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Transposon Tn5 Mutagenesis in *Acetobacter* sp. HA

CHUN, HONG-SUNG, BYUNG-KWON LEE, JONG-PHIL PARK, SOOK-YOUNG LEE, HYEON-SOOK CHEONG, JUNG-SUP LEE, JIN-CHEOL YOO[1], HONG-SUB KIM[2] AND SUNG-JUN KIM*

Department of Genetic Engineering, [1]Department of Pharmacy, and [2]Department of Biology, Chosun University, Kwangju 501-759, Korea

An efficient and convenient method of introducing transposable elements into acetic acid bacteria was developed by the method of conjugal transfer. The ampicillin-resistant strain, *Acetobacter* sp. HA, was selected to be conjugated with two *E. coli* strains, WA803 containing pGS9 and AC8001 harboring pJB4JI. The Tn5 containing suicide vector pGS9 or pJB4JI, was transferred from *E. coli* to *Acetobacter* sp. HA and kanamycin-ampicillin-resistant transconjugants obtained at high frequencies. The conjugal frequencies of pGS9 and pJB4JI were 6.20×10^{-1} and 2.79×10^{-1} per recipient, respectively. The transfer method was applied on four different strains of *Acetobacter*. The conjugal transfer frequencies ranged from 2.00×10^{-2} to 4.45×10^{-8} per recipient in the three strains. Some transconjugants tested were found to contain Tn5 DNA in their genomes and this was confirmed by Southern blot analysis. This is the first study which shows that Tn5 mutagenesis can be applied to successfully isolate mutants of *Acetobacter* genus.

The genus Acetobacter is not well characterized genetically, but its industrial and scientific importance in vinegar production has resulted in the growing interest in its genetics (Inoue *et al.*, 1989; Fukaya *et al.*, 1990; Schroder *et al.*, 1991). To make the *Acetobacter* more genetically useful, recombinant DNA techniques have been applied. As a result, host-vector systems, spheroplast fusion, and an efficient conjugal transfer method for Acetobacter have been developed (Fukaya *et al.*, 1985a, 1985b; Inoue *et al.*, 1985; Okumura *et al.*, 1985; Fukaya *et al.*, 1989).

One powerful and direct mean of approaching the elucidation of gene function is the transpositional mutagenesis with Tn5 (Calos, 1980; Berg and Berg, 1987). The transposon Tn5 has been used extensively as a tool for mutagenesis in many enteric and non-enteric Gram-negative species (Berg and Berg, 1983). It is one of the best characterized transposons and integrated random target sites (Berg, 1987). Since transposons themselves do not have autonomous replicons, it is reasonable to expect that if they are delivered efficiently into a cell by means of a plasmid vehicle that will not maintain itself (a suicide vehicle), it would allow the selection of the rare transposon-insertion derivatives with the use of the antibiotic-resistance marker contained within the transposon.

On the other hand, methods for mutagenesis using transposable elements have not been exploited in the genetic studies of *Acetobacter.* At present, the only report on the introduction of transposon into *Acetobacter* is that by Valla *et al.* (1986), who showed transposon Tn1 insertion into the plasmids of *Acetobacter xylinum.*

In a recent investigation, *Acetobacter* sp. HA has been isolated from the Korean rice wine fermented vinegar which proved to be a Gram-negative, acidophilic methanol-utilizing, short-rod-shaped organism that grows aerobically in the acidic pH range (Chun and Kim, 1993). In this study, we describe the efficient method for the conjugal transfer of transposon Tn5 to the chromosome of *Acetobacter* sp. HA. Consequently, we present evidence to indicate that Tn5 mutagenesis is a viable method for the mutant isolation in this bacterium.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Acetobacter* sp. HA was mainly used in this study. Other *Acetobacter* strains were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The media used for *Acetobacter* were a mannitol medium (0.5% yeast extract, 0.3% pep-

*Corresponding author
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Table 1. Strains and Plasmids used.

Strain or Plasmid	Genotype or Phenotype	Source or Reference
Bacteria		
Acetobacter aceti	wild type	ATCC 23746
A. methanolicus	MB58^T, isolated from septic methanol-yeast process	ATCC 43581
A. pasteurianus	wild type	ATCC 9428
A. xylinum	wild type	ATCC 23769
Acetobacter sp. HA	spontaneous Ap^r derivative of a natural isolate from Korea	This lab. (Chun and Kim, 1993)
E. coli		
AC8001	arg his Δlac Str^r Km^r Gm^r (pJB4JI=pPH1JI::Mu::Tn5)	Zink et *al*, 1984
WA803	met thi incN rep p15A replicon Cm^r Km^r (pGS9::Tn5)	Selvaraj *et al*, 1983
HB101	hsdR hsdM pro leu thi lacY end Ol$^-$ recA Str^r	Maniatis *et al*, 1989
Plasmids		
pRZ102	ColE1::Tn5	Jorgensen *et al*, 1979
pRK2013	IncP Km^r tra PK2$^+$ rep RK2 repE1	Figurski *et al*, 1979

tone, 2.5% mannitol, pH 6.0), a YPG (1% yeast extract, 1% peptone, 3% glycerol), a YPD (1% yeast extract, 1% peptone, 3% glucose), and an AE (1% yeast extract, 1% peptone, 1% glucose, 3% ethanol, 1% acetic acid), and 1.5% of agar was added to make a solid media. *Acetobacter* strains were cultured at 30°C for 3 days and all of the *Escherichia coli* strains were grown at 37°C. For the selection of acetic acid-sensitive mutants by mutagenesis, an aldehyde indicator medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g, pararosaniline 6.25 mg, sodium bisulfite 250 mg, ethanol 20 m*l* per liter) was used (Conway *et al.*, 1987).

Mating Procedure

Bacterial matings were made on the YPG agar plate. Tn5 mutagenesis was carried out using *E. coli* AC8001 (pJB4JI) or WA803 (pGS9) as the donor strain and *Acetobacter* sp. HA, a spontaneous ampicillin resistant strain, as the recipient. To cross the *E. coli* harboring Tn5 and *Acetobacter* sp. HA, 2 m*l* of an exponentially grown culture of donor *E. coli* was mixed with an equal volume of the culture of *Acetobacter* sp. HA grown at 30°C for 3 days in a YPG medium. To complete the mating in broth, the mixture was allowed to stand for 4 hr at 30°C. Alternatively, when the mating was done on a filter, the mixture was filtered onto a nitrocellulose membrane (0.45 μm pore size, 47 mm diameter, Gelman Sciences, Ann Arbor, MI, USA). The filter was incubated at 30°C on the surface of a YPG agar plate for 4 hr. Cells were then resuspended in 4 ml of saline (0.85% NaCl) and appropriate dilutions were spreaded on the selective plate (AE medium containing 100 μg/m*l* of kanamycin and 100 μg/m*l* of ampicillin). Donor and recipient cells were counterselected by using their drug susceptibility. Donor cells were counterselected by 100 μg/m*l* of ampicillin, and recipient cells were counterselected by 100 μg/m*l* of kanamycin. Conjugation/Tn5 mutagenesis frequencies were calculated as the number of transconjugants/recipient cells after conjugation.

Screening of Mutants Defective in Alcohol Dehydrogenase Production

A YPDECa medium (5 g yeast extract, 5 g Polypeptone, 10 g glucose, 30 ml ethanol, and 5 g calcium carbonate per liter) and an aldehyde indicator medium were used for the direct selection of mutants that do not produce alcohol dehydrogenase. The indicator plates were prepared by adding 8 ml of pararosaniline (2.5 mg/m*l* of 95% ethanol) and 100 mg of sodium bisulfite to 400 m*l* batches of precooled (45°C) Luria agar.

Isolation and Manipulation of Chromosomal DNA

Acetobacter sp. HA was grown in an AE medium containing ampicillin (100 μg/m*l*), and the transconjugants were cultured in an AE medium supplemented with kanamycin (100 μg/m*l*) and ampicillin (100 μg/m*l*). The cells (1 g wet weight) were washed once with a TESS buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl, 25% sucrose, pH 8.0) and suspended in 6 m*l* of the same buffer. Two m*l* of lysozyme solution (5 mg/m*l* lysozyme in TESS buffer) was added and mixed, and the mixture was incubated for 30 min at 37°C. Then 1 m*l* of pronase E solution (5 mg of pronase E per m*l* of TESS buffer) was added and the mixture was incubated for 30 min at 30°C. After the addition of 1 m*l* of 10% sodium dodecyl sulfate (SDS), the solution was mixed vigorously. For further purification, the DNA was sedimented with a CsCl-ethidium bromide density gradient.

Restriction Analysis and Southern Hybridization

The DNA was digested with restriction enzymes as described by Maniatis *et al.* (1989). Agarose gel electrophoresis was carried out as described previously (Okumura, 1985). The DNA restriction fragments were separated by agarose (0.7%, w/v) gel electrophoresis. After ethidium bromide staining and denaturation, the DNA was transferred to nitrocellulose membranes (Sigma Inc., USA) in the presence of 20×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) by the Southern blot standard procedure (Southern, 1975). The DNA was fixed to the membranes by incubation at 80°C for 2 hr. The labelling of probes and their subsequent filter hybridization and detection were carried out using the Digoxigenin non-radioactive DNA labelling and detec-

tion kit (Boehringer Mannheim., Germany) in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

Conjugal Transfer of Tn5 into *Acetobacter* sp. HA
Table 2 shows the frequencies of pGS9 and pJB4JI into *Acetobacter* sp. HA. Mating was performed either in broth or on a filter as described in the Materials and Methods. Transfer from donor plasmids to *Acetobacter* sp. HA occured at frequencies ranging from 6.24×10^{-8} (pJB4JI used as the donor) to 2.72×10^{-6} (pGS9 used as the donor) when it was carried out in broth. In filter matings, transconjugants were observed at frequencies of approximately 2.79×10^{-1} (pJB4JI used as a donor) to 6.20×10^{-1} (pGS9 used as a donor). This result suggests that the filter matings have a much higher transfer efficiency than the broth matings.

P-type plasmid, pJB4JI, contains both bacteriophage Mu and transposon Tn5. When a plasmid containing bacteriophage Mu is introduced to a Mu-sensitive enteric bacterium, zygotic induction occurs and the recipient cell does not survive. In contrast, pJB4JI fails to produce viable Mu bacteriophage, suggesting that this vector should be an safe and efficient vector for the introduction of transposons (Ely and Croft, 1982). In this study, we provide a means of introducing Tn5 into an *Acetobacter* sp. HA by conjugation with the P-type suicide plasmid pJB4JI which carries the Tn5 inserted into prophage Mu. Donor plasmid pJB4JI is not stable after being transferred from *E. coli*, presumably due to the prescence of Mu (Zink *et al.*, 1984). This results in the loss of plasmid and the segregation of markers, thereby allowing selection of $Km^rGm^sAp^r$ transconjugants in which Tn5 has potentially transposed. The transfer of pJB4JI from *E. coli* into *Acetobacter* sp. HA occured at a high frequency since the presence of Mu does not result in the killing of the recipient cells. From these results, it is likely that the Tn5 only transferred into the chromosomal DNA but did not maintain the Mu-containing plasmid in *Acetobacter* sp. HA.

It was also found that the another suicide vehicle pGS9, p15A group plasmid with N-transfer system and transposon Tn5 is more effective for *Acetobacter* sp. HA than for *Rhizobium* (Selvaraj, 1983). All these results suggested that the N type plasmid transfer system would be applicable to *Acetobacter* sp. HA.

In addition, no plasmid could be found in *Acetobacter* sp. HA and transconjugants (data not shown). This could be presumed as the result of pGS9 and pJB4JI not having been properly introduced into the chromosome of the recipient cell.

Conjugal Transfer under Various Conditions
Table 3 shows the conjugal transfer frequencies with pGS9 and pJB4JI using the recipients grown in a medium containing mannitol, glucose (YPD), glycerol (YPG), or ethanol (AE) as the carbon source. As shown in Table 3, glycerol seems the most suitable. Transfer from donor plasmid pGS9 to *Acetobacter* sp. HA revealed the highest frequency (6.20×10^{-1}) in the YPG medium and this frequency was 134 times higher compared to the frequency found in the mannitol medium (0.46×10^{-2}). When pJB4JI was used as the donor, the frequency in the YPG medium (2.79×10^{-1}) was 53 times higher compared to the frequency in mannitol medium (0.53×10^{-2}).

The effects of mating time on the conjugal transfer frequency when the filter mating method is used, are shown in Figs. 1 and 2. Conjugal transfer frequencies of pGS9 and pJB4JI were maximum when 6 and 8 hr elapsed, respectively. The number of conjugants per filter was almost constant between the 6th and the 8th hour, and then it decreased. From these results,

Table 2. Conjugal transfer frequencies of pGS9 and pJB4JI into *Acetobacter* sp. HA[a].

Mating conditions	Plasmid	Frequency (conjugants/recipient)
In broth	pGS9	2.72×10^{-6}
	pJB4JI	6.24×10^{-8}
On filter	pGS9	6.20×10^{-1}
	pJB4JI	2.79×10^{-1}

[a] Exponentially grown cultures of donor strains were mixed with equal volumes of exponentially grown cultures of *Acetobacter* sp. HA. In broth mating, incubated for stationary for 4 hour and then spread on selective medium after appropriate dilution. On filter mating, the mixture was filtered, incubated for 4 hour on a YPG agar at 30°C, and then spread on selective medium after appropriate dilution. Transconjugants were screened on an AE agar containing the antibiotics (100 μg/*ml* of ampicillin and 100 μg/*ml* of kanamycin).

Table 3. Effects of carbon sources on conjugation frequencies in *Acetobacter* sp. HA with pGS9 and pJB4JI[a].

Carbon sources	Plasmids	Recipient (cells/filter)	Conjugant (cells/fliter)	Frequency (conjugant/recipient)
Mannitol	pGS9	4.8×10^{7}	2.20×10^{5}	0.46×10^{-2}
	pJB4JI	4.8×10^{7}	2.56×10^{4}	0.53×10^{-2}
Glucose	pGS9	2.4×10^{6}	1.20×10^{4}	0.50×10^{-2}
	pJB4JI	2.4×10^{6}	3.40×10^{4}	1.42×10^{-2}
Glycerol	pGS9	5.6×10^{6}	3.45×10^{6}	6.20×10^{-1}
	pJB4JI	5.6×10^{6}	1.56×10^{6}	2.79×10^{-1}
Ethanol	pGS9	1.0×10^{7}	0.62×10^{6}	0.62×10^{-1}
	pJB4JI	1.0×10^{7}	0.80×10^{5}	0.80×10^{-2}

[a] The ratio of recipient cells to donor cells was approximately 1:50.

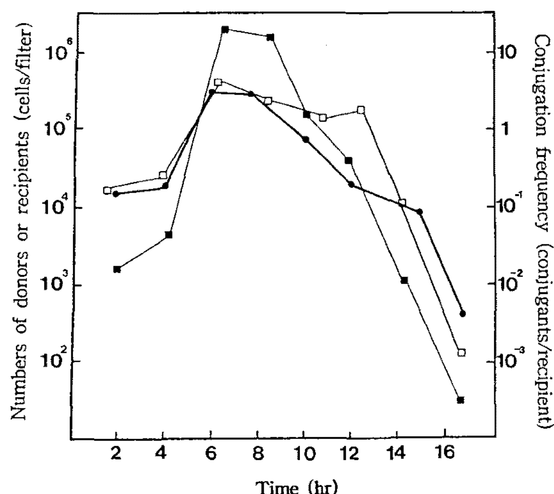

Fig. 1. Effect of mating time on conjugation frequency. *Acetobacter* sp. HA was conjugated with pGS9 by the filter mating method. ●, number of recipients; ■, number of donors with pGS9; □, conjugation frequency with pGS9.

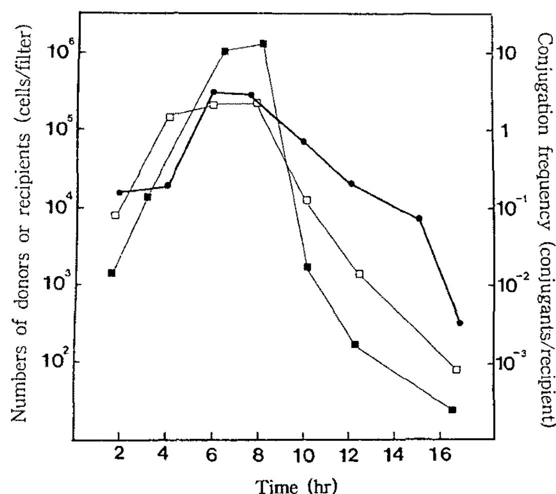

Fig. 2. Effect of mating time on conjugation frequency. *Acetobacter* sp. HA was conjugated with pJB4JI by the filter mating method. ●, number of recipients; ■, number of donors with pJB4JI; □, conjugation frequency with pJB4JI.

it was determined that the most suitable procedure for introducing Tn5 into *Acetobacter* sp. HA is as follows. *Acetobacter* sp. HA should be grown in a YPG medium, and mating should be performed on a filter for 6 to 8 hr.

Table 4 shows the conjugal transfer frequencies of Tn5 into various *Acetobacter* strains. The plasmids pGS9 and pJB4JI containing Tn5 were efficiently introduced into *Acetobacter* strains except *A. methanolicus* ATCC 43581 and *A. pasteurianus* ATCC9428. It is suggested that Tn5 can be introduced into many strains of *Acetobacter* at high frequencies by conjugation.

Identification of ADH-Deficient Mutants

Mixtures of pararosaniline and bisulfite are often referred to as Schiff reagents and have been widely used: to detect aldehyde; to detect sugars in glycoproteins after periodic acid oxidation; and in broth tests for organisms such as *Zymomonas mobilis* which secrete aldehydes (Conway *et al.*, 1987). These components were incorporated into a solid medium which was relatively nontoxic and could be used to identify clones expressing enzymes which produce aldehyde. Ethanol diffused into the cells, where it could be converted to acetaldehyde by alcohol dehydrogenase (ADH). The leuco dye served as a sink, reacting with acetaldehyde to form a Schiff base which was intensely red. This reaction was expected to shift the equilibrium for alcohol dehydrogenase toward aldehyde production. The red product was still quite soluble and diffused into the surrounding agar. ADH-deficient mutants with pGS9 appeared on this indicator plate ranging from white to medium shades of red, depending upon the level of background

Table 4. The frequency of conjugal transfer of Tn5 in *Acetobacter* strains using filter mating method[a]

Strains	Frequency (conjugants/recipient)	
	pGS9	pJB4JI
A. aceti ATCC23746	3.30×10^{-3}	1.06×10^{-4}
A. methanolicus ATCC43581	--- ND*	--- ND
A. pasteurianus ATCC9428	1.09×10^{-7}	4.45×10^{-8}
A. xylinum ATCC23769	2.00×10^{-2}	0.48×10^{-2}

* ND, not determined. [a] Experiments were performed as described in footnote a of Table 2.

color in the plate (Fig. 3).

In addition, colonies selected by aldehyde indicator plates were transfered onto a YPDECa plate containing allyl alcohol to validate the result of the aldehyde indicator plates (data not shown). Since aldehydes, in which the keto group is conjugated with a double or triple bond, are potent protein-alkylating agents, allyl alcohol ($CH_2=CHCH_2OH$) was an effective suicide substrate for ADH (Lutstorf *et al.*, 1968). Although *Acetobacter* sp. HA was sensitive to very low concentrations of allyl alcohol, Tn5-induced mutants were found to be resistant to high concentrations, which suggests that it no ADH activity had been produced.

Physical Characterization of Transconjugants

The objective of this research was to determine whether the Tn5 mutagenesis with pGS9 and pJB4JI could be utilized in the genetic studies of *Acetobacter* sp. HA. To confirm whether chromosomal Tn5 insertions were generated in *Acetobacter* sp. HA, *Eco*RI-digested DNA from several randomly picked kanamycin and ampicillin resistant Tn5 mutants, were separated by agarose

Fig. 3. Aldehyde indicator plate.
After overnight incubation, ADH positive clones (arrowheads) showed intensely red color, surrounded by a zone of dye diffusion. Tn5-induced ADH negative mutants with pGS9 appeared white to pink and showed a halo of dye clearing.

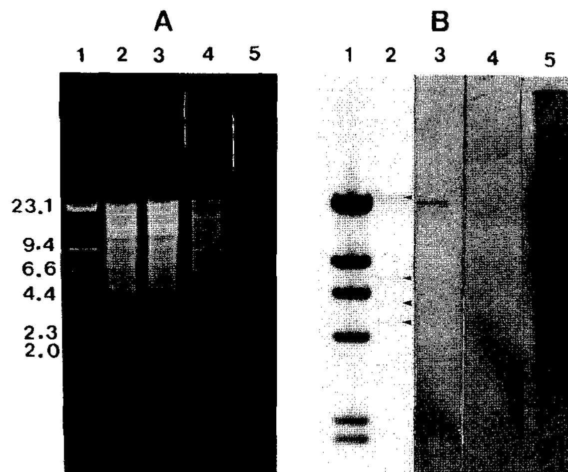

Fig. 4. Southern hybridization analysis of genomic DNA isolated from the *Acetobacter* sp. HA and transconjugant obtained by Tn5 mutagenesis.
Lane 1, Size markers (kb) from wild-type bacteriophage lambda DNA digested with *Hin*dIII; Lane 2, transconjugants with pJB4JI; lane 3, transconjugant with pGS9; lane 4, *Eco*RI-digested genomic DNA of *Acetobacter* sp. HA; Lane 5, pRZ102. **A**, Agarose gel of *Eco*RI-digested genomic DNA isolated from transconjugants and pRZ102. **B**, Hybridization pattern of corresponding lanes with Tn5 probe labelled with digoxygenin. The bands indicated by arrowheads, present in lane 2 of **B**, corresponds in size to 5-kb, 6-kb, 7.5-kb, and 24-kb bands of low fluorescence intensity.

gel electrophoresis and analyzed by Southern hybridization using the digoxigenin-labelled Tn5 (pRZ102; ColE1:: Tn5) probe described in Materials and Methods. As shown in Fig. 4, Tn5 was present in the chromosomal DNA of each strains tested.

As is apparent from Fig. 4, use of the Tn5 probe resulted in single band being observed for one mutant with pGS9. This mutant contained Tn5 insertions into an approximate 23-kb *Eco*RI fragment. As *Eco*RI does not cut within Tn5, this result indicate that this mutant contains a single copy of the transposon.

In most cases only a single insertion of Tn5 was detected; however, it is fairly common for Tn5 mutagenesis to generate anomalous mutant clones (Simon, 1989) and it is evident from the result the mutant with pJB4JI contained four insertions (Fig. 4). In this case, hybridization with Tn5 probe (pRZ102; ColEI::Tn5) revealed the four bands correspond in size approximate 5-kb, 6-kb, 7.5-kb, and 24-kb *Eco*RI fragment. Such multiple Tn5 insertions are difficult to detect without an elaborate genetic system, and from this observation it can be concluded that the mutant somewhat unstable and can readily translocate to new sites within the chromosome. This uncommon result is likely attributed to imprecise exision of Tn5 after transposition and may be associated with bacteriophage Mu (Zink *et al.*, 1984). It has been proposed that abortive replication of Mu results in this exceptional multiple Tn5 insertions (Ely et *al.*, 1982).

This disparity possibly reflects the strain-dependent variations inherent in a Tn5 mutagenesis system (Simon, 1989). Despite the relative inefficiency of the insertion in the chromosome of *Acetobacter*, transposon Tn5 mutagenesis seems to be an effective method of random mutagenesis in this bacterium. This procedure may be used to complement cloning and manipulating of genes in *Acetobacter*, by providing a facile technique of mutant isolation that has many advantages when compared with chemical mutagenesis.

Based on the data we have presented, transposon mutagenesis with the plasmid pGS9 has potential as a tool for constructing single gene Tn5 insertions in *Acetobacter* sp. HA.

The present study presents the first evidence which indicates that the transposition of Tn5 occurs in *Acetobacter* sp. HA and that Tn5 mutagenesis may be applied to successfully isolate mutants of this bacterium. For ease of subsequent molecular genetic analysis and manipulation, it is desirable that a Tn5 mutagenesis procedure generate single chromosomal Tn5 insertions.

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