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Site-Directed Insertion and Deletion Mutagenesis with Cloned Fragments in *Escherichia coli*

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A mutation of a cloned gene that has been made by introducing a transposon or some other selectable genetic determinant can be crossed into the gene's original replicon by linearizing the cloned DNA and transforming a *recB recC sbcB* mutant. A number of applications of this method are described with genes of either chromosomal or plasmid origin.

We have frequently found it useful to carry out a genetic manipulation—such as inserting a transposon, creating a deletion, or constructing a fusion—on a cloned fragment of DNA and then to reintroduce the altered DNA back into its original site in either the *Escherichia coli* chromosome or in a plasmid. We report here that, in many cases, this can be accomplished readily by transforming a linear DNA fragment containing the alteration into a *recB recC sbcB* mutant and selecting for the desired recombinant. We initially reported the use of this technique to generate a particular Tn*5* insertion mutant of pKM101 (20) and have since found it to be of general applicability.

Two other methods have been reported for achieving this same end, but both have certain limitations. The first approach that has been described for crossing selectable markers from recombinant plasmids into chromosomal or plasmid replicons is based on the use of plasmid incompatibility to displace the resident plasmid. This procedure has been used extensively with *Rhizobium* spp. and *Agrobacterium* spp. (2, 14), but to date its successful use has only been reported with recombinant plasmids derived from IncP vectors. The second method (3) is based on the transfer of genetic determinants from *polA*$^+$-dependent plasmids to the chromosome of a *polA* mutant of *E. coli*. In principle, either selectable or nonselectable markers can be transferred from the recombinant plasmid; however, we have encountered instances in which we were unable to apply this technique successfully. The method has the further limitations that only recombinant plasmids constructed with *polA*$^+$-dependent cloning vectors can be used and that mutations that are lethal in combination with *polA* (such as *recA*) cannot be recombined from the plasmid into the chromosome without constructing a merodiploid.

We rationalized that, if *E. coli* were transformed with a fragment of linear DNA containing a selectable marker flanked by homologous DNA, the cell could acquire the selectable marker by a double recombination event (20). The selectable marker could be introduced into the interior of the fragment by (i) inserting a transposon, (ii) cloning in a selectable marker with or without the deletion of some DNA, or (iii) constructing a fusion in which the inserted DNA contains not only the DNA creating the fusion, but also a selectable marker. Strain JC7623, a *recB21 recC22 sbcB15* strain was chosen since it can be transformed by linear DNA (12, 19). The *recB recC* mutations inactivate exonuclease, preventing it from degrading the linear DNA (15), whereas the *sbcB* mutation restores recombination proficiency to a strain which carries *recB recC* mutations (4, 8). In addition, strains carrying *recB recC* and *sbcB* mutations are extremely poor at supporting the replication of many plasmids, including ColE1 and most of its derivatives (1, 6, 13, 18), so that even if the linearized, transformed molecules are able to recircularize in vivo, they cannot be stably maintained.

We have recently used this technique to introduce a drug resistance determinant into the chromosomal *recA* gene to create an allele of *recA* that can be readily transduced into new strains. The procedure used is shown in Fig. 1. pJS33 (provided by R. Brent) is a Ap^r Tc^s derivative of pBR322 which carries the *recA* gene on a 3.3-kilobase *Bam*HI fragment. A 4-kilobase *Pst*I fragment containing a Cm^r gene (derived from pDPT427 [16]) was cloned into a *Pst*I site that lies within the *recA* structural gene. (This fragment also contains a Sp^r determinant, but for reasons that are not understood, this gene is expressed poorly in this clone, and Sp^r was therefore not used in subsequent procedures.) Approximately 2 μg of this plasmid, pGW2152, was digested with *Nar*I, a restriction nuclease that cuts the plasmid only in the vector DNA, and used to transform strain JC7623 to Cm^r. About 50 colonies were obtained, of which 12 were selected for further study. Five of these were determined to be Ap^s, extremely UV^s, and recombination deficient and to grow poorly, whereas the other seven were Ap^s, UV^r, and recombination proficient and grew well. These latter strains were not characterized further. All of the Ap^s UV^s strains so characterized were determinant not to contain detectable plasmid DNA. P1 lysates were made from three of these strains, and these were found to cotransduce Cm^r and UV^s with 100% efficiency. When a strain containing a *srl*::Tn*10* was transduced to Cm^r, about 50% of the transductants were *srl*$^+$ and Tc^s. All these results would be expected of a strain containing a Cm^r determinant within the *recA* structural gene. Since we did observe some *recA*$^+$ colonies it appears to be important to screen for the loss of function of the gene of interest. Marinus et al. (10) have recently made use of this strategy to introduce a Tn*9* into the *dam* gene. Others (7) have recently used the method to create a deletion that spans the *recA* and *alaS* loci.

We have also used this technique to obtain selectable markers closely linked to, but outside of, a chromosomal gene of interest. Specifically, we isolated a Tn*5* insertion (11) adjacent to the *metK* gene of pK8, a derivative of pBR322 carrying *met*K (17). This plasmid was linearized with *Eco*RI, which cuts at one of the junctions between the vector DNA

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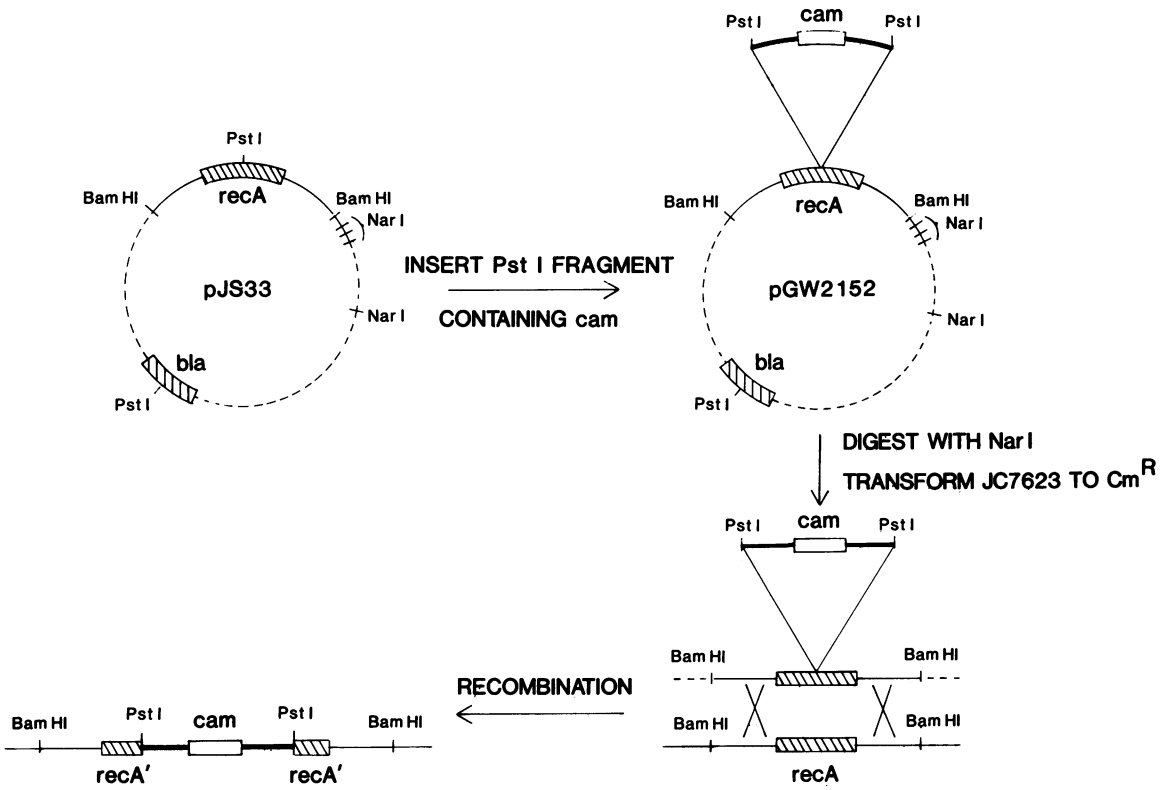

FIG. 1. Creation of a mutation in the chromosomal *recA* gene by insertion of a 4.0-kilobase *Pst*I restriction fragment coding for Cm^r. This fragment was inserted into the *Pst*I site of the *recA* gene of pJS33. The resulting plasmid, pGW2152, was digested with *Nar*I and used to transform JC7623 to Cm^r. Cross-hatched boxes represent the *recA* and *bla* genes, solid bars represent DNA inserted into the *Pst*I site of *recA*, solid lines indicate chromosomal sequences, and dashed lines indicate vector DNA.

and the cloned chromosomal DNA, and used to transform JC7623 to Km^r. P1 lysates of seven Km^r colonies were prepared, and one lysate was found to cotransduce the Km^r marker with *serA*, a gene linked to *metK*. The low frequency of recombination (one out of seven) compared with the frequency of the presumed transposition of the Tn*5* (six out of seven) may be due to the fact that the Tn*5* inserted into pKA8 about 200 to 300 bases from one end of the chromosomal DNA, allowing for only a small stretch of homologous DNA on one side of the insertion. In subsequent crosses, the insertion linked to *serA* was found to be cotransduced with *metK* at a frequency of about 95%. We have isolated a strain containing this insertion and *metK86* (5), and we have used it to transduce *metK86* by selection for Km^r.

We have also used this technique extensively to mutagenize various plasmid-coded genes. It was first used to obtain a Tn*5* insertion in the *nuc* gene of plasmid pKM101; *nuc* codes for a periplasmically localized endonuclease (9, 20). The plasmid pGW1502, which carries a 6-kilobase piece of pKM101 DNA including *nuc*, was mutagenized with Tn*5*, and a *nuc*::Tn*5* derivative was obtained (20). Two micrograms of DNA of pGW1502 *nuc-1210*::Tn*5* were linearized with *Eco*RI, which cuts only in the vector DNA, and used to transform JC7623(pKM101) to Km^r. Approximately 200 transformants were obtained. Since pKM101 is self-transmissable, 40 of these derivatives were assayed for their ability to transfer Km^r by conjugation; 36 of these were able to do so and thus carried a Tn*5* insertion in their plasmid DNA. The plasmid DNA from four of these was examined and was determined to have exactly the restriction pattern expected if the Tn*5* mutation had been recombinationally transferred to pKM101 by a double crossover. Using identical techniques, we have subsequently introduced Tn*5* into eight loci of pKM101 that affect pilus synthesis into one locus affecting entry exclusion and into two loci coding for lethal gene products (21–23).

In principle, this method ought to be adaptable to mutagenizing any chromosomal or plasmid gene. We have not formally determined the amount of homologous DNA required for recombination, although we succeeded in recombining, without difficulty, a Tn*5* into the *eex* locus of pKM101 (22) with a plasmid that had 0.3- and 2.4-kilobase regions of homologous DNA flanking the insertion.

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