An easily modifiable conjugative plasmid for studying horizontal gene transfer

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Published in:
Plasmid

DOI:
10.1016/j.plasmid.2022.102649

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

Document license:
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Citation for published version (APA):
Original Research Article
An easily modifiable conjugative plasmid for studying horizontal gene transfer

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ARTICLE INFO
Keywords:
Conjugative plasmid
Genetic engineering
β- red recombination system
Tn7 transposons

ABSTRACT
Horizontal gene transfer is an important mechanism in bacterial evolution and can occur at striking frequencies when mediated by mobile genetic elements. Conjugative plasmids are mobile genetic elements that are main drivers of horizontal transfer and a major facilitator in the spread of antibiotic resistance genes. However, conjugative plasmid models that readily can be genetically modified with the aim to study horizontal transfer are not currently available. The aim of this study was to develop a conjugative plasmid model where the insertion of gene cassettes such as reporter genes (e.g., fluorescent proteins) or antibiotic resistance genes would be efficient and convenient. Here, we introduced a single attTn7 site into the conjugative broad-host-range IncP-1 plasmid pJK5 in a non-disruptive manner. Furthermore, a version with lower transfer rate and a non-conjugative version of pJK5-attTn7 were also constructed. The advantage of having the attTn7 sites is that genes of interest can be introduced in a single step with very high success rate using the Tn7 transposition system. In addition, larger genetic fragments can be inserted. To illustrate the efficacy of the constructed pJK5 plasmids, they were complemented with sfGFP (a gene encoding superfolder green fluorescent protein) in addition to seven different β-lactamase genes representing the four known classes of β-lactamases.

1. Introduction

Horizontal gene transfer (HGT) is an important facilitator of bacterial evolution and when mediated by mobile genetic elements, such as conjugative plasmids, HGT can occur at striking frequencies (Sørensen et al., 2005). Conjugative plasmids are especially known for their role in spreading antibiotic resistance genes (Che et al., 2021) and virulence factors (Ghigo, 2001), however, while this underlines their importance, there are still many unknowns about the biology of plasmids and HGT in general. Here we constructed a conjugative plasmid which easily can be complemented with genetic cassettes to investigate various aspects of HGT including plasmid biology, transfer dynamics, accessory genes, stability, etc. Molecular engineering of conjugative plasmids can have numerous objectives such as, complementing conjugative plasmids with reporter genes that can be used to study transfer dynamics at the single cell level (Pinilla-Redondo et al., 2018), testing what genes can be transferred stably by conjugation, factors that affect the success of HGT, and metagenome engineering and cargo delivery by conjugation. Though, many other applications can be imagined.

Tn7 transposons have been widely used in bacterial genome engineering enabling gene complementation and expression (Peters and Craig, 2001). When transposition is mediated by TnsABC-D the Tn7 transposon can be inserted specifically into chromosomes at an attTn7 site, which typically is located downstream of the bacterial glutamine synthetase (glmS) gene of gram-negative bacteria which is highly conserved (Peters and Craig, 2001). Choi et al. constructed the widely used mini-Tn7 system where the genetic material one wishes to transpose is flanked by inverted repeats named Tn7L and Tn7R on a transfer plasmid. Tn7L and Tn7R are recognized by the transposase complex TnsABCD, encoded on the helper plasmid, and then inserted at the chromosomal attTn7 (Kyoung Hee Choi et al., 2005). The bacterial Tn7-transposon-based delivery systems have important advantages: (i) It has high affinity integration at attTn7 sites; (ii) both small and large DNA fragments can be integrated; (iii) integration at a defined target site can be done without any deleterious effects or preparatory genetic modification; and (iv) the inserted transposon is maintained without antibiotic selection (Kyoung H. Choi and Schweizer, 2006; Kyoung Hee Choi et al., 2005; Kyoung Hee Choi and Kim, 2009; Remus-Emsermann et al., 2016). Later derivatives of the mini-Tn7 system include pGRG36 where Tn7L and Tn7R associated multiple cloning sites and the transposase...
complex TnsABC-D were integrated into a single temperature-sensitive vector, which furthered the ease by which Tn7-based gene complementation can be done (McKenzie and Craig, 2006).

Another widely used gene integration system is the λ phage derived Red recombination system, a mutagenesis method that through homologous recombination allows defined insertions of genes, deletions, or point mutations in bacteria and fungi (Chaveroche, 2000; Datsenko and Wanner, 2000). With this method, efficient recombination can be achieved between polymerase chain reaction (PCR) products and a target replicon by induction of the λ phage Red operon, provided that the linear DNA obtained by PCR has ca. 36-nt or larger flanking extensions that are homologous to the target DNA. The Red operon encodes three genes, *gam, exo* and *beta*. *Gam* prevents the intracellular exonucleases from digesting the linear DNA introduced into the bacteria. *Exo* will degrade linear dsDNA starting from the 5’ end and generate ssDNA, and *Beta* protects the ssDNA produced by the *Exo* and promotes its annealing to the complementary ssDNA target in the replicon (Datsenko and Wanner, 2000). The great advantage of the Red recombination system is that mutagenesis can be done specifically at any genomic site (Datsenko and Wanner, 2000).

Here we utilized the λ Red recombination system to insert an *attTn7* site into a model conjugative plasmid *pJK5*, in a non-disruptive manner. *pJK5* belongs to the IncP-1 incompatibility group (Bahl et al., 2007a) and has a very broad-host ranges (Klümper et al., 2015). It transfers at high frequencies to many proteobacteria but has also been reported to transfer to gram-positive bacteria (Klämper et al., 2015) and to the *Escherichia coli* DH5α, MG1655 (Klämper et al., 2015) and has a single temperature-sensitive replicon by induction of the λ Red operon (Klämper et al., 2015, McKenzie and Craig, 2006).

2. Material and methods

2.1. Plasmids and strains

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (VWR Life Science) at 30 °C, 37 °C or 42 °C. Antibiotics (Sigma-Aldrich) were used at the following concentration: Ampicillin (Amp, 100 μg/ml), kanamycin (Kan, 50 μg/ml), tetracycline (Tet, 15 μg/ml), gentamicin (Gen, 15 μg/ml), rifampicin (Rif, 100 μg/ml), nalidixic acid (Nal, 100 μg/ml), chloramphenicol (Chl, 30 μg/ml), Cefotaxime (Ctx, 2 μg/ml), and Meropenem, (Mem, 0.5 μg/ml). Plasmid DNA was extracted from overnight cultures using the Plasmid Mini AX kit (A&A Biotechnology). Primers (TAG Copenhagen A/S) used in this study are in Table 2.

2.2. Electroporation

5 ml of *Escherichia coli* cultures were grown to mid-log phase (OD600 approximately 0.6) at 30 °C and washed twice with 1 ml of ice-cold 10% v/v glycerol. Cells were then resuspended in 50 μl of ice-cold 10% glycerol. DNA (100–150 ng PCR fragment or 10 ng plasmid) was added to the cell suspension and electroporated in a 0.1 cm Gene Pulser® cuvette (Bio-Rad) at 1.8 kV for ~ 5 ms on a MicroPulser Electroporator (Bio-Rad). 1 ml of LB medium was added immediately after electroporation and the cells were incubated for 1.5 h at 30 °C. Finally, the cells were plated on LB agar plates containing selective antibiotics and incubated overnight at 30 °C.

2.3. Construction of plasmid pJK5-attTn7

Plasmid p-attTn7 was synthesized by ThermoFisher Scientific based on a provided design, as further described in the results section (Fig. 1). Using p-attTn7 as template, the *attTn7-FRT-aac3-FRT* fragment was amplified by PCR using primers *attTn7GenR* and *attTn7GenF*. These primers included overhangs with 40 bp homology to the pJK5 target region. The two tandem flippase recognition target (FRT) sites are used for later flippase (FLP) to delete the DNA sequence between these two sites (Schlake and Bode, 1994). 100–150 ng of the linear PCR product was transformed by electroporation into competent *E. coli* DH5α cells already harboring both the helper plasmid pKD46 and the target plasmid pJK5. Here the λ Red recombinant protein expressed by the helper helper plasmid pKD46 recombined the *attTn7-FRT-aac3-FRT* fragment into pJK5 by homologs recombination. Successfully integration of *aac3* rendered strains resistant to gentamicin. Selection was thus done on LB agar medium containing Amp, Tet, and Gen. Growing colonies were screened by colony PCR using the *attTn7GenR* and *attTn7GenF* primers. Positive colonies were grown in LB with Tet and Gen at 42 °C overnight to remove pKD46. Post plasmid curing, it was ensured that colonies were sensitive to Amp.

Hereafter, to remove the antibiotic resistance selection marker *aac3*, the helper plasmid pFLP2 expressing the FLP recombinase was introduced (Kyoung Hee Choi and Schweizer, 2005). Plasmid pFLP2 was transformed into the *E. coli* DH5α cells containing the now constructed pJK5-attTn7-FRT-aac3-FRT by electroporation, to obtain plasmid pJK5-attTn7. FLP excised the gene *aac3* between the two inversely repeated FRT sites by cleavage and re-ligation, leaving a single FRT site. Clones that grew on LB agar medium with Tet but did not grow in the presence of Gen were selected and verified by colony PCR with primers *attTn7GenR* and *attTn7GenF*. After flippase mediated removal of *aac3*, LB agar plates containing 5% w/v sucrose were used to counter-select against pFLP2 which encoded *aac3*. Cells containing only pJK5-attTn7 were whole-genome sequenced by Nanopore sequencing.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temperature</th>
<th>Antibiotic resistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>37 °C</td>
<td>–</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>37 °C</td>
<td>KanR, RifR, NatR</td>
<td>(Madsen et al., 2016)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>37 °C</td>
<td>KanR</td>
<td>(Klümper et al., 2015)</td>
</tr>
<tr>
<td><em>pGRG36</em></td>
<td>30 °C, temperature-sensitive plasmid</td>
<td>AmpR</td>
<td>Conjugal plasmid Transposition vector</td>
</tr>
<tr>
<td><em>pKD46</em></td>
<td>30 °C, temperature-sensitive plasmid</td>
<td>AmpR</td>
<td>Expression of Red recombinase</td>
</tr>
<tr>
<td><em>pKD3</em></td>
<td>37 °C</td>
<td>AmpR, cat (ChlR)</td>
<td>(Datsenko and Wanner, 2000)</td>
</tr>
<tr>
<td><em>pFLP2</em></td>
<td>37 °C</td>
<td>AmpR</td>
<td>Confers resistance to chloramphenicol</td>
</tr>
<tr>
<td><em>p-attTn7</em></td>
<td>37 °C</td>
<td>aac3 (GenR)</td>
<td>(Hoag et al., 1998)</td>
</tr>
<tr>
<td><em>p-sGFP</em></td>
<td>37 °C</td>
<td>tetA(TetR)</td>
<td>Confers sGFP fluorescence protein</td>
</tr>
</tbody>
</table>

Table 2

Strains and plasmids used in this study.
Table 2
Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attTn7Gen(^a)_F</td>
<td>5' - GGTTGGATCTCTGTTGTGGAT -3'</td>
<td>1501 bp</td>
</tr>
<tr>
<td>attTn7Gen(^a)_R</td>
<td>5' - CCAAGATGGATACAGACG -3'</td>
<td></td>
</tr>
<tr>
<td>CD-cat-F</td>
<td>5' - TTGATCTGCTGGGCGGCGGCGGCTGCTGGTGGTGAACGCTGCTGTTC -3'</td>
<td>1113 bp</td>
</tr>
<tr>
<td>CD-cat-R</td>
<td>5' - CCCGGCAATGGCAGCCAGGCGGCAGGAGGGGTATAGGGAATTAGCCGTCGATC -3'</td>
<td></td>
</tr>
<tr>
<td>NC-cat-F</td>
<td>5' - TAAATAGCTCGACGATTTGAGTTCTATGAGCAATGAGA-3'</td>
<td>1113 bp</td>
</tr>
<tr>
<td>NC-cat-R</td>
<td>5' - AATCCGATTCCTTCTGGAATTTATGATGATGAACTGAAATGAGA-3'</td>
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<tr>
<td>psfGFp-F</td>
<td>5' - ATGAGCAAGAAGAGAAACT -3'</td>
<td>714 bp</td>
</tr>
<tr>
<td>psfGFp-R</td>
<td>5' - TTTGTTAGACGCTATCCATGCG -3'</td>
<td></td>
</tr>
<tr>
<td>pGRG36-F</td>
<td>5' - ATCAAGTGATAGCGGCGCGG -3'</td>
<td>563 bp</td>
</tr>
<tr>
<td>pGRG36-R</td>
<td>5' - GCAAGGGAATTTTAAGCTGAACGCTAATCTTCCAGAAGA-3'</td>
<td></td>
</tr>
<tr>
<td>blaTmo-F</td>
<td>5' - ACTTGCGAATTAGAATCGACCTGACGATCCTGAGGTGGTAC -3'</td>
<td></td>
</tr>
<tr>
<td>blaTmo-R</td>
<td>5' - CAAAGGGGTCTTACGATGATTCTGATGAGTCGTAATCTTCCAGAAGA-3'</td>
<td></td>
</tr>
<tr>
<td>psfGFP-F</td>
<td>5' - ATGGAGAAAGAAAATCGCTGCAGGCGGCTGCTGTTC -3'</td>
<td></td>
</tr>
<tr>
<td>psfGFP-R</td>
<td>5' - TTTGTTAGACGCTATCCATGCG -3'</td>
<td></td>
</tr>
<tr>
<td>Fig. 1. Stepwise engineering of pKJK5-attTn7. (a) The backbone of the conjugative broad-host-range IncP-1 plasmid pKJK5 and the region of insertion. (b) Schematic diagram of the attTn7 fragment used. (c) Schematic diagram of the plasmid pKJK5-attTn7-aac3 after insertion of the attTn7-aac3 cassette into plasmid pKJK5. (d) Schematic diagram of the plasmid pKJK5-attTn7 after removal of resistance gene aac3. (e) Colony PCR used to verify insertions. The primers attTn7Gen(^a)-F/R were used for PCR here. Lane M is DNA marker (NEB 1 Kb DNA Ladder), lane N shows the PCR product from negative control (ddH(_2)O), lane P shows the PCR product from plasmid pKJK5-attTn7-aac3, lanes 1 and 2 show the PCR products from two clones of pKJK5-attTn7. Figures (a), (b), (c) and (d) were generated with SnapGene software 6.0.2 (GSL Biotech).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
carried out on R.9.4 MiniION flowcells (Oxford Nanopore Technologies) for up to 48 h. Libraries were prepared using the Rapid Barcoding Sequencing Kit (Oxford Nanopore Technologies, SQK-RBK004) following the manufacturer’s instructions.

2.4. Construction of conjugation deficient plasmid pKJK5_CT-attTn7 and non-conjugative plasmid pKJK5_NC-attTn7

The ρ. Red homologous recombination method was also used to construct two additional versions of pKJK5-attTn7. One that transfers at lower frequencies, referred to as pKJK5_CT-attTn7, and a non-conjugative version referred to as pKJK5_NC-attTn7. Plasmid pK3 (Daniels and Wanner, 2000) was used as template to amplified the chloramphenicol acetyltransferase gene (cat) including FRT sites flanked by 40 bp homology overhangs (1113 bp). The primers CD-cat-F/R and primers NC-cat-F/R were used here respectively. The cat fragment was integrated into the traC gene (pKJK5 nt position 36,168–37,226, AM261282) of pKJK5-attTn7 using ρ. Red homologous recombination as described above. Hereafter, the plasmid pLP2 was used to remove the tra region including the cat gene (pKJK5 nt position 33,431–37,226) hereby obtaining pKJK5_CT-attTn7. Similarly, to obtain pKJK5_NC-attTn7, the cat fragment was integrated upstream of the traK gene (pKJK5 nt position 43,767–45,046) of pKJK5-attTn7, hereafter, the same approach was used to remove the tra-raj regions including the cat gene (pKJK5 nt position 33,431–45,046). Colony PCRs with primer sets attTn7Gen-F and CD-cat-F/R-NC-cat-R were used to screen candidate colonies for pKJK5_CT-attTn7 and pKJK5_NC-attTn7, respectively. pKJK5_CT-attTn7 specific PCR products were purified and confirmed by Sanger sequencing (Eurofins Genomics). pKJK5_NC-attTn7 was whole-genome sequenced using the Illumina MiSeq platform. Sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1096) and sequenced with 2 × 250 base paired-end reads on the Illumina MiSeq platform (Illumina) according to the manufacturer’s protocol. Sequencing results were analyzed in CLC Genomic Workbench V7.5.1.

2.5. Construction of mini-Tn7 pGRG36-based delivery vectors for bla-sfGFP complementation

To verify that integration could be done at the attTn7-site of the plasmids, the superfolder green fluorescent protein (sfGFP) gene and seven different β-lactamase genes were cloned into helper plasmid pGRG36 to subsequently be used as delivery into pKJK5-attTn7 and pKJK5_NC-attTn7. sfGFP flanked by tetA was amplified by PCR from p-sfGFP, using primers psfGFP-F/P and psfGFP-R. This fragment was cloned into pGRG36 at the Smal restriction site following the manufacturer’s protocol (Smal, New England Biolabs and T4 DNA ligase, Thermo Scientific) resulting in pGRG36-sfGFP. Hereafter, 7 β-lactamase genes originating from wildtype plasmids (blaTEM-1 (GeneBank Accession No. AY599232), blaCTX-M-15 (8ZF94, an wastewater isolate in our lab), blaCMY-2 (https://www.ncbi.nlm.nih.gov/biocat?p=2010583205), blaNDM-5 (an isolate from chicken cloaca), blaKPC-2 (ATCC® BAA-1705™), blaOXA-181 (isolated from hospital), blaampC (https://www.ncbi.nlm.nih.gov/biocat?term=ampC)) were cloned into 7 different pGRG36-sfGFP vectors at the ZraI restriction site. Primers blaTEM1-F/R, blaCTXM15-F/R, blaCMY2-F/R, blaKPC2-F/R, blaOXA181-1-F/R, blaampC-F/R were used to obtain genes blaTEM-1, blaCTX-M-15, blaCMY-2, blaKPC-2, blaOXA-181, blaampC, respectively. Upon insertion of the bla genes the tetA gene was excised. Clones were selected on LB agar medium incubated at 30 °C containing appropriate antibiotics. Verification was done by colony PCR using the aforementioned primer sets.

2.6. Insertion of different bla-sfGFP cassettes into pKJK5-attTn7 and pKJK5_NC-attTn7 using Tn7 transposition

Firstly, the plasmids pKJK5-attTn7 and pKJK5_NC-attTn7 were transformed by electroporation to Escherichia coli MG1655-lacIq-mcherry (Klümper et al., 2015) competent cells, where the chromosomal attTn7-site is blocked by the lacIq-mcherry gene cassette, ensuring that Tn7 transposition inserts the bla-sfGFP cassettes into the attTn7-site of pKJK5-attTn7 and pKJK5_NC-attTn7.

Secondly, vectors pGRG36-sfGFP and pGRG36-sfGFP-bla (100–150 ng) were transformed by electroporation to E. coli MG1655-lacIq-mcherry competent cells containing the conjugative plasmid pKJK5-attTn7 or pKJK5_NC-attTn7. pGRG36 is a convenient Tn7 transposon vector that encodes transposition genes traABC under the control of the arabinose-inducible regulator araC and Pbad promoter. pGRG36 also carries the temperature sensitive pSC101 replication origin and an ampicillin resistance gene (McKenzie and Craig, 2006). For each of the 14 constructs, three colonies that grew on LB agar medium with Amp and Tet at 30 °C, single colonies were grown in LB broth supplemented with 0.1% w/v L-arabinose overnight at 30 °C with shaking. Hereafter, cultures were re-streaked onto LB agar medium and incubated overnight at 42 °C with the aim of curing the different pGRG36 vectors after the bla-sfGFP cassettes insertion. Finally, clones were screened and verified by colony PCR with primers psfGFP-F/R and primers pGRG36-F/R.

2.7. Solid surface filter conjugation assay

Overnight cultures of donor strains (E. coli MG1655/pKJK5, E. coli MG1655/pKJK5-attTn7, E. coli MG1655/pKJK5_CT-attTn7, and E. coli MG1655/pKJK5_NC-attTn7) and a recipient strain (E. coli MG1655-KanR, RifR-NalR) were washed twice with PBS, and the OD600 of all cultures were adjusted to 0.5 with PBS. Donors and recipients were mixed at a ratio of 1:1 and immediately 50 μl was added onto a 0.2 μm mixed cellulose ester filter which was placed on top of LB agar medium. These were incubated for 20 h at 37 °C. After incubation, cells were transferred to a tube with 5 ml PBS by repeatedly pipetting cells off the filter. 100 μl of each of these suspensions were spread evenly onto agar plates with antibiotics as detailed below and incubated overnight at 37 °C. Transfer efficiencies were calculated as transconjugants per donor (Sørensen et al., 2005). LB agar plates containing Tet were used to count CFUs of the donors. LB agar plates containing Tet, Nal, and Rif were used to count CFUs of transconjugants. Three biological replicates were performed for each experiment and three technical replicates were made for each biological replicate.

2.8. Growth curves

E. coli strains carrying plasmids pKJK5, pKJK5-attTn7, pKJK5_CT-attTn7, or pKJK5_NC-attTn7 were cultured overnight in LB broth with Tet at 37 °C. Then LB broth was used to make 105-fold dilutions of the overnight cultures, and 200 μl diluted cultures were added to wells of a 96-well microtiter plate. Finally, the plate was incubated in a spectrophotometer (Bio Tek ELx808™ Absorbance Microplate Reader) at 37 °C overnight, with continuously shaking, and OD600 was measured every 15 min. Nine replicates were done for each strain (three biological replicates each with three technical replicates).

2.9. Flow cytometry

Flow cytometry was conducted to verify sfGFP expression using a BD FACS AriaIIa (BD Biosciences) with a 488 nm excitation laser and FITC (530/30 nm band-pass filter) detector. Wild-type strain E. coli MG1655 was used as a negative control. For sample treatment, 5 μl of the overnight cultured bacterial solution was added to 1 ml of PBS to ensure reaching ~3000 evt/s. The threshold for forward scatter (FSC) was 1200, for side scatter (SSC) was 200, and the gating strategy was...
consistent with our previous study (Olesen et al., 2022). Data was acquired and analyzed using the BD FACSDiva software v.6.1.3.

2.10. Antibiotic sensitivity

Inhibitory concentrations of antibiotics for all strains were determined using the agar dilution method (Wiegand et al., 2008). Overnight cultures were diluted to 10^4 CFU/ml. 10 μl from each suspension were spotted onto LB agar plates containing a series of antibiotics (0–10 μg/ml MEM, 0–10 μg/ml CTX, 0–200 μg/ml AMP) to determine the minimum inhibitory concentration. The agar plates were incubated at 37 °C for 16 h and the lowest antibiotic concentration that inhibited visible bacterial growth was regarded as inhibitory. These experiments were repeated independently three times.

2.11. Statistical analysis

All the comparisons of mean values between conditions (e.g., OD_{600}) were performed using one-way ANOVA with post-hoc Tukey tests calculated using R 4.0 (Team, 2013). Growth curves were analyzed with the R package Growthcurver (Sprouffske and Wagner, 2016).

3. Results

3.1. Engineering an easily modifiable conjugative plasmid pKJK5-attTn7

Here, an attTn7-site was introduced into conjugative IncP-1 plasmid pKJK5 (Fig. 1a). Based on the genome sequence of pKJK5, we chose to insert the attTn7-site between nt 33,431–33,440 (Fig. 1a), as this was at the interface between plasmid backbone and accessory genes with little risk of functional disruption. The region may have encoded a terminator associated with traC, however, a new terminator was added with the attTn7 fragment (Fig. 1b). The inserted attTn7-FRT-aac3-FRT fragment was flanked on both sides with strong terminators to diminish expression spillover both from flanking genes and from genes to be inserted into the attTn7-site (Fig. 1c). aac3 was introduced alongside the attTn7-site and was subsequentially removed by FLP recombinase-mediated excision via the flippase recognition target sites that flanked aac3 (Fig. 1d and Fig. 2a). Colony PCR and full genome sequencing verified pKJK5-attTn7 had been constructed successfully (Fig. 1e).

3.2. Conjugation deficient plasmid pKJK5_{CD}-attTn7 and non-conjugative plasmid pKJK5_{NC}-attTn7

In addition to the conjugative pKJK5-attTn7 plasmid, two additional versions of pKJK5-attTn7 were constructed. One that transfers at lower frequencies, referred to as pKJK5_{CD}-attTn7, and a non-conjugative version referred to as pKJK5_{NC}-attTn7. These were made as they can be used as comparative controls in various experimental setups. Previous studies have shown that making a knock-out in traF, which is in operon and upstream of traC, reduces the conjugation transfer frequency of pKJK5 (Bahl et al., 2007b; Roder et al., 2021) Therefore, it was assumed that deleting traC would in a similar manner create a conjugation deficient version of pKJK5-attTn7. To create a version that was not able to conjugate the traC-I region was deleted. This region encodes core proteins for relaxosome formation which is essential for the transfer of plasmid DNA during conjugation (Adamczyk and Jagura-Burdzy, 2003; Bahl et al., 2007a). An FRT-cat-FRT fragment was introduced upstream of traC to create pKJK5_{CD}-attTn7 (Fig. 3a) and upstream traI (and traK) to create pKJK5_{NC}-attTn7 (Fig. 3b) using λ Red recombination. Hereafter, FLP recombination was used to remove the regions between the FRT-site associated with attTn7 and those introduced with cat. This resulted in the deletion of the traC-FRT-cat-FRT region constructing pKJK5_{CD}-attTn7 and the deletion of traCDEFGHI-FRT-cat-FRT region construction.
Fig. 3. Stepwise engineering of plasmids pKJK5CD-attTn7 and pKJK5NC-attTn7. (a) FRT-cat-FRT was inserted into pKJK5-attTn7 upstream traC. Hereafter, Fpl recombination was used to remove traC-FRT-cat-FRT constructing conjugation deficient plasmid pKJK5CD-attTn7. (b) FRT-cat-FRT was inserted into pKJK5-attTn7 upstream traI. Fpl recombination was used to remove traCDEFGHI-FRT-cat-FRT constructing non-conjugative plasmid pKJK5NC-attTn7. (c) Schematic diagram of the FRT-cat-FRT fragment. (d) Colony PCR to verify the construction of pKJK5CD-attTn7 and pKJK5NC-attTn7. Lanes M are 1 kb DNA ladders (Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder). Lanes 1, 2, 3, and 4 show the PCR product from negative control (ddH₂O), plasmid pKJK5-attTn7, pKJK5CD-attTn7-cat, and pKJK5CD-attTn7 with primers CD-cat-F/R, respectively. Lanes 5, 6, and 7 show the PCR product from plasmid pKJK5CD-attTn7-cat, pKJK5CD-attTn7, and negative control (ddH₂O) with primers attTn7Gen⁵-F and CD-cat-R, respectively. Lanes 8, 9, 10, and 11 show the PCR product from negative control (ddH₂O), plasmid pKJK5-attTn7, pKJK5NC-attTn7-cat, and pKJK5NC-attTn7 with primers NC-cat-F/R, respectively. Lanes 12, 13, and 14 show the PCR product from plasmid pKJK5NC-attTn7-cat, pKJK5NC-attTn7, and negative control (ddH₂O) with primers attTn7Gen⁵-F and NC-cat-R, respectively. Figures (a), (b) and (c) were generated with SnapGene software 6.0.2 (GSL Biotech).
constructing pKJK5_NC-attTn7 (Fig. 2b). Removal of traC in pKJK5_CD-attTn7 and traC-1 in pKJK5NC-attTn7 was verified by colony PCRs (Fig. 3d).

3.3. Growth and conjugal transfer frequencies

To investigate if the genetic modification applied to the plasmids had an immediate effect on their host, growth curves of the same host complemented with the different plasmids were done. Growth curves were made of strains E. coli MG1655/pKJK5, E. coli MG1655/pKJK5-attTn7, E. coli MG1655/pKJK5_CD-attTn7, and E. coli MG1655/pKJK5NC-attTn7 (Fig. 4a). No significant difference was found between growth rates or the doubling times between the strains (P < 0.05, one-way ANOVA with post-hoc Tukey test).

Next, we compared conjugal transfer frequencies of the different constructed plasmids in addition to the unmodified pKJK5 plasmid. We used the strains E. coli MG1655/pKJK5, E. coli MG1655/pKJK5-attTn7, E. coli MG1655/pKJK5_CD-attTn7, and E. coli MG1655/pKJK5NC-attTn7 as donors, and strain E. coli MG1655-Kan^R/Rif^R/Nal^I as recipient (Fig. 4b-c). We found that the insertion of the attTn7 site did not affect conjugal transfer as transfer frequencies of wildtype plasmid pKJK5 and pKJK5-attTn7 were similar (P = 0.739, one-way ANOVA with post-hoc Tukey test). The transfer frequency of traC deletion plasmid pKJK5_CD-attTn7 was significantly reduced (P < 0.05, one-way ANOVA with post-hoc Tukey test) rendering it conjugation deficient. No transconjugants were observed in transfer experiments with pKJK5NC-attTn7 showing that this plasmid had lost its ability to conjugate.

3.4. Gene complementation at the attTn7-site of the constructed plasmids

To test the efficacy of the attTn7 complemented plasmids, 7 different β-lactamase genes (bla_TEM-1, blaCTX_M-15, blaCMY-2, blaNDM-5, blaKPC-2, blaOXA-181, blaAmpC) flanked by sfGFP were inserted into pKJK5-attTn7 using the mini-Tn7 delivery vector pGRG36. Since the attTn7 site typically also exists in the bacterial chromosome, E. coli MG1655-lacF- mcherry (Klümper et al., 2015) was used as background when performing complementation. In E. coli MG1655-lacF-mcherry the chromosomal attTn7-site was occupied by the lacF-mcherry cassette preventing further chromosomal transposition events. PCR, gel electrophoresis, and Sanger sequencing was used to verify that the plasmids has been successfully constructed (Fig. 5a). The different β-lactamase genes and sfGFP were also inserted into the non-conjugative plasmid pKJK5NC-attTn7, illustrating the high efficacy of gene complementation into the attTn7-sites located on the constructed plasmids presented here (Fig. 5a).

To verify the expression of the inserted genes, the inhibitory concentrations of the different strains were tested (Table 3). The inhibitory concentrations towards relevant β-lactamases of all strains carrying the different bla genes in pKJK5-attTn7, were significantly higher than those of the wildtype E. coli MG1655 (Table 3). To test the expression of sfGFP, green fluorescence was measured by flow cytometry using E. coli MG1655-lacF-mcherry/pKJK5-attTn7::sfGFP-KPC2 as a representative (Fig. 5b-c). Here the constitutively expressed lacF represses the P_O1 promoter of sfGFP. To counteract this 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to relieve the repression of sfGFP by lacF (Dahlberg et al., 1998; Lanzer and Bujard, 1988). Lastly, we tested if the blaKPC-2::sfGFP insertion influenced conjugation frequencies of the plasmid (Fig. 4b-c). We found that the blaKPC-2 and sfGFP were expressed as expected and transfer frequencies were similar to the wildtype plasmid pKJK5 (P = 0.592, one-way ANOVA with post-hoc Tukey test).

![Fig. 4. Growth and conjugal transfer frequencies.](image-url)
Discussion

Here we present a suite of engineered plasmids that were developed to advance research on HGT and plasmid biology. We introduced an attTn7-site into conjugative broad-host-range IncP-1 plasmid pJK5 using the λ phage derived Red recombination system. Hereafter, we demonstrate the ease at which genes of interest can be inserted into the attTn7-site with high efficacy using the bacterial mini-Tn7 transposon site-specific recombination system. We utilized vector pGRG36 which contains a multiple cloning site for easy complementation, and it allows insertion of large fragments. It is known that Tn7 possesses transposition immunity, which counteracts Tn7 from transposing into attTn7 sites already occupied by Tn7 (DeBoy and Craig, 1996), reducing the chance of at least 104 fold (McKenzie and Craig, 2006). The construction of the 14 different plasmids with different bla genes and sfGFP (pJK5-sfGFP-bla) illustrates that, e.g., when wanting to study a series of functional genes in the context of a conjugative plasmid, the introduction of an attTn7-site reduced time spent engineering and overall costs.

Examples of studies where genetic complementation of plasmids has been central include those where conjugative plasmids have been complimented with fluorescent proteins such as GFP providing valuable insight into e.g. host range (Anjum et al., 2019; Olesen et al., 2022). One could imagine complimenting and generating plasmids with several different fluorescent proteins, using the pJK5-attTn7 plasmids presented here, to study dissemination dynamics and their impact on cell lineages and ecology in line with Jasinska et al. (Jasinska et al., 2020), or use the pJK5-attTn7 plasmids when developing and assessing new reporter systems with different fluorescent proteins, promoters, and regulatory elements (Johns et al., 2018; Klümper et al., 2015; Krol et al., 2010; Röder et al., 2021). We imagine the pJK5-attTn7 plasmids will be useful in future studies investigating the horizontal and vertical transfer

Table 3
The minimum inhibitory concentration (MIC) of strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Meropenem (μg/ml)</th>
<th>Cefotaxime (μg/ml)</th>
<th>Ampicillin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MG1655 WT</td>
<td>0.125</td>
<td>&lt; 0.05</td>
<td>20</td>
</tr>
<tr>
<td>E. coli MG1655/pJK5::sfGFP</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::OXA-181</td>
<td>/</td>
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<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::KPC-2</td>
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<tr>
<td>E. coli MG1655/pJK5::NDM-5</td>
<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::CMY-2</td>
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<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::CTX-M-15</td>
<td>/</td>
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<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::ampC</td>
<td>/</td>
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<tr>
<td>E. coli MG1655/pJK5::TEM-1</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>E. coli MG1655/pJK5::Bla</td>
<td>0.125</td>
<td>&lt; 0.05</td>
<td>20</td>
</tr>
</tbody>
</table>
| (μg/ml), /" means not been tested.

Fig. 5. Verification of insertion of bla-sfGFP cassettes into pJK5-attTn7 and pJK5-attTn7. (a) PCR verification of the correct insertion of the different bla-sfGFP cassettes into pJK5-attTn7 and pJK5-attTn7. Lanes 1, 2, 3, 4, 5, 6, and 7 verify insertion of blaKPC-2, blaNDM-5, blaOXA-181, blaCTX-M-15, blaampC, blaCMY-2, and blaTEM-1, respectively, into pJK5-attTn7. Lane 8 a 1 kb DNA Ladder (Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder). Lanes 9, 10, 11, 12, 13, 14, and 15 verify insertion of blaKPC-2, blaNDM-5, blaOXA-181, blaCTX-M-15, blaampC, blaCMY-2, and blaTEM-1, respectively, into pJK5-attTn7. (b) Image showing green fluorescence under ultraviolet light due to expression of the sfGFP by E. coli MG1655/pJK5-attTn7::blaKPC-2-sfGFP. E. coli MG1655/pJK5-attTn7 was used as a negative control. (c) Green fluorescence due to expression of the sfGFP by E. coli MG1655/pJK5-attTn7::blaKPC-2-sfGFP detected by flow cytometry. E. coli MG1655/pJK5-attTn7 was used as a negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
of genes. This is an important topic and studies have, for example, focused on antibiotic resistance genes (Sakamoto et al., 2022) and metal resistance genes (Cyrilique et al., 2021), but many other genes are equally relevant to study from this perspective (Jain et al., 1999; Madsen, 2020). Another use of conjugative plasmids is as delivery vectors when attempting to engineer microbial communities (sometimes referred to as metagenomic engineering) (Ronda et al., 2019). E.g., Song et al. delivered a CRISPR-Cas12a system into a gut microbiome to selectively kill pathogens (Song et al., 2022). Our system could be used for similar approaches and the advantage would be that one could try many different types of CRISPR-Cas systems and/or spaces (or other systems) and compare pros and cons between them, by easily and efficiently complementation at the attTn7-site. These but a few ways we imagine the pKJK5-attTn7 plasmids could be useful for future applications but countless more are evidently possible.

The mini-Tn7-based transposon system is typically used for the insertion of single gene cassettes, however, innovative approaches such as mini-Tn7 sites/integration systems (attTn7-sites) have been developed to integrate multiple gene cassettes into bacterial genomes. This is an important topic and studies have, for example, referred to as metagenomic engineering) (Ronda et al., 2019).

Another use of conjugative plasmids is as delivery vectors focused on antibiotic resistance genes (Sakamoto et al., 2022) and metal resistance IncP-1 plasmids. Acta Biochim. Pol. 50 (2), 425–453. https://doi.org/10.18388/abp.2003.1666


