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ORIGINAL PAPER

A method for generating precise gene deletions and insertions in *Escherichia coli*

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Abstract A simple and general method for disrupting chromosomal genes and introducing insertions is described. This procedure involves eliminating wild-type bacterial genes and introducing mutant alleles or other insertions at the original locus of the wild-type gene. To demonstrate the utility of this approach, the *tig* gene of *Escherichia coli* was replaced by homologous recombination with a cassette containing the chloramphenicol resistance gene and the *sacB* gene. The cassette was then removed and the *tig* mutant alleles were moved into the native *tig* location. Sequencing and Western blotting results demonstrated that insertions or deletions can be introduced precisely in *E. coli* using our approach. Our system does not require extra in vitro manipulations such as restriction digestion or ligation, and does not require use of specific plasmids or strains which are used to prevent false positive transformants caused by template plasmid transformation. This technique can be used widely in bacterial genome analysis.

Keywords λ-Red recombineering system · pKD3S · Counterselection · Counter-selectable marker

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Introduction

Homologous DNA recombination systems are a basic analytical tool used in functional analysis of bacterial genes. A large number of methods such as the RecBCD-deficient system (Dabert and Smith 1997; El Karoui et al. 1999), RecA-mediated recombination (Imam et al. 2000; Lalioti and Heath 2001) and suicide vectors (Hamilton et al. 1989; Posfai et al. 1999) have been used to generate recombinant DNA constructs in vivo. However, these recombination methods require very long flanking regions of linear homologous DNA or construction of temperature-sensitive plasmids to carry homologous sequences, and thus require extensive in vitro manipulations before recombination. Creating specific changes in chromosomes is thus time-consuming, and the frequency of recombinants is low (Court et al. 2002).

In contrast to the above techniques, bacteriophage-encoded recombination systems, for example, the rac-encoded RecET system (Muyrers et al. 1999; Zhang et al. 1998, 2000) and the bacteriophage λ-Red recombination system (Datsenko and Wanner 2000; Yu et al. 2000), provide some advantages. These methods do not rely on in vitro manipulations using restriction enzymes or DNA ligases, and can efficiently recombine sequences with homologies as short as 36–50 base pairs. The Red system is similar to the RecET system, but has been shown to be 50–100 times more efficient in *Escherichia coli* (Zhang and Huang 2003).

Datsenko and Wanner set up a simple and highly efficient Red system which allows the replacement of a chromosomal

sequence with a selectable antibiotic resistance gene that is generated by PCR using primers with 36-nt homology extensions, and successfully constructed 13 different disruptions of chromosomal genes (Datsenko and Wanner 2000). In a further study, 3,985 in-frame deletions mutants were obtained using this system (Baba et al. 2006). This system has not only been used in the systematic construction of single gene knockout mutants in *E. coli* (Baba et al. 2006; Datsenko and Wanner 2000; Kao et al. 2005), but has also been successfully applied in other bacteria such as *Salmonella* and *Yersinia* (Doublet et al. 2008; Ellermeier et al. 2002; Sun et al. 2008; Uzzau et al. 2001). However, Flippase Recognition Target (FRT) sites are introduced into genomic DNA after recombination using this system. A scar containing an FRT site is left even if the resistance gene is eliminated by using a helper plasmid expressing FLP (Flippase recombination enzyme). These scars could be problematic under certain conditions and chromosomal rearrangements might result from FLP-promoted recombination events between FRT sites at different loci (Datsenko and Wanner 2000).

The *sacB* gene encodes a levansucrase that transfers fructosyl residues from sucrose to different cellular acceptors. Since expression of SacB is lethal in a wide range of Gram negative bacteria (Quandt and Hynes 1993), it has been used widely in gene replacement (Lalioti and Heath 2001; Link et al. 1997; Oswald et al. 1999). Here, in order to extend the use of Datsenko and Wanner's Red system, we used the *sacB* gene of *Bacillus subtilis* to reconstruct a template plasmid which contained both positive- and counter-selectable markers.

To verify the effectiveness of this modified system, mutant alleles were introduced to replace the native *tig* gene which encodes trigger factor (TF) in *E. coli*. TF consists of 432 amino acids, and limited proteolysis has shown that TF contains three distinct domains from N-terminal to C-terminal, in which the N-domain (N, aa 1–145) is essential for ribosome binding, the M-domain (M, aa 146–251) displays PPIase activity and the C-domain (C, aa 252–432) plays an important role in TF chaperone activity (Baram et al. 2005; Hesterkamp et al. 1997; Merz et al. 2006; Stoller et al. 1996; Zarnt et al. 1997). By using positive and counter selection, we have made scarless mutations of the *tig* gene including point mutations, domain truncation mutations and clean deletions in *E. coli*.

Materials and methods

Bacterial strains and plasmids

E. coli strain BW25113 harbors plasmid pKD46 which expresses λ-recombination proteins γ, β and *exo* under the control of the arabinose-inducible P_{araB} promoter. The strain BW25141 harbors plasmid pKD3 which contains a chloramphenicol-resistant (*cat*) gene (Table 1).

Plasmids pRSETa-NM, pRSETa-NC, and pRSETa-MC which express different TF domain truncation mutants, and plasmid pQE60-I195G which contains a point mutation (isoleucine at position 195 is changed into glycine) have been described previously (Fan et al. 2008, 2009; Liu and Zhou 2004).

Amplification primers

The names of PCR primers, their sequences and functions are listed in Table 2.

Plasmid construction

The primers sacB3 and sacB4 were used to amplify the *sacB* gene from *B. subtilis* and the PCR product was digested by *Bsp*T104I. The resultant fragment was ligated into plasmid pKD3 which had been digested by *Bsp*T104I and dephosphorylated by CIAP. The ligation mixture was transformed into BW25141 according to standard procedures using $CaCl_2$.

Preparation of electrocompetent cells and generation of recombinants

BW25113 cells harboring plasmid pKD46 were cultured at 30°C in LB medium supplied with 100 μg ml^{-1} ampicillin until OD_{600} was 0.2. L-arabinose was added to a final concentration of 0.1 M and cultures were then grown to an $OD_{600} = 0.6$. After chilling on ice for 15 min, cells were centrifuged for 5 min at 4000*g* (4°C), washed three times with ice-cold 10% glycerol and then resuspended in 10% glycerol.

The BW25113 strain was transformed by electroporation with the PCR product generated using primers K-tigA and K-tigB. cat^+ strain clones were screened on plates of LB containing chloramphenicol and then characterized by PCR with a pair of primers (Gtig1 and Gtig2) which are located upstream and downstream of the *tig* gene. The resultant strain was confirmed by sequencing and designated as Z416.

Electrocompetent Z416 cells were prepared as described above and were then transformed with products amplified using the primers K-Nn/K-Mc (template: pRSETa-NM), K-Nn/K-Cc (template: pRSETa-NC), K-Mn/K-Cc (template: pRSETa-MC), K-Nn/K-Mc (template: pQE60-I195G), or K-tign/K-tigc (template: the annealing and extension product from tign and tigc). Sucrose-resistant strains were screened on LB containing 10% sucrose and characterized using PCR with the primers Gtig1 and Gtig2.

Table 1 Bacterial strains and plasmids

Strains or plasmids	Relevant genotype or characteristics	Reference
Strains		
BW25113	*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568*	Datsenko and Wanner (2000)
BW25141	*lacI*^{q} *rrnB3* Δ*lacZ4787* Δ*phoBR580 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 galU95* Δ*endA9 uidA*(*DMluI*)::*pir*(*wt*) *recA1*	Datsenko and Wanner (2000)
Z416	BW25113[Δ*tig*::(*sacB-cat*)]	This study
Z500	BW25113[Δ*tig*]	This study
M09-NM	BW25113[*tig*$\Delta_{252–432}$]	This study
M09-NC	BW25113[*tig*$\Delta_{146–251}$]	This study
M09-MC	BW25113[*tig*$\Delta_{1–145}$]	This study
M09-I195G	BW25113[*tig*-I195G]	This study
Plasmids		
pKD46	*araBp-gam-bet-exo*, *bla*(ApR), *repA101*(ts), *oriR101*	Datsenko and Wanner (2000)
pKD3	*oriR6K*$_\gamma$, *bla*(ApR), *cat*, *rgnB*(Ter)	Datsenko and Wanner (2000)
pKD3S	*oriR6K*$_\gamma$, *bla*(ApR), *cat*, *sacB*, *rgnB*(Ter)	This study
pKD3V	*oriR6K*$_\gamma$, *bla*(ApR), *cat*, *sacB*, *rgnB*(Ter)	This study
pQE60-I195G	*ColE1* PT5 *lacO* Amp^r	Liu and Zhou (2004)
pRSETa-NM	*ColE1* PT7 Amp^r	Fan et al. (2008)
pRSETa-NC	*ColE1* PT7 Amp^r	Fan et al. (2009)
pRSETa-MC	*ColE1* PT7 Amp^r	Fan et al. (2008)

Table 2 Oligonucleotide primers used in this study

Primer name	Sequence[a]	Function
Gtig1	acaccgtctttgcctctcct	Located upstream and downstream of the *tig* gene
Gtig2	atctcgttcgccgctgtat	Used to confirm *tig* mutants
sacB3	cgTTCGAAcctgccgttcactattatt (*Bsp*T104I)	Used to amplify the *sacB* gene from *Bacillus subtilis*
sacB4	gcTTCGAAaatgccaataggatatc (*Bsp*T104I)	
K-tigA	taagagttgaccgagcactgtgatttttttgaggtaacaagtgtaggctggagctgcttcg	The template used was pKD3S. The PCR product contained the *sacB-cat* cassette and the flanking regions of *tig*
K-tigB	ggcctttgtgcgaatttagcgcgttatgctgcgtaaattacatatgaatatcctccttag	
K-Nn	taagagttgaccgagcactgtgatttttttgaggtaacaagatgcaagtttcagttgaaacc	The templates used were pRSETa-NM, pRSETa-NC, pRSETa-MC and pQE60-I195G. PCR products were used for generating *tig* gene domain truncation mutations and the point mutation
K-Mn	taagagttgaccgagcactgtgatttttttgaggtaacaagatgaaacagcaggcgacctgg	
K-Mc	ggcctttgtgcgaatttagcgcgttatgctgcgtaaattattctgcagtcagttccgg	
K-Cc	ggcctttgtgcgaatttagcgcgttatgctgcgtaaattacgcctgctggttcatc	
tign	caacaacctgtgcttgcggggtaagagttgaccgagcactgtgatttttttgaggtaacaagtttacgcagc	tign and tigc overlap. Their annealing and extension product was used as the template for K-tign and K-tigc. The final product was used for generating the *tig* gene complete deletion
tigc	aaaagcccaccacctggcggtgacgggcctttgtgcgaatttagcgcgttatgctgcgtaaacttgttacct	
K-tign	caacaacctgtgcttgcgg	
K-tigc	aaaagcccaccacctggcg	

[a] The nucleotides that are homologous to regions adjacent to the *tig* gene (marked as H1 or H2 in Fig. 2) are *underlined*. The sequences of restriction endonuclease sites are in *capitals*, and the restriction endonuclease is shown in *parentheses* after the sequence

PCR products were then cloned into a pMD18-T vector (Takara) and sequenced to confirm that no other mutations, such as frameshift mutations, had been introduced.

Western blotting

Whole-cell bacterial lysates of wild-type and mutant strains were analyzed on a 12% polyacrylamide-SDS gel. The gel was blotted onto nitrocellulose, and then probed with anti-TF polyclonal antibody and HRP-conjugated goat anti-rabbit IgG (Liu and Zhou 2004).

Results

Since the regions flanking the *Bsp*T104I site in the plasmid pKD3 lack a regulatory sequence for gene expression, the *sacB* open reading frame together with the *sacB* promoter was amplified from *B. subtilis* using primers sacB3 and sacB4 and ligated into pKD3. The *sacB* gene can be inserted into pKD3 in two directions, and so two kinds of ligation products were obtained in this way and designated as pKD3S and pKD3V (Fig. 1). Both plasmids were transformed into BW25141 strains and all transformants exhibited sucrose sensitivity. pKD3S was chosen at random as the template plasmid for subsequent experiments.

Our strategy for introducing *tig* mutant alleles consisted of two steps. (1) The *tig* gene was first disrupted by positive chloramphenicol selection for a 3.7 kb PCR fragment containing the *sacB-cat* cassette, and then (2) the *sacB-cat* cassette was replaced by insertions of *tig* mutant allele fragments by sucrose counterselection against *sacB* (see Fig. 2).

A PCR product was generated using the long primers K-tigA and K-tigB that included 40-nt *tig* homology extensions and 20-nt priming sequences, with pKD3S as the template, and then transformed into a BW25113 strain carrying pKD46. The resultant strain Z416 was Δ*tig*, chloramphenicol resistant and sucrose sensitive. The genotype of Z416 was verified by PCR with the primers Gtig1 and Gtig2 that annealed to the upstream and downstream regions of the *tig* gene, and the size of the product observed on 1% agarose gel was in agreement with the predicted size of 3.7 kb (the size of the product from the wild-type strain would be 1.9 kb; Fig. 2). The genotype of Z416 was further confirmed by sequencing.

Primer pairs K-Nn/K-Mc, K-Nn/K-Cc, K-Mn/K-Cc, K-Nn/K-Mc, and K-tign/K-tigc were used to generate PCR fragments that contained different mutations of the *tig* gene. The 5′ ends of the primers K-Nn, K-Mn, K-Mc and K-Cc were similar to those of K-tigA or K-tigB, each consisting of a 40-nt *tig* flanked region; and the 3′ ends were the priming regions complementary to their respective templates. A 20-nt overlap segment between the primers tign and tigc covered the head and tail flanking regions of the *tig* gene locus. The annealing and extension product from the primer pair tign/tigc was used as the template for the primers K-tign and K-tigc, and the final product was used for generating a *tig* gene clean deletion mutant (see Table 2). Z416 cells were transformed with products amplified from the above primer pairs, and sucrose-resistant colonies were screened. Whole-cell PCR analysis of the transformants using the primers Gtig1 and Gtig2 was used to verify the transformants, and the observed lengths of the PCR products corresponded with the predicted lengths. The genotypes of the resultant strains were also further confirmed by sequencing, and the strains were named as M09-NM (C domain of TF was deleted), M09-NC (M domain of TF was deleted), M09-MC (N domain of TF was deleted), M09-I195G (the amino acid

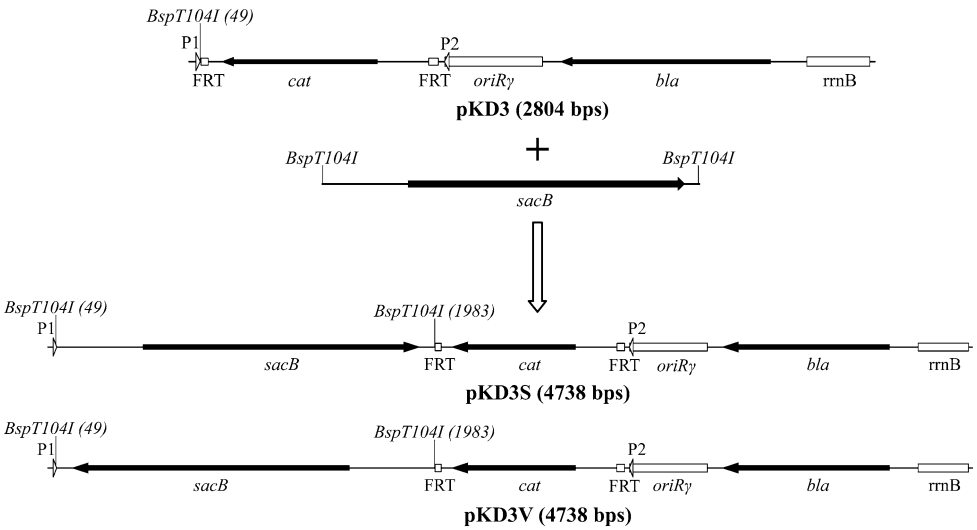

Fig. 1 Construction of templates. Plasmids are shown as linear representations. P1 and P2 are primering sites (see Datsenko and Wanner 2000), and the primers KtigA and KtigB were used to amplify the *sacB-cat* cassette from pKD3S

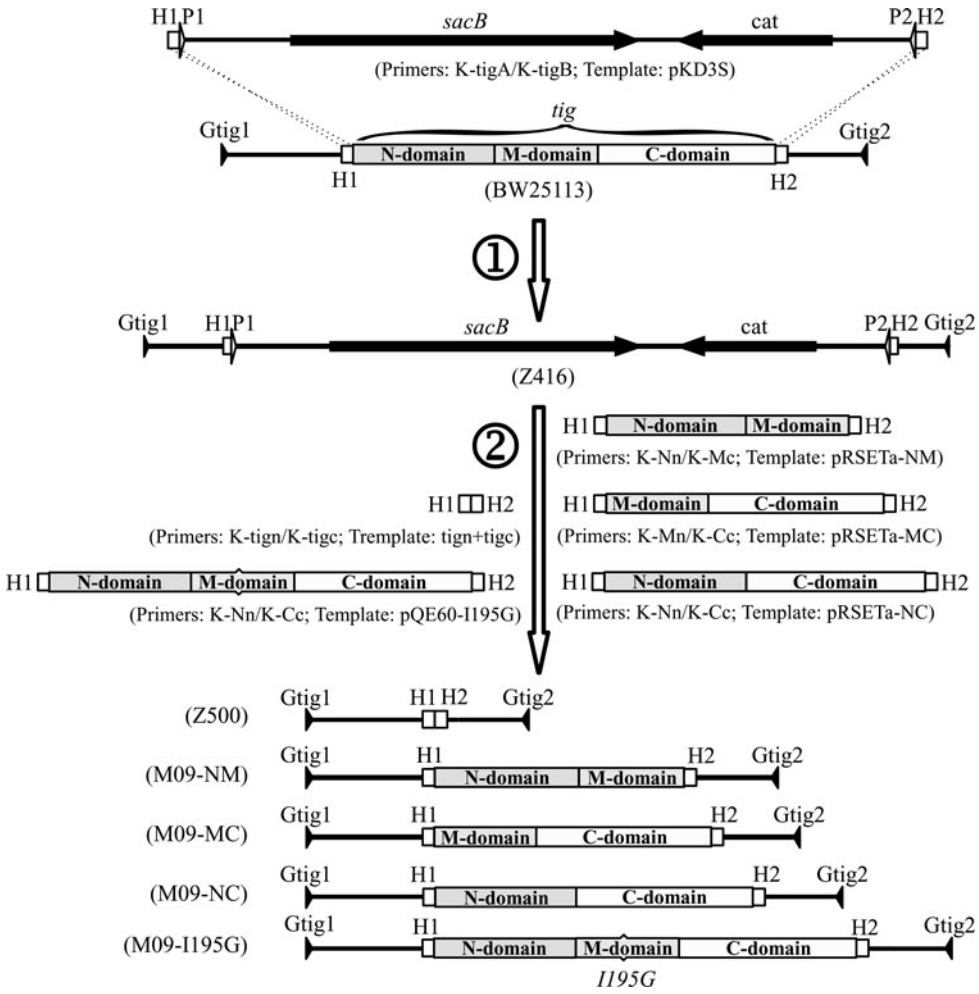

Fig. 2 Introduction of *tig* mutant alleles. The *tig* gene was replaced by a 3.7 kb PCR fragment containing the *sacB-cat* cassette resulting in the strain Z416; the *sacB-cat* cassette was then removed by insertion of *tig* mutant allele fragments, and strains containing TF mutants were obtained. H1 and H2 are homologous to regions adjacent to the *tig* gene. Gtig1 and Gtig2 are the primers that annealed to the upstream and downstream regions of the *tig* gene. The PCR products used to disrupt the *tig* gene and to remove the *sacB-cat* cassette from the native *tig* locus are shown, and primer pairs and templates are listed in parentheses

residue I at 195 was changed into G) and Z500 (the entire *tig* gene was deleted; Fig. 2).

Western blotting indicated that the TF domain truncation mutants and the point mutant were expressed in their hosts. No band was observed for Z500 which was a TF clean deletion mutant (Fig. 3).

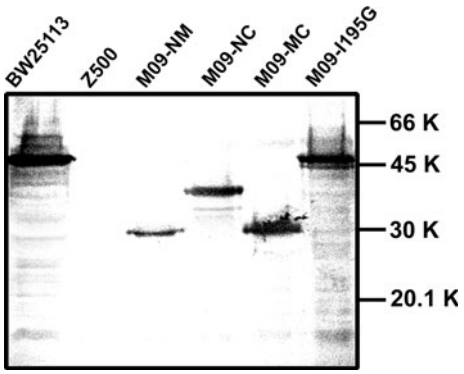

Fig. 3 Whole-cell bacterial lysates of wild-type and mutant strains were separated by 12% SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane and probed with an anti-TF polyclonal antibody and HRP-conjugated goat anti-rabbit IgG

Discussion

Driven by the needs of protein function research, gene inactivation or gene knockout by homologous recombination has emerged as a major addition to existing technologies. In contrast to classical methods, homologous recombination based on phage systems has a unique advantage in that neither construction of suicide plasmids nor in vitro enzyme manipulations such as restriction digestion and ligation are required.

Datsenko and Wanner's Red system is a powerful tool in gene inactivation due to its efficiency and simplicity, since it can catalyze recombination using very short regions of sequence homology. However, this system cannot introduce mutations into chromosomal genes, and gene disruption does not result in a clean deletion. When eliminating the antibiotic resistance gene which is used to replace the target gene, an 82- to 85-nt scar is left in place of the disrupted gene.

We prevented this problem by making clean deletions of target genes via a positive counter-selection method using

two-step recombination. We have extended the use of Datsenko and Wanner's system so that other mutations such as point mutations and domain truncation mutations can also be generated in the same manner. We demonstrated the use of this method by introducing different mutations of the *tig* gene into *E. coli* BW25113 (see Fig. 2).

It should be noted that the plasmid used as a template in our method was designed bearing in mind the fact that template plasmids intermingle with linear PCR products and can be transformed into host strains. If the template plasmid is able to replicate in the host, a high proportion of false positive transformants without gene disruptions but harboring the template plasmid will be produced. In order to assess the effect of template plasmid transformation, we used a pMD18-T vector carrying the *cat-sacB* fragment as the template to generate a linear PCR product to destroy the *tig* gene in *E. coli* as described in the Sect. "Materials and Methods". A total of 30 colonies that exhibited chloramphenicol resistant were selected. However, sequencing results indicated that the host's native *tig* gene was unchanged and further experiments revealed that all these false positive transformants just harbored the pMD18-T vector carrying the *cat-sacB* cassette.

There have been some previous reports of two-step recombination methods for scarless gene replacement; however, no general scheme for overcoming the problem of template plasmid transformation resulting in a high proportion of false positive transformants has been designed. Some ingenious approaches for avoiding this problem have been described, for example, Muyrers et al. used a plasmid carrying the *ColE1* replication origin as a template while the host harbored another plasmid carrying the same replication origin which would prevent the template plasmid from invading (Muyrers et al. 1999), and Sun et al. used a plasmid derived from pUC18 as a template when the host was *Yersinia pestis* in which pUC18 cannot replicate (Sun et al. 2008). However, the applicability of these methods is limited to specific types of plasmids or bacterial species. Alternatively, *Dpn*I can be used to digest the template plasmid after PCR amplification, however, extra manipulation in vitro is thus required and the plasmid may sometimes escape *Dpn*I digestion (Datsenko and Wanner 2000; Zhang et al. 1998).

To circumvent template transformation, we constructed the special template plasmid pKD3S which contains a conditional (*oriR*γ) replicon that requires the trans-acting $\prod$ protein (the *pir* gene product) for replication. The plasmid pKD3S is not maintained in most strains but can be maintained in *pir*$^+$ hosts such as BW25141, thus ensuring that our system can be used in various strains to avoid false positive transformants caused by template plasmid transformation without any additional manipulations. Resistant colonies carrying template-like plasmids were markedly reduced by this means, and no false positive transformants were detected in our study.

In this study we successfully obtained scarless mutations of the *tig* gene including a point mutation, domain truncation mutations and a clean deletion in *E. coli*, and these mutant strains have been used in the study of the structure and function of the *E. coli* trigger factor in vivo. The system we developed to disrupt chromosomal genes and introduce mutant alleles into native locations of wild-type bacterial genes has wide applications especially in bacterial genome analysis since the λ-Red recombination proteins, the counter selection marker *sacB* and the conditional replicative *oriR*γ plasmid pKD3S can be applied in an extensive range of hosts. This technique should be readily extendible to other bacterial species.

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