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SHORT COMMUNICATION

Differentially Marked IncP-1β R751 Plasmids for Cloning via Recombineering and Conjugation

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Abstract

We demonstrate here for the first time the use of an IncP-1β plasmid, R751, as a gene capture vehicle for recombineering/conjugation strategies to clone large segments of bacterial genomes (20 – 100 + Kb). We designed R751 derivatives containing alternative markers for greater flexibility when using the R751 vehicle across different bacteria. These markers are removable if desired as part of the cloning procedure (with no extra steps needed). We demonstrated utility via cloning of 38 and 22 kb genomic segments from *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, respectively. The plasmids expand the options available for use in recombineering/conjugation-based cloning applications.

Key words: IncP-1, R751, FRT, FLP, Pdu, MCP

IncP-1 plasmids have facilitated numerous studies on the promiscuous nature of plasmid-based genetic elements in nature and have allowed broad range transfer of genes across a variety of cell types (Trieu-Cuot et al. 1987; Heinemann and Sprague 1989; Pansegrau et al. 1994; Thorsted et al. 1998; Waters 2001). The IncP-1 group is divided into five subgroups termed α, β, δ, ε, and γ based largely on phylogenetic analysis (Pansegrau et al. 1994; Thorsted et al. 1998; Norberg et al. 2011; Sen et al. 2013). In this report, we demonstrate the first-time use of an IncP-1β plasmid (R751) as a gene capture vehicle via the FRT-Capture technique. Recombineering-based approaches such as FRT Capture and other techniques allow the convenient cloning and/or manipulation of large DNA fragments using PCR and associated insertional/recombination steps (Wilson and Nickerson 2006; Narayanan and Chen 2011; Zeng, Zang, et al. 2017; Zeng, Hao, et al. 2017; Graf et al. 2018; Zeng et al. 2018). The development of a range of plasmid vehicles for these techniques improves their application and utility (Datsenko and Wanner 2000; Quick et al. 2010; Santiago et al. 2011; Wang et al. 2016; Bubnov et al. 2018). Since R751 encodes only a single resistance marker for trimethoprim resistance and this marker may not be suitable in certain bacteria due to background resistance, we engineered a series of R751 derivatives containing additional markers (such a series of IncP-1β plasmids does not exist in the literature to our knowledge). The use of the FRT-Capture technique using a choice of R751 plasmid vehicles is a robust, flexible, and convenient option for the cloning and transfer of large genomic segments in bacteria.

R751 is a self-transmissible IncP-1β plasmid encoding Tp-R that is 53.3 Kb in size and fully sequenced (Thorsted et al. 1998). To utilize this plasmid as a gene capture vehicle in a recombineering/conjugation-based approach like FRT-Capture and other techniques, we engineered R751 derivatives containing FRT sites and a range of different antibiotic resistance markers termed R751 Km, R751 Cm, and R751 Sp (Table I). We used standard Lambda Red recombination to insert the markers and FRT sites in the R751 *qacE* gene, an accessory efflux pump gene located next to the *dhfr* gene encoding Tp-R (Thorsted et al. 1998; Datsenko and Wanner 2000). Briefly, PCR primers were designed to amplify the Km-R, Cm-R, and Sp-R genes from pKD4, pKD3, and pJW102, respectively, such that the PCR products contained homology to the R751 *qacE* gene for insertion via recombineering (Datsenko and

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Table I
Plasmids used in this study.

Plasmid	Reference
R751	(Thorsted et al. 1998)
R751 Km	this study
R751 Cm	this study
R751 Sp	this study
pCP20	(Datsenko and Wanner 2000)
pKD3	(Datsenko and Wanner 2000)
pKD4	(Datsenko and Wanner 2000)
pKD46	(Datsenko and Wanner 2000)
pJW102	(Quick et al. 2010)

Wanner 2000; Quick et al. 2010). Lambda Red recombination was used for recombineering with PCR products as described previously (Datsenko and Wanner 2000; Quick et al. 2010). The sequence of the PCR primers for this recombineering were as follows: P1*qacE*: AGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGTGTAGGCTGGAGCTGCTTC; P2*qacE*: TTTGCCCATGAAGCAACCAGGCAATGGCTGTAATTATGACCATATGAATATCCTCCTTAGTTCC. The same primers as listed could be used for each template (pKD4, pKD3, and pJW102). The PCR products were electroporated into the *E. coli* strain TOP10 containing both R751 and pKD46, the latter plasmid expressing the Lambda Red recombination products for DNA insertion. The transformants were selected on media containing the appropriate antibiotic, and pooled colonies from the transformation were used as donors in a conjugation to the *E. coli* recipient strain MG1655 (Blattner et al. 1997). Tranconjugants were selected on M9 minimal medium containing the appropriate antibiotic (since recipient strain MG1655 is prototrophic and the donor TOP10 is auxotrophic). The plasmid DNA from selected transconjugants was isolated and confirmed via PCR analysis and DNA sequencing. To confirm that conjugation was not affected by these manipulations, we compared the conjugation frequency of R751 Km, R751 Cm, and R751 Sp to the control R751 in separate conjugation experiments (Fig. 1A). The results showed no difference between the new R751 derivatives and WT R751 in conjugation ability. In addition, plasmid stability assays showed no difference between R751 Km, R751 Cm, and R751 Sp and the WT R751 for plasmid maintenance under non-selective conditions (data not shown).

The FRT-Capture technique is diagrammed in Fig. 1B. This technique allows for convenient *in vivo* cloning of large, intact genomic segments (20 – 100 Kb +) (Santiago et al. 2011; Graf et al. 2018). This allows large gene systems to be cloned and subsequently transferred to a range of other bacterial recipients for evolutionary studies, complementation analysis, and bacterial engineering applications (Wilson and Nickerson 2006; Blondel et al. 2010; Graf et al. 2018). To test the new R751 derivatives as cloning vehicles in the FRT-Capture technique, we targeted two separate regions for cloning in *S.* Typhimurium and *E. coli*. The *S.* Typhimurium *pdu* region is 38 Kb in size and contains 43 genes that code for the formation of a protein microcompartment (MCP) that houses associated Pdu enzymes to catalyze the metabolism of 1,2 PD (Chowdhury et al. 2014; Bobik et al. 2015). The *E. coli rimL* region is 22 Kb and contains *rimL* (an acetyltransferase), *ydcI* (a DNA binding gene regulator), and numerous other genes of unknown function (Blattner et al. 1997; Jennings et al. 2011). For both regions, FRT sites were inserted into locations flanking the target genes (using standard recombineering) such that a Km-R gene would be removed with the genes upon excision via FLP recombinase (Fig. 1B) (Datsenko and Wanner 2000). In the presence of one of the R751 derivatives (R751 Sp is shown in Fig. 1B as an example), the excised target genes would be inserted into the plasmid via FLP, and then this molecule is isolated via conjugation to a differentially marked recipient strain. For cloning the *S.* Typhimurium *pdu* genes, the cloning plasmid was R751 Sp and the target DNA strain was χ3477 containing FRT sites flanking the *pdu* genes such that a Km-R marker would be excised with the *pdu* genes (as diagrammed in Fig. 1B) (Graf et al. 2018). For cloning the *rimL* region from *E. coli*, the cloning plasmid was R751 Cm and the target DNA strain was TOP10 containing FRT sites similarly flanking the *rimL* region (inserted at the b1422 and b1444 genes) (Blattner et al. 1997). The Ap-R plasmid pCP20, which expresses the FLP

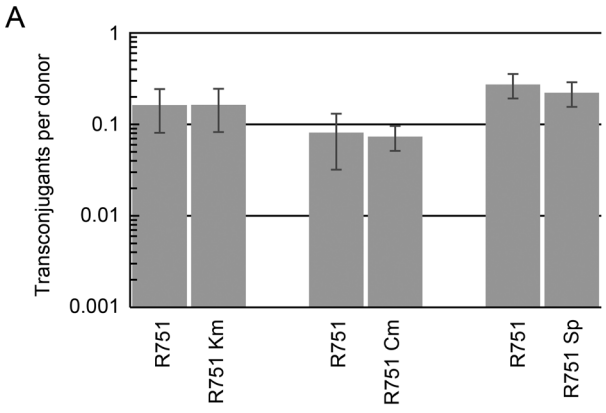

Fig. 1. Characterization of R751 plasmid derivatives.

Panel A: Conjugation frequency (transconjugant per donor) of R751 derivatives compared to WT R751. Each conjugation was performed with different recipients with appropriate counterselective markers, and each R751 derivative is compared to the associated R751 control for that corresponding recipient performed simultaneously.

recombinase, was electroporated into competent target DNA strains containing the R751 derivative, and colonies were selected on either LB Sp Km Ap or LB Cm Km Ap for the *pdu* or *rimL* clonings, respectively. Pooled colonies from a given electroporation were used as donor to the *E. coli* recipient strain TOP10 Rif (Graf et al. 2018), and transconjugants were selected on either LB Rif Sp Km (for the *pdu* cloning) or LB Rif Cm Km

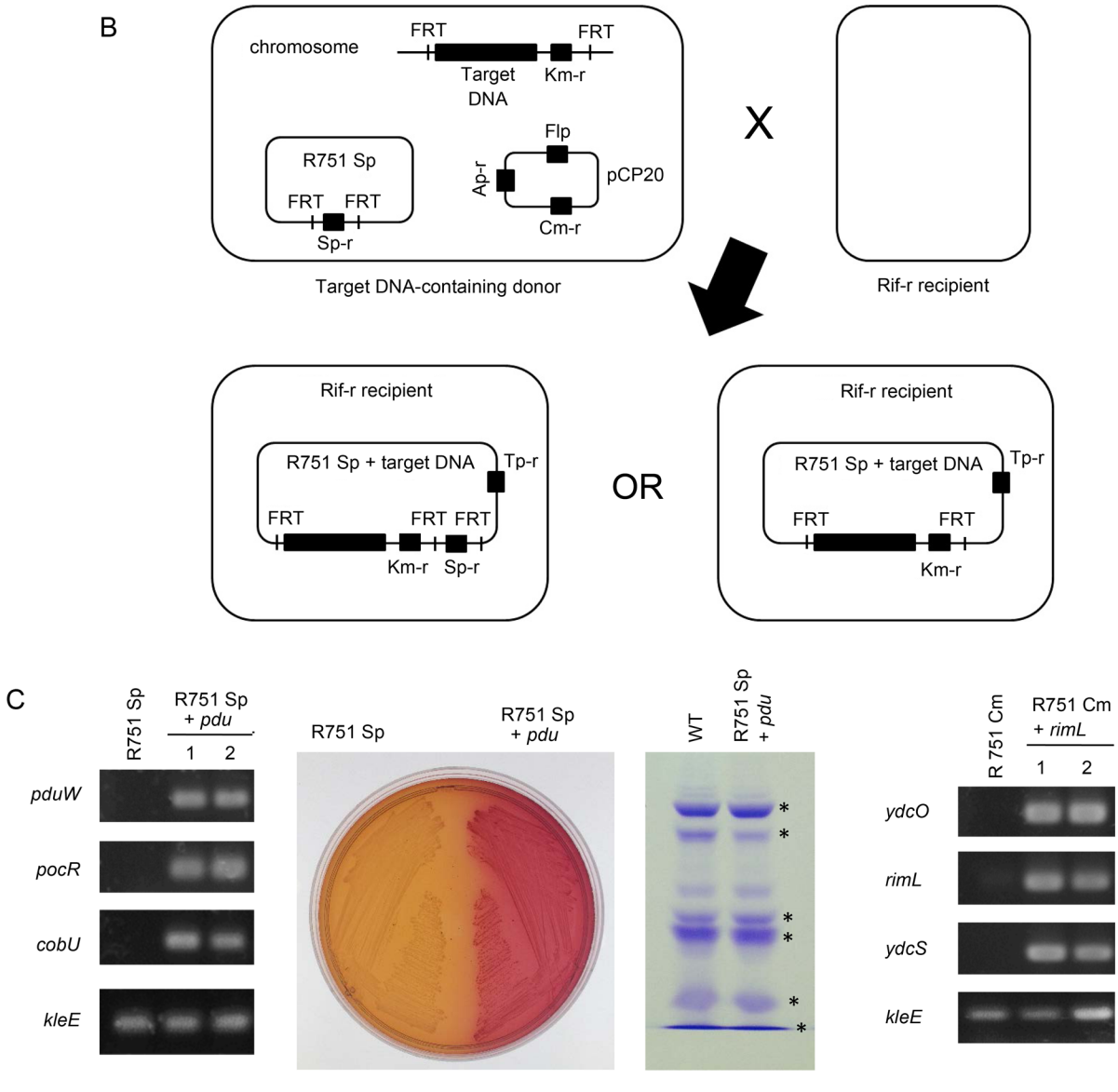

Fig. 1. Characterization of R751 plasmid derivatives.

Panel B: Diagram of the FRT-Capture technique using plasmid R751 Sp as the cloning vehicle. The Sp-r marker can either be retained (via selection for Sp-r) or removed (by using solely Tp-r as the R751 plasmid selection) via this procedure (see text for details). Please, note that when the Sp-r marker is retained, insertion of the target DNA could occur on either side of the Sp-r marker (only one such insertion is shown). The insertion location can be easily verified using PCR or DNA sequencing of the plasmid.

Panel C: Left-most picture: R751 Sp + *pdu* plasmid DNA was isolated and used as a template in PCR reactions using primers hybridizing to the *pduW, pocR*, and *cobU* genes. Primers hybridizing to the R751 Sp plasmid vector (*kleE* gene) were used as control. PCR products were run on 1.5% agarose and stained with SYBR Safe stain. The lanes labeled "1" and "2" are separate isolates of R751 Sp + *pdu*.

Middle two pictures: *E. coli* TOP10 Rif strains containing either R751 Sp or R751 Sp + *pdu* were streaked onto MacConkey agar containing 1,2 PD as carbon source and supplemented with coenzyme B12. Red colony color indicates the expression of the *pdu* genes and metabolism of 1,2 PD. In addition, intact MCPs were isolated from TOP10 Rif (R751 Sp + *pdu*) and approximately 15 micrograms were run on an SDS-PAGE gel and stained with Coomassie. Asterisks on the gel photo indicate bands of known Pdu MCP proteins. Corresponding negative control strains display no bands (or a very faint non-MCP background band) via this analysis (data not shown).

Right-most picture: R751 Cm + *rimL* plasmid DNA was isolated and used as a template in PCR reactions using primers hybridizing to the *ydcO, rimL*, and *ydcS* genes, and the samples were analyzed as above. The lanes labeled "1" and "2" are separate isolates of R751 Cm + *rimL*.

(for the *rimL* cloning). In regard to the efficiency of this process, when using approximately 5×10^7 cells of both the electroporated target DNA strain and the TOP10 Rif recipient, we regularly obtain hundreds of transconjugant colonies (each representing independent clones).

After isolation of R751 Sp + *pdu* and R751 Cm + *rimL*, we used PCR to confirm the presence of the indicated genes (located at 5', center, and 3' locations in these regions) on these clones (Fig. 1C). Plasmid DNA from individual transconjugants was isolated and screened using PCR and relevant phenotypic assays (Fig. 1C). PCR primers used to confirm the presence of cloned *pdu* and *rimL* genes and the R751 *kleE* gene were as follows: *pduW*5': TATGGCAGATGCGCAGGTGACAATTAAGAC; *pduW*3': TGACAACAAATCACCCGTAATGCGCTGAGT; *pocR*5': GCAGGTTCGTTTAAGTAATGACGTGGAGCT; *pocR*3': ATAGACATGTGAGGCGACATCCTCAAGACG; *cobU*5': ACCTCATCCGCCGCTGCCGCCAGTCGTTGG; *cobU*3': CTTAATTGGCGATGCGCCGCAGGTACTGTA; *ydcO*5': GCCGCGTCTCGCTCACGCTCATTATGCAGC; *ydcO*3': GATCGTCATCGCGCAAGGTGACGTTGTCAC; *rimL*5': AAGCGAATCACTTGAATTACATGCTGTTGC; *rimL*3': CTCAGCCTGTTTCAGGCAACCTTCAAGGAT; *ydcS*5': CAGCAGCCTGTGTGCGCTCAGCATGACAAT; *ydcS*3': GCCTTTATTGCTCTTGCCGTCCGGCAGATT; *kleE*5': CGCGGTCAGTGCCGCGAAGTACGCCAGGAA; *kleE*3': TGGCACACCGTAACCATGCTTCCGAGTGGG.

For R751 Sp + *pdu*, we also used MacConkey agar containing 1,2 PD as a carbon source to confirm *pdu* gene expression and functional MCP formation from this plasmid (Fig. 1C) (Graf et al. 2018). In addition, we used an MCP isolation procedure to confirm recovery of intact MCP particles from an R751 Sp + *pdu* strain (analyzed via SDS-PAGE and Coomassie staining) (Fig. 1C) (Graf et al. 2018). Briefly, for MCP isolation, we harvested cells via centrifugation from 10 ml of stationary phase culture (grown in the presence of 1,2 PD), resuspended the cells in 4 ml of buffer A (50 mM Tris-HCl pH = 7.5, 500 mM KCl, 12.5 mM MgCl2, 5 mM beta-mercaptoethanol, 100 micrograms/ml lysozyme, 2 units/ml DNase I, 30% B-PER lysis reagent), and allowed lysis to occur over 1 hour at room temperature with gentle tube inversion. After the sample was centrifuged at 12 000 × g to remove the insoluble fraction, we recovered the supernatant and centrifuged this at 16 000 × g to pellet the MCPs. The pelleted MCPs were washed, resuspended in 150 µl buffer B (50 mM Tris-HCl pH = 7.5, 50 mM KCl, 5 mM MgCl2), and stored at minus 80°C until SDS-PAGE analysis. Taken together, the PCR and phenotypic assays demonstrate the successful utilization of R751 derivatives as gene capture vehicles in a recombineering/conjugation approach to clone large genomic segments from different species.

A convenient feature of the R751 derivatives reported here is that the alternative marker on each can be removed during the FRT-Capture process and replaced with the target DNA (a deletion/replacement of the marker) (Fig. 1B). This is achieved with high efficiency when selection for the marker is removed during the steps for FRT-Capture. To perform the deletion/replacement, the same procedure as above is followed, but trimethoprim resistance (Tp-R) is used as the plasmid selection (as opposed to Sp-R or Cm-R in the above examples). When this is done, transconjugants can be screened for loss of Sp-R or Cm-R (using the examples above), which would have been removed in the donor strain via FLP from pCP20 (with 100% efficiency in our hands).

The range of different marker combinations found on the R751-derived cloning vehicles allows great flexibility for use in FRT-Capture and other similar approaches, and the deletion/replacement option allows convenient removal of a given alternative marker during this process. We emphasize the underdeveloped potential in using recombineering/conjugation-based systems to clone large genomic segments from bacterial genomes. This allows multi-gene systems that function together to be obtained on a single intact fragment that is easily isolated and transferred for subsequent applications. This will have increasing relevance in the post-genomic era as we discover novel large gene systems that can function independently in different bacteria for beneficial microbial bioengineering and evolutionary studies.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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