A simple method for construction of pir+ Enterobacterial hosts for maintenance of R6K replicon plasmids

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Abstract

Background: The R6K replicon is one of the best studied bacterial plasmid replicons. Replication of the R6K plasmid and derivatives harboring its y origin of replication (oriR6K) is dependent on the pir gene-encoded π protein. Originally encoded by R6K, this protein is usually provided in trans in hosts engineered to support replication of plasmids harboring oriR6K. In Escherichia coli this is commonly achieved by chromosomal integration of pir either via lysogenization with a λpir phage or homologous recombination at a pre-determined locus.

Findings: Current methods for construction of host strains for oriR6K-containing plasmids involve procedures that do not allow selection for presence of the pir gene and require cumbersome and time-consuming screening steps. In this study, we established a mini-Tn7-based method for rapid and reliable construction of pir+ host strains. Using a curable mini-Tn7 delivery plasmid, pir expressing derivatives of several commonly used E. coli cloning and mobilizer strains were isolated using both the wild-type pir+ gene as well as the copy-up pir-116 allele. In addition, we isolated pir+ and pir-116 expressing derivatives of a clinical isolate of Salmonella enterica serovar Typhimurium. In both E. coli and S. enterica serovar Typhimurium, the presence of the pir+ wild-type or pir-116 alleles allowed the replication of oriR6K-containing plasmids.

Conclusions: A mini-Tn7 system was employed for rapid and reliable engineering of E. coli and S. enterica serovar Typhimurium host strains for plasmids containing oriR6K. Since mini-Tn7 elements transpose in most, if not all, Gram negative bacteria, we anticipate that with relatively minor modifications this newly established method will for the first time allow engineering of other bacterial species to enable replication of plasmids with oriR6K.

Background

The y origin of replication of the broad-host-range plasmid R6K (oriR6K) has been used to construct conditionally replicative cloning and transposon delivery vectors too numerous to cite them all, with some of the most well-known vectors described in the late 1980s and early 1990s [1-6]. Replication of these vectors requires the π protein encoded by the pir gene which on R6K is located next to the y origin of replication [7,8]. For maintenance of conditionally replicative plasmids that contain oriR6K, but lack pir, the π protein is expressed in trans from pir located on a compatible plasmid or, most frequently, on a λ phage or a gene inserted into the chromosome via homologous recombination at a predetermined locus [9]. In cells harboring a wild-type pir+ gene, oriR6K containing plasmids are maintained at 15 copies or less depending on size of the oriR6K plasmid and pir gene source. A number of pir mutations have been identified that alter plasmid copy number, for example the pir-116 allele [10]. In cells harboring this allele integrated into the chromosome, oriR6K containing plasmids are maintained at a copy number of about 250 per cell which compares to 15 copies per cell when the pir+ allele is integrated at the same chromosomal locus [9]. The host range for oriR6K-containing plasmids is limited because construction of strains supporting their replication in a variety of bacterial species requires a pir+ gene source. A number of plasmids described in the late 1980s and early 1990s [1-6] have been identified that alter plasmid copy number, for example the pir-116 allele [10]. In cells harboring this allele integrated into the chromosome, oriR6K containing plasmids are maintained at a copy number of about 250 per cell which compares to 15 copies per cell when the pir+ allele is integrated at the same chromosomal locus [9]. The host range for oriR6K-containing plasmids is limited because construction of strains supporting their replication in a variety of bacterial species requires a pir+ gene source. A number of plasmids described in the late 1980s and early 1990s [1-6] have been identified that alter plasmid copy number, for example the pir-116 allele [10]. In cells harboring this allele integrated into the chromosome, oriR6K containing plasmids are maintained at a copy number of about 250 per cell which compares to 15 copies per cell when the pir+ allele is integrated at the same chromosomal locus [9]. The host range for oriR6K-containing plasmids is limited because construction of strains supporting their replication in a variety of bacterial species requires a pir+ gene source. A number of plasmids described in the late 1980s and early 1990s [1-6] have been identified that alter plasmid copy number, for example the pir-116 allele [10]. In cells harboring this allele integrated into the chromosome, oriR6K containing plasmids are maintained at a copy number of about 250 per cell which compares to 15 copies per cell when the pir+ allele is integrated at the same chromosomal locus [9]. The host range for oriR6K-containing plasmids is limited because construction of strains supporting their replication in a variety of bacterial species requires a pir+ gene source.
replication involves methods that do not allow selection for presence of the pir gene and require cumbersome and time-consuming screening steps. To allow expansion of plasmid host range to customized genetic strain backgrounds we therefore developed a mini-Tn7-based method for rapid and reliable construction of enterobacterial pir\(^+\) host strains.

**Results and discussion**

**Development of a mini-Tn7 based ori\(_{R6K}\) chromosomal insertion system**

We sought to employ the mini-Tn7 method described by McKenzie and Craig [11] for chromosomal insertion of pir alleles in the absence of selection. For this purpose, the pir\(^+\) and pir-116 genes were cloned into the mini-Tn7 delivery vector pGRG36 (Figure 1) and published procedures [11] followed in an attempt to transpose the cloned pir genes into the chromosomes of various *E. coli* strains. However, in some strains, despite repetition and exhaustive PCR screening, this method proved ineffective for this purpose as the majority of colonies obtained after completion of the procedure did not contain the desired mini-Tn7 insertions or did not result in any insertions, for unexplained reasons. We therefore designed a method that allows positive selection of strains containing chromosomally inserted pir alleles (Figure 2). The rationale for this method is to establish the delivery vector with a temperature-sensitive replicon (ts), here pSC101ori\(_{ts}\), at permissive temperature (30°C), then introduce an ori\(_{R6K}\) reporter plasmid at 37°C, creating conditions at which the mini-Tn7 delivery vector is cured and replication of the reporter plasmid is dependent on the presence of a chromosomally-integrated pir gene. After verification of the desired mini-Tn7-pir insertions the reporter plasmid is then cured using sucrose counter-selection.

**Mini-Tn7 insertion of pir genes in *E. coli* and *S. enterica* serovar Typhimurium**

Following the procedure outlined in Figure 2, we readily obtained mini-Tn7-pir insertions in several commonly used *E. coli* laboratory cloning and mobilizer strains—DH5\(_{a}\), JM108, MC4100, SM10, RH03—and a clinical *S. enterica* serovar Typhimurium isolate. Exconjugants examined by PCR contained the desired insertion, either mini-Tn7-pir\(^+\) or mini-Tn7-pir-116 (Figure 3A). The plasmid copy number of pR6KT2 was greatly elevated in pir-116-containing *E. coli* DH5\(_{a}\) and *S. enterica* serovar Typhimurium 14028S host strains when compared to the same strains containing chromosomally inserted wild-type pir\(^+\) (Figure 3B). An alternative to employing an ori\(_{R6K}\) reporter plasmid is to use mini-Tn7-pir elements that contain a Km\(^+\) selection marker that after verification of desired inserts can be removed using *Saccharomyces cerevisiae* Flp recombinase-mediated site-specific excision, followed by curing of the Flp recombinase expression plasmid. We have successfully used this strategy in *E. coli*. Both strategies require equal time and effort.

**Conclusions**

We have developed simple and effective strategies for engineering of pir expressing strains of Enterobacteriaceae.
These strategies allow extension of the host range of ori\textsubscript{R6K} containing plasmids to virtually any enterobacterial strain, something that was, to date, only possible using relatively cumbersome and time-consuming methods, e.g. isolation of \textit{\lambda pir} lysogens or chromosomal insertion of cloned \textit{pir} alleles via site-specific recombination at a predetermined locus [9]. Mini-Tn7 elements insert at naturally evolved \textit{attTn7} sites that are usually located in intergenic regions downstream of conserved \textit{glmS} genes [12-16]. This alleviates the need for selecting potential insertion sites not affecting bacterial fitness when choosing recombinant DNA strategies for \textit{pir} allele insertion into a bacterial genome. Since mini-Tn7 elements transpose in most, if not all, Gram negative bacteria, we anticipate that with relatively minor modifications this newly established method will for the first time allow engineering of other bacterial species to enable replication of plasmids with ori\textsubscript{R6K g}. As described, the procedure relies on availability of ts replicons which may limit its applicability to bacteria that can tolerate the non-permissive temperatures needed...
for plasmid curing. The methods described here were
developed for Enterobacteriaceae which, like most bac-
teria, can tolerate 37°C, a temperature at which most plas-
mids with ts replicons, including pSC101ori_t, are readily
cured. For bacteria with growth temperature optima less
than 37°C, the described strategy will not work and require
appropriate modifications, i.e. inclusion of different coun-
ter-selection markers, for example sacB [17]. Lastly,
though many manipulations described in this paper use
conjugations as means for introduction of plasmids into
cells, some them could also be done by plasmid transfor-
mation which would alleviate the need for counter-selection
strategies required for bacterial matings. We,
however, consistently find that conjugations are equally
convenient and more efficient means of plasmid transfer
than transformation.

Methods
Bacterial strains, media and growth conditions
Bacterial strains used in this study are listed in Table 1.
Bacteria were routinely grown at 37°C in Luria Bertani
broth Lennox (LB) [18] or on LB agar purchased from
MO BIO Laboratories, Carlsbad, CA. The sacB-containing
ori_{RbsK} reporter pR6KT2 was cured by plating plas-
mid-containing cells on yeast extract-tryptone (YT)
sucrose medium containing 10 g/l yeast extract (Difco,
Detroit, MI), 16 g/l tryptone (Fisher Scientific, Fairlawn,
NJ), 16 g/l Bacto agar (Becton, Dickinson and Company,
Sparks, MD) and 15% sucrose (w/v) [19]. Strains contain-
ing temperature-sensitive (TS) plasmid derivatives were
grown at 30°C (permissive temperature) for plasmid
maintenance and 37°C or 42°C (non-permissive tem-
perature) for plasmid curing. Antibiotics were added at
the following concentrations: 100 μg/ml ampicillin (Amp),
10-15 μg/ml gentamicin (Gm) and 35 μg/ml
kanamycin (Km) for E. coli and S. enterica serovar
Typhimurium harboring plasmids or for selection of
chromosomally-integrated mini-Tn7 elements. Antibio-
tics were purchased from EMD Biosciences, San Diego,
CA (Gm) and Sigma, St. Louis, MO (Amp and Km).

For E. coli strain RHO3, media were supplemented with dia-
minopimelic acid (DAP; LL-, DD-, and meso-isomers;

Table 1 Bacterial strains used in this study

| Bacterial strains E. coli | Relevant genotype | Source or reference |
|---------------------------|-------------------|---------------------|
| DB31                      | F' gyrA62 endA1 glnV44 Δ(sorl-recA) microB mrr hsdS20 (F mK) ara14 galK2 lacY1 proA2 rpsL20 xylS Δeu mtl1 | Invitrogen |
| PIR1                      | Δ(argF-lac)169 psoSA1 rpsA1 creC510 hsdR514 endA recA1 u1d1ΔMull;pir-116 | Invitrogen |
| PIR2                      | Δ(argF-lac)169 psoSA1 rpsA1 creC510 hsdR514 endA recA1 u1d1ΔMull;pir' | Invitrogen |
| RHO3                      | SM10 (pir-116) ΔsdwΔFT pirΔphoAΔFT | [19] |
| DH5α                      | F' q80 lacZ2M15 ΔlacZ2Y-argF(U169 deoR recA1 endA1 hsdR17(ω mK) phoA glnV44 | [20] |
| JM108                     | ΔcroA recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δlac-proAB | [21] |
| MC4100                    | F' araD139 Δ(argF-lac)169 hiDS301 Δ(fruK-yeiR)725 (fruA25) relA1 rpsL150 rbiR2Δ2 Δ(fimB-fimE)632 ΔdeoC1 | [22,23] |
| SM10                      | thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA1 integrated RPA-2 Tc' μMu aphA' (Km') (RPA-2 is RPA ΔTn1) | [24] |
| SBr1                      | ΔHis6::mini-Tn7-pir' | This study |
| SBr2                      | JM108::mini-Tn7-pir' | This study |
| SBr3                      | MC4100::mini-Tn7-pir' | This study |
| SBr4                      | SM10::mini-Tn7-pir' | This study |
| MaH1                      | ΔHis6::mini-Tn7-pir-116 | This study |
| MaH2                      | JM108::mini-Tn7-pir-116 | This study |
| MaH3                      | MC4100::mini-Tn7-pir-116 | This study |
| MaH4                      | SM10::mini-Tn7-pir-116 | This study |
| MaH5                      | DH5α::mini-Tn7-pir-116-FKm | This study |
| RHD4                      | RHO3::mini-Tn7-pir-116-FKm | This study |
| RHD5                      | RHO3::mini-Tn7-pir-116-FRT | This study |
| S. enterica serovar Typhimurium |  |
| 14028S                    | Wild Type | ATCC |
| SDR1                      | 14028S::mini-Tn7-pir' | This study |
| SDR2                      | 14028S::mini-Tn7-pir-116 | This study |

1 The pir gene carried by p pir has a truncation that removes the coding region for the carboxy-terminal 30 amino acids of the μ protein. Despite this truncation a
pir phage maintains plasmids with at the same copy number as cells carrying a wild-type pir gene [9].

2 The pir-116 allele is dominant over the pir gene carried by the lysogenic λ phage and leads to increased copy number of ori_{RbsK} containing plasmids.
DNA and genetic methods

Isolation of plasmid DNA

Plasmid DNAs were isolated from E. coli and S. enterica serovar Typhimurium by using a Fermentas GeneJET Plasmid MiniPrep Kit (Fermentas, Glen Burnie, MD).

Transposition of mini-Tn7

The respective mini-Tn7 delivery vectors were transformed into E. coli mobilizer strain RHO3 [19]. Conjugation of delivery plasmid into E. coli and S. enterica serovar Typhimurium strains was achieved by biparental mating using previously described methods [19] with some minor modifications. Briefly, RHO3 donor and E. coli and S. enterica serovar Typhimurium recipient cultures were grown overnight at 30°C (pGRG36-based donor strains) or 37°C (recipient strains) in LB medium with the appropriate nutritional (DAP) and antibiotic (Amp) supplements for RHO3 with the mini-Tn7 delivery vector. One ml of donor and recipient were placed into separate 1.7 ml microcentrifuge tubes and harvested by centrifugation in a microcentrifuge for 30 s at 13,400×g and room temperature. Cells were washed twice in 1 ml LB medium and then re-suspended in 200 μl of LB medium. Equal volumes (25 μl) of each cell suspension were transferred to a cellulose acetate membrane (13 mm diameter; 0.45 μM pore size; Sartorius Stedim, Bohemia, NY) sitting on an LB agar plate containing 400 μg/ml DAP and 0.5% arabinose. After overnight incubation at 30°C, the membrane was transferred to a microcentrifuge tube containing 1 ml of LB and cells dislodged by centrifugation in a microfuge for 30 s at 13,400×g and room temperature. After removing the membrane, cells were washed twice in 1 mL LB and then re-suspended in 200 μl of LB medium. The entire sample was placed on an LB-agar plate with 100 μg/ml Amp and 0.5% arabinose, and a portion streaked for single colonies with an inoculating loop. The plates were incubated at 30°C overnight or until single colonies were clearly discernable.

A single purified colony was then used as recipient for the oriR6K<sup>−</sup>, reporter pr6KT2. This plasmid was introduced via biparental mating from RHO3 as described above, except that recipient cells were grown in the presence of Amp and arabinose and RHO3/pr6KT2 cultures were grown in the presence of Gm and DAP. After overnight incubation at 30°C mating mixtures were recovered and plated on LB with 15 μg/ml Gm at 37°C to cure the temperature-sensitive mini-Tn7 delivery vector and select for pr6KT2. Purified colonies were patched on LB, LB + Gm and LB + Amp to confirm the loss of the mini-Tn7 delivery vector (Amp susceptibility) and presence of pr6KT2 (Gm resistance). After verification of mini-Tn7 insertions by PCR, pr6KT2 was cured by streaking a single colony on YT medium with sucrose and XGluc, and incubating overnight at 37°C. Single colonies were patched on LB and LB + Gm plates to confirm the loss of the plasmid. The pir gene insertions in the resulting strains were then re-confirmed by PCR and sequencing of the resulting DNA fragments.

When using the mini-Tn7-<i>pir</i>-FKm vectors, the protocol for conjugation was as described above for mini-Tn7 delivery without antibiotic selection. Exconjugants were grown overnight in LB + DAP and arabinose at 30°C, and Km<sup>−</sup> transposon insertions were selected by plating conjugation mixtures on LB plates with 35 μg/ml Km followed by incubation at 42°C to cure the delivery plasmid. The Km<sup>−</sup> marker can optionally be deleted from the strain with the mini-Tn7-<i>pir</i>-FKm insertion by transformation with pFLP2 (or any other Flp recombinase-expressing plasmid such as pCP20 [25]), testing transformants for Km susceptibility and then curing pFLP2 by plating on sucrose-containing media following previously described protocols [17].

Confirmation of mini-Tn7-<i>pir</i> insertions

Insertions of mini-Tn7-<i>pir</i> at attTn7 in E. coli and S. enterica serovar Typhimurium were performed by colony PCR using DNA in boiling preparation lysates as templates. These lysates were obtained by transferring separate colonies to individual sterile microcentrifuge tubes containing 30 μl of sterile water and boiling for 10 min. Using 6 μl of these boiling preparations and Taq DNA polymerase from New England Biolabs, PCR reactions were performed in a total volume of 50 μl. Primer pairs 2372 (5'-GATGCTGGTGCGAAGCTGTC) & 2373 (5'-GATGACGGTTTGTCACATGGAG) and 2374 (5'-CAGCAACGCCCCATGCACA) & 2375 (5'-AAACCATCGCGCGGAAACAA) were used for E. coli and S. enterica serovar Typhimurium, respectively. In each case, the entire PCR reaction was analyzed on a 1% agarose gel. Expected PCR fragment sizes are 678 bp for E. coli without a mini-Tn7 insertion and 2,539 bp for derivatives containing mini-Tn7-<i>pir</i><sup>+</sup> and mini-Tn7-<i>pir</i>-116. Fragment sizes for S. enterica serovar Typhimurium without and with mini-Tn7-<i>pir</i> insertions are 485 bp and 2,345 bp, respectively. When utilizing mini-Tn7-<i>pir</i>-FKm, the fragment sizes obtained with strains containing <i>pir</i><sup>+</sup> or <i>pir</i>-116 insertions change by +1,470 bp when the Km<sup>−</sup> marker is present or by +145 bp after its Flp-mediated excision.
Table 2: Plasmids used in this study

| Plasmids       | Description                                      | Reference/Source       |
|----------------|--------------------------------------------------|------------------------|
| pGRG36         | Amp<sup>+</sup>, mini-Tn7 delivery vector with TS replicon | [11]                   |
| pGRG36GW       | Amp<sup>+</sup>, Km<sup>+</sup>, Gateway version of pGRG36 | This study             |
| pDONR221       | Cm<sup>+</sup>, Km<sup>+</sup>, Gateway cloning vector | Invitrogen             |
| pDONR221pir    | Km<sup>+</sup>, pir<sup>+</sup> donor vector       | This study             |
| pDONR221pir-116| Km<sup>+</sup>, pir-116 donor vector              | This study             |
| pGRG36pir      | Amp<sup>+</sup>, pir<sup>+</sup> delivery vector   | This study             |
| pGRG36pir-116  | Amp<sup>+</sup>, pir-116 delivery vector          | This study             |
| pGRG36pir-116-FKm | Amp<sup>+</sup>, Km<sup>+</sup>, pir-116-FKm delivery vector | This study             |
| pFH2M4         | Amp<sup>+</sup>, Km<sup>+</sup>, pFH2M [26] with XbaI and SpeI sites removed and PacI site introduced | This study             |
| pR6KT2         | Cm<sup>+</sup>, Suc<sup>+</sup>, ori<sub>Bak</sub> reporter plasmid | This study             |

*The DNA sequence of pGRG36 can be obtained from GenBank under accession number DQ460223. Sequences of other plasmids constructed in this study are available from the authors upon request.

Plasmid construction

Plasmids used in this study are listed in Table 2. The Gateway-compatible mini-Tn7 delivery vector pGRG36GW was constructed by cloning of a 1,770-bp Stul-Xhol fragment from pUC18-mini-Tn7-Gm-GW [16] between the Smal and Xhol sites of pGRG36 [11], followed by transformation into the gvrA662 strain DB3.1.

The pir<sup>+</sup> and pir-116 Gateway donor clones were constructed as follows. First, the pir<sup>+</sup> and pir-116 genes were PCR-amplified from E. coli strain PIR2 (pir<sup>+</sup>) and PIR1 (pir-116) chromosomal DNA templates using HiFi Taq polymerase (Invitrogen) and primers 1333 (5'-TGAGGGTCGCAGAACATTACA) & 1334 (5'-ACC TGGGTGGACGATATCAC). The resulting 1,264-bp PCR fragments were cloned into the TA cloning vector pCR2.1 (Invitrogen) to create pir<sup>+</sup> and pir<sup>+</sup>-116. Next, attB sequences were attached to the respective pir gene segments using PCR with primers 1558 (5'-GGGGACAAAGTTTGTACAAAAAGCGAGCTTGA GCGTCGAGACATTACA; the attB1 sequence is underlined) & 1559 (5'-GGGACCACCTTTGATACAA GAAAGCTGGTCAGACACATTACAC; the attB2 sequence is underlined), and pCR2.1-pir and pCR2.1-pir-116 DNA as templates. This generated 1,323-bp DNA fragments that were recombined into pDONR221 using Gateway BP clonase reactions which created pDONR221pir and pDONR221pir-116, respectively. Finally, the inserts from pDONR221pir and pDONR221pir-116 were transferred to pGRG36GW using LR Gateway recombination to yield pGRG36pir and pGRG36pir-116. The pGRG36pir-116-FKm plasmid was constructed by cloning a 1,470-bp SaI fragment from pFKM4 containing a Km<sup>+</sup> gene flanked by Flp recombinase target (FRT) sites into the Xhol site of pGRG36pir-116.

The ori<sub>Bak</sub> reporter pR6KT2 was constructed as follows. First, the ori<sub>Bak</sub> and oriT regions were PCR-amplified from pUC18R6KT-mini-Tn7T [16] using Taq polymerase (NEB) and primers 2298 (5'-ATTCCCGG GAGGCCCCACTTCAAGAATCTC) & 2299 (5'-TAAT CCCGGGCTTCCGTTCTCTCGTCTCA). The resulting 824-bp amplicon was cloned into pCR2.1 to create pCR2.1-R6KoriT. Second, the 901-bp XbaI fragment from non-methylated pPS856 [17] DNA obtained by isolating the plasmid from dam dcm E. coli JM110 [21] was ligated with the 6.5-kb SpeI and XbaI digested backbone of pEXKm5 [19] to construct pEXGm5B. Third, the 915-bp ori<sub>Bak</sub> and oriT XbaI and Eco53K1 fragment was released from pCR2.1::R6KoriT and ligated with the 6.2-kb XbaI and Smal digested pEXGm5B backbone to construct pR6KTSCE. Finally, pR6KTSCE was digested with BamB1 and FspI and the 5.9-kb backbone was re-circularized to construct the reporter vector pR6KTS2.

All plasmid constructions were verified by restriction digest and DNA sequence analysis.

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Authors' contributions

BK and SB performed most of the experiments, JP, IM, EB, SL, KM and KHC contributed to plasmid construction and performed pilot experiments. BK and RKS designed and supervised experiments, and BK, RKS and HPS drafted the final manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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