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

Tania S. Darphorn, Stanley Brul, Benno H. ter Kuile

**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.

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

**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 to 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
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 

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

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Donor plasmids</th>
<th>Beta-lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 2082</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncX&lt;sub&gt;4&lt;/sub&gt;, IncX&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL, p&lt;sub&gt;FRAP&lt;/sub&gt;</td>
<td>bla&lt;sub&gt;SHV-12&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt; (3&lt;sup&gt;x&lt;/sup&gt;)</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3153</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncX&lt;sub&gt;4&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3156</td>
<td>IncB&lt;sub&gt;0&lt;/sub&gt;/K/Z, IncX&lt;sub&gt;4&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3170</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB, IncFIC/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3171</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB, IncFIC/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3227</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3221</td>
<td>IncFIB/FIL, p0111</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 en&lt;sup&gt;x&lt;/sup&gt;</td>
<td>E. coli 3277</td>
<td>IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3288</td>
<td>IncFIA/FIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
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<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3301</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncX&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 en&lt;sup&gt;x&lt;/sup&gt;</td>
<td>E. coli 3308</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli MG1655 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli 3310</td>
<td>IncB&lt;sub&gt;0&lt;/sub&gt;/K/Z, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli JW3686 kan&lt;sup&gt;b&lt;/sup&gt;</td>
<td>E. coli MG1655 pI&lt;sub&gt;inc&lt;/sub&gt;3170</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncI&lt;sub&gt;1&lt;/sub&gt;, IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>E. coli JW3686 chlor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E. coli JW3686 pI&lt;sub&gt;inc&lt;/sub&gt;3170</td>
<td>IncI&lt;sub&gt;1&lt;/sub&gt;, IncI&lt;sub&gt;1&lt;/sub&gt;, IncI&lt;sub&gt;1&lt;/sub&gt;, IncFIB/FIL</td>
<td>bla&lt;sub&gt;SHV-2&lt;/sub&gt;, bla&lt;sub&gt;TEM&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
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 plnC3170) was initially mated with *E. coli* JW3686 (kan<sup>R</sup>). The transfer was continued by using a transconjugant as the new donor strain (*E. coli* JW3686 plnC3170) mating with *E. coli* MG1655 (chlor<sup>R</sup>) 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 endpoints 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/](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) and assembly errors were corrected using Pilorn 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 (Bolikovitch 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 Pilorn 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.

### 2.5. Data analysis

The scaffold sequences were screened for resistance genes and their incompatibility group using CGEs ResFinder 4.0 (Bortolata 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](http://snapgene.com)), CLC Genomics Workbench 21 ([https://digitalinsights.qiagen.com](https://digitalinsights.qiagen.com)) and BLAST ([http://www.ncbi.nlm.nih.gov/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* scaffold sequences were corrected using Pilorn 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 (Bolikovitch 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 Pilorn version 1.21 (Walker et al., 2014).

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### Table 3

<table>
<thead>
<tr>
<th>Genes with mutations</th>
<th>Specific mutation</th>
<th>Transconjugant strain from donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pilIV rearrangement</td>
<td>See Fig. 1</td>
<td>3170, 3171, 3227, 3301, 3308</td>
</tr>
<tr>
<td>Mobile genetic element</td>
<td>IS26</td>
<td>R45W</td>
</tr>
<tr>
<td></td>
<td>G184N</td>
<td>2082, 3301</td>
</tr>
<tr>
<td></td>
<td>N184G</td>
<td>3288 (2×)</td>
</tr>
<tr>
<td></td>
<td>intAI</td>
<td>3277</td>
</tr>
<tr>
<td></td>
<td>F81L (intAI)</td>
<td>3277</td>
</tr>
<tr>
<td></td>
<td>T31A (intAI)</td>
<td>3277</td>
</tr>
<tr>
<td></td>
<td>C55 (intAI)</td>
<td>3277</td>
</tr>
<tr>
<td>Tn431</td>
<td>N422K</td>
<td>2082</td>
</tr>
<tr>
<td>IS91</td>
<td>Q179H, M210L, S232A</td>
<td>3153</td>
</tr>
<tr>
<td>arDA</td>
<td>D786</td>
<td>3153</td>
</tr>
<tr>
<td>sbA</td>
<td>F sub to Colib sb</td>
<td>3156</td>
</tr>
<tr>
<td>PsbA</td>
<td>P6T</td>
<td>3156</td>
</tr>
<tr>
<td>dam</td>
<td>A20G, F38Q, G90S</td>
<td>3171</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Donor plasmids</th>
<th>Transconjugant plasmids</th>
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</thead>
<tbody>
<tr>
<td></td>
<td># Inc types</td>
<td># Inc types</td>
</tr>
<tr>
<td>2082</td>
<td>5, X4, X1, FIB/FII, FIB (phage)</td>
<td>1, X4</td>
</tr>
<tr>
<td>3153</td>
<td>3, X4, FII</td>
<td>1</td>
</tr>
<tr>
<td>3156</td>
<td>B/O/K/Z, X4, FIB/FII</td>
<td>1, B/O/K/Z</td>
</tr>
<tr>
<td>3170</td>
<td>3, FIB, FIC/FII</td>
<td>1</td>
</tr>
<tr>
<td>3171</td>
<td>2, FII</td>
<td>1</td>
</tr>
<tr>
<td>3227</td>
<td>2, FIB/FII</td>
<td>1</td>
</tr>
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<td>3231</td>
<td>N, FIB/FII, P0111</td>
<td>1, N</td>
</tr>
<tr>
<td>3277</td>
<td>FIB/FIC</td>
<td>1, FIB/FIC</td>
</tr>
<tr>
<td>3301</td>
<td>3, X1, FIB/FII</td>
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<tr>
<td>3308</td>
<td>4, Y, FIB, FIC/FII</td>
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</tr>
<tr>
<td>3310</td>
<td>B/O/K/Z, FIB/FIC</td>
<td>1, B/O/K/Z</td>
</tr>
</tbody>
</table>

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stains, donor strains obtained from meat purchased in supermarkets and a general recipient strain were co-incubated for 1 h. Transconjugants observed only once, but significantly changed the plasmid (Table 3). Some mutations occurred multiple times, while others were 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 IncI plasmids and a variable region. The variable region consists of several 19-bp repeat sequences and four segments that can switch directions and positions (A-A, B-B, C-C and D-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 ISEc9, class A beta-lactamase gene blaCTX-M-1 and cryptophan 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 inculcations 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 IS26, 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 IS26 transposases in transconjugant E. coli 3288 where the asparagine is changed to glycine. In all cases IS26 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 IS1, 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: CSS. Two more mobile genetic elements showed mutations, TnAsI and IS91. For transconjugant E. coli 2082 the mutation in TnAsI resulted in amino acid change N422K. Multiple mutations in IS91 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, psb, single-stranded DNA-binding protein (ssb) and adenosine-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 D78Q (Table 3). PsbB 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 cline, including the genes ardA, psiB, M210L, S232A.

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 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, tetB, 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, ISEc9 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 and the type of the recipient cell. A study by Brouwer et al. (2019) suggested that there is no obvious consensus arrangement that would be necessary for recognition and adhering to a recipient cell (Koman et al., 1994). It consists of a constant region and variable region (Koman et al., 1997). Different rearrangements can change the specificity, hence this variable region is responsible for recipient recognition (Koman 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 the final transconjugants. This suggests that only one configuration confers maximal transfer efficiency.

In rare cases 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; Talemon 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 Tn10 and transposon Tn721 (Miriagou et al., 2006; Partridge et al., 2018). The Tn3-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 plv. Short incubation times enhance the transfer of plasmids adapted to the specific selective environment, while longer 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.

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