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A Series of Vectors with Alternative Antibiotic Resistance Markers for Use in Lambda Red Recombination

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A target bacterial strain of interest for use in Red-based recombineering may already encode resistance to antibiotic markers used with current Red recombination tools, such that the resistance cannot be removed. Such cases include those where markers are needed to maintain an unstable genetic element co-resident in the strain or those where the genetic source of resistance is not known. We report the availability of PCR templates with FRT-flanked mutagenesis cassettes and plasmids encoding Red recombination functions that contain marker combinations not currently available on widely disseminated lambda Red molecular reagents. The functionality of these convenient alternative tools is demonstrated.

Keywords: Lambda Red recombination, recombineering, trimethoprim, spectinomycin, streptomycin

The lambda Red recombination system has become a popular and convenient approach for creating mutations in bacteria [3, 8, 10, 11]. The method of Datsenko and Wanner [3] is particularly widely regarded, and their molecular reagents have become disseminated among molecular biologists. The system functions by inserting a PCR product, engineered to contain 30–50 bp extensions homologous to a DNA target, into a bacterial genome. Two popular plasmid templates used to create the PCR products are pKD3 and pKD4, which allow the amplification of PCR products containing chloramphenicol and kanamycin resistance, respectively, using primer sequences termed P1 and P2 that flank the markers [3]. To decrease background issues, these plasmids are suicide plasmids and do not replicate unless provided *trans*-acting replication proteins in specific bacterial strains. The PCR products also contain FRT sites flanking the resistance marker such that the marker can be deleted using the FLP recombinase after the insertion has been established. The lambda Red recombination proteins Gamma, Beta, and Exo are expressed from a plasmid resident in the target bacterial strain to facilitate the initial insertion of the PCR product. The ampicillin-resistant, temperature-sensitive plasmid pKD46 is widely used to express the lambda Red recombination proteins from an arabinose-inducible promoter to facilitate recombination and then self-curing after the target mutation is established [3]. This system has been adapted for use in a range of Gram-negative bacterial genera including *Escherichia* [3, 11], *Salmonella* [10], *Yersinia* [4, 9], *Shigella* [2], *Serratia* [7], *Klebsiella* [5], and *Pseudomonas* [6].

Certain situations exist where antibiotic resistance encoded by current lambda Red reagents is either intrinsically present in a target strain from an unknown source (which creates background) or is required to be present in the target strain background to maintain other co-resident genetic elements (which may be unstable). In these cases, the antibiotic resistance cannot be removed and thus prevents use of those markers if they are encoded by lambda Red recombination tools of choice or other reagents used in recombineering approaches. Convenient recombineering tools encoding alternative antibiotic resistance would expand options to researchers designing experiments using strains with other markers already present in the background. Here, we report the availability of plasmid templates that can be used to produce PCR products encoding resistance to trimethoprim or spectinomycin/streptomycin for use in Red-catalyzed recombineering. In addition, we have constructed a series of plasmids expressing Red recombination functions that encode resistance to kanamycin, chloramphenicol, trimethoprim, or spectinomycin/streptomycin. The functionality of these plasmids in their corresponding roles in lambda Red recombination is demonstrated here using both plasmid and chromosomal target genes. To our knowledge, these are the first reported Red suicide plasmid PCR templates to encode trimethoprim resistance and spectinomycin/streptomycin resistance (the latter from a single marker gene). In addition, to our knowledge, these are the first

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reported Red function helper plasmids encoding this range of antibiotic markers using a temperature-sensitive pSC101 replicon and arabinose-inducible Red functions. These vectors provide convenient novel options in cases where current Red reagents are unable to be used.

Plasmid pJW101 was constructed by PCR-amplifying the dihydrofolate reductase gene (encoding resistance to trimethoprim) from the transposon EZ-Tn5-DHFR (Epicentre, Madison, WI, U.S.A.) with 40-bp homology extensions such that it inserted into the Cm-r gene of plasmid pKD3 in the presence of pKD46. Plasmid pJW102 was similarly constructed using the PCR-amplified Sp/Sm-r gene from plasmid pVEX1212 [1]. Both pJW101 and pJW102 are chloramphenicol sensitive with complete deletions of the Cm-r gene (including promoter) and replacement with the markers encoding resistance to trimethoprim and spectinomycin (and streptomycin), respectively (Fig. 1A). Primers P1 and P2 can be used to produce engineered PCR products containing FRT sites flanking the marker genes (just as with pKD3). We tested the utility of these plasmid templates by amplifying PCR products with 40-bp extensions containing homology to the *bla* gene of pKD46 from pKD3, pKD4, pJW101, and pJW102. This gives a convenient direct comparison of the ability of these different PCR products to serve as recombination substrates for lambda Red functions by inserting the gene directly into pKD46 (since many more transformants arise with this protocol compared with chromosomal insertion). The number of *E. coli* transformants resistant to the corresponding antibiotic after introduction of the PCR product (representing integration into pKD46) was comparable for all the plasmid templates (Table 1, first section). Plasmid DNA was isolated from representative transformants for each template, and the correct integration structure was verified for each using PCR analysis and DNA sequencing (Table 1 and data not shown). The results indicate that PCR products obtained from pJW101 and pJW102 behave similarly to those obtained from pKD3 and pKD4. Chromosomal gene integrants in *S. typhimurium* and *E. coli* were also obtained using PCR products from pJW101 and pJW102 (Table 1, second section). These isolates were used to verify that the markers produced from these plasmids could be successfully removed using the Flp recombinase expressed from plasmid pCP20 (Table 1, second section).

The sizes of the PCR products obtained from pJW101 and pJW102 with primers engineered using P1 and P2 (and 40-bp extentions) are 1,056 and 2,084 bp, respectively. Concentrations of antibiotics used to select for integrants after electroporation of PCR products into bacterial cells were 100 μg/ml for trimethoprim and 250 μg/ml for spectinomycin. The copy numbers of pJW101 and pJW102 were comparable to that of pKD3 using semiquantitative agarose gel analysis of plasmid DNA isolated using the Qiagen Spin Mini prep kit (Qiagen, Valencia, CA, U.S.A.)

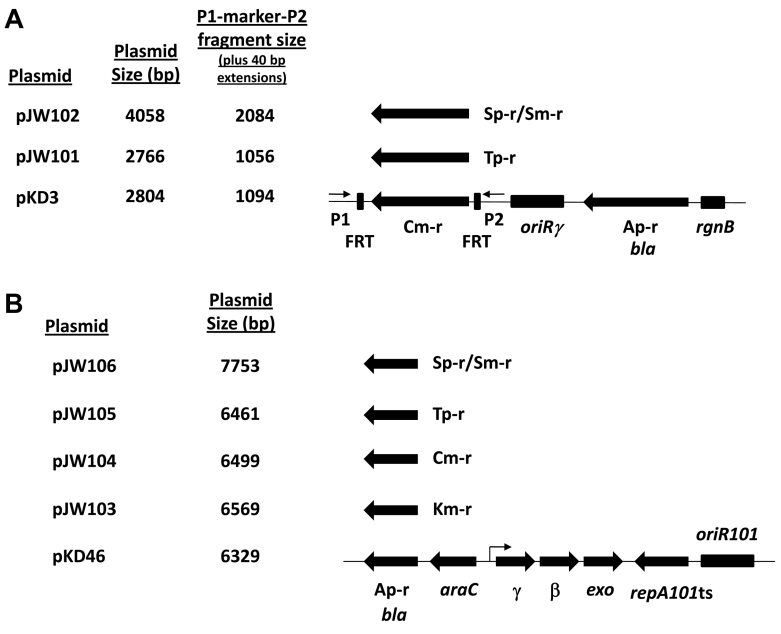

Fig. 1. Maps of plasmids constructed in this study.
A. The Cm-r gene of pKD3 was deleted and replaced with a Tp-r gene and a Sp-r/Sm-r gene to create plasmids pJW101 and pJW102, respectively. Tp-r and Sp-r/Sm-r cassettes can be PCR-amplified from these templates using primer sequences P1 and P2 (from reference [3]). These cassettes contain FRT sites for subsequent deletion of the antibiotic resistance markers after genomic insertion. The rgnB region indicates a transcriptional terminator. The sizes of the plasmids and the corresponding P1–P2 PCR products are provided (maps not drawn to scale). **B**. The *bla* gene of plasmid pKD46 was deleted and replaced with Km-r, Cm-r, Tp-r, and Sp-r/Sm-r genes to create plasmids pJW103, pJW104, pJW105, and pJW106, respectively. The sizes of the resulting plasmids are provided (maps not drawn to scale).

Table 1. Utilization of vectors reported in this study.

Source of marker	Marker tested	# Integrants	% Correct integration (of 4 screened)	Targeted gene
pKD4	Km	5680	100	*bla E. coli*
pKD3	Cm	5536	100	*bla E. coli*
pJW101	Tp	3540	100	*bla E. coli*
pJW102	Sp	4320	100	*bla E. coli*
Chromosomal marker tested	# Colonies screened	# Resistance marker deleted	% Deleted	Chromosomal gene mutation tested
Km (from pKD4)	50	50	100	STM0606 *S. typhimurium*
Cm (from pKD3)	50	50	100	STM0459 *S. typhimurium*
Tp (from pJW101)	50	50	100	STM1414 *S. typhimurium*
Sp (from pJW102)	50	50	100	b1422 *E. coli*
Plasmid tested	# Integrants screened	% Correct integration	Targeted gene	
pKD46	4	100	STM4315 *S. typhimurium*	
pJW103	4	100	STM0459 *S. typhimurium*	
pJW104	4	100	STM1625 *S. typhimurium*	
pJW105	4	100	STM0859 *S. typhimurium*	
pJW106	4	100	STM0859 *S. typhimurium*	

(data not shown). All plasmid and chromosomal integrations were performed in the *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA, U.S.A.) or *S. typhimurium* strain χ3477 (generously donated by Roy Curtiss III) as indicated.

The *bla* gene on plasmid pKD46 was used as a target for insertion of markers amplified from pKD4, pKD3, pJW101, and pJW102 in such a way that deletion of the entire *bla* gene (including promoter) and replacement with the corresponding marker was obtained (Fig. 1B). This produced the ampicillin-sensitive plasmids pJW103, pJW104, pJW105, and pJW106, respectively. To test the function of these plasmids, each was used to facilitate successful insertion of engineered PCR products into genes of the *S. typhimurium* genome (Table 1, third section). The correct structure of these insertions was verified using PCR analysis of chromosomal DNA isolated from representative transformants for each experiment indicating correct targeting catalyzed by these vectors (Table 1, third section and data not shown). Temperature sensitivity of each plasmid was verified, and the copy numbers of the plasmids were found to be comparable to that of pKD46 (data not shown). The sizes of pJW103, pJW104, pJW105, and pJW106 are 6,569, 6,499, 6,461, and 7,753 bp, respectively (Fig. 1B).

Nucleotide sequences of new gene constructs are deposited using GenBank accession numbers GU589571 (pJW101), GU589572 (pJW102), GU589573 (pJW103), GU589574 (pJW104), GU589575 (pJW105), and GU589576 (pJW106). Nucleotide sequences of primers used to obtain the engineered PCR products for targeted mutations described in this study are as follows: (i) to insert the Tp-r gene into pKD3, aagatcacttcgcagaataaataaatcctggtgtccctgtctgtctcttatacacatctcaacc (tpkd35) and cactcatcgcagtactgttgtattcattaagcatctgccggaca ggtttgcgaatccgttgc (tp3kd3); (ii) to insert the Sp-r/Sm-r gene into pKD3, aagatcacttcgcagaataaataaatcctggtgtccctgtgacgt cccatggccattcgaattc (kd3sp5) and cactcatcgcagtactgttgtattc attaagcatctgccgggcgtcggcttgaacgaattg (3spkd3); (iii) to insert the pKD4 Km-r gene into pKD46 *bla*, tacattcaaatatgtatccg ctcatgagacaataaccctgctactgggctatctggacaaggg (5blakm) and taatcagtgaggcacctatctcagcgatctgtctatttcggcgtcgcttggtcggtca tttcg (3kmbla2); (iv) to insert the pKD3 Cm-r gene into pKD46 *bla*, tacattcaaatatgtatccgctcatgagacaataaccctgcatat gaatatcctccttagttcc (5cmbla) and taatcagtgaggcacctatctcagc gatctgtctatttcggtgtaggctggagctgcttc (3blacm); and (v) to insert the pJW101 Tp-r gene and pJW102 Sp-r/Sm-r gene into pKD46 *bla*, we used the 5cmbla and 3blacm primers since they use the same P1 and P2 homology for template hybridization.

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