Constructing Lambda Red-Swaps in *Salmonella*

LINEAR RECOMBINATION

Homologous recombination is very efficient in many organisms. For example, short PCR products have been used extensively to generate gene replacements in the yeast *Saccharomyces cerevisiae* (reviewed in [Que and Winzeler 2002; http://www.openbiosystems.com/productPage.php?pageType=yeast.knockout&q=0]). Very short regions of homology (from 25 to 50 bp) are sufficient for efficient recombination in this system, with nearly 95% of the transformed cells carrying the desired gene replacement. In contrast, short linear DNA fragments are poor substrates for recombination in many bacteria.

Efficient recombination between short, linear DNA fragments and the bacterial chromosome can be catalyzed by taking advantage of phage recombination systems (reviewed in [Court et al. 2002]). The phage λ Red locus encodes a system that promotes homologous recombination. The λ Red locus includes three genes: *bet* (aka ß), *exo*, and *gam* (aka γ) [Datsenko and Wanner 2000; Murphy1998; Yu et al. 2000; Poteete and Fenton 2000; Kuzminov 1999]. Exo is a 5'-3' exonuclease that degrades the 5' ends of linear DNA molecules. Bet is a single-stranded DNA binding protein that binds to the single stranded 3' ends generated by Exo and promotes annealing to complementary DNA [Kuzminov 1999; Murphy1998; Yu 2000; Poteete and Fenton 2000]. Gam binds to the host RecBCD complex and inhibits its exonuclease activity [Murphy 1991; Murphy 1998; Karu 1975].

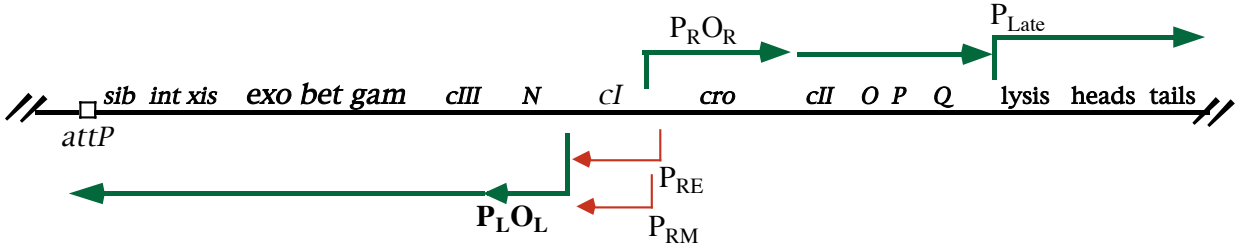

Figure 1. Organization of the phage λ Red genes.

Use of the λ Red system to promote gene replacement in *E. coli* using linear DNA molecules as the substrate for recombination was first described by Murphy [Murphy 1998; Murphy et al. 2000]. In the original system the Red functions were provided from a plasmid or from the *E.coli* chromosome. In the plasmid construct the Gam gene was included to inactivate host RecBCD exonuclease. In the chromosomal construct, the host *recBCD* genes were replaced with the λ Red genes, so Gam was not necessary. The recombination efficiency was tested using restriction fragments or PCR products with long regions of homology (>1Kb). Gene replacement occurred at a high frequency in the presence of the Red genes in both constructs, but the frequency of recombination was higher when the Red functions were expressed from the chromosome. The frequency of recombination with the λ Red system was higher than the frequencies obtained with *recBC sbcBC*, *recBC sbcA*, or *recD* hosts, which have been widely used to promote homologous

recombination with linear DNA molecules in *E. coli* [Biek and Cohen 1986; Jasin and Schimmel 1984; Russell et al. 1989; Winans et al.1985].

An analogous system was developed that used the recombination functions of the cryptic lamboid prophage Rac (RecET recombinase) to disrupt plasmid genes or to generate chromosomal deletions, but this system also required long regions of homology (>138 bp) [Zhang et al. 1998]. The Rac system is the platform for a broad patent on this approach (see [http://container.pharmalicensing.com/licensing/displicopp/1781]).

The evidence for λ Red-promoted gene replacement with PCR products prompted the modification of this approach to facilitate recombination with shorter DNA fragments [Datsenko and Wanner 2000; Yu et al. 2000]. Although Murphy provided the first evidence for PCR-generated gene replacements in *E. coli*, recombination with PCR products containing short regions of homology was unsuccessful. Two independent methods, which take advantage of λRed-promoted recombination, have been recently developed to promote gene disruptions with PCR products containing very short regions of homology (30 to 50 bp). An exchange made by any of these methods is generically described as a "Red-swap". A figure showing the basic steps of the procedure is shown below.

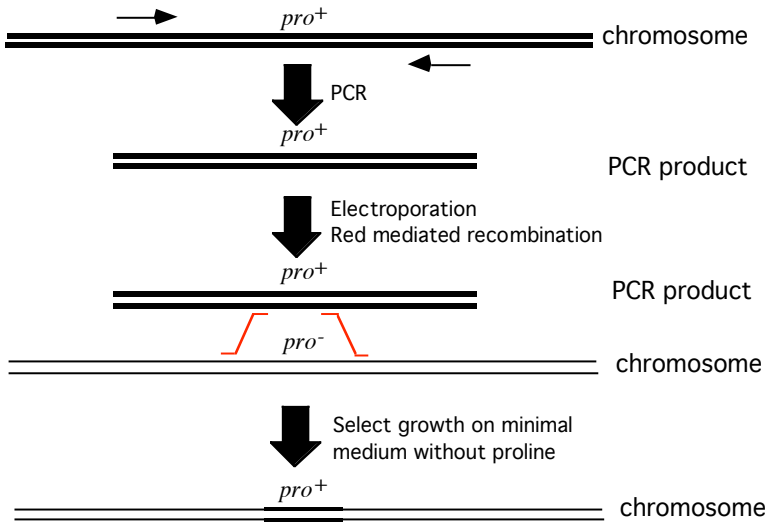

Figure 2. The basic red-swap protocol.

The method developed by Court and colleagues [Yu et al. 2000; Ellis et al. 2001] uses a defective prophage, which expresses the *bet*, *gam*, and *exo* genes from λP_L under the control of the *cI857* (Ts) repressor. In this system, expression of the Red genes is induced by shifting the temperature to 42˚C for approximately 7 to 20 min. The high level expression of the Red genes from P_L causes cell death within 60 min. The frequency of recombination becomes saturated at DNA concentrations of about 300 molecules per cell. Under optimum conditions, recombination efficiencies up to 0.1% of surviving cells have been reported.

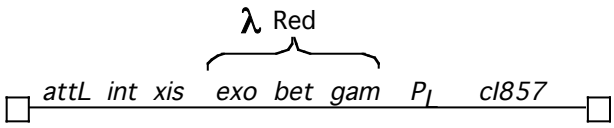

Figure 3. The Court approach. Red genes are expressed from a defective λ phage under control of the temperature sensitive cI857 repressor.

An alternative method developed by Wanner and colleagues [Datsenko and Wanner 2000], expresses the λ Red genes under the control of an arabinose-inducible promoter from a temperature-sensitive (Ts), low copy number plasmid. Expression of the Red functions from the P_{BAD} promoter facilitates tight regulation with induction by the addition of arabinose.

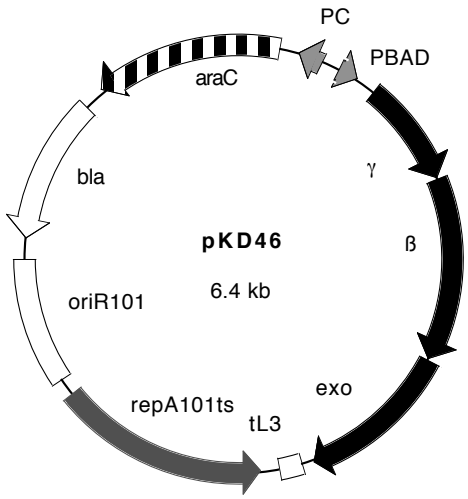

Figure 4. The Wanner approach. Red genes are expressed from the plasmid vector pKD46 under the control of the arabinose P_{BAD} promoter.

The linear DNA fragments used for recombination can be generated by a variety of means, including purified restriction fragments, PCR products, or oligonucleotides. The fragment should include 20-50 bp of homology at each end. The effect of homology is shown if the following figure.

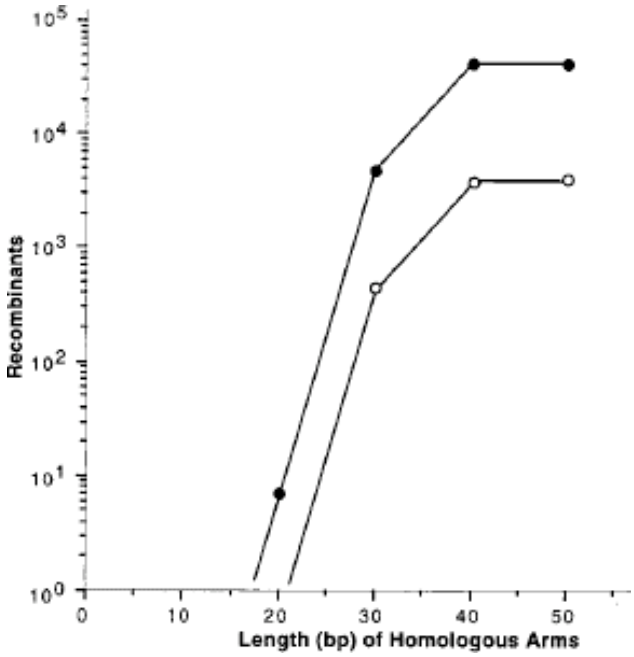

Figure 4. The effect of homology on recombination frequency mediated by the Red. (From [Yu et al. 2001]). Closed circles indicate results in a *recA*$^+$ host and open circles indicate results in a *recA* mutant host.

Although the λ Red enzymes allow recombination of short linear DNA fragments, the frequency is sufficiently low that a selection is needed to obtain the desired recombinants. In some cases it is possible to select for loss of a chromosomal phenotype (e.g. an auxotrophic requirement or a counter-selectable marker such as *sacB* or *tetA*) [Gomez-Curet 2001]. Alternatively, it is possible to select for the desired recombinants by including a dominant selectable marker on the linear DNA (e.g. an antibiotic resistance marker) . Plasmid templates have been constructed for the production of PCR fragments flanking a selectable antibiotic resistance marker. Three π-dependent suicide plasmids (pKD3, pKD4, and pKD13) which lack homology to the *E. coli* chromosome, were constructed to use as templates for PCR amplifications that generate a selective marker flanked by short regions of homology (36 to 50 bp) to the target gene. The presence of a conditional replication origin, reduces the background from resistant colonies carrying aberrant-PCR products that are homologous to plasmid sequences [Datsenko and Wanner 2000]. FRT sites flanking the antibiotic resistance markers facilitate excision of the selective marker by expressing FLP recombinase from an easily curable plasmid. Removal of the markers by FLP-mediated recombination generates identical "scars" in pKD3- and pKD4-generated gene disruptions. These scars generate stop codons in all 6 reading frames. In addition they contain a ribosome-binding site and start codon facilitating the creation of non-polar gene disruptions. pKD13 do not contain translation signals and can be primarily used for single gene or operon disruptions. However, the scars provide homology for subsequent rounds of recombination using templates from these plasmid vectors.

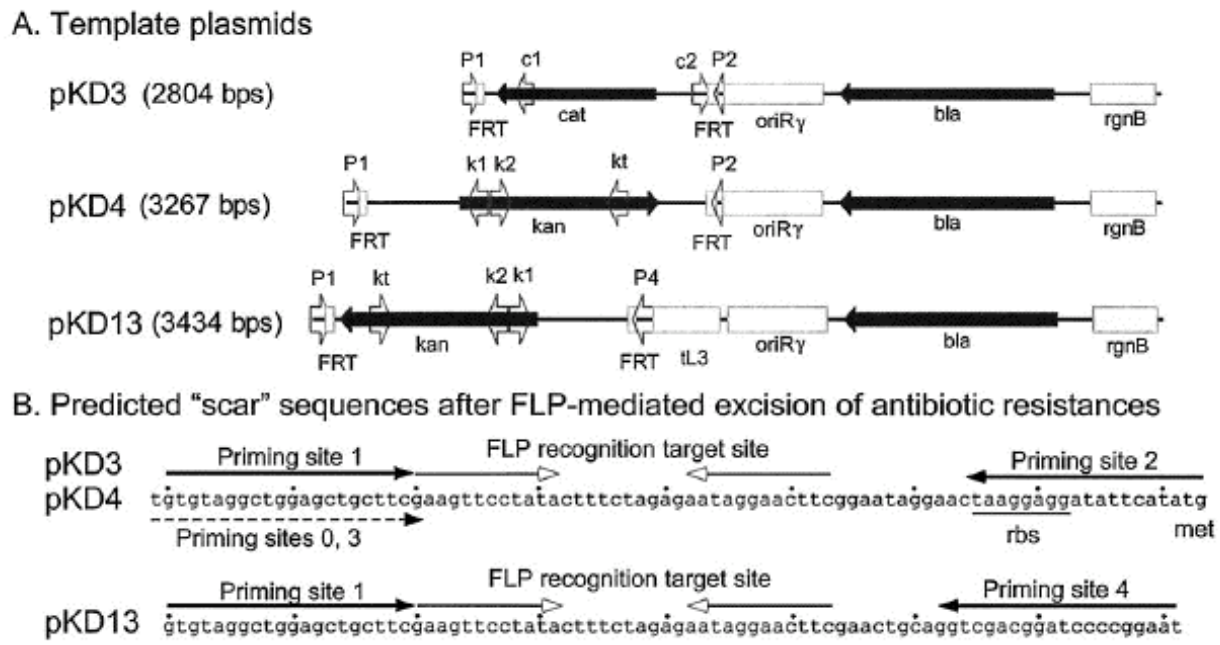

Figure 5. Plasmids designed for generated antibiotic resistance mutations. The backbone for these plasmids is related to the π-dependant plasmid pGP704 [http://www.salmonella.org/vectors/pgp704/]. Linear representations of the template plasmids are shown in (A). Arrowheads indicate locations and orientations of priming sites. P1: priming sites 0, 1, and 3; P2: priming site 2; P4: priming site 4. c1, c2, k1, k2, and kt: common test primers. Sequences remaining after FLP-mediated excision of the antibiotic resistance genes are shown in (B). Priming sites 0, 1, and 3 begin at nucleotides 1, 2, and 3, respectively. Priming sites 2 and 4 begin at the left or right ends, as shown. Arrows with open arrowheads show the nearly perfect FRT site inverted repeats. The ribosome binding site (rbs) and methionine (met) start codon are marked. (From [Datsenko and Wanner 2001])

Because the Red genes may promote undesired events, it is important to remove these genes from the cell once the desired recombinants have been obtained. It is possible to eliminate the λ vector carrying the Red genes in the Court system by simply replacing the prophage with a small PCR fragment flanking the att site. The use of a temperature sensitive plasmid replicon in the Wanner system allows for easy elimination of the Red functions by growing the transformants at 42˚C in the absence of selection. However, whenever possible it is a good idea to backcross the mutations into a clean strain. When the recombinants encode a selectable phenotype, this can be done by directly growing transducing phage on the recombinants and using the phage to transduce a recipient selecting for the desired phenoytpe .

Although the λ Red system has been most widely used in *E. coli* and *Salmonella*, it works in other bacteria and eukaryotic cells as well [Copeland et al. 2001; Sergueev et al. 2001]. It seems likely that limiting caveat is that the Red genes must be appropriately expressed in the recipient cell.

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Primer Design for Red-Swap Experiments

For optimal recombination using double-stranded templates, we typically design primers that hybridize to the complementary strands with 35 bp of perfect homology with the target sequence and 25 bp of perfect homology with the antibiotic resistance locus. These primers are used to amplify the antibiotic resistance locus via PCR prior to use for homologous recombination.

For single-stranded recombination, we typically use 70- to 100-mers with at least 35 bp of perfect homology to the each side of the target sequence.

It is useful to blast the primer sequences against the complete genome sequence to determine whether there is substantial homology to alternative sites.

Red-swap Protocol

1. Start an overnight of LT2 / pKD46 in 2 ml of LB + Amp. Incubate approximately 8 hr.
2. Subculture the o/n in fresh LB Amp + 10-50 mM arabinose at a 1:100 dilution (i.e. 250 ul of overnight culture in 25 ml fresh media). Grow to an OD of ~0.4-0.5. (typically 4 hr for Typhimurium).
3. Prepare cell culture for electroporation (as per attached electrocompetent cell protocol
4. Electroporate PCR product (previously prepared) into cells (as per attached protocol
5. Recover electroporated cells for 1-2 hr in rich medium at 30°C.
6. Remove cells from incubator and spin down in 1.5 ml microfuge tube, 5 min at maximum speed.
7. Decant the supernatant and resusspend the pellet in 500 ul of LB broth.
8. Plate out 100 ul of cell solution on selective media (with appropriate antibiotic).
9. Incubate selection plates overnight at 37°C.
10. Replica print to screen colonies for auxotrophies.
11. Perform colony PCR as final screen (as per colony PCR protocol).
12. Load and run PCR products onto a 1.0-1.5% agarose gel .
13. Analyze results.

Colony PCR Protocol

1. Using a yellow-tip with end sealed in a flame, resuspend a single colony in 10 µl PBS (phosphate buffered saline).
2. "Boil" on PCR machine at 99° for 5 min.
3. Remove 8 µl of PBS/cell solution.
4. Add the following rreaction mixture to the 2 µl remaining

2.0 µl	cell/PBS soln
5.0 µl	MgCl2 (25 mM)
5.0 µl	Taq buffer
0.8 µl	dNTP (100mM)
2.0 µl	Primer #1 (50 µM)
2.0 µl	Primer #2 (50 µM)
1.0 µl	Taq Polymerase
32.2 µl	dH_2O
50.0 µl	Total volume

[Addition of 0.5 µl BSA (10 mg/ml) sometimes improves the yield]

5. Run PCR on the sample as follows (optimal conditions will vary depending upon the primers used):

1 Cycle	30 Cycles			1 Cycle	Hold
95°C	95°C	60°C	68°C	72°C	4°C
1 min	15 sec	15 sec	2 min*	7 min	