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NOTES

Mini-Tn*5* Transposon Derivatives for Insertion Mutagenesis, Promoter Probing, and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria

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A collection of Tn*5*-derived minitransposons has been constructed that simplifies substantially the generation of insertion mutants, in vivo fusions with reporter genes, and the introduction of foreign DNA fragments into the chromosome of a variety of gram-negative bacteria, including the enteric bacteria and typical soil bacteria like *Pseudomonas* species. The minitransposons consist of genes specifying resistance to kanamycin, chloramphenicol, streptomycin-spectinomycin, and tetracycline as selection markers and a unique *Not*I cloning site flanked by 19-base-pair terminal repeat sequences of Tn*5*. Further derivatives also contain *lacZ*, *phoA*, *luxAB*, or *xylE* genes devoid of their native promoters located next to the terminal repeats in an orientation that affords the generation of gene-operon fusions. The transposons are located on a R6K-based suicide delivery plasmid that provides the IS*50*$_R$ transposase *tnp* gene in *cis* but external to the mobile element and whose conjugal transfer to recipients is mediated by RP4 mobilization functions in the donor.

The Tn*5* transposon has proven to be of great utility for the insertion mutagenesis of a variety of gram-negative bacteria (1, 2, 23, 30, 31). In an accompanying report (11), we describe the construction of mini-Tn*5* (and mini-Tn*10*) derivatives with nonantibiotic selection markers as vectors for the cloning of genes and their stable chromosomal integration in selected host bacteria. Such cloning vectors were devised primarily for the engineering of bacteria destined for environmental applications. Here we describe further mini-Tn*5* derivatives constructed for other purposes, namely, derivatives carrying various standard selectable antibiotic resistance markers for genetic analysis and engineering of strains for contained use as well as derivatives carrying different types of reporter genes for generating gene and operon fusions.

The delivery system employed for all the mini-Tn*5* transposons is the pUT plasmid that we describe in the accompanying paper (11). pUT is a derivative of pGP704 (25; J. Mekalanos, personal communication) and has as its origin of replication the π protein-dependent origin of plasmid R6K (13); it is only maintained in π protein-producing bacteria, e.g., in a λ*pir* lysogen of *Escherichia coli* K-12. It also carries the origin of transfer, *oriT*, of plasmid RP4, which results in its efficient conjugal transfer to recipient strains from donor strains expressing RP4-conjugative functions like *E. coli* SM10(λ*pir*) (25). pUT additionally carries *tnp**, a mutant *tnp* gene of IS*50*$_R$ that lacks *Not*I sites and that encodes the transposase needed for transposition of the mini-Tn*5* elements.

The first series of mini-Tn*5* elements is depicted in Fig. 1. Each element consists of an *Sfi*I cassette containing an antibiotic resistance gene and a single *Not*I site outside of the cassette that can be used for cloning foreign DNA fragments; flanking these two features are the 19-base-pair I and O Tn*5* ends. The cassettes conferring resistance to tetracycline, streptomycin-spectinomycin, and chloramphenicol are derived from omega interposons (8) and contain T4D phage gene 32 transcriptional terminators as well as stop codons in the three reading frames flanking the antibiotic resistance gene; therefore, they generate strongly polar mutations at their sites of insertion. In addition to the previously described mini-Tn*5* kanamycin element contained in pUT/Km (11), three additional Km^r derivatives were constructed. Two of these carry the kanamycin resistance gene of transposon Tn*903* in opposite orientations (33), whereas a third contains the kanamycin resistance interposon originally isolated from Tn*5* (8). One important feature of these elements is that, as a result of the loss of the transposase-cognate inhibitor (14) along with the pUT delivery system after transposition, a single recipient strain can be used for repeated insertion events with differentially marked minitransposons. The presence of the unique *Not*I restriction site within the mobile elements also permits their use as antibiotic resistance transposon cloning vectors for the chromosomal integration of foreign genes (11).

A second series of mini-Tn*5* elements was devised to generate random operon (type I) and gene (type II) fusions with a variety of reporter genes (Fig. 2). Mini-Tn*5lacZ1* has a promoterless *trp'-'lacZ* fusion with its Shine-Dalgarno sequence downstream of the Tn*5* I end of the transposon to generate operon fusions. Mini-Tn*5lacZ2* creates carboxy-terminal gene fusions with the interrupted chromosomal gene and *lacZ* when inserted in the proper reading frame. These fusions begin with an *'lacZ* moiety at codon 9, which is separated from the interrupted gene by a 49-base-pair linker sequence composed of the 19-base-pair I end of Tn*5* and an additional 30 base pairs resulting from the different steps in the construction. Although Tn*3* (32), Tn*5* (15, 16, 20,

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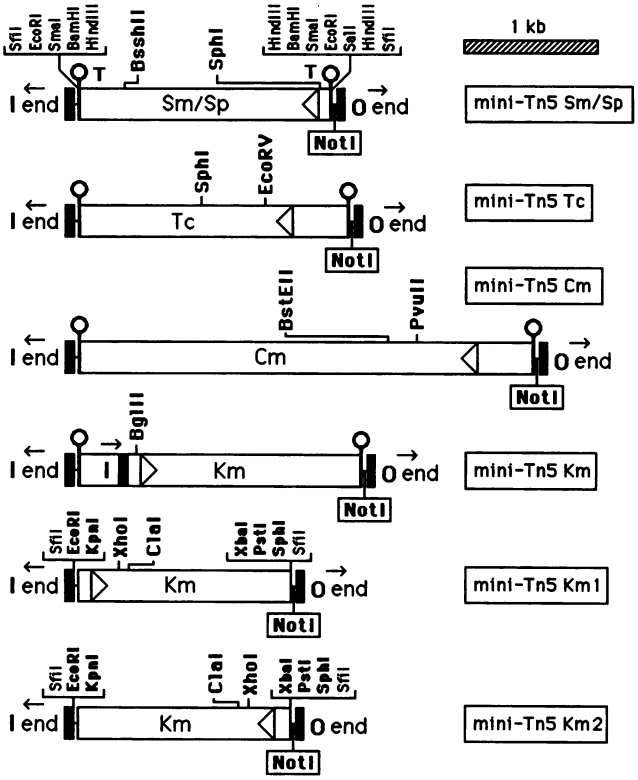

FIG. 1. Structure of mini-Tn*5* elements. Transposons were constructed in vitro by standard recombinant DNA techniques (19). The determinants for streptomycin-spectinomycin (Sp/Sm), tetracycline (Tc), chloramphenicol (Cm), and kanamycin (Km) resistances were obtained as *Eco*RI fragments from the plasmids bearing them as interposons (8). These fragments were subsequently cloned into the single *Eco*RI site of p18Sfi (11), excised as an *Sfi*I fragment, and inserted between the Tn*5* 19-base-pair termini in pUT (11) so that the mobile unit is present in all cases as an *Xba*I-*Eco*RI (partial) portion of the delivery plasmid. The resulting elements were named mini-Tn*5Sp/Sm*, mini-Tn*5Tc*, mini-Tn*5Cm*, and mini-Tn*5Km*, respectively. Mini-Tn*5Km* has one more I end at the left extreme of the kanamycin resistance gene, which itself originates from Tn*5* (8). These four transposons carry strong transcriptional terminators (labeled with a T and a circle) flanking the resistance gene as well as all the restriction sites indicated in mini-Tn*5Sm/Sp* at their termini. Two further mini-Tn*5Km* transposons were constructed that contained the Km^r determinant of Tn*903*, which was excised as a 1.7-kilobase (kb) *Bam*HI fragment from the mini-Tn*10Km* of phage λ1105 (33), and inserted into the *Bam*HI site of pUC18Sfi (11) in both orientations before introduction into the pUT plasmid. The resulting elements mini-Tn*5Km1* and mini-Tn*5Km2* do not carry terminators flanking the resistance gene. Unique sites within the transposons are marked in boldface type, but note that some of them might not be unique in the delivery plasmid pUT (11). All elements shown can be used for insertional mutagenesis or as transposon vectors for the cloning of DNA fragments flanked by *Not*I sites (readily isolated by cloning DNA fragments first into the pUC18 derivatives p18Not and pUC18Not; see reference 11 and the text for explanations).

26, 31), Tn*9* (12), and Mu phage (9, 27) derivatives for the generation of *lacZ* fusions in gram-negative bacteria have been already described, the simplicity of the mini-Tn*5lacZ1* and mini-Tn*5lacZ2* elements makes them easier to use. A mini-Tn*5phoA* transposon was also constructed. This element generates active type II fusions with periplasmic and exported proteins in the same fashion as Tn*phoA* (21, 22), while being only half of its size as a result of the trimming off

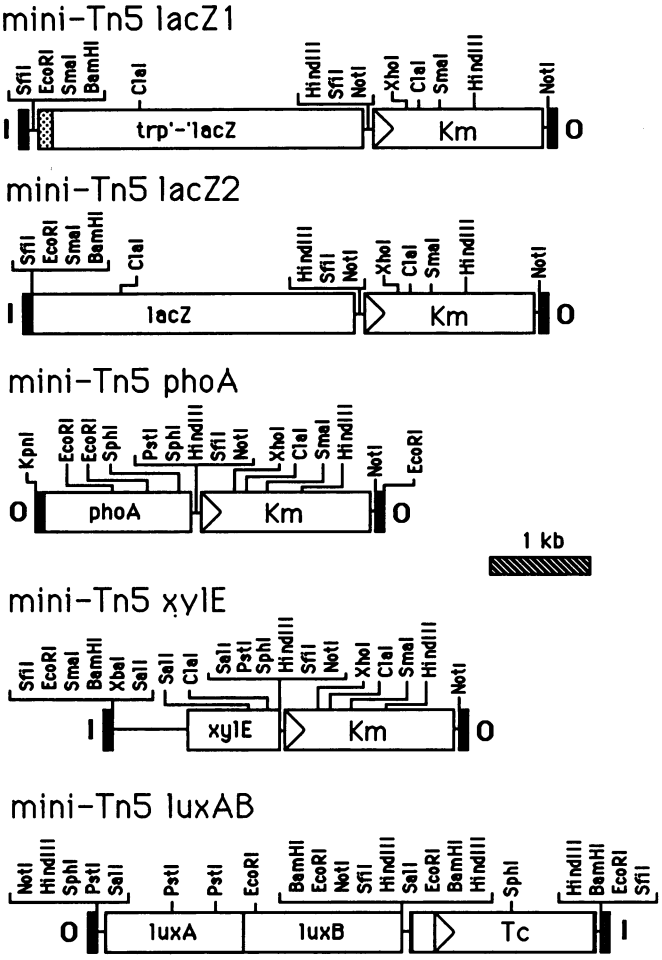

FIG. 2. Organization of promoter-probe minitransposons. Different reporter genes were cloned in the proper orientation at permissive sites next to one of the terminal ends of the mini-Tn*5* elements described above. Since the termini have neither transcriptional terminators nor stop codons in several of the possible frames, they allow generation of type I or II gene fusions (depending on the element used) with target genes. The figure summarizes the structures of the elements. Mini-Tn*5lacZ1* was made by cloning a promoterless *trp'-'lacZ* fusion with pRZ5605 (18) into p18Sfi as a 3.3-kb *Eco*RI-*Hin*dIII (the latter being formerly a *Dra*I site) fragment, with subsequent excision as an *Sfi*I restriction fragment and insertion at the *Sfi*I site of pUT/Km (11). The same strategy was used to create mini-Tn*5lacZ2*, but in this case the *lacZ* structural genes devoid of either transcriptional or translational signals are those of pMLB1034 (29). Construction of mini-Tn*5phoA* involved the cloning of a 1.5-kb *Xba*I-*Bst*EII fragment of pPHO7 (4, 10), containing the structural *phoA* gene into *Xba*I-*Hin*cII-digested pUC18Sfi, and subsequent transfer of an *Xba*I-*Sfi*I fragment to the corresponding sites of pUT/Km. This reconstructs the 5′ terminus of the original Tn*phoA* transposon (10, 22) and leaves the mobile element flanked by two Tn*5* O ends. The *xylE* gene present in mini-Tn*5xylE* was introduced as a partial 1.7-kb *Sal*I fragment of the TOL plasmid of *P. putida* (kindly provided by A. Wasserfallen) into p18Sfi and further cloned into the corresponding site of pUT/Km (11). Finally, mini-Tn*5luxAB* was made by reconstructing the promoterless *luxAB* unit from pFIT001 and pPALE001 (17) as a 3.2-kb *Sal*I-*Bam*HI fragment, which was cloned into the corresponding sites of pUC18Not and further cloned at the *Not*I site of the mini-Tn*5Tc* element. All minitransposons are present as partial *Xba*I-*Eco*RI fragments in the delivery plasmid pUT/Km (11), except mini-Tn*5luxAB*, which is inverted in respect to the rest. Deduced restriction sites present in the mobile elements are indicated.

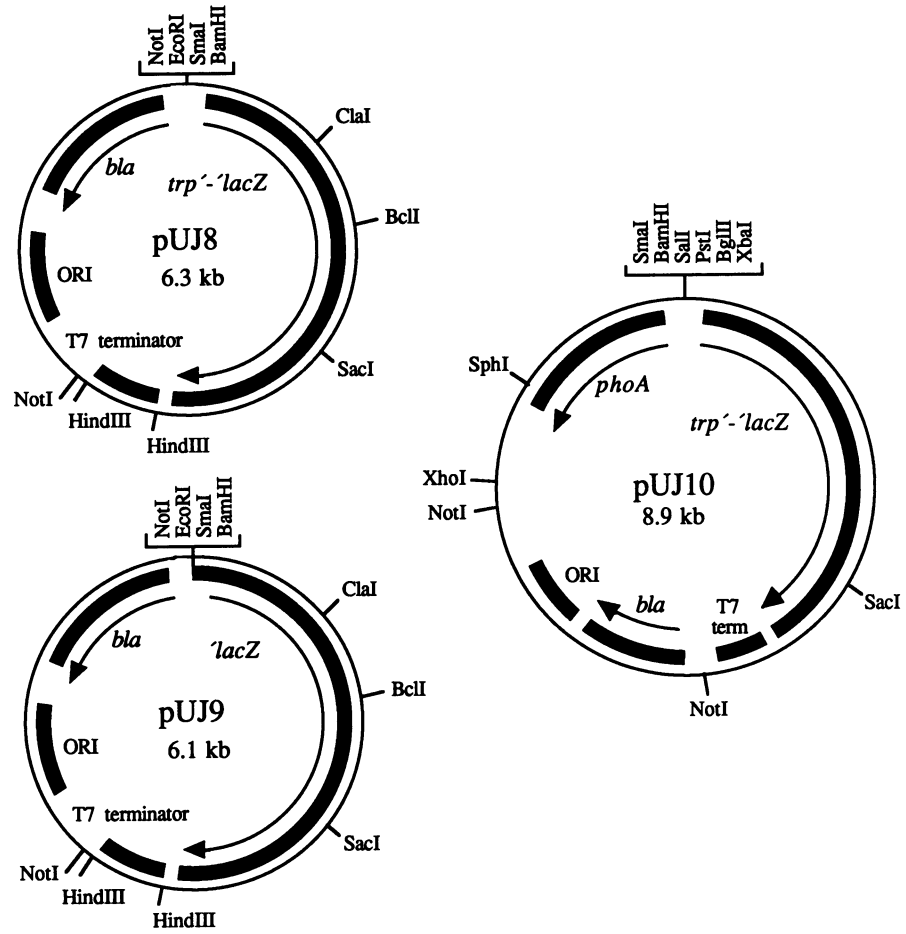

FIG. 3. Auxiliary plasmids for insertion of gene fusions into the chromosomes of target bacteria. pUJ8 allows the generation in vitro of operon fusions with *lacZ* (type I) in a fashion identical to that of other plasmids like pRZ5605 (18), whereas pUJ9 is used for type II fusion construction identical to that of pMLB1034 (29). Both plasmids, however, allow the excision of the fusions obtained as a *Not*I fragment, cloning of the fusions at the *Not*I site of the mini-Tn*5* elements (Fig. 1), and its eventual chromosomal integration through transposition to the chromosome. The same is true for pUJ10, which allows the construction in vitro of divergent type I fusions to *lacZ*/*phoA* reporters and their insertion in the chromosome as *Not*I fragments. The *Not*I fragment of pUJ8 (devoid of inserts) containing the *lacZ* gene is 4.1 kb, that of pUJ9 is 3.9 kb, and the corresponding fragment in pUJ10 with both *phoA* and *lacZ* is 6.7 kb. The other *Not*I fragment, containing in all cases an origin of replication and an ampicillin resistance gene derived from pUC18, is 2.2 kb. Strong T7 phage transcriptional terminators (T) have been added in all cases downstream of the *lacZ* sequence at a former *Dra*I site.

of nonessential sequences during construction. To complete the series, mini-Tn*5xylE* and mini-Tn*5luxAB* elements were developed also. These produce type I fusions to catechol 2,3-dioxygenase of *Pseudomonas putida* (34) and to the luciferase genes of *Vibrio harveyi*, respectively (3, 7, 17). These two reporter genes are convenient alternatives to *lacZ* in a variety of applications (5). We have used all these elements to generate random fusions in *P. putida*.

A typical insertional mutagenesis of a target strain with the elements described above was carried out as follows. Recipient and donor strain *E. coli* SM10(λ*pir*) (25) harboring the pUT derivative with the minitransposon were grown overnight at 30 to 37°C in LB medium (24) in the presence of the required antibiotics. Samples of 50 to 100 μl of each culture were mixed in 5 ml of sterile 10 mM $MgSO_4$ and then filtered through a 1.3-mm-diameter Millipore type HA 0.45μ filter (or an equivalent). The filter was then placed on the surface of an LB plate and further incubated for at least 8 h. The cells grown on the filter surface were then suspended in 5 ml of sterile 10 mM $MgSO_4$ (or 0.85% NaCl if selection is made for Tc^r), and 0.1 to 0.5 ml of the suspension was plated on a medium that counterselects the donor strain and selects recipient cells carrying the transposon marker. With *P. putida*, 100 to 1,000 exconjugants that had acquired the minitransposon antibiotic resistance were obtained per plate, which corresponds to a calculated operational transposition frequency (11) higher than 10^{-6}. Optimal conditions for insertional mutagenesis may have to be adjusted when other strains or species are used as recipients.

Analysis of *P. putida* exconjugants reveals that more than 90% of them arise from authentic transposition of the mini-Tn*5* rather than from cointegration of the whole delivery plasmid into the target chromosome. This is not true when *E. coli* K-12 cells are mutagenized with the R6K-based transposon delivery system described here. We have observed that a significant induction of phage harbored by the donor strain *E. coli* SM10(λ*pir*) takes place during mating, lysogenizing some target strains and therefore impairing the suicide donation system (25). Depending on the recipient strain and the antibiotic used for selection, 10 to 90% of *E. coli* exconjugants may carry the markers of the delivery plasmid. This at present limits the use of the mini-Tn*5* elements in *E.*

coli K-12 with the currently available donor strain *E. coli* SM10(λ*pir*) (25).

In addition to the mini-Tn*5* elements described above, three auxiliary plasmids were constructed (Fig. 3). Two of them, pUJ8 and pUJ9, permitted application of the mini-transposons to the study of the regulation in monocopies of *lacZ* fusions initially generated in vitro. pUJ8 has the same *Eco*RI-*Sma*I-*Bam*HI polylinker of pRZ5605 (18) and produces transcriptional fusions with a promoterless *trp'-'lacZ*. Similarly, pUJ9 has the same *Eco*RI-*Sma*I-*Bam*HI sequence present in pMLB1034 (29) immediately upstream of a promoterless truncated *'lacZ* gene that specifies white colonies on medium with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside when transformed into *lac* mutant *E. coli* strains. Insertion in vitro of an appropriate restriction fragment leads to the generation of a productive *lacZ* gene fusion that is detectable on indicator media. Both pUJ8 and pUJ9 have a strong T7 terminator (6) immediately downstream of the *lacZ* sequence. In both cases, the fusions generated plus their corresponding regulatory signals can be excised as a *Not*I fragment and inserted into any of the mini-Tn*5* derivatives described above for subsequent chromosomal integration. If oriented properly in the delivery plasmid, the *lacZ* fusion is bracketed by two strong termination signals, and hence its regulation will be independent of the site of insertion in the chromosome. Finally, plasmid pUJ10 was created to permit the analysis in monocopy of divergent promoters. This plasmid is a derivative of pCB267 (28), and it has several unique sites allowing insertions of restriction fragments to generate in a single step divergent transcriptional fusions with *lacZ* and *phoA* reporter genes. Like pUJ8 and pUJ9, the unit containing the divergent fusions can be excised also as a *Not*I restriction fragment and subsequently cloned in a mini-Tn*5* for a final chromosomal integration.

The simplicity and variety of the mini-Tn*5* transposons described here will simplify genetic analysis and gene cloning in different gram-negative bacteria. The modular nature of their construction, which allows a facile interchange of functional determinants, provides them with considerable versatility and facilitates their further development and tailoring for specific purposes.

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