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Self-Transmissible IncP R995 Plasmids with Alternative Markers and Utility for Flp/FRT Cloning Strategies

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The IncP plasmid R995 has been a useful self-transmissible, broad-host-range vector for a number of applications including the recombinase/conjugation-based cloning of large genomic DNA segments. However, R995 derivatives (or related plasmids) expressing a wide range of different resistance markers and Flp recombinase target sites do not exist in the literature. In addition, documented strategies for applying such plasmids in cloning applications that take advantage of conjugation for the convenient isolation and recovery of constructs are extremely limited. Here, we report a new series of R995 plasmids with alternative markers to increase options for applications in backgrounds already expressing resistance to a particular antibiotic(s). These R995 plasmids have been engineered to contain FRT sites that can be used for recombinase-based cloning. We demonstrate the utility of this approach by cloning 20 kb regions from the *Salmonella* Typhimurium and *Escherichia coli* genomes and by cloning DNA from an exogenous plasmid source. To our knowledge, this represents the first systematic engineering of an intact, self-transmissible IncP plasmid with a series of alternative antibiotic markers and FRT sites.

Keywords: IncP, R995, Flp, FRT, broad host range, self-transmissible, VEX-capture

Plasmids of the IncP compatibility group are known for the broad-host-range activity of their replication and conjugation systems [1, 7, 10]. IncP plasmid R995 has been used in several previous studies as a self-transmissible vector for cloning large bacterial genomic segments and to study IncP plasmid biology [2, 3, 6, 9, 11, 13, 15]. The self-transfer of R995 is extremely advantageous when used during bacterial genomic DNA cloning procedures that involve the "capture" of excised fragments, since the clone can be isolated away from background upon conjugation to a suitable recipient. This is particularly applicable for recombinase-based methods that can excise any targeted genomic fragment as a circular, non-replicating molecule. In addition, the clone is then able to be easily transferred to other recipients of choice for subsequent studies. However, examples of self-transmissible, broad-host-range vectors designed for these approaches are currently very limited in the literature, and the associated cloning-conjugation methods are extremely underdeveloped overall as a convenient technique. During use of plasmid R995, we (and others) have encountered situations where marker choice has become a problem owing to inherent background resistance or markers already present in the strain/DNA elements used for recombineering. Moreover, we have found that the use of more than one antibiotic (and corresponding markers) in combination can help to alleviate background issues that are observed when either is used alone. Here, we report a new series of R995 plasmids with alternative markers to increase options for applications in backgrounds already expressing resistance to a given antibiotic(s). These plasmids can also be used to provide greater marker choice in studies aimed at understanding the biology of IncP plasmids in a range of Gram-negative genera, possibly with co-resident plasmids encoding antibiotic resistance. These R995 plasmids also contain FRT sites that can be used for cloning using recombinase-based approaches. We demonstrate the utility of this strategy by cloning 20 kb regions from the *Salmonella* Typhimurium and *Escherichia coli* genomes and by cloning DNA from an exogenous plasmid source introduced into the R995-containing strain. To our knowledge, this represents the first systematic engineering of an intact, self-transmissible IncP plasmid with a series of alternative antibiotic markers and FRT sites.

Plasmid R995 is naturally resistant to tetracycline (*tetRA*) and kanamycin (*aphA*) and is referred to as "R995" in the

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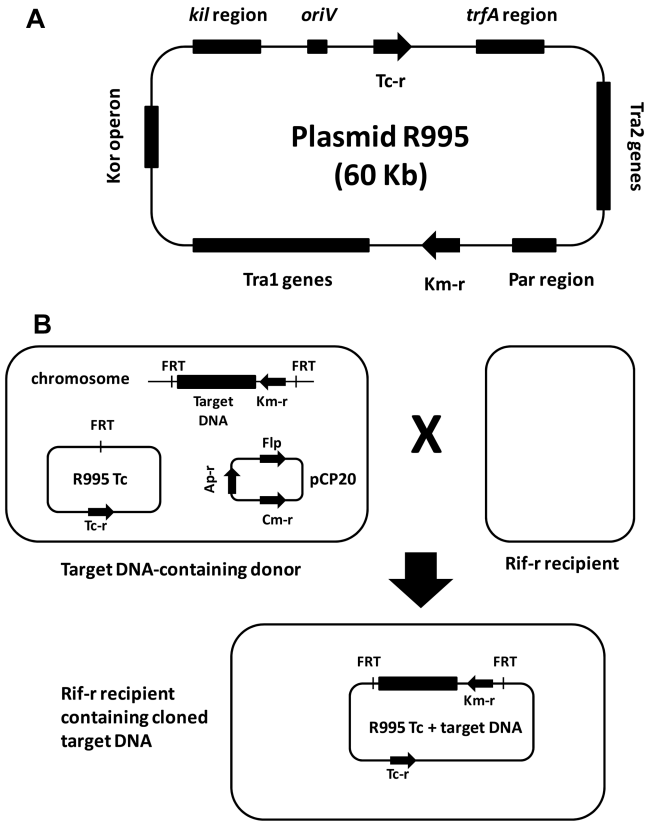

Fig. 1. Plasmid R995 and cloning strategy.
(**A**) Plasmid R995. A circular map of the self-transmissible, broad-host-range IncP plasmid R995 is shown. This plasmid is naturally Tc-r (*tetRA*) and Km-r (*aphA*). R995 derivatives constructed in this study are listed in Table 1. (**B**) Recombinase/conjugation strategy to clone bacterial genomic regions using R995. A targeted DNA region in a bacterial genome is flanked by FRT sites and an antibiotic resistance marker, as diagrammed, using lambda Red recombination. An R995 derivative containing an FRT site is transferred to this strain *via* conjugation, and then the Flp-expressing plasmid pCP20 is introduced *via* electroporation. The electroporation outgrowth culture can be used directly as a donor for conjugation with a Rif-r recipient strain. Alternatively, the electroporation can be plated on media containing Tc and Km, and the colonies can be used as donors. The conjugation is plated on media containing Rif, Tc, and Km to select recipients that have obtained the cloned target DNA on R995. The transconjugants can be used to confirm the clone and as a donor for transfer of the clone to other bacterial strains for subsequent studies.

text (Fig. 1A). The modified derivatives of R995 containing different marker combinations that deviate from the natural plasmid are noted with the markers in the plasmid name. For example, "R995 Km Cm" encodes resistance to both kanamycin and chloramphenicol. We first engineered plasmids in which either the Tc or Km markers were singly replaced with Tp, Cm, or Sp to produce plasmids R995 Tc Tp, R995 Tc Cm, R995 Tc Sp, R995 Km Tp, R995 Km Cm, and R995 Km Sp (Table 1). These plasmids were constructed using the lambda Red technique and PCR verification as described previously [4, 8]. Primer sequences used for this technique are indicated

Table 1. Strains and plasmids.

Strain/plasmid	Description	Reference
14028	*S.* Typhimurium	ATCC
TOP10	*E. coli*	Invitrogen
R995	IncP plasmid, Tc-r, Km-r	[2, 7]
R995 Tc Cm	Δ*aphA*::FRT-Cm-FRT	This study
R995 Tc Tp	Δ*aphA*::FRT-Tp-FRT	This study
R995 Tc Sp	Δ*aphA*::FRT-Sp-FRT	This study
R995 Km Cm	Δ*tetRA*::FRT-Cm-FRT	This study
R995 Km Tp	Δ*tetRA*::FRT-Tp-FRT	This study
R995 Km Sp	Δ*tetRA*::FRT-Sp-FRT	This study
R995 Tc	Δ*aphA*::FRT	This study
R995 Km	Δ*tetRA*::FRT	This study
R995 Cm	Δ*tetRA*::FRT, Δ*aphA*::Cm-r	This study
R995 Tp	Δ*tetRA*::FRT, Δ*aphA*::Tp-r	This study
R995 Sp	Δ*tetRA*::FRT, Δ*aphA*::Sp-r	This study
pKD3	PCR template for Cm-r	[4]
pJW101	PCR template for Tp-r	[8]
pJW102	PCR template for Sp-r	[8]
pCP20	Flp-expressing plasmid, Ap-r, Cm-r	[4]
pCE36	Km-r, *lacZY*, R6K *ori*-based suicide plasmid	[5]

in the GenBank submission for each construct (see below). Next, we constructed R995 derivatives containing just a single marker and single FRT site. To make plasmids R995 Tc and R995 Km, we used Flp recombinase to remove the Sp marker from plasmid R995 Tc Sp and the Cm marker from plasmid R995 Km Cm, respectively. To construct R995 Cm, R995 Tp, and R995 Sp, the Km gene in R995 Km was replaced with Cm, Tp, and Sp, respectively, such that the marker was not associated with FRT sites. Please see Table 1 for a list of all R995 derivatives constructed in this study. The DNA sequences of the modified regions of these plasmids can be obtained using the following GenBank accession numbers: JN243341 (R995 Tc Cm), JN243342 (R995 Tc Tp), JN243343 (R995 Tc Sp), JN243344 (R995 Km Cm), JN243340 (R995 Km Tp), JN243345 (R995 Km Sp), JN243346 (R995 Tc), JN243347 (R995 Km), JN243348 (R995 Cm), JN243349 (R995 Tp), JN243350 (R995 Sp).

Previous studies have utilized R995 to capture excised genomic fragments using homologous recombination [3, 11–15]. However, the R995 derivatives reported here can also be used to capture such fragments *via* Flp recombinase activity. To demonstrate the utility of Flp/FRT-based cloning using R995, we targeted two different 20 kb regions in the *S.* Typhimurium and *E. coli* genomes for cloning as shown in Fig. 1B. First, we inserted a single FRT site at the *S.* Typhimurium STM4315 and *E. coli ydcI* genes by using the Cm-r and Sp-r genes from pKD3 and pJW102, respectively, and then subsequent Flp/FRT-based marker removal. This procedure results in a single FRT

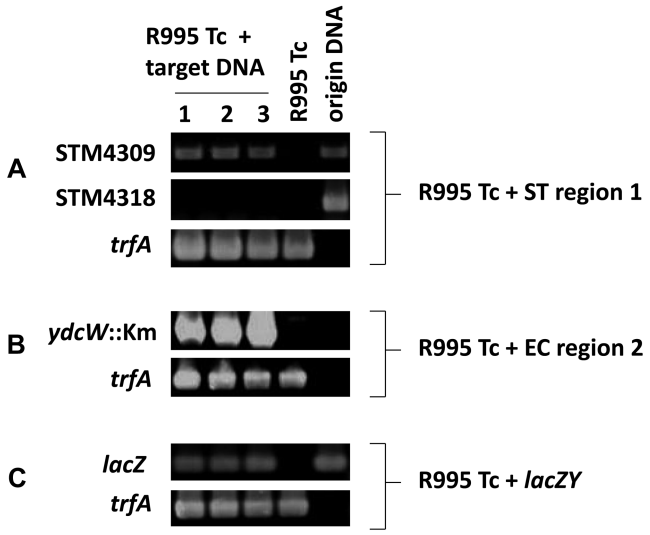

Fig. 2. Cloning results.
(**A**), (**B**) Genomic DNA clones. Regions of 20 kb target DNA from the *S.* Typhimurium and *E. coli* genomes were cloned into R995 Tc using the strategy in Fig. 1B. The regions corresponded to coordinates 4537678–4559787 (**A**, ST region 1) and 1492971–1513642 (**B**, EC region 2) for the *S.* Typhimurium and *E. coli* genomes, respectively. Primers for the indicated genes were used to verify the clones *via* PCR. STM4309 is found in the middle of the *S.* Typhimurium target DNA. STM4318 is located adjacent to the *S.* Typhimurium target DNA but outside of the FRT sites and is not predicted to be cloned on this fragment. The *trfA* gene is located on the R995 vector plasmid. The *ydcW*::Km product is obtained using primers that give a unique product only if the target region is correctly cloned onto R995 (and thus is not seen in the "R995 Tc" or "origin DNA" samples). Please note that additional different primer sets were also used to show correct cloning of both fragments into R995 Tc (data not shown). (**C**) Cloning of exogenous DNA introduced into an R995 Tc-pCP20-containing strain. An *E. coli* strain containing R995 Tc and pCP20 was electroporated with non-replicating DNA containing an FRT site, the *lacZY* genes, and a Km-r gene as described in the text. Primers hybridizing to the *lacZ* gene were used for PCR. Please note that other primer sets were used in addition to those shown to verify correct cloning of this DNA into R995 Tc (data not shown).

site located at the indicated gene position in each genome. Next, we inserted a Km-r marker associated with a single FRT site at the *S.* Typhimurium *adiY* and *E. coli ydcW* genes, both located 20 kb from the first FRT site insertions. We then conjugatively transferred R995 Tc into these strains and electroporated the Flp-expressing plasmid pCP20 into the R995 Tc-containing strains. After outgrowth of cells post-electroporation, we then set up a mating between the electroporated cells (which served as donor) and the *E. coli* recipient TOP10 Rif to isolate R995 Tc plasmids that had "captured" the excised circular molecule of targeted genomic DNA. Transconjugants from this mating were selected on LB Rif Tc Km. We found that either directly using the outgrowth culture in the mating or first plating the outgrowth on LB Km Tc and using those colonies for mating gave successful results. We isolated plasmid DNA from the transconjugants and confirmed the presence of inserted target genomic DNA using PCR analysis (Fig. 2).

In addition to cloning large excised genomic DNA targets, this approach can be used to clone FRT-containing DNA molecules that are transformed into an R995-containing strain expressing Flp. To demonstrate this approach, we cloned the *lacZY* genes onto R995 Tc by electroporating a non-replicating circular molecule encoding *lacZY* and Km-r (termed pCE36) into a strain containing both R995 Tc and pCP20. Cells were selected on LB Km media, and the R995 plasmid was conjugated to a fresh strain. The plasmid DNA was then isolated and verified *via* PCR for the *lacZY* insert (Fig. 2). Thus, two separate approaches can be used to clone FRT-containing DNA (chromosomal or plasmid) into R995 derivatives.

Plasmids that contain two FRT sites (which flank a given marker, as noted in Table 1) can also be used to clone DNA as described above by either single-step deletion/replacement of the marker or by retaining the marker *via* selection (data not shown). We also note that the original R995 plasmid (and thus all derivatives) used in this study contains a single *Xba*I site located in the *kilC* region that can be used for cloning inserts as described previously [2, 12, 14]. These inserts can be used for homologous recombination-based cloning protocols, such as the VEX-Capture technique, which allow other alternatives for cloning large genomic segments [12, 14]. Finally, we emphasize the underdeveloped potential to use the recombinase/conjugation approach for cloning large regions from bacterial genomes as a means to obtain several genes (that may function together) on a single fragment that is easily isolated and transferred for downstream applications.

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