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DOI
10.1016/j.plasmid.2022.102640

Publication date
2022

Document Version
Final published version

Published in
Plasmid

Plasmid 122 (2022) 102640

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Original Research Article

Genetic editing of multi-resistance plasmids in *Escherichia coli* isolated from meat during transfer

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ARTICLE INFO

Keywords:
Horizontal gene transfer
Mobile genetic element
Resistance plasmid
Antimicrobial
Genetic rearrangement

ABSTRACT

Resistance plasmids mediate the rapid spread of antimicrobial resistance, which poses a threat to veterinary and human healthcare. This study addresses the question whether resistance plasmids from *Escherichia coli* isolated from foodstuffs always transfer unchanged to recipient *E. coli* cells, or that genetic editing can occur. Strains containing between one and five different plasmids were co-incubated with a standard recipient strain. Plasmids isolated from transconjugant strains were sequenced using short and long read technologies and compared to the original plasmids from the donor strains. After one hour of co-incubation only a single plasmid was transferred from donor to recipient strains. If the donor possessed several plasmids, longer co-incubation resulted in multiple plasmids being transferred. Transferred plasmids showed mutations, mostly in mobile genetic elements, in the conjugative transfer gene *pilV* and in genes involved in plasmid maintenance. In one transconjugant, a resistance cluster encoding tetracycline resistance was acquired by the IncI1 plasmid from the IncX1 plasmid that was also present in the donor strain, but that was not transferred. A single plasmid transferred twelve times back and forth between *E. coli* strains resulted in a fully conserved plasmid with no mutations, apart from repetitive rearrangements of *pilV* from and back to its original conformation in the donor strain. The overall outcome suggests that some genetic mutations and rearrangements can occur during plasmid transfer. The possibility of such mutations should be taken into consideration in epidemiological research aimed at attribution of resistance to specific sources.

1. Introduction

Antimicrobial resistance is spread fast and effectively by resistance plasmids (Carattoli, 2013; Levy and Marshall, 2004; Lopatkin et al., 2017; Sommer et al., 2017). This poses a serious risk for veterinary and human healthcare as infections with resistant pathogens are becoming increasingly difficult to treat (Tacconelli et al., 2018). In livestock, resistance genes on plasmids encoding for resistance to beta-lactam antibiotics and/or tetracycline are especially widespread (Kaesbohrer et al., 2019; Stine et al., 2007; Verraes et al., 2013). The application of these antibiotics in livestock farming causes the selection and spread of resistance plasmids (CDC, 2021). In the end, genes located on such plasmids can transfer to human healthcare (Mughini-Gras et al., 2019). Resistance plasmids are known to spread from livestock human healthcare by a variety of routes (EFSA, 2021). To better understand the dynamics of plasmid transfer, it is crucial to know what exactly is transferred during conjugation and whether plasmids can sometimes undergo genetic editing during the event.

Plasmids are usually classified by incompatibility group (Couturier et al., 1988; Thomas, 2014). Incompatibility of plasmids refers to the notion that 2 plasmids of the same class in one cell cannot co-exist due to competition for the same replication system. (ES)BL and tetracycline resistance are strongly associated with IncF and IncI-type plasmids (Rozwandowicz et al., 2018). Plasmids typically harbor a conserved and a variable region (Orlek et al., 2017). The conserved region mainly consists of genes encoding for conjugation, replication, and maintenance (Fernandez-Lopez et al., 2016; Zhang et al., 2019), while the variable region contains accessory genes such as resistance genes (Orlek et al., 2017).

Most *Escherichia coli* strains isolated from meat destined for the

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https://doi.org/10.1016/j.plasmid.2022.102640
Received 14 July 2022; Accepted 18 July 2022
Available online 21 July 2022

consumer market harbored multiple plasmids (Darphorn, Bel, et al. 2021). IncI, IncX and IncF type plasmids were represented most in this subsample, while IncB, IncN and IncR type plasmids were found more scarcely. The plasmids harbored a wide variety of resistance genes encoding for ESBL, BL, tetracycline, aminoglycoside, sulphonamide, fluoroquinolone, and chloramphenicol resistance. Resistance plasmids and resistance genes in *E. coli* from veterinary or human healthcare have been studied extensively (Carattoli, 2013; Rozwandowicz et al., 2018; van Hoek et al., 2011). There are many types of these plasmids in databases that show high similarity to each other but differ in their variable region showing different clusters of resistance genes, while the constant region is fully conserved. In other cases, small mutations or rearrangements are known for common genes in the conserved region such as *pilV* for IncI-type plasmids (Komano et al., 1987; Sekizuka et al., 2017). The question thus arises whether these mutations or variations can come about during transfer? Especially when a donor strain already harbors multiple plasmids with multiple resistance gene clusters, this would be a logical consequence of the known genetic processes. If they occur, do these changes happen mostly in the variable regions, or in conserved regions? In addition, longer co-incubation of donor and recipient strains could have different effects on the plasmids and so could repeated back and forth transfer of one plasmid.

To answer these questions, a set of donors, *E. coli* strains isolated from foodstuffs, was co-incubated with a general recipient to obtain transconjugants using a standardized transfer procedure. This method can be adapted to also obtain transconjugants after longer co-incubation and continuous back and forth transfer. The plasmids of the transconjugants were isolated and sequenced using short and long read technologies and compared to the plasmids of the donor strain to detect any mutations or rearrangements.

2. Materials & methods

2.1. Strains used

The strains used in experiments are shown in Table 1. The *E. coli* plasmid containing strains were isolated from foodstuffs by the Dutch Food and Consumer Product Safety Authority (NVWA), characterized by Wageningen Bioveterinary research (WBVR) and donated by Dr. Kees Veldman of WBVR. These strains originated from turkey, bovine or chicken meat and were selected for having minimally beta-lactam resistance. The NVWA originally established the resistance with a sensitivity test (National Institute for Public Health and the Environment, 2020) and this was confirmed in this study using MIC assays (Schuurmans et al., 2009). Plasmid presence was verified by the detection of incompatibility groups and further characterization (Darphorn et al., 2021a; Garcia-Fernandez et al., 2009). The chloramphenicol resistant (chlor^R) *E. coli* MG1655 YFP (kindly provided by MB Elowitz) (Elowitz et al., 2002) was used as common recipient strain for transfer experiments as described below. Another *E. coli* MG1655 strain was evolved to build up enrofloxacin resistance by exposure to stepwise increasing concentrations over a period of two weeks. This strain was used as an alternative recipient whenever donor strains were already resistant to chloramphenicol, as was the case for *E. coli* 3277 and 3308.

2.2. MIC measurements

MIC values were used throughout the study to confirm the presence of resistance plasmids. MIC was measured as described by Schuurmans et al. (2009) in 96-well plates in a ThermoScientific Multiskan FC spectrophotometer plate reader. Plates were shaken and kept at 37 °C in a final volume of 150 μl with a starting OD595 of 0.05 for 23 h. Antibiotic concentrations stepwise increasing by a factor of 2 and ranging from 1 μg/ml to 2048 μg/ml were used. The lowest concentrations that limited final OD to 0.2 or less was reported as the MIC. MICs of the transconjugants were determined for ampicillin, amoxicillin, tetracycline, kanamycin, enrofloxacin and chloramphenicol.

2.3. Transfer experiments

Transconjugants were obtained by performing transfer experiments. The donor and recipient cells were grown overnight in defined minimal mineral medium containing 55 mM glucose with a pH of 6.9 and a buffer of 15.6 g/L $Na_2H_2PO_4$ (Evans et al., 1970). In preliminary experiments a standardized mating procedure was designed that eliminated the effects of growth and yielded reproducible numbers of transconjugants (Darphorn et al., 2022). The overnight cultures were starved to deplete the cells of glucose by centrifuging the cells at 4400 rpm for 15 min, discarding the spent medium and adding new minimal medium without glucose. Cells were incubated for 4 h at 37 °C to ensure starvation. Starvation ensured that all cells growing on the double selective plates are true transconjugants and not further growth of original transconjugants. The transfer experiment is initiated by mixing donor and recipient cells in a 1:1 ratio at a density of approximately 2 10^8 cells/ml in minimal medium without glucose. The cells were co-incubated for 1 h at 37 °C while shaken at 200 rpm. Appropriate dilutions of the mixture were pipetted onto selective LB plates (1% NaCl; 0.5% yeast extract; 1% g bactotryptone and 2% agar). The selective plates contained either ampicillin (amp) to differentiate the donor, chloramphenicol (chlor) or enrofloxacin (enr) to select the recipient or both amp and either chlor or enr to distinguish the transconjugants. Final concentration of antibiotics in the plates was 64 μg/ml. Stock solutions of 10 mg/ml antibiotics were filter sterilized and stored at 4 °C for maximum up to 2 weeks. Transconjugants were isolated from plates and tested for MIC before storing in

Table 1
Recipient strains with their corresponding chromosomal resistance as mated with donor strains containing plasmids. Listed are number of plasmids (#), replicon types and beta-lactamase genes found in these donor strains.

Recipient		Donor	Donor plasmids		
Strain	Resistance	Strain	#	Replicon type	Beta-lactamase
E. coli MG1655	chlor^R	*E. coli* 2082	5	IncI1, IncX4, IncX1, IncFIB/FII, p_{phage}	bla_{SHV-12}, bla_{TEM} (3×)
E. coli MG1655	chlor^R	*E. coli* 3153	3	IncI1, IncX4, IncFII	$bla_{CTX-M-1}$
E. coli MG1655	chlor^R	*E. coli* 3156	3	IncB/0/K/Z, IncX4, IncFIB/FII	bla_{CMY-2}, bla_{TEM-1B}
E. coli MG1655	chlor^R	*E. coli* 3170	3	IncI1, IncFIB, IncFIC/FII	$bla_{CTX-M-1}$
E. coli MG1655	chlor^R	*E. coli* 3171	2	IncI1, IncFII	bla_{CMY-2}
E. coli MG1655	chlor^R	*E. coli* 3227	2	IncI1, IncFIB/FII	$bla_{CTX-M-2}$, bla_{TEM-1B}
E. coli MG1655	chlor^R	*E. coli* 3231	3	IncN, IncFIB/FII, p0111	bla_{TEM}, bla_{SHV-12}
E. coli MG1655	enr^R	*E. coli* 3277	1	IncFIB/FIC	$bla_{CTX-M-55}$
E. coli MG1655	chlor^R	*E. coli* 3288	1	IncFIA/FIB/FII	$bla_{CTX-M-15}$
E. coli MG1655	chlor^R	*E. coli* 3301	3	IncI1, IncX1, IncFIB/FII	$bla_{CTX-M-1}$
E. coli MG1655	enr^R	*E. coli* 3308	4	IncI1, IncY, IncFIB, IncFIC/FII	$bla_{CTX-M-1}$
E. coli MG1655	chlor^R	*E. coli* 3310	2	IncB/0/K/Z, IncFIB/FIV	bla_{CMY-2}
E. coli JW3686	kan^R	*E. coli* MG1655 pIncI3170	1	IncI1	$bla_{CTX-M-1}$
E. coli MG1655	chlor^R	*E. coli* JW3686 pIncI3170	1	IncI1	$bla_{CTX-M-1}$

glycerol stocks at −80 °C. To examine the effect of the length of co-incubation on the number of plasmids transferred, *E. coli* 2082, which contains 5 plasmids, and MG1655 were co-incubated for 1 and 24 h. Three isolates from 1-h co-incubation and two isolates from a 24-h co-incubation were randomly picked for plasmid isolation.

To examine whether a single plasmid undergoes changes after multiple back and forth transfers, a transconjugant containing the IncI1 plasmid of *E. coli* 3170 (*E. coli* MG1655 pIncI3170) was initially mated with *E. coli* JW3686 (kan^R). The transfer was continued by using a transconjugant as the new donor strain (*E. coli* JW3686 pIncI3170) mating with *E. coli* MG1655 (chlor^R) as recipient. These steps were repeated so that in the end the plasmid had been transferred back and forth between these strains for a total of 12 transfers. Transconjugant strains were tested for MIC and for positivity in the indole test. The indole test was used to confirm that the isolated transconjugant derived from the intended recipient and in that way to rule out the, unlikely, possibility that the donor strain had obtained de novo resistance to the selective antibiotic. Plasmids were isolated at the beginning and end-points of the repeated transfer experiment.

2.4. DNA isolation and sequencing

Plasmid isolation of the transconjugant and donor strains listed in Tables 1, 2 and 3 were carried out using the Qiagen Plasmid Maxi Kit. The cell pellets from 400 mL of overnight culture were used as substrate for this kit. DNA obtained were checked for purity with Nanodrop. Samples with low concentration or contaminations were purified with ethanol precipitation. Sequencing was performed by BaseClear B.V. (Leiden, The Netherlands). Most samples were sequenced using a hybrid method with the Illumina NovaSeq 6000 and PacBio systems, obtaining short and long read data. Samples shown by analysis of short reads to contain a single plasmid were sequenced using Illumina short reads only, as assembly was straightforward in this case. For samples containing multiple plasmids hybrid assembly was needed to distinguish the separate plasmids with confidence. Hybrid assembly was performed by first improving the quality of the Illumina reads by trimming of low-quality bases using BBDuk, part of BBMap suite version 36.77 (Bushnell B., http://sourceforge.net/projects/bbmap/). High-quality reads were assembled into contigs using ABySS version 2.0.2 (Jackman et al., 2017). The long reads were mapped to the draft assembly using BLASR version 1.3.1 (Chaisson and Tesler, 2012). Based on these alignments, the contigs were linked together and placed into scaffolds. The orientation, order, and distance between the contigs were estimated using SSPACE-LongRead version 1.0 (Boetzer and Pirovano, 2014). Using Illumina reads, gapped regions within scaffolds were closed using GapFiller version 1.10 (Boetzer and Pirovano, 2012). Finally, assembly errors and the nucleotide disagreements between the Illumina reads and scaffold sequences were corrected using Pilon version 1.21 (Walker et al., 2014). Illumina only data was assembled in a slightly different manner. The quality of Illumina reads was improved using the error correction tool BayesHammer (Nikolenko et al., 2013). Error-corrected reads were assembled into contigs using SPAdes version 3.10 (Bankevich et al., 2012). The order of contigs, and the distances between them, were estimated using the insert size information derived from an alignment of the paired end reads to the draft assembly. Consequently, contigs were linked together and placed into scaffolds using SSPACE version 2.3 (Boetzer et al., 2011). Gapped regions within scaffolds were closed using GapFiller version 1.10 (Boetzer and Pirovano, 2012) and assembly errors were corrected using Pilon version 1.21 (Walker et al., 2014).

The tools for sequencing and assembling plasmids are not yet as reliable as those for genomic DNA. Plasmids are more variable and have more sequences such as repeats, transposable elements, etc., that are difficult to analyze. For this reason, we discarded data that could be interpreted in more than one way. In addition, we examined the literature to determine whether presumed changes were observed before as an extra reliability check. Sequences were only reported when the sequencing quality reached minimally Q30, indicating that virtually all reads will be correct, without errors or ambiguities. Actually, almost all sequences scored over Q35. The minimal coverage was 30, but most ranged in the hundreds to (ten)thousands. Plasmids from strains that had been used often and thus were grown for many generations, were unchanged and experimental replicates invariably had identical mutations, ruling out random errors and misreading as origin of observed mutations. Furthermore, we ascertained that the conclusions were supported by data obtained by several different methods.

Table 2
Number of plasmid types (#) and their Inc. classification as found in the *E. coli* donor and transconjugant strains. There can be more than 1 type within one plasmid.

Strain	Donor plasmids		Transconjugant plasmids	
	#	Inc types	#	Inc types
2082	5	I1, X4, X1, FIB/FII, FIB (phage)	1	X4
3153	3	I1, X4, FII	1	I1
3156	3	B/O/K/Z, X4, FIB/FII	1	B/O/K/Z
3170	3	I1, FIB, FIC/FII	1	I1
3171	2	I1, FII	1	I1
3227	2	I1, FIB/FII	1	I1
3231	3	N, FIB/FII, P0111	1	N
3277	1	FIB/FIC	1	FIB/FIC
3288	1	FIA/FIB/FII	1	FIA/FIB/FII
3301	3	I1, X1, FIB/FII	1	I1
3308	4	I1, Y, FIB, FIC/FII	1	I1
3310	2	B/O/K/Z, FIB/FIC	1	B/O/K/Z

Table 3
Mutations found in the plasmids isolated from the transconjugant strain as compared to the plasmid of the donor strain.

Genes with mutations		Specific mutation	Transconjugant strain from donor
pilV rearrangement		See Fig. 1	3170, 3171, 3227, 3301, 3308
Mobile genetic element	IS*26*	R45W	2082
		G184N	2082, 3301
		N184G	3288 (2×)
	insAB	F81L (*insA*)	3277
		T31A (*insA*), C5S (*insB*)	3288
	Tn*As1*	N422K	2082
	IS*91*	Q179H, M210L, S232A	3153
ardA		D78G	3153
ssb		F *ssb* to ColIb *ssb*	3156
psiB		P6T	3156
dam		A20G, F38Q, G90S	3171

2.5. Data analysis

The scaffold sequences were screened for resistance genes and their incompatibility group using CGEs ResFinder 4.0 (Bortolaia et al., 2020) and PlasmidFinder 2.1 (Carattoli et al., 2014). Full annotation was performed afterwards using RAST 2.0 (Aziz et al., 2008). The annotated sequences of the donor and transconjugant strains were compared using Snapgene viewer 5.3.1 (from Insightful Science; available at http://snapgene.com), CLC Genomics Workbench 21 (https://digitalinsights.qiagen.com) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The accession numbers are given in Table 4.

3. Results

To examine which plasmids can be transferred between related *E. coli*

Table 4
accession numbers of the plasmids in Genbank.

accession number	name
MW390511	pESBL2057-IncX4
MW390512	pESBL2057-IncI
MW390513	pESBL2073-IncI
MW390514	pESBL2073-IncX4
MW390515	pESBL2082-IncI
MW390516	pESBL2082-IncX1
MW390517	pESBL2082-IncF
MW390518	pESBL2082-IncX4
MW390519	pESBL2082-IncF(phage)
MW390520	pESBL3153-IncF
MW390521	pESBL3153-IncX4
MW390522	pESBL3153-IncI
MW390523	pESBL3156-IncB/O/K/Z
MW390524	pESBL3156-IncF
MW390525	pESBL3156-IncX4
MW390526	pESBL3171-IncF
MW390527	pESBL3171-IncI
MW390528	pESBL3203-IncF
MW390529	pESBL3203-unknown IncI
MW390530	pESBL3215-phage plasmid
MW390531	pESBL3215-IncI
MW390532	pESBL3215-IncX4
MW390533	pESBL3215-IncF
MW390534	pESBL3227-IncF
MW390535	pESBL3227-IncI
MW390536	pESBL3231-p0111
MW390537	pESBL3231-IncN
MW390538	pESBL3231-IncF
MW390539	pESBL3277-IncF
MW390540	pESBL3284-IncF
MW390541	pESBL3288-IncF
MW390542	pESBL3301-IncX1
MW390543	pESBL3301-IncI
MW390544	pESBL3301-IncF
MW390545	pESBL3310-IncB/O/K/Z
MW390546	pESBL3310-IncF
MW390547	pESBL3311-IncX1
MW390548	pESBL3311-IncF
MW390549	pESBL3311-IncR
MW390550	pESBL3312-IncX1
MW390551	pESBL3312-IncF
MW390552	pESBL3312-IncR

stains, donor strains obtained from meat purchased in supermarkets and a general recipient strain were co-incubated for 1 h. Transconjugants were isolated from selective plates and sequenced using short and long read sequencing techniques. The results indicate that in (almost) all cases only a single type of plasmid transfers from the donor to the recipient strain, regardless of the number of different plasmids that the donor harbors (Table 2). In the case of six out of seven donor strains harboring an IncI1-type plasmid with in addition one or more other plasmids, this IncI1 plasmid was the plasmid transferred. Only transconjugant strain 2082 had the IncX4 plasmid transferred instead, even though the IncI1 was also present in the donor cell. Both strains harboring an IncB/0/K/Z-type plasmid had this specific plasmid transferred. IncF-type plasmids were only transferred to the recipient strain from the two donor strains that exclusively harbored this type of plasmid. Most plasmids acquired several mutations during the transfer process. Some mutations occurred multiple times, while others were observed only once, but significantly changed the plasmid (Table 3).

3.1. Mutations

The most common mutations were rearrangements of the *pilV* gene observed in five out of twelve strains (Table 3). The *pilV* gene was only present in IncI1 plasmids, where it is part of an operon that encodes a thin pilus that facilitates transfer (Komano et al., 1994). Five out of the six IncI-type plasmids transferred underwent some rearrangement (Fig. 1). The *pilV* gene has a constant region that is the same for all IncI1 plasmids and a variable region. The variable region consists of seven 19-bp repeat sequences and four segments that can switch directions and positions (A-A', B-B′, C-C′ and D′). All five donors in this subset showed a different rearrangement of *pilV* and it was sometimes split into two parts with some genes in between. The genes in between the *pilV* regions were the same in the plasmids of *E. coli* strains 3170 and 3308 and comprised mobile genetic element IS*Ec9*, class A beta-lactamase gene $bla_{CTX\text{-}M\text{-}1}$ and tryptophan synthase. In *E. coli* 3170, however, these genes sometimes switched direction while flanked by segments A-B and C of *pilV* (Fig. 1B). In theory, *pilV* rearrangement could also occur during regular growth of the plasmid containing strain. Before and after frequent serial inoculations of the most used donor strains, amounting to considerable growth, no rearrangements were observed.

Mutations during transfer also regularly appeared in mobile genetic elements (MGEs) (Table 2). Out of twelve transconjugant strains, five had mutations compared to their donor in one or more mobile genetic element. Mobile genetic elements mediate the movement of DNA within the genome (Frost et al., 2005). The mutations were found in four different types of mobile genetic elements. Most mutations were found in IS*26*, as three strains showed one or more mutations in its transposase. Four of these mutations were similar as both transconjugant *E. coli* 2082 and 3301 showed an amino acid change for position 184 where glycine was converted to asparagine, while the opposite happened for two IS*26* transposases in transconjugant *E. coli* 3288 where the asparagine is changed to glycine. In all cases IS*26* was near a beta-lactamase gene.

For both transconjugants of IncF plasmids, mutations were found in *insAB* (Table 3). *insA* and *insB* are part of IS*1*, which is known for various kinds of genomic rearrangements (Sekino et al., 1995). In both transconjugant *E. coli* 3277 and 3288 multiple point-mutations were found. However, in the case of transconjugant *E. coli* 3277, only one mutation resulted in an amino acid change: in *insA* F81L. In transconjugant *E. coli* 3288 the mutations caused two amino acid changes, one in *insA*: T31A, and one in *insB*: C5S. Two more mobile genetic elements showed mutations, Tn*As1* and IS*91*. For transconjugant *E. coli* 2082 the mutation in Tn*As1* resulted in amino acid change N422K. Multiple mutations in IS*91* of transconjugant *E. coli* 3153 caused three amino acid changes: Q179H, M210L, S232A.

A set of four interesting mutations were found in other strains: in *ardA, psiB,* single-stranded DNA-binding protein (*ssb)* and adenine-specific methyltransferase (*dam*). ArdA facilitates anti-restriction in the recipient cell (Read et al., 1992; Thomas et al., 2003). In *E. coli* 3153, the mutation resulted in an amino acid change D78G (Table 3). PsiB can inhibit the SOS response of the recipient cell (Althorpe et al., 1999; Golub et al., 1988). In *E. coli* 3156 the mutation resulted in an amino acid change: P6T. Ssb has the function to bind to single-stranded DNA (ssDNA) and is thought to increase plasmid stability when located in a plasmid (Porter and Black, 1991; Jain et al., 2012) as well as being essential for DNA replication in *E. coli* (Meyer et al., 1979). The recombination found in this gene was quite drastic, as the gene changed from a *ssb* gene mostly similar to that of F plasmids to one resembling the *ssb* gene in IncI plasmid ColIb (Howland et al., 1989). Adenine-specific methyltransferase or *dam* methylates adenine at specific GATC sites (Hattman et al., 1978). A more specific role in plasmids is thus far unknown. In the *E. coli* 3301 plasmid these mutations resulted in three amino acid changes: A20G, F38Q, G90S.

3.2. Acquisition of TET cluster

After co-incubation of *E. coli* strain 3301 and *E. coli* MG1655, the resulting transconjugant strain had an additional special mutation occurring in the transferred IncI1 plasmid (Fig. 2). Both the donor and transconjugants plasmids were sequenced and assembled with a hybrid method of Illumina and PacBio data. The transferred IncI1-plasmid contained an extra cluster of genes involved in resistance to tetracycline, including the genes *tetA*, *tetR*, a permease of the DMT superfamily and 2 mobile genetic elements flanking the resistance genes (Fig. 2C).

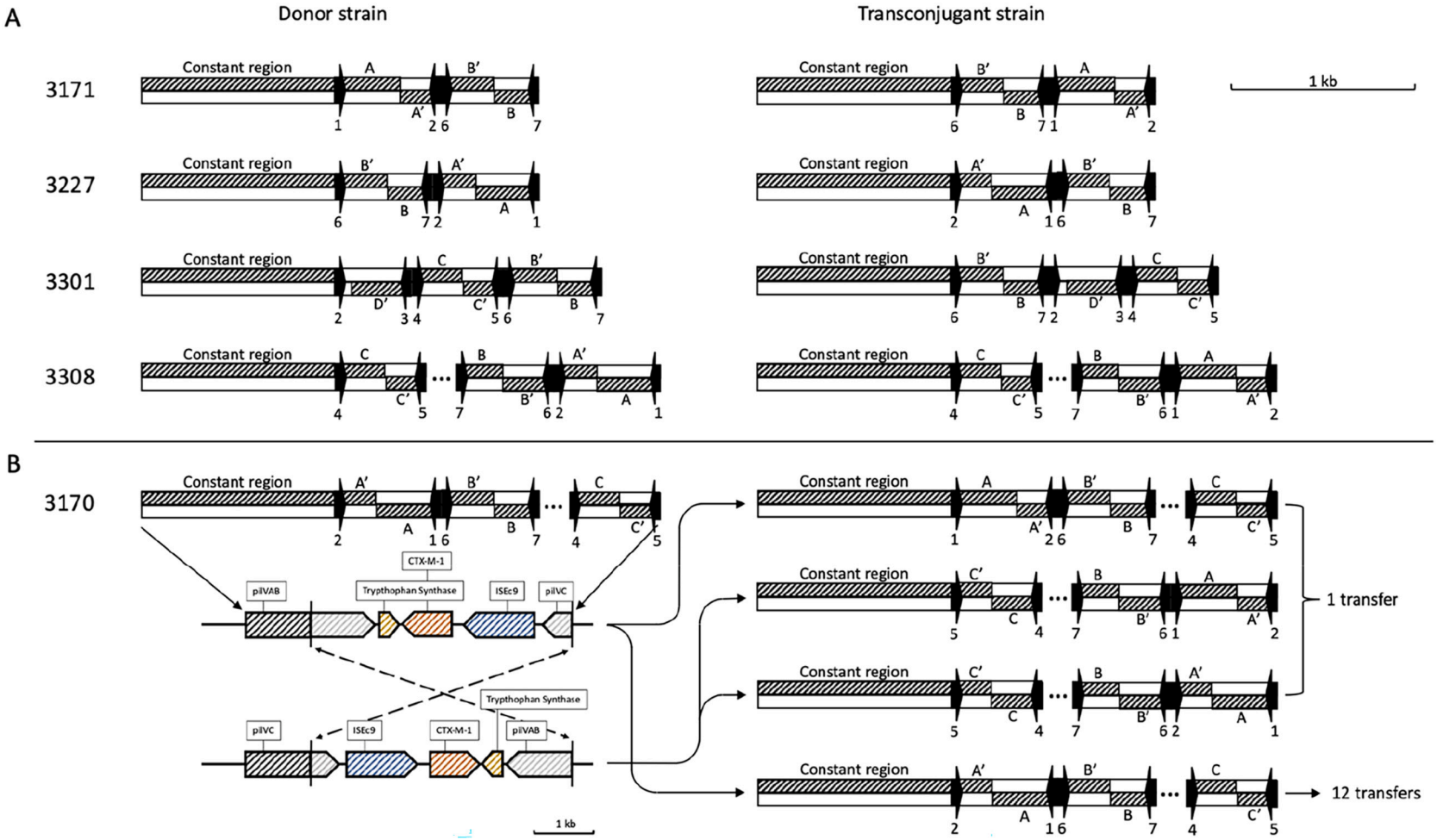

Fig. 1. Different rearrangements of the *pilV* gene in different *E. coli* strains before (donor strains) and after (transconjugant strains) a transfer event with IncI1-type plasmids. The *pilV* gene consists of a constant region and a variable region that can be built out of segments A-A', B-B′, C-C′ and D′ as well as seven 19-bp repeat sequences (black arrows). The variable region can be split from each other and have genes in between, which is indicated with three dots between the segments. Part **A** shows the rearrangement of *pilV* after 1 h after one transfer event. Part **B** shows the rearrangement for *E. coli* 3170 after one and after twelve consecutive transfers of its IncI1 plasmid. The genes in between the two *pilV* regions are shown for *E. coli* 3170. The region, comprising the genes: tryptophan synthase, *CTX-M-1*, IS*Ec9* and *pilV* segments C and AB, switches direction after one transfer and is reversed back after twelve transfers.

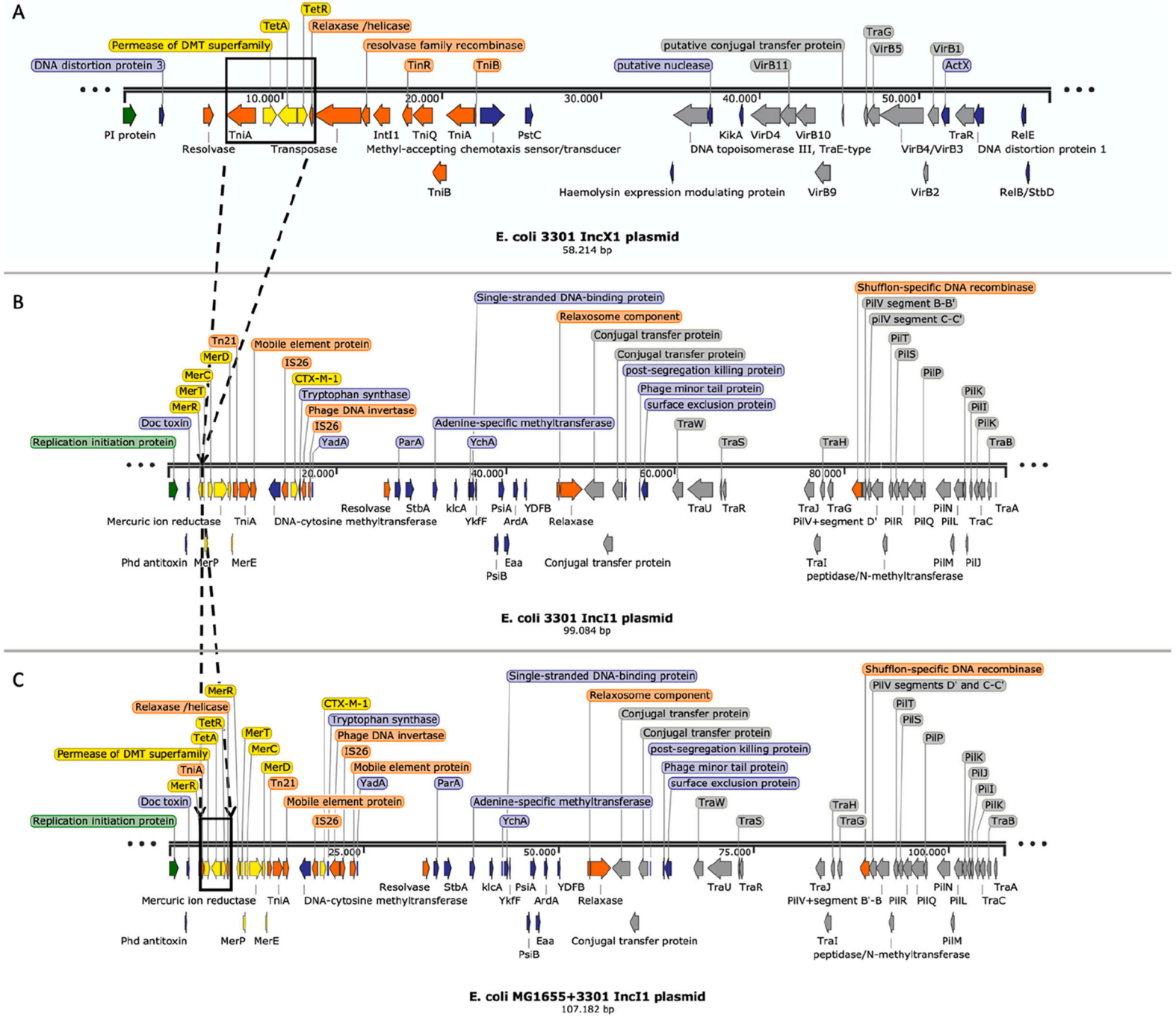

Fig. 2. IncX1 (**A**) and IncI1 (**B**) plasmids as found in *E. coli* 3301 and IncI1 plasmid as found in transconjugant *E. coli* MG1655 that was co-incubated with *E. coli* 3301 (**C**). Transfer genes are highlighted in grey, mobile genetic elements are highlighted in orange, resistance gene are shown in yellow, and all other genes are shown in blue/purple. The black square represents a cluster of genes that was transferred from the IncX1 plasmid of the donor into the IncI1 plasmid that ended up in the transconjugant strain, which includes two mobile genetic elements and three genes associated with tetracycline resistance (*tetA*, *tetR* and a permease of DMT superfamily). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The cluster most likely translocated from another plasmid that was present in the donor strain: IncX1 (Fig. 2B), as this cluster was only present in the IncX1 plasmid (Fig. 2A). This TET cluster is mostly plasmid-mediated rather than chromosomal (Grossman, 2016). The cluster's new position in the IncI1 plasmid is within another resistance cluster of genes coding for mercury resistance.

3.3. Effect of multiple transfers on pilV gene

The IncI1 plasmid originating from *E. coli* strain 3170 was transferred multiple times back and forth between to recipient strain *E. coli* MG1655 and *E. coli* JW3686, to see whether repeated back and forth transfer would alter the plasmid composition. Over 12 transfers the IncI1 plasmid stayed almost identical, except for the *pilV* gene. The *pilV* gene was rearranged in transfer 12 as compared to transfer 1. This was a reversal of what happened in the earlier rearrangement seen during transfer from the donor strain to its first transconjugant strain (Fig. 1B). As a result, the configuration of the *pilV* gene in the plasmid after 12 transfers was the same as in the donor strain *E. coli* 3170.

3.4. Longer co-incubation

Several transconjugants were isolated from transfers with *E. coli* 2082 as donor and *E. coli* MG1655 as recipient. The initial MICs varied for tetracycline and kanamycin after 24 h co-incubation as opposed to 1 h. Plasmids from 5 different transconjugant strains were sequenced and analyzed to determine whether different, more, or edited plasmids had transferred to the recipient strain. The outcome was that after 1 h only the IncX4 plasmid was isolated with the exact same mutations as seen before in the MGEs, while after 24 h the IncI1 and IncX1 were transferred together with the IncX4 plasmid.

4. Discussion

One of the conclusions from this study is that not all plasmids from a donor that contains several, are transferred during conjugation. Relatively short co-incubation of 1-h resulted in the transfer of only one plasmid from the donor even if the donor harbored more than one conjugative plasmid. The most transferred plasmid was the IncI-type plasmid. Which plasmid is transferred depended mostly the presence of an (extended spectrum) beta-lactamase gene on that specific plasmid (Darphorn, Bel, et al. 2021), since the selective plates contained that type of antibiotic. In one strain two plasmids harbored an (extended spectrum) beta-lactamase gene, but only the IncX4 plasmid was successfully transferred. IncX-type plasmids can on average transfer faster than IncI plasmids (Alderliesten et al., 2020) and especially IncX4 plasmids have shown high transfer rates when compared to IncF plasmids (Lo et al., 2014). Thus, the nature of the selective pressure and the transfer speed both influence the chance of successful transfer. Longer co-incubation results in the transfer of more plasmids to a single recipient (Benz et al., 2021).

Another conclusion is that plasmid transfer is, at least in some cases, accompanied by small mutations. These mutations were mostly related to the transfer system such as *pilV* and to the stability of the plasmid in its host with genes *ardA* (Read et al., 1992; Thomas et al., 2003), *ssb* (Jain et al., 2012; Meyer et al., 1979; Porter and Black, 1991) and *psiB* (Althorpe et al., 1999; Golub et al., 1988). The mutations in *ardA*, *ssb* and *psiB* are interesting since they form a leading region in IncI and IncB-type plasmids and because they are the first genes expressed in the recipient cell with the help of a single stranded promoter (Althorpe et al., 1999; Bates et al., 1999). These genes counteract the first response of the recipient cell by stopping the SOS-response with *psiB*, the restriction of the plasmid with *ardA* (Read et al., 1992; Thomas et al., 2003) and promote DNA-replication and plasmid stability with *ssb*. In particular, *ssb* in F plasmids can delete the chromosomal *ssb* complement and replace its function in order to enhance the survival of the plasmid within the host, since *ssb* is essential for DNA replication (Porter and Black, 1991). These mutations could be beneficial to the first response. The *ardA* and *psiB* mutations have not been described before. In the case of *ssb* an unexpected change happens where the gene changes in type. The newly obtained type may not have the same function for the plasmid as the F *ssb* gene (Howland et al., 1989). Thus, the gene might have lost its ability to improve the stability of the plasmid in the host cell.

Mutations also happen frequently in mobile genetic elements. These elements can mediate the movement of DNA within a genome (Frost et al., 2005). IS*26*, found in this study near (extended spectrum) beta-lactamases, has been associated with the transposition of (extended spectrum) beta-lactamases such as $bla_{\text{CTX-M-1}}$ (Dolejska et al., 2013), $bla_{\text{CTX-M-15}}$ (Partridge et al., 2011; Smet et al., 2010) and bla_{TEM} (Bailey et al., 2011) which are found in transconjugant *E. coli* 3301, 3288 and 2082 respectively (Darphorn et al., 2021a). It is suggested that IS*26* plays an important role in the structural rearrangements within a plasmid and facilitates the mobilization of fragments from other plasmids (Smet et al., 2010). The G184N mutation is known as the most common mutation found for IS*26* and enhances activity of its transposase (Pong et al., 2019). In this study we can see this mutation go both ways. Transconjugants for *E. coli* 2082 and 3301 had an enhanced transposase activity, while transconjugant *E. coli* 3288 had decreased activity. The mutated gene could thus increase or decrease the movement of resistance genes, in particular beta-lactamases, within the genome of the host cell. The transposase of IS*1*, which consists of a protein from frameshifted *insAB* (Sekino et al., 1995), is also associated with transposition of beta-lactamases (Darphorn et al., 2021b). IS*1* can downregulate the expression of $bla_{\text{CTX-M}}$ (Fernandez et al., 2007). In short, the mutations discussed above can be understood in the framework of their function.

Tn*As1* can mobilize *mcr-5* and tetracycline resistance (Kieffer et al., 2019; Li et al., 2021). Within the IncX4 plasmid found in this study the transposase is closer to a gene of the *aadA* family that encodes for aminoglycoside resistance. IS*91* family transposases have been reported as potential mediators of multiple resistance genes as part of class 1 integrons and use rolling circle replication to mobilize adjacent DNA (Toleman et al., 2006). The gene is part of an integron in transconjugant *E. coli* 3153 that is located next to another gene of the *aadA* family and thus could boost its mobilization.

PilV is known to exhibit an array of rearrangements (Sekizuka et al., 2017). The *pilV* gene is the last gene in an operon of *pil* genes that encode a thin pilus that facilitates transfer of IncI-type plasmids (Komano et al., 1994; Komano et al., 1987). The *pilV* gene itself encodes for the tip adhesin of the pilus that is responsible for recognizing and adhering to a recipient cell (Komano et al., 1994). It consists of a constant region and variable region (Komano et al., 1987). Different rearrangements can change the specificity, hence this variable region is responsible for recipient recognition (Komano et al., 1994). In the present dataset the donor plasmids differ in their arrangement of the *pilV* gene, as not all contain the same set of segments. As a result, the arrangement of the *pilV* gene in transferred plasmids is not analogous, even though the recipient is the same in all transfer experiments. Therefore, it seems that there is no obvious consensus arrangement that would be necessary for recognizing the recipient strain. A study by Brouwer et al. (2019) suggested that *pilV* can rearrange constantly depending on different growth conditions, highlighting the dynamics of the *pilV* gene. Something similar happened several times during transfer of an IncI plasmid in this study, as *pilV* changed as part of the process. This process is not random, as during twelve consecutive back and forth transfers of an IncI plasmid of *E. coli* strain 3170, the *pilV* gene changed after the initial transfer, but had been converted back to its original configuration in in the final transconjugants. This suggests that only one configuration confers maximal transfer efficiency.

In rare occasions resistance genes mediated by mobile genetic elements can also transfer from plasmid to plasmid before transferring into a new host, as in the case of the tetracycline cluster in *E. coli* strain 3301.

Mobile genetic elements can mediate the transposition of resistance genes (Fernandez et al., 2007; Li et al., 2021; Partridge et al., 2011; Pong et al., 2019; Preston et al., 2004; Toleman et al., 2006; Darphorn et al., 2021b). In this study this process was replicated under experimental conditions. Tetracycline resistance gene clusters have been found widespread in plasmids, such as cluster Tn*1O* and transposon Tn*1721* (Miriagou et al., 2006; Partridge et al., 2018). The Tn*3* associated with the cluster in this study is also found more and more associated with tetracycline resistance (Miriagou et al., 2006). This wide spread of tetracycline resistance clusters suggests many transposition events with tetracycline resistance genes have taken place over time between many different plasmids, as illustrated by one such event in this study.

In conclusion, plasmid transfer in *E. coli* isolated from meat destined for the consumer is a dynamic process. Plasmids can change some of their configuration during transfer to ensure accuracy and stability in the recipient as well as to increase resistance to antimicrobials. However, once the recipient and circumstances such as antibiotic stress are similar, the change in the transferred plasmid is minimal and only related to *pilV*. Short incubation times enhance the transfer of plasmids adapted to the specific selective environment, while longer co-incubation can result in the spread of multiple plasmids to a singular host regardless of specificity.

Author contributions

TD and BtK conceived the project. SB assisted in the design of experiments. TD performed experiments and analysis of the data. TD and BtK wrote the manuscript. All authors critically reviewed the manuscript and approved the final version.

Declaration of Competing Interest

The authors have no competing interests to declare.

Acknowledgements

We thank Kees Veldman and Ben Wit for providing the resistant strains and Sarah Duxbury for assistance in identifying proper analysis programs and all three for stimulating discussions. The students Mireille Nulkes, Siem de Haan, Lisa Daniels, Sidra Kashif, Selin Alpagot, Hannah Boersma and Eva Kozanli performed transfer experiments, MICs and plasmid isolations in the framework of an internship. We thank Belinda Koenders-van Sintanneland and Keshia Bel for the help with transfer experiments and plasmid isolations. This study was financed by a subsidy from the Netherlands Ministry of agriculture. The sponsor had no role designing the study nor in the collection, analysis and interpretation of data nor in writing the manuscript or in the decision to submit the article for publication.

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