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Highly Efficient Method for Introducing Successive Multiple Scarless Gene Deletions and Markerless Gene Insertions into the *Yersinia pestis* Chromosome▽

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An efficient two-step recombination method for markerless gene deletion and insertion that can be used for repetitive genetic modification in *Yersinia pestis* was developed. The method combines λ Red recombination and counterselective screening (*sacB* gene) and can be used for genetic modification of *Y. pestis* to construct live attenuated vaccines.

Suicide vectors introduced by conjugation or electrotransformation have been used to generate knockout mutants in *Yersinia* spp. (3, 13, 18, 20). The transformation efficiency in *Yersinia* for these plasmids is typically low, which makes introducing mutations difficult and laborious. Thus, developing an easy and highly efficient method to generate mutations in the chromosome of *Yersinia pestis* would be a useful tool to facilitate research on this pathogen.

The λ Red system is a simple method for disrupting chromosomal genes using PCR products in *Escherichia coli* (7, 14, 15) and *Salmonella enterica* (12). Subsequently, Derbise et al. introduced an improvement by using long flanking sequences to disrupt genes in *Yersinia pseudotuberculosis* (9). However, disrupting genes by either method leaves antibiotic markers (17) or FLP recombination target (FRT) site scars in the bacterial chromosome (7). Antibiotic resistance markers cannot be used when working with a select agent, such as *Y. pestis* (Centers for Disease Control and Prevention, unpublished report), or for construction of live vaccines. The presence of FRT scars, typically 82 to 85 bp in length, could become problematic if one were to use this system to introduce multiple mutations. In the standard system, each incoming PCR product encodes a selectable antibiotic resistance marker flanked by FLP sites, the source of FRT scars. Therefore, the scars could serve as recombinational hot spots at each successive step in strain construction, reducing the frequency of obtaining the desired insertion (7). Use of the Flp recombinase in a cell that carries two nearby scars could lead to an unwanted deletion of the intervening sequence (7). Finally, the presence of multiple scars in the chromosome could lead to chromosomal rearrangements or deletions resulting from recombination events between FRT scars, even in the absence of the Flp recombinase (7). This latter possibility is of concern to the FDA and could therefore hinder licensure of a live bacterial vaccine possessing multiple mutations constructed using this method.

Some researchers have successfully used suicide vectors to disrupt genes in *Y. pestis* (1, 18). We were unable to delete the *relA* and *spoT* genes from *Y. pestis* using either suicide vectors or the standard λ Red method. Here, we have combined elements of both systems to develop a highly efficient method for introducing gene deletions free of antibiotic markers and FRT scars and for markerless gene insertion.

Tables 1 and 2 list the bacterial strains, plasmids, and oligonucleotide primers used in this study. *Y. pestis* strains were grown in heart infusion broth (HI broth) and on plates containing HI broth, Congo red, and agar at 30°C (1). For screening, *Y. pestis* electroporants were spread or streaked onto Tryptose blood agar (TB agar) plates containing 10 μg/ml chloramphenicol (Cm) and/or 5% sucrose. *Y. pestis* was grown at 30°C for 24 h with shaking (liquid media) or for 48 h (solid media). General DNA isolation and enzymatic manipulation were performed as described previously (19). The targeted region of each of the final constructs was verified for the expected DNA sequence by PCR and DNA sequence analyses.

A general outline of our method is shown in Fig. 1. Deletion of a target gene is accomplished in two steps. In the first step (Fig. 1A), a linear DNA fragment carrying the *cat* (chloramphenicol resistance gene) and *sacB* genes flanked by long stretches (~500 bp) of DNA homologous to the regions flanking the deletion site is prepared. The DNA fragment is electroporated into the desired host containing plasmid pKD46, which encodes the genes required for λ Red recombination (Table 1) (7). The targeted gene is then replaced by homologous recombination. Cells carrying the desired insertion/deletion are selected on media containing Cm. In the next step (Fig. 1B), a second DNA fragment encoding the desired deletion is prepared and used to electroporate pKD46-containing host cells. Replacement of the *cat-sacB* cassette is selected for on media containing sucrose (13). Sucrose-resistant electroporants are then screened for sensitivity to chloramphenicol and by PCR for the deletion. This process can be repeated as many times as necessary to construct a strain with multiple deletion mutations. When all the mutations have been made, pKD46 can be removed by growing the mutant at 37°C in the appropriate medium (3).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Relevant genotype or annotation[a]	Source, reference, or derivation
Bacterial strains		
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
Y. pestis strains		
KIM6$^+$	Pgm$^+$; pMT1 pPCP1; cured of pCD1	1
χ10003	Δ*relA233*	*Y. pestis* KIM6$^+$
χ10004	Δ*relA233* Δ*spoT85 Y. pestis* KIM6$^+$	This study
χ10020	Δ*relA233* ΔP_{spoT}::*spoT-1*::TT *araC* P_{BAD} *spoT+1 Y. pestis* KIM6$^+$	This study
Plasmids		
pUC18	For cloning and sequencing	22
pKD46	λ Red recombinase expression plasmid	7
pKD3	Template for amplifying the *cat* gene	7
pRE112	Template for amplifying the *sacB* gene	10
pYA3700	TT *araC* P_{BAD} cassette plasmid; Ap^r	6
pYA4373	The *cat-sacB* cassette was inserted in the PstI and SacI sites of pUC18	pUC18
pYA4452	The *'gmk-rpoZ* (the *spoT* upstream sequence from −1 to −749 bp) and *spoT'* (585 bp of the N-terminal fragment of the *spoT* gene from the start codon TTG) were put into SphI and PstI sites and XhoI and EcoRI sites of pYA3700, respectively	pYA3700
pYA4453	The *cat-sacB* cassette from pYA4373 was ligated into the PstI site of pYA4452	pYA4452
pYA4566	The *'barA-y0809-y0810-mazG'* fragments ligated by overlapping PCR and containing restriction endonuclease sites (PstI and SacI) near the center of the fragment were cloned into EcoRI and HindIII sites of pUC18	pUC18
pYA4567	The *'barA-y0809-y0810-mazG'* fragments ligated by overlapping PCR without any exogenous intervening sequences were cloned into EcoRI and HindIII sites of pUC18	pUC18
pYA4568	The *cat-sacB* cassette from pYA4373 was ligated into PstI and SacI sites of pYA4566	pYA4566

[a] TT, transcription terminator.

To demonstrate the feasibility of this system, we constructed a *relA* gene deletion in *Y. pestis* strain KIM6$^+$ as outlined in Fig. 2. Plasmids encoding *relA* deletion cassettes with and without a *cat-sac* cassette insertion were constructed by overlapping PCR (Table 1). To prepare a *red*$^+$ host, plasmid pKD46 was electroporated into *Y. pestis* KIM6$^+$ (5). *Y. pestis* KIM6$^+$(pKD46) cells were electroporated with 1 μg of PCR-amplified, gel-purified *cat-sacB* fragment flanked by long regions homologous to genes just upstream and downstream of *relA*. Approximately 1,000 Cm^r transformants were obtained. We randomly picked 30 colonies and verified that all had the expected insertion by PCR (data not shown). Electrocompetent cells were prepared from a sucrose-sensitive isolate and electroporated with ap-

TABLE 2. Oligonucleotide primers used in this work

Oligonucleotide primer	Sequence[a]
Cm-F	5′CGCGGATCCAACTTCATTTAAATGGCGCGCCTTACG3′ (BamHI)
Cm-R	5′CGAACTGCAGGGCGCGCCTACCTGTGACGGAAG3′ (PstI)
SacB-F	5′CGGGGATCCGCCCTTATGTGTAAAGGGCAAAGTGTATACTTTG3′ (BamHI)
SacB-R	5′CGGGAGCTCTTATTTGTTAACTGTTAATTGTCCTTG3′ (SacI)
relA-U1	5′CGGAAGCTTCATCATGCGCGCGCGAAGACTGTATTTG3′ (HindIII)
relA-U2	5′**GAGCTCGGTACCAGCCTGCAGGCATGC**AACTTCTCCCTACTTTGCGACCTCT3′ (SacI and PstI)
relA-D1	5′**GCATGCCTGCAGGCTGGTACCGAGCTC**GCTTTTTAATCTGGACGGCGATTAC3′ (PstI and SacI)
relA-D2	5′CGGGAATTCCTAATCGCATGGCAAACGTCATCAAAG3′ (EcoRI)
relA-U3	5′TCGCCGTCCAGATTAAAAAGCAACTTCTCCCTACTTTGCGACCTCTG3′
relA-D3	5′CAGAGGTCGCAAAGTAGGGAGAAGTTGCTTTTTAATCTGGACGGCGA3′
relA-V1	5′ATGCTCAAGTATCAGTTTATCGGCTTCCAG3′
relA-V2	5′GTGGGGAATAACCTGAATAGTCGCACCC3′
Cm-V	5′GTTGTCCATATTGGCCACGTTTA3′
spoT-U1	5′CTTGAGCATGCCAAAGTATTTGAAAATTACTACG3′ (SphI)
spoT-U2	5′GAAACTGCAGAGGCAGACTCGCAGTCTAATTAACG3′ (PstI)
spoT-P1	5′CGGCTCGAG***GGAGTG***AAACG***TTG***TACCTGTTTGAAAGCCT3′ (XhoI)
spoT-P2	5′CGGGAATTCGATAGAGTGCTTCAAAACCCAG3′ (EcoRI)
spoT-V1	5′AGGGTATATCAACCTAATCAACTGAAACG3′
spoT-V3	5′CAGAGAGTTTACGCCCATTTCCTAATGCATG3′
araC-V	5′CATCCACCGATGGATAATCGGGTA3′

[a] The sequences of restriction endonuclease sites are underlined, and the restriction endonuclease(s) is shown in parentheses after the sequence. The nucleotides in boldface type show the reverse complementary region between primers relA-U2 and relA-D1. The nucleotides in italic boldface type show the Shine-Dalgarno sequence and the TTG start codon.

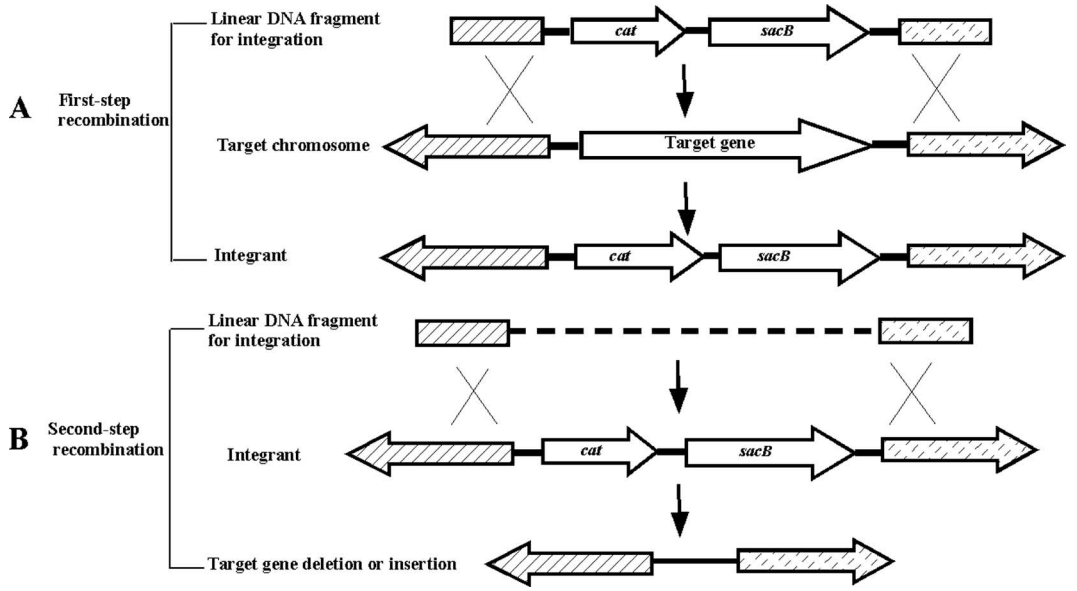

FIG. 1. Schematic strategy for markerless deletion of a target chromosomal gene by two-step recombination. Cross-grained regions represent homology between the integration cassettes and sequences flanking the target gene. (A) A DNA fragment carrying the *cat-sacB* genes flanked by two long regions homologous to the DNA sequences bordering the target site is integrated into the chromosome to disrupt or delete the target gene(s). (B) A DNA fragment carrying the desired deletion or insertion flanked by two long regions homologous to the DNA sequences bordering the target sites directs replacement of the *cat-sacB* genes through homologous recombination.

proximately 1 μg of a linear DNA containing the Δ*relA233* deletion and DNA sequences flanking the *relA* gene.

We obtained ~300 sucrose-resistant colonies and randomly selected 30 colonies. Twenty-six were PCR positive for the expected deletion, an efficiency of ~86%. A single colony isolate was chosen and designated Δ*relA233* KIM6$^+$(pKD46). Plasmid pKD46 was cured by growth at 37°C to yield strain χ10003.

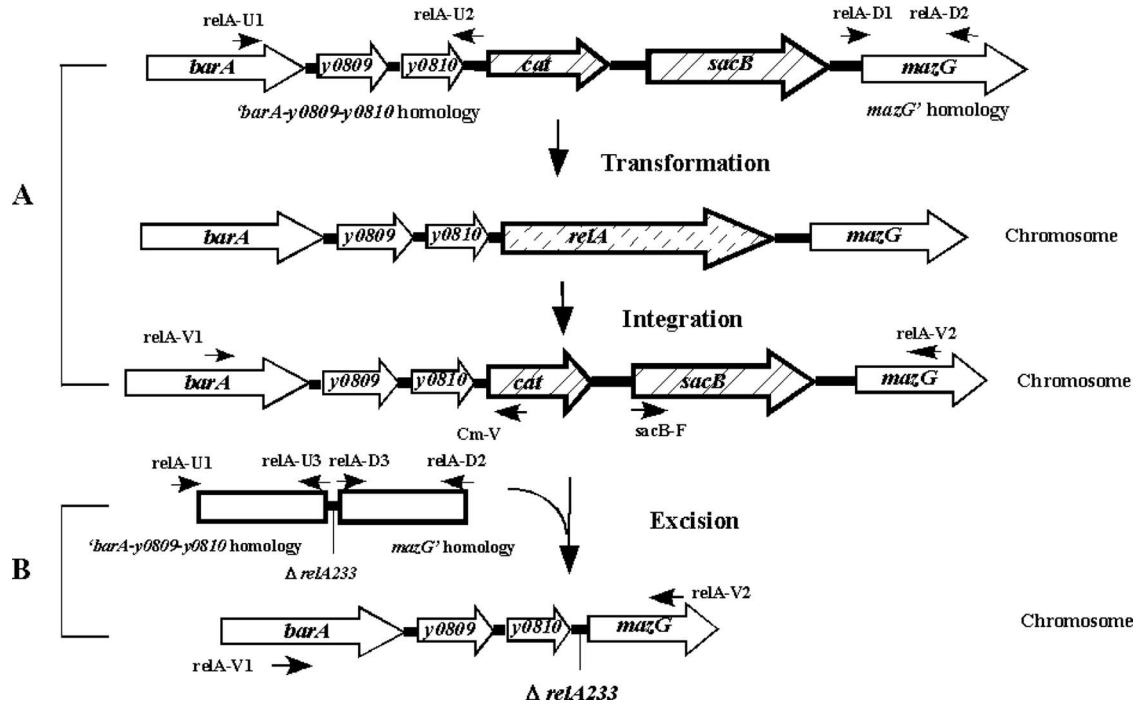

FIG. 2. Construction of a Δ*relA* mutation in *Y. pestis* KIM6$^+$. (A) A DNA fragment encoding a *cat-sacB* cassette flanked by long regions homologous to genes just upstream and downstream of *relA* is introduced into *Y. pestis* KIM6$^+$(pKD46). Selection on media containing chloramphenicol yields a strain in which *relA* is replaced by the *cat-sacB* cassette. The positions of oligonucleotide primers, such as relA-U1, relA-D1, relA-V1, and Cm-V, are indicated. (B) A DNA fragment containing the *relA233* deletion and flanking regions adjacent to *relA* is introduced into the *Y. pestis* KIM6$^+$(pKD46) *cat-sacB* strain. After selection on media containing sucrose, the *cat-sacB* cassette is replaced by the fragment containing the Δ*relA233* deletion.

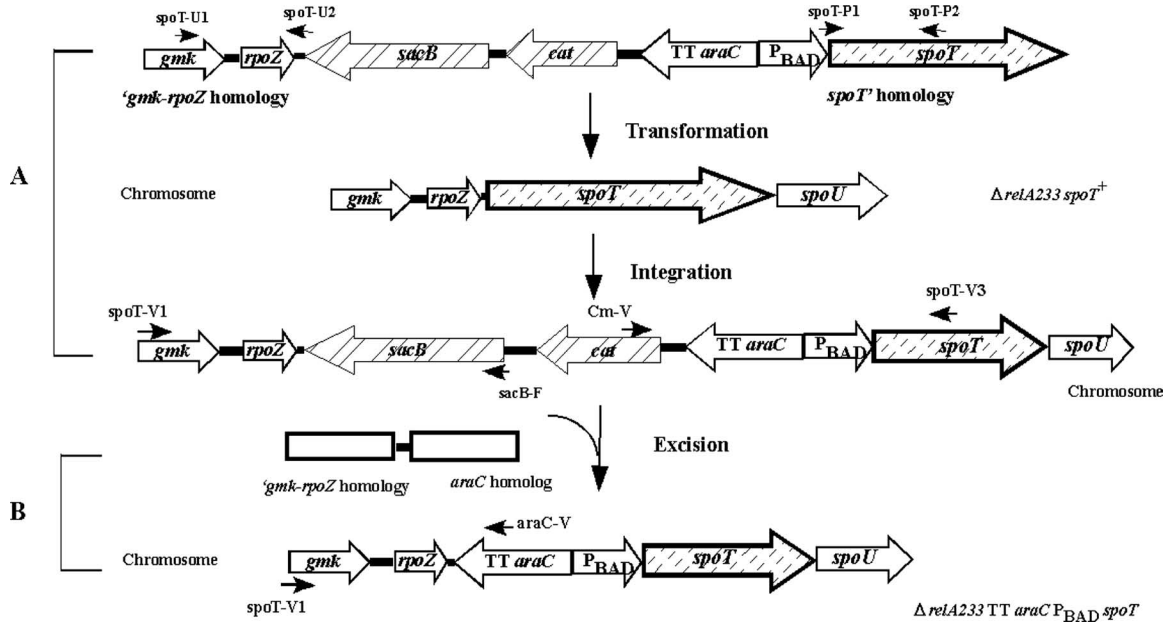

FIG. 3. Construction of an arabinose-regulated *spoT* gene in *Y. pestis* KIM6$^+$. The transcription terminator (TT) *araC* P_{BAD}-Shine-Dalgarno (SD) sequence was inserted upstream of the *spoT* gene in the chromosome of Δ*relA233* KIM6$^+$(pKD46). The steps involved are analogous to those described in the legend to Fig. 2. The positions of oligonucleotide primers, such as spoT-U1, spoT-P1, spoT-V1, and Cm-V, are indicated. (A) Insertion of *sacB-cat* TT *araC* P_{BAD}-SD *spoT* just after *rpoZ*. (B) Excision of the *sacB-cat* cassette.

To further validate this methodology, we introduced a second deletion, Δ*spoT85*, into strain Δ*relA233* KIM6$^+$(pKD46) using a similar strategy. The efficiency at each step was similar to those reported above. Plasmid pKD46 was cured from a single colony isolate of the resulting strain to yield χ10004. These results show that this is a highly efficient method for introducing one or more gene deletions into *Y. pestis*.

Because of our interest in studying the effects of *spoT* on virulence in *Y. pestis*, we wanted to construct a strain in which *spoT* expression could be regulated at will. The *spoT* gene is expressed in an operon after *rpoZ* (8). We inserted the arabinose-regulated P_{BAD} promoter (11) between the *rpoZ* and *spoT* genes as outlined in Fig. 3. To ensure translation of *spoT*, we included the ribosome-binding sequence from the *E. coli* K-12 *araB* gene (GGAGTG) (21) 5 bp upstream of the start codon. The yields from this construction were similar to those described above.

HHS/CDC regulations dictate that antibiotic markers cannot be introduced into select agents, such as wild-type *Y. pestis*. Therefore, for these studies, we used the pCD1-cured KIM strain, *Y. pestis* KIM6$^+$, which is not a select agent. However, due to the fact that the final constructs are marker-free after curing pKD46, plasmid pCD1 can be reintroduced into these strains to evaluate the effects of the introduced mutations on virulence.

We have described a strategy to generate scarless deletion and insertion mutations into the *Y. pestis* chromosome with high efficiency. In addition, we have eliminated the need to introduce a plasmid such as pCP20 that carries the Flp recombinase (4) which constitutes an additional electroporation and subsequent plasmid curing cycle. By obviating this need, plasmid pKD46 does not have to be cured and reintroduced for each mutagenic step. Therefore, multiple genes can be successively deleted more rapidly than with the traditional λ Red method. While this new method should be applicable to any *Y. pestis* strain, we note that for workers using *Y. pestis* strains that carry plasmid pCD1, curing of plasmid pKD46 should be done in medium containing 2.5 mM calcium (2). We have also successfully applied our strategy to other gram-negative bacteria, such as *E. coli* and *Salmonella enterica* serovars Typhi, Typhimurium, and Paratyphi A, demonstrating the broad utility of this method.

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