here as R16Δ. Recently, a sequence of wild type R16 plasmid, which was still conjugative and was obtained from a stock culture from the 1950s, was published (Moran and Hall, 2019). This plasmid is substantially bigger in size than R16Δ sequenced in this study. Besides parts of conjugation and pilus formation genes, R16Δ lacks a region (20,942 bp) containing a nickase, plasmid SOS inhibition proteins A and B and hypothetical proteins.

In this study we used three IncZ plasmids together with the R16Δ plasmid, which was originally used to describe the phenomenon of dislodgement (Tschäpe and Tietze, 1983). Dislodgement, which can be mistaken with incompatibility, was defined as a rapid exclusion of a resident plasmid after superinfection (Coetzee et al., 1972). Two previous studies examined dislodgement between IncB/O and IncZ plasmids: Tschäpe and Tietze used only wild-type plasmids, while Praszkier et al. used multicopy pBR322-derivatives of IncB/O and IncZ plasmids with minireplicon versions (Praszkier et al., 1991; Tschäpe and Tietze, 1983). Similar results were obtained, but these were interpreted differently, resulting in different conclusions. The first group reported dislodgement of the plasmids, while the latter designated IncB/O and

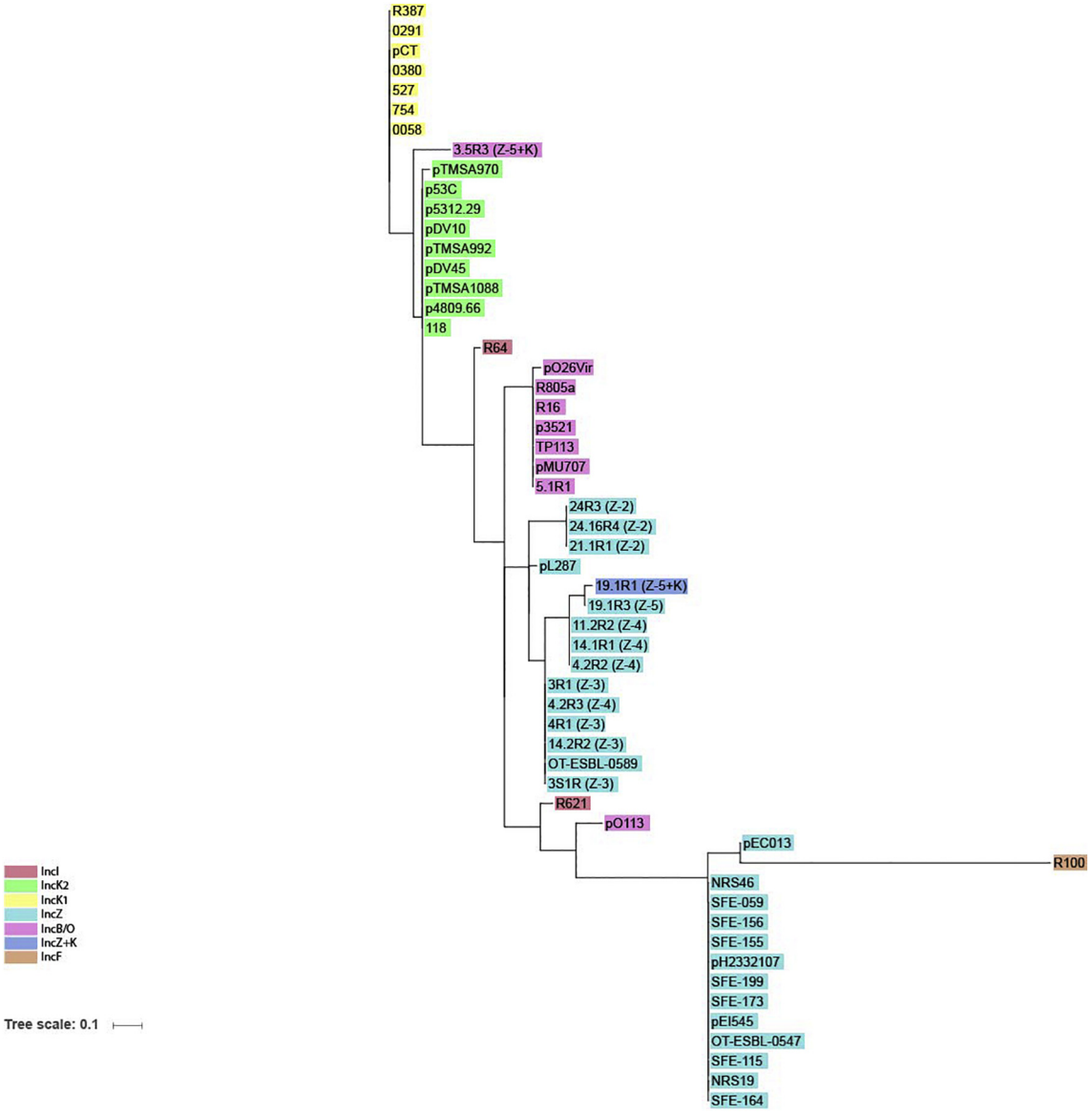

Fig. 6. RNAI-based phylogenetic tree of I-complex plasmids. Plasmid names are coloured based on the incompatibility group in which they were organised in the original studies, based phenotypic incompatibility typing, PBRT or in silico PBRT. See supplementary table (S1) for accession numbers.

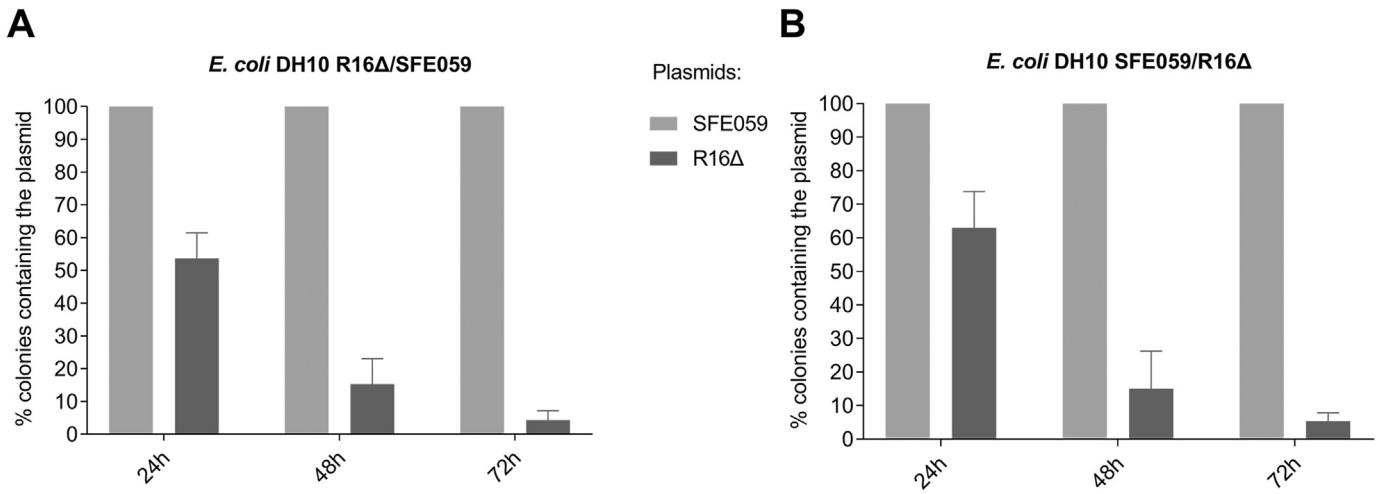

Fig. 7. Stability of single IncZ (pSFE-059) and IncB/O (R16Δ) and combination of both plasmids in *E. coli* DH10B R16Δ/SFE059 and *E. coli* SFE059/R16Δ grown without antibiotic pressure. Stability experiments were performed in triplicate. Bars represent standard deviation.

IncZ plasmids as incompatible.

In this study we created a bacterial strain carrying the IncB/O plasmid R16Δ and the IncZ plasmid pSFE-059. Both plasmids were completely stable in a single plasmid population after three culturing rounds. However, plasmid R16Δ was highly unstable and almost completely lost from a heteroplasmid population created using electroporation, whereas plasmid pSFE-059 was 100% stable after three rounds of consecutive batch cultures, in the absence of selective pressure. One of the reasons only plasmid R16Δ was lost might be its inability to conjugate. Plasmid pSFE-059, even if lost from the cell, can be re-introduced to plasmid-free cells via conjugation. Therefore, we did not only observe a rapid loss of the resident plasmid from the heteroplasmid population, which would indicate dislodgement, as it was suggested in previous studies, but specifically of the IncB/O plasmid that was tested. Compatibility of five other IncB/O and IncZ plasmid pairs was tested (Table 3). We were unable to create a bacterial strain carrying both plasmids from any of these pairs using conjugation or electroporation. Plasmid pSFE-059, which was present in one cell together with R16Δ, was not detected in any cells with any other IncB/O plasmid. These results suggest that the plasmid pair of pSFE-059 - R16Δ was exceptional and does not represent the relationship between IncB/O and IncZ plasmids. Therefore, our results do not support the hypothesis that the relationship between IncB/O and IncZ plasmids should be considered as dislodgement. Overall, the results demonstrate that IncB/O and IncZ plasmids are incompatible.

In this study we created phylogenetic trees of I-complex plasmids: based on the *repA* gene and the RNAI region. As shown in Fig. 4, the stem loop structure of the RNAI molecule differs between plasmids from different groups, while they are strongly conserved within plasmid groups. This is in line with previous findings (Zhang et al., 2019; Moran and Hall, 2019; Moran et al., 2015; Praszkier et al., 1991). To our knowledge this is a first attempt to summarize and confirm the work that was previously published on I-complex plasmids on a larger sample set. The *repA*-based phylogenetic tree shows a clear clustering of plasmids: the first clusters contains IncK1, IncB/O and IncI plasmids and the second cluster contains IncK2 and IncZ plasmids. Recently Zhang et al. also published a *repA*-based phylogenetic tree of I-complex plasmids (Zhang et al., 2019). Even though both trees were constructed on plasmid sets with a minimal overlap, the clustering of plasmids is the same as described in this study. The RNAI-based tree shows completely different clustering as compared to the *repA*-based tree. IncK1 and IncK2 plasmids are phylogenetically distant from IncB/O, IncZ and IncI plasmids. Phylogenetic group discrepancies between RNAI-based and other conserved plasmid genes-based analysis were shown for plasmid R471 (Carattoli et al., 2015). According to the core genome ExcA-, TraX- and TraY-based phylogenetic trees, this plasmid belongs to the IncL plasmids group, but RNAI-based analysis position it within IncM1 cluster. Seven types of amplicons of IncZ plasmids were published previously (Moran et al., 2015; He et al., 2019). Six out of seven types were included in the phylogenetic analysis presented in this study. Although here we used only the RNAI sequence instead of the whole amplicon, similar clustering was seen as previously described except for plasmid 4.2-R3, which was typed as Z-4, but clustered here together with Z-3 plasmids. In the light of previous findings, showing that IncZ plasmids may have formed by recombination of IncFII and IncI1α plasmids, (Kato and Mizobuchi, 1994), we decided to include IncFII plasmid R100 in our analysis. Plasmid R100 clusters closely with IncZ plasmids, which shows that the findings of this study are in line with previous reports (He et al., 2019). The results confirm on a larger sample set that the evolution of the plasmid replication initiation protein (RepA) and incompatibility determinant (RNAI), which are both part of the replication system, can be independent of each other. Additionally, previously published manuscript showed a high mosaicism of IncB/O plasmid backbones. Analysis performed in this manuscript prove similar mosaicism of IncZ plasmids backbones. Additional research is needed to determine if other members of I-complex plasmid group show similar high mosaicism.

This manuscript combines classical incompatibility testing with modern sequence and phylogenetic analysis. It highlights that sequence-based plasmid types do not necessarily correspond with plasmid incompatibility groups designated using phenotypic methods. Both methods provide valuable information, which can be used for different purposes. Phenotypic incompatibility provides an insight about the possibilities of plasmid transmission and possible combinations of different plasmids present in one cell, while plasmid sequence types have a higher resolution and allow us to associate and compare plasmids with certain hosts or regions. We believe that both typing methods are valuable and they both should be used in typing newly discovered or redefined plasmid groups. A good illustration are recent studies in which compatibility of the IncLM and IncA/C plasmid groups were revised, based on phenotypic incompatibility experiments, resulting in splitting the groups into IncL and IncM groups and IncA and IncC groups, respectively (Carattoli et al., 2015; Ambrose et al., 2018).

In conclusion, the results suggest that the IncB/O and IncZ plasmids are incompatible and, based on phenotypic tests, could therefore be considered one plasmid group. This is in contrast to the different lineages that have been observed using sequence-based techniques. Additional research, including more plasmids of the different lineages, should be performed to confirm our findings.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2020.102502.

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