Engineering Gram-negative Microbial Cell Factories Using Transposon Vectors

by

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Abstract

The construction of microbial cell factories *à la carte* largely depends on specialized molecular biology and synthetic biology tools, needed to re-program bacteria for modifying their existing functions or for bestowing them with new-to-Nature tasks. In this protocol, we document the use of a series of broad-host-range mini-Tn*5* vectors for the delivery of gene(s) into the chromosome of Gram-negative bacteria and the generation of saturated, random mutagenesis libraries for studies of gene function. The application of these tailored mini-transposon vectors, which could be also used for chromosomal engineering of a wide variety of Gram-negative microorganisms, is demonstrated in the platform environmental bacterium *Pseudomonas putida* KT2440.

Key words: Mini-transposon, Tn*5* transposon, *Pseudomonas putida*, *Escherichia coli*, Synthetic biology, Metabolic engineering, Microbial cell factory, Genome editing

1. Introduction

Mini-transposon vectors allow for the stable insertion of foreign DNA into the chromosome of many types of Gram-negative bacterial targets (*1,2*). Tn*5*-derived elements (*3*) present clear advantages over the use of their plasmid-based counterparts for the random interruption of gene(s), or for the introduction and expression of heterologous genes into several bacterial species. These features include, but are not limited to, (i) maintenance of the corresponding transgenes without antibiotic selective pressure, (ii) long-term stability of the constructs and re-usability of the functional DNA parts, and, furthermore, (iii) mini-Tn*5*–based vectors admit cloning and chromosomal delivery of considerably long DNA fragments (which would be cumbersome to manipulate in other DNA delivery tools). As the transposase gene *tnpA* is lost following each transposition event (*4,5*), one added value of mini-Tn*5* vectors is the possibility to use them recursively in the same microbial host, provided that they bear different selection markers. Since the TnpA transposase tends to act in *cis* (*6*), it promotes the insertion of DNA sequences borne by the plasmid irrespective of any previous DNA insertions in a given chromosome. These features allow for the delivery and integration of various DNA cargoes into the same target genome. However, the original layout of such mini-transposon vectors was not exempt of downsides. One of them is the unavoidable inheritance of long, non-functional DNA fragments stemming from the intricate cutting-and-pasting DNA methods available at the time when the original vectors were constructed. These procedures were also afflicted by the presence of an excessive and inconvenient number of non-useful restriction sites scattered along the plasmids, and the suboptimal transposition machinery encoded therein.

Martínez-García *et al*. (*7*) thoroughly revisited the original mini-Tn*5* transposon vector concept. The most attractive features of the mini-Tn*5*–aided mutagenesis procedure have been enhanced while each of its drawbacks [identified along >20 years of use in many independent laboratories worldwide (*8*)] has been eliminated. The functional modules that constitute the vector (including the mosaic elements, MEs) have been edited to minimize the length of the corresponding DNA fragments, improving their functionality and making them entirely modular and exchangeable. The final product was the entirely synthetic plasmid construct termed pBAM1 (*b*orn-*a*gain *m*ini-transposon). This design was soon followed by a series of synthetic, modular broad-host-range mini-Tn*5* plasmids derived from pBAM1. These vectors, termed pBAMDs vectors (*9*), enable the possibility of easy cloning and

subsequent chromosomal insertion of functional DNA cargoes with three different and interchangeable antibiotic resistance markers. Another set of pBAM1-derivative plasmids, termed pBELs and pBEXs vectors (*10*), were designed to exploit the possibility of delivering DNA cargoes under the control of regulated gene expression modules (i.e., $LacI^Q/P_{trc}$ in pBELs vectors or XylS/*Pm* in pBEXs vectors). Furthermore, the antibiotic-resistance determinants in the mini-transposon modules of the pBELs and pBEXs vectors can be removed by means of the FLP recombinase from *Saccharomyces cerevisiae* (*11*). In all the cases presented above, the functional parts of the mini-transposon vectors can be easily swapped by digestion with the appropriate restriction enzymes, allowing for the easy shuffling of each DNA element as needed. Finally, the multiple cloning site of all the mini-transposon vectors share the same set of restriction sites, which eases the subcloning of DNA cargoes by making them compatible with plasmids from the *S*tandard *E*uropean *V*ector *A*rchitecture (SEVA) initiative (*12,13*).

The expansion of the available mini-transposon tools is a step forward in our efforts to purposely engineer microbial cell factories, mainly based on environmental bacteria. *Pseudomonas putida* KT2440 is a robust host for strong oxidative bioreactions (*14-16*), it exhibits the GRAS (i.e., *g*enerally *r*ecognized *a*s *s*afe) status (*17*), and it has the inherent ability to grow on a wide range of (often, difficult-to-degrade) substrates (*18-21*). Re-wiring its extant genetic features to extend its metabolic potential –or even introducing new-to-Nature functions– is a task continuously undergoing in our laboratory. In the present protocol, we detail all the experimental steps needed to either (i) construct random mutant libraries by mini-Tn*5* insertions to explore gene-function relationships, or (ii) deliver a DNA cargo into a target chromosome, with the option of FLP-catalyzed removal of the antibiotic resistance determinant.

2. Materials

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids and vectors used in this protocol are described in Table 1 and 2, respectively.

2.2. Culture media and reagents preparation

Unless otherwise stated, all the culture medium components and chemicals described below were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Whenever appropriate, please follow all waste disposal regulations when disposing waste materials.

1. LB medium is used as the nutrient-rich culture medium in routine cultivations of both *P*. *putida* and *Escherichia coli*. The components of LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl) were dissolved and brought up to 1 L with deionized H_2O, and sterilized by autoclaving (20 min at 121°C and 1.05 kg/cm^2). This culture medium can be indefinitely stored at room temperature protected from light.
2. Nutritional selection is employed as a general strategy to counter-select for *P*. *putida*. M9 minimal medium, supplemented with sodium citrate at 0.2% (w/v) as the sole carbon source (*see* Note 1) and $MgSO_4$ at 2 mM, is used for this purpose since *E*. *coli* cannot grow on citrate. A 10× stock of M9 salts is prepared by dissolving 42.5 g of $Na_2HPO_4{\cdot}2H_2O$, 15 g of KH_2PO_4, 2.5 g of NaCl, and 5 g of NH_4Cl in deionized H_2O up to a final volume of 500 mL. A 20% (w/v) sodium citrate solution is prepared by dissolving 20 g of anhydrous sodium citrate in deionized H_2O up to 100 mL, and a 1 M $MgSO_4$ solution is prepared by dissolving 12 g of anhydrous $MgSO_4$ in deionized H_2O up to 100 mL. All these solutions are separately sterilized by autoclaving as indicated above, and can be indefinitely stored at room temperature. Components are mixed and diluted as appropriate with deionized H_2O to prepare M9 minimal medium immediately prior to use.
3. To elaborate solid media [containing 1.5% (w/v) agar], add 15 g of bacteriological agar (e.g., BactoAgar™; Becton-Dickinson Diagnostics Co., Sparks, MD, USA) to 1 L of LB medium and autoclave it as indicated above. In the case of M9 minimal medium plates, prepare a 1.6% (w/v) agar suspension in deionized H_2O, autoclave it separately from the other medium stock solutions, and then mix an adequate amount of this suspension with the rest of the M9 minimal medium components to reach a final agar concentration of 1.4% (w/v). Antibiotics and other additives are added when the molten agarized medium reaches ca. 50°C. Distribute the agarized culture media in plastic Petri dishes (25 mL of molten culture medium per 90-mm Petri dish), and let the medium solidify at room temperature. Culture medium plates are prepared freshly immediately prior to use, but they can be stored at 4°C (ideally for no longer than 1 week, especially if antibiotics were

added to the agarized culture medium).

4. All the antibiotics needed for bacterial selection in this protocol are prepared as concentrated stock solutions in deionized H_2O at the concentrations indicated, sterilized by filtration (0.45 μm), and stored at –20 ºC for several months (*see* Note 2). The concentration of the stock solutions is as follows: ampicillin (Ap), 150 mg/mL; kanamycin (Km), 50 mg/mL; streptomycin (Sm), 50 mg/mL; gentamicin (Gm), 10 mg/mL; and carbenicillin (Cb), 500 mg/mL. The chloramphenicol (Cm) stock solution is prepared at 30 mg/mL in 100% (v/v) ethanol. Unless indicated otherwise, all the antibiotic stock solutions are considered to be 1000× concentrated.
5. To prepare 1× phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, and 137 mM NaCl, pH = 7.0), dissolve 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 0.2 g of KCl, and 8 g of NaCl in 800 mL of deionized H_2O, adjust the pH at 7.0 by dropwise addition of 6 N HCl, and bring the volume to 1 L with deionized H_2O. Sterilize this buffer by autoclaving as indicated above, and store it at room temperature.
6. In order to obtain electrocompetent cells of *P. putida*, prepare a 300 mM sucrose solution by dissolving 10.27 g of sucrose in deionized H_2O up to a final volume of 100 mL, sterilize by filtration (0.45 μm), and keep at room temperature.
7. To wash and prepare bacterial cells for mating, dilute the 1 M $MgSO_4$ solution described above with sterile deionized H_2O to obtain a 10 mM $MgSO_4$ solution.
8. To maintain bacteria as frozen stocks, use 20% (v/v) glycerol in LB medium. Prepare this solution by adding 118 mL of 85% (v/v) glycerol to 382 mL of LB medium, sterilize by filtration (0.45 μm), and keep this solution at room temperature protected from light.

2.3. DNA and general molecular biology techniques

1. To purify plasmids from bacteria, we normally use the QIAprep Spin Miniprep™ kit (Qiagen Inc., Valencia, CA, USA) by following the manufacturer's instructions.
2. Colony PCR is routinely used as the source of template DNA for amplifications. Fresh bacterial colonies (i.e., incubated for <24 h) are taken straight from the agar plate with a sterile toothpick and dispersed into the PCR reaction tube containing deionized H_2O (*see* Note 3).
3. Go*Taq*™ Flexi DNA polymerase (Promega Corp., Madison, WI, USA) is routinely used for PCR amplifications; however, any other DNA polymerase can be used for this purpose by following the

specific manufacturer's indications.

4. Prepare a 10 mM stock solution of deoxynucleotide triphosphates (dNTPs) containing equimolar amounts of dATP, dCTP, dGTP, and dTTP in milliQ water (resistivity ≥18 MΩ/cm at 25°C); and store the solution at –20 ºC for up to 8-12 months.
5. The NucleoSpin™ Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) or the ExoSAP-IT™ PCR product cleanup kit (USB Molecular Biology, Affymetrix Ltd., Santa Clara, CA, USA) are used for purification of PCR products.
6. Agarose gel electrophoresis is routinely used to identify, quantify, and purify DNA fragments. Basic protocols are given elsewhere (*22*), and have to be adjusted for each specific purpose depending on the application.

2.4. Primers used to localize and sequence the mini-transposon insertion point

All the oligonucleotides needed for arbitrary PCR amplifications are indicated below, along with the mini-transposon vectors used for the chromosomal insertions. Primers are purchased from Sigma-Aldrich Co., as desalted, lyophilized DNA, and resuspended in the appropriate volume of milliQ H_2O to obtain 5 μM primer solutions. These solutions are aliquoted and stored at –20°C for several months.

2.4.1. Primers for arbitrary PCR amplifications

Common to all vectors	(i) ARB6: 5'-GGC ACG CGT CGA CTA GTA C*NN NNN NNN NN*A CGC C-3'. This primer is used in arbitrary PCR, round 1 (*23*). In this sequence, *N* represents any nucleotide.
	(ii) ARB2: 5'-GGC ACG CGT CGA CTA GTA C-3'. This primer is used in arbitrary PCR, round 2 (*23*).

2.4.2. Primers specific to the ME-O end of each mini-transposon

pBAMD1-2	(i) ME-O-Km-Ext-F: 5'-CGT CTG TTT CAG AAA TAT GGC AT-3'. This primer is used in arbitrary PCR, round 1 (*9*).
	(ii) ME-O-Km-Int-F: 5'-ATC TGA TGC TGG ATG AAT TTT TC-3'. This primer

	is used in arbitrary PCR, round 2, and for sequencing to map the integration point (*9*).
pBAMD1-4	(i) ME-O-Sm-Ext-F: 5'-CTT GGC CTC GCG CGC AGA TCA G-3'. This primer is used in arbitrary PCR, round 1 (*9*). (ii) ME-O-Sm-Int-F: 5'-CAC CAA GGT AGT CGG CAA AT-3'. This primer is used in arbitrary PCR, round 2, and for sequencing to map the integration point (*9*).
pBAMD1-6	(i) ME-O-Gm-Ext-F: 5'-GCA CTT TGA TAT CGA CCC AAG T-3'. This primer is used in arbitrary PCR, round 1 (*9*). (ii) ME-O-Gm-Int-F: 5'-TCC CGG CCG CGG AGT TGT TCG G-3'. This primer is used in arbitrary PCR, round 2, and for sequencing to map the integration point (*9*).
pBELG and pBELK	(i) pBEL-ME-O-Ext-F: 5'-CTG CGA CAT CGT ATA ACG TTA CTG GTT TC-3'. This primer is used in arbitrary PCR, round 1 (*10*). (ii) pBEL-ME-O-Int-F: 5'-GGG CGC TAT CAT GCC ATA CCG-3'. This primer is used in arbitrary PCR, round 2 (*10*).
pBEXG and pBEXK	(i) pBEX-ME-O-Ext-F: 5'-CTT CTT ACA TTT GGG ACG CTT CGC TG-3'. This primer is used in arbitrary PCR, round 1 (*10*). (ii) pBEX-ME-O-Int-F: 5'-CCT TCC GAC ACC CTG CGT CAA TG-3'. This primer is used in arbitrary PCR, round 2 (*10*).

2.5.3. Primers specific to the ME-I end of each mini-transposon

pBAMD1-2	(i) pBAM-ME-I-Ext-R: 5'-CTC GTT TCA CGC TGA ATA TGG CTC-3'. This primer is used in arbitrary PCR, round 1 (*7*). (ii) pBAM-ME-I-Int-R: 5'-CAG TTT TAT TGT TCA TGA TGA TAT A-3'. This primer is used in arbitrary PCR, round 2, and for sequencing to map the

	integration point (*7*).
pBAMD1-4	(i) ME-I-Sm-Ext-R: 5'-ATG ACG CCA ACT ACC TCT GAT A-3'. This primer is used in arbitrary PCR, round 1 (*9*). (ii) ME-I-Sm-Int-R: 5'-TCA CCG CTT CCC TCA TGA TGT T-3'. This primer is used in arbitrary PCR, round 2, and for sequencing to map the integration point (*9*).
pBAMD1-6	(i) ME-I-Gm-Ext-R: 5'-GTT CTG GAC CAG TTG CGT GAG-3'. This primer is used in arbitrary PCR, round 1 (*9*). (ii) ME-I-Gm-Int-R: 5'-GAA CCG AAC AGG CTT ATG TCA-3'. This primer used in arbitrary PCR, round 2, and for sequencing to map the integration point (*9*).

2.4.4. Primers specific to the mini-transposon vector backbone

Oligonucleotides annealing to specific sequences in SEVA vectors (*12,13*) are routinely used to detect the presence of the Tn*5*-bearing mini-transposon vector backbone in transconjugant cells. If the plasmid is present in such cells, a PCR amplification using primers PS4 [5'-CCA GCC TCG CAG AGC AGG-3'] and PS5 [5'-CCC TGC TTC GGG GTC ATT-3'] generates a DNA amplicon of 225 bp.

2.4.5. Primers to confirm elimination of antibiotic resistances by ectopic expression of the FLP recombinase

When employing the specialized pBELs or pBEXs mini-Tn*5* vectors (*10*), the presence or the absence of antibiotic resistance genes can be easily assessed by colony PCR with primers c*FRT*-Ab-R (5'-GAG AAT AGG AAC TTC GGA ATA GG-3') in combination with either cKm-F (5'-CGG AAT GCT ATG CAG ACG-3', when using pBELK or pBEXK) or cGm-F (5'-CCC GTA TGC CCA ACT TTG-3', when using pBELG or pBEXG). The corresponding expected amplicon lengths are 736 bp (for pBELG and pBEXG) and 535 bp (for pBELK and pBEXK).

2.5. Other laboratory material and equipment and standard procedures

1. Bacteria are routinely grown aerobically in 10-mL plastic test tubes (e.g., 16×100 mm, round bottom tubes; E&K Scientific Products Inc., Santa Clara, CA, USA) containing 3 mL of the corresponding liquid culture medium. *P. putida* KT2440 is incubated at 30°C and *E. coli* strains are incubated at 37°C with rotary agitation at 170 rpm.
2. For electroporation, 2-mm gap width cuvettes (e.g., Gene Pulser™/Micropulser™ electroporation cuvettes; Bio-Rad Laboratories Inc., Hercules, CA, USA) and a bacterial electroporation system (e.g., MicroPulser™; Bio-Rad Laboratories Inc.) are used.
3. For filter-assisted bacterial matings, we recommend the use of mixed cellulose esters filter disks of 0.45-μm pore-size and 25-mm diameter (EMD Millipore Corp., Billerica, MA, USA), Millipore SX0002500 Swinnex™ Syringae filter holders for 25-mm diameter filters, 20-mL Luer-lock™ tip syringes (Becton-Dickinson Diagnostics Co.), and blunt-end filter forceps (e.g., XX6200006P forceps; EMD Millipore Corp.) to manipulate the filter discs.
4. Thermocycler (e.g., T100™ Thermal Cycler; Bio-Rad Laboratories Inc.).
5. Sterile, round plastic Petri dishes (e.g., Nunc™ Lab-Tek™ Petri dishes; Thermo Fisher Scientific Inc., Waltham, MA, USA), either of regular size (i.e., diameter = 90 mm) or bigger plates (i.e., diameter = 140 mm), for screening of colonies or during the generation of random mutant libraries.
6. To spread bacteria onto agar plates, use 5-10 sterile 3-mm diameter glass beads (VWR International, Radnor, PA, USA). Once glass beads are used, immediately dispose them off into a flask with 70% (v/v) ethanol. To recover the glass beads for subsequent use, rinse them twice with deionized H_2O, let them dry overnight at room temperature, and sterilize the beads by autoclaving.

3. Methods

The mini-transposon vectors described in the present protocol have two principal uses: (i) the generation of libraries of random mutants to correlate a particular and observable phenotype to a specific gene (*1,7,8,24*); or (ii) the introduction of heterologous gene(s) randomly into the chromosome of a target Gram-negative bacterium (*2,9,10*). In the first application, a typical random mutagenesis protocol involves three steps: (i) *delivery* of the non-replicative plasmid bearing the mini-transposon into a recipient strain, (ii) *selection* of transconjugants carrying the transposon, and (iii) *storing* the

library for future uses or directly *identifying* the insertion point of the mini-transposon in the genome of selected recipient cells. In the second case, when the objective is to introduce heterologous DNA into a bacterial genome, a previous step is included in which the gene (or genes) of interest has to be cloned into the multiple cloning site of the mini-Tn*5* delivery plasmid. Steps (i), (ii), and (iii) are then followed as described in the specific procedure below. Finally, when using any of the pBELs or pBEXs mini-Tn*5* plasmids (*10*) to deliver a DNA cargo under a controlled expression system, a final extra step is added *ad libitum* to remove the antibiotic marker of the mini-Tn*5* cassette.

The first step of the protocol (i.e., introduction of the mini-transposon plasmid into the recipient strain), could be done either by mating or electroporation. Specific protocols to perform either delivery technique are described below, and a specific application example, in which plasmid pBAMD1-2 (*9*) was employed to generate a library of random insertion mutants of *P. putida* KT2440, is discussed. The main steps of the protocol are summarized in Fig. 1.

*3.1. Mini-Tn*5 *delivery into* P. putida *by conjugation*

Whenever possible, we recommend to use this delivery method since it is more efficient than electroporation of plasmids. Conjugation requires cell contact to transfer DNA from a donor cell to a recipient strain. To establish such intimate contact, donor bacteria produce the conjugative pilus (i.e., a type IV secretion system) that ultimately retracts, bringing both cells together. A number of proteins of the donor bacterial cell form a bridge between both donor and recipient cells forming a mating pair (i.e., Mpf proteins, for *m*ating *p*air *f*ormation). Then, the *relaxosome* (i.e., a complex formed by a relaxase and auxiliary proteins) recognizes the origin of transfer (*oriT*) sequence and move one strand of the target DNA to the recipient cell [for a review on the biology behind this process, please see Zechner *et al*. (*25*) and Ilangovan *et al*. (*26*) and references therein].

To perform a conjugation experiment, one just needs to bring together the donor cell (i.e., bearing the mini-transposon plasmid), the recipient cell (i.e., the target bacterium), and a helper bacterial strain to assist and catalyze the mating process. The mating helper is an *E. coli* strain that provides the conjugation machinery. Typically, this molecular machinery is derived from the IncPα plasmid RP4 (also known as RK2 or RP1), and involves the mobilization (*mob*) and transfer (*tra*) functions (*27,28*),

supplied *in trans*. There are two basic types of helper strains, which express the *mob*/*tra* functions either (i) in a plasmid (e.g., *E*. *coli* HB101 carrying plasmid pRK600; Table 1 and 2), or (ii) integrated in the genome (e.g., *E*. *coli* S17-1 λ*pir*, *E*. *coli* SM10 λ*pir,* or *E*. *coli* MFD λ*pir*; Table 1). When using the former type of helper *E*. *coli* strain, the user needs to include three bacterial strains in the mating process (i.e., setting up a triparental mating). A triparental mating offers the possibility of changing the donor *E*. *coli* strain (which should contain the *pir* gene as a λ*pir* lysogen, e.g., *E*. *coli* CC118 λ*pir* or DH5α λ*pir*) in order to favor counter selection of transconjugants as needed. In the case of performing a biparental mating, the protocol is exactly the same as per the triparental mating procedure, but using only two bacterial strains in the mixture (i.e., the recipient strain and the mobilizing donor cell, that in addition to the mini-Tn*5* plasmid also has the *mob*/*tra* functions integrated in the genome).

As indicated above, in the present protocol we describe a triparental mating using *P. putida* KT2440 as the recipient strain and pBAMD1-2 as the mini-Tn*5* delivery plasmid (*9*). In order to properly generate a random mutagenesis library when working with other recipient bacterial species, it is important to perform several previous tests to determine the optimal experimental conditions for successful DNA transfer, since the expected number of transconjugant colonies depends on several factors such as the nature of the recipient species, the initial amount of recipient cells, the mixing ratio of recipient to donor cells, and the mating incubation time. With the help of these prior experiments, the user should be able to set the appropriate experimental conditions and to estimate the number of plates needed to obtain a saturated library.

1. To prepare the mating mixture, grow the following strains overnight as indicated:
   (i) Donor: *E*. *coli* CC118 λ*pir* (carrying plasmid pBAMD1-2) grown in LB medium added with Ap at 150 μg/ml. Incubate for 18 h at 37ºC with rotary agitation. These cells bear the mobilizable and non-replicative plasmid with the Tn*5* mini-transposon (*see* Note 4).
   (ii) Mating helper: *E*. *coli* HB101 (carrying plasmid pRK600) grown in LB medium added with Cm at 30 μg/ml. Incubate for 18 h at 37ºC with rotary agitation. This bacterium provides the plasmid with the mobilization (*mob*) and transfer (*tra*) functions, encoded in plasmid pRK600.
   (iii) Recipient: *P. putida* KT2440 grown in LB medium at 30ºC with rotary agitation (*see* Note 5).
2. Measure the optical density at 600 nm (OD_{600}) of the bacterial cultures and adjust the bacterial suspensions to an OD_{600} of 1 with PBS in a final volume of 1 mL in a 1.5-mL Eppendorf tube.

3. Centrifuge the cultures at 7200×*g* for 2 min at room temperature, discard the supernatant, and re-suspend the sediment in 1 mL of 10 mM $MgSO_4$ to wash the cells.
4. Mix the three bacterial suspensions in a 1:1:1 ratio (i.e., 150 μL of each suspension) in a test tube containing 4.55 mL of 10 mM $MgSO_4$. The final OD_{600} should be ≈ 0.03 (*see* Note 6).
5. Pass the 5-mL cell suspension through a filter disk (0.45-μm pore-size, 25-mm diameter) using a 20-mL sterile syringe (*see* Note 7). Discard the flow-through and laid the filter, in sterile conditions, onto an LB medium agar plate (cells facing up). Incubate the plate containing the filter (lid facing up) at 30 ºC during the desired mating time (4 h, 6 h, or even 24 h) (*see* Note 8).
6. Gently take the filter from the agar plate with tweezers [blunt-end filter forceps, previously sterilized by quickly dipping them in 70% (v/v) ethanol and flaming] and place it in a 10-mL test tube containing 5 mL of 10 mM $MgSO_4$.
7. Re-suspend the cells in the mating mixture from the filter by vigorous vortexing (at least for 1 min) and plate appropriate dilutions (*see* Note 9) onto M9 minimal medium plus citrate at 0.2% (w/v) and Km at 50 μg/mL (i.e., selective culture medium for transconjugant *P. putida* cells harboring the mini-transposon) (*see* Notes 10 and 11).

*3.2. Mini-Tn*5 *delivery into* P. putida *by electroporation*

If no other choice is available (or just for cases when a DNA cargo is to be integrated into a target genome, where higher frequencies are not that important as they are for the construction of mutant libraries) then electrotransformation is the preferred alternative, mainly due to the fastness and simplicity of the protocol thereof. This technique is based in the transient permeabilization of the cell membrane, that allows for the entry of DNA after applying a high electric field (*24,29,30*).

1. Inoculate a 100-mL Erlenmeyer flask containing 20 mL of LB medium with *P. putida* KT2440 from a fresh LB medium agar plate (or directly from a frozen stock, by scrapping the surface of the stock with a sterile toothpick). Let the cells grow overnight (e.g., 18-24 h) aerobically (170 rpm) at 30ºC.
2. Transfer the saturated culture to a 50-mL Falcon tube and centrifuge it at 3220×*g* for 10 min at room temperature.
3. Discard the supernatant, add 10 mL of 300 mM sucrose and softly resuspend the cell sediment; then, centrifuge the suspension at 3220×*g* for 10 min at room temperature.

4. Remove the supernatant and add 1 mL of 300 mM sucrose, resuspend the cells, and transfer the suspension to a 2-mL sterile Eppendorf tube. Centrifuge at 7200×*g* for 3 min at room temperature.
5. Remove the supernatant, add 800 μL of 300 mM sucrose, resuspend the cells, and centrifuge the suspension at 7200×*g* for 3 min at room temperature. Repeat this washing step once more.
6. Remove the supernatant and add 500 μL of 300 mM sucrose to resuspend the sediment and to obtain a concentrated cell suspension (after the final resuspension step, the concentration of electrocompetent bacteria should be ≈ 5×10^{10} cells/mL).
8. Transfer 100 μL of the electrocompetent cell suspension to a 1.5-mL sterile Eppendorf tube and add ≈ 500 ng of plasmid pBAMD1-2 (in < 10 μL). Pipet the plasmid DNA-cell suspension mix to a 2-mm gap width electroporation cuvette. Care has to be taken to avoid the formation of bubbles at this step, which would reduce the overall efficiency of the electroporation process.
9. Place the cuvette in the MicroPulser™ apparatus, set the electroporation program to EC2, and proceed to electroporate. With these working conditions and using an optimum electric pulse (a single pulse of 2.5 kV with a field strength of 12.5 kV/cm), a time constant (τ) between 4 and 5 ms should be obtained.
10. Immediately after the electric shock, add 900 μL of LB medium to the cuvette and then transfer the cells to a sterile 1.5-mL Eppendorf tube. Incubate the cells aerobically for 3 h at 30 ºC.
11. Spread dilutions of the cell suspension obtained in the step above onto LB medium agar plates containing Km at 50 μg/mL. Since no *E. coli* cells are used in this procedure, there is no need for nutritional selection as performed in mating experiments.

3.3. Isolation and mapping the mini-transposon genomic insertion landing sites

1. If specifically looking for particular phenotypes, select interesting colonies based on a trait (e.g., morphology or color) different from that observed in the wild-type cells, and streak them with a sterile toothpick onto both (i) M9 minimal medium plates containing 0.2% (w/v) citrate and 50 μg/mL Km, and (ii) M9 minimal medium plates containing 0.2% (w/v) citrate and 500 μg/mL Ap. This process is aimed to differentiate between genuine transposition events (i.e., Km^R colonies) from spurious mini-Tn*5* plasmid co-integration incidents (i.e., Km^R and Ap^R colonies). Incubate the plates overnight at 30ºC.
2. Select Km^R and Ap^S clones. Also, use colony PCR amplifications with oligonucleotides PS4 and

PS5 to confirm the absence of the delivery plasmid backbone.

3. Re-streak selected colonies several times onto M9 minimal medium plates containing 0.2% (w/v) citrate and 50 μg/mL Km to make sure of working with pure isolated clones.
5. Make a frozen stock in 20% (v/v) glycerol in LB medium of the selected mutants and store the resulting stocks at –80 ºC. Bacterial frozen stocks can be prepared by growing the cells of interest onto LB medium plates (with the appropriate antibiotics as necessary) overnight, and adding 2 mL of 20% (v/v) glycerol in LB medium thereafter. Cells are gently scrapped from the surface by using a sterile glass rod (i.e., a Drigalski spatula). One mL of the resulting suspension is then transferred into a cryotube (e.g., a 1.8-mL Nunc™ CryoTubes™ cryogenic vial, round bottom). Cells can be stored at –80°C under these conditions for several years without significant loose of viability, provided that the bacterial stock is not repeatedly frozen and thawed.
6. Take mutant clones from the frozen stock and streak the cells onto LB medium agar plates containing 50 μg/mL Km. Grow the cells overnight at 30°C.
7. In order to genetically analyze the transconjugants, firstly choose one of the mini-transposon ends (i.e., ME-I or ME-O) to determine its insertion place in the genome and then perform arbitrarily-primed colony PCR (*31*). The DNA sequence of the primers needed to perform arbitrarily primed PCR amplifications when using the different mini-Tn*5* plasmids is described in *Section 2.4.* (*see* Note 12).
8. Prepare a PCR reaction mix on ice as per the following recipe. Note that most of the components indicated in the recipe are provided along with the commercial *Taq* DNA polymerase. Thoroughly vortex each concentrated solution before pipetting into the PCR reaction mix.
   - 5 μL of 5× Green or Colorless Go*Taq*™ reaction buffer
   - 1.5 μL of 25 mM $MgCl_2$
   - 0.5 μL dNTPs (10 mM)
   - 0.5 μL of dimethyl sulfoxide (when performing amplifications from high G+C DNA templates)
   - 1 μL of 5 μM arbitrary primer
   - 1 μL of 5 μM mini-transposon primer (i.e., ME-I or ME-O)
   - 0.2 μL of 5 U/μL Go*Taq*™ Flexi DNA polymerase
9. Aliquot 15.3 μL of sterile deionized H_2O into each PCR tube.
10. Transfer fresh colonies from agar plates directly into the PCR reaction tube with a sterile toothpick.
11. Distribute 9.7 μL of the PCR reaction mix into each PCR tube.

12. The primers needed for round 1 of the arbitrarily primed PCR amplification are ARB6 together with the external ME-I or ME-O primers (i.e., either ME-I-Ext or ME-O-Ext).
13. The settings for round 1 of the arbitrarily primed PCR amplification are as follows:
    - 5 min at 95ºC
    - 30 s at 95ºC, 30 s at 30ºC, and 1.5 min at 72ºC (6×)
    - 30 s at 95ºC, 30 s at 45ºC, and 1.5 min at 72ºC (30×)
14. Directly take 1 μL of the PCR after running round 1 (i.e., no need to check for positive amplifications in an agarose gel) and use it as the template for round 2 of arbitrary PCR. In this round, use primer ARB2 together with the internal ME-I or ME-O primers (i.e., either ME-I-Int or ME-O-Int) (*see* Note 13). Prepare the PCR reaction mix for round 2 as indicated in step 8 above.
15. The settings for round 2 of the arbitrarily primed PCR amplification are as follows:
    - 1 min at 95ºC
    - 30 s at 95ºC, 30 s at 52ºC, and 1.5 min at 72ºC (30×)
    - 4 min at 72ºC
16. Clean up the PCR product from the second round of the arbitrary PCR amplification using either the NucleoSpin™ Gel and PCR clean-up kit or the ExoSAP-IT™ PCR product cleanup kit.
17. Send the DNA product to sequence (*32,33*) with the ME internal primer used in round 2 of the arbitrary PCR.
18. Analyze the sequencing results. Start by identifying the DNA sequence of the mini-transposon end (i.e., either ME-I or ME-O) (*see* Note 14), and then trim that part and select the rest of the DNA sequence. Use the BlastN program (*34*), available on-line at www.pseudomonas.com/blast/set (*35*), to map the precise genomic coordinates of the mini-transposon insertion (*see* Note 15).
19. Once an interesting mutant is spotted, in which the phenotype-gene has been identified, it is always recommended to complement that mutant back with the identified gene(s) to rule out the occurrence of polar effects, since mini-Tn*5* insertions are known to alter the expression of neighbouring genes (*36,37*).

*3.4. Eliminating the antibiotic resistance marker of specialized mini-Tn*5 *vectors*

When using any of the pBELs or pBEXs mini-Tn*5* vectors (Table 2) to introduce heterologous DNA under the control of an expression system (i.e., $LacI^Q/P_{trc}$ or XylS/*Pm*) (*10*), the genes conferring

resistance to Km (*aphA*) or Gm (*aacC1*) in these transposons can be removed as they are flanked by FLP recombinase target (*FRT*) sequences (*38*). This layout offers the possibility to the user of eliminating that marker by means of ectopic expression of the FLP recombinase from *Saccharomyces cerevisiae* using plasmid pFLP2 (Table 2). The expression of the FLP recombinase in plasmid pFLP2 is driven by the strong, rightward λ promoter (located within the *FLP-cI857* intergenic region) and is regulated by the temperature-sensitive, *cI857*-encoded λ repressor (*39*).

1. Select a transconjugant *P. putida* clone in which the insertion place of the mini-transposon has been successfully localized.
2. Introduce plasmid pFLP2 (*see* Note 16) into this selected clone by either mating or electroporation as described above.
3. Plate the cells on M9 minimal medium plates added with sodium citrate at 0.2% (w/v) and Cb at 500 μg/mL. Incubate the plates overnight at 30ºC. If no discernible colonies are observed after this incubation period, try lowering the Cb concentration to 350 μg/mL.
4. Select two or three independent colonies and re-streak them on M9 minimal medium plates added with sodium citrate at 0.2% (w/v) and Cb at 500 μg/mL. Incubate the plates overnight at 30ºC.
5. Pick single colonies and check for Km or Gm sensitivity and Cb resistance in LB medium plates containing these antibiotics. Double check for the removal of the antibiotic gene by colony PCR using the primers described in *Section 2.4.5*. (i.e., c*FRT*-Ab-R and either cKm-F or cGm-F). Such PCR should give no amplification. If possible, use primers annealing within the gene(s) delivered in the mini-transposon cassette to conduct a colony PCR of the antibiotic-sensitive clone to make sure that the gene(s) of interest have been stably inserted into the target chromosome.
6. Cure plasmid pFLP2 from the selected clone by performing several (at least three) cycles of growth in LB medium without any antibiotic.
7. Plate cells onto M9 minimal medium plates added with 0.2% (w/v) sodium citrate.
8. Pick single colonies and double re-streak onto M9 minimal medium plates added with 0.2% (w/v) sodium citrate plus Cb at 500 μg/mL.
9. Select Cb-sensitive clones and store them as frozen stocks at –80ºC.

*3.5. Preparing and storing a mutant library of mini-Tn*5 *insertions in* P. putida

After obtaining a random mutagenesis library, it is always useful to save it for later analyses. The steps below indicate the procedure to store the library after introduction of plasmid pBAMD1-2 in strain KT2440 as explained in the preceding sections.

1. Spread dilutions of the triparental mating mixture onto selective agar plates in order to obtain an approximate number of ≈ 3000 transconjugant colonies per plate in a regularly sized (i.e., 90 mm) Petri dish. Estimate the number of plates needed to obtain a non-saturated mutant library as indicated by Liberati *et al*. (*37*).
2. Add 2.5 mL of LB medium containing 20% (v/v) glycerol to each overnight-incubated plate and, with the aid of an inoculation loop or a Drigalski spatula gently scrap the cells from the agar surface. Tilt the plate and collect 1-mL of the bacterial suspension with a micropipet (*see* Note 17).
3. Mix the liquid content collected from all the plates, aliquot the resulting suspension in several cryotube vials, and store the library as a series of frozen stocks at –80ºC.

4. Notes and troubleshooting

1. The appropriate culture medium composition has to be defined to select against *E*. *coli* donor/mating helper cells when using other bacterial species as the target strain. As a general rule, try to make use of specific carbon sources in which only the recipient strain grows or take advantage of the auxotrophies of the *E*. *coli* donor/mating helper cells [e.g., most of the laboratory *E*. *coli* strains need thiamine·HCl to grow (*40*)].
2. Avoid repeated freezing and thawing of antibiotic solutions as they may lose effectiveness. We routinely distribute the stock solutions in 0.5-mL working aliquots that are used just a couple of times before discarding them.
3. If no amplification is obtained through colony PCR, genomic DNA can be isolated with a commercial kit (e.g., UltraClean™ Microbial DNA isolation kit; MoBio Laboratories Inc., Carlsbad, CA, USA) and used as the template for amplifications.
4. It is very important to grow the donor bacterial strain in the presence of the antibiotic for which the plasmid backbone carries a resistance gene (e.g., Ap) to avoid inadvertent selection of transposed

donor cells. Note that there is a vector derived from pBAM1 which carries a promoterless *gfp* gene (plasmid pBAM1-GFP), which allows for the visual inspection of successful *gene*::*gfp* fusions after the transposition event.

5. In some cases, incubating the recipient strain at high temperatures (40ºC-42ºC) for a few hours before mating is known to increase the efficiency of the process by inactivating its endogenous DNA restriction machinery.
6. Different ratios of the bacterial strains to be included in the triparental mating could also be tested if needed (e.g., by increasing the amount of donor cells). To do this, simply adjust the volume of each bacterial suspension appropriately with 10 mM $MgSO_4$ to bring the final volume to 5 mL, and proceed as indicated. In the case of integrating DNA cargoes into the bacterial genome, where there is no need of the high numbers of transconjugant colonies usually required for random mutant libraries, one can use just 100 μL of each overnight cultures (i.e., without adjusting the OD_{600} of the individual cultures).
7. If a filter system for bacterial matings is not available, one can simply mix the three bacterial strains in a 1.5-mL Eppendorf tube (e.g., 150 μL of each bacterial suspension adjusted at OD_{600} = 1), centrifuge the cells at 7200×*g*, discard the supernatant, and resuspend the sediment in 25 μL of 10 mM $MgSO_4$ (i.e., a small buffer volume to maximize cell contact). The 25-μL mating mix can be laid onto a 0.45-μm filter disc onto an LB medium plate, or be directly spotted onto the surface of an LB medium plate. In the later case, cells can easily recovered using an inoculation loop and resuspended in 10 mM $MgSO_4$ before plating on a selective culture medium.
8. When creating non-saturated random mutant libraries it is better to use shorter incubation times to maintain cell divisions of transconjugants to a minimum.
9. Depending on the purpose of the experiment, different Petri dishes sizes could be used to recover more transconjugant cells per plate. Adjust the plating volume accordingly.
10. It is also important to plate (i) the donor strain, (ii) the mating helper, and (iii) the recipient strain onto the selective culture medium used to recover transconjugants. These three bacterial strains should not grow in the selective culture medium (i.e., they are used as negative controls).
11. Take into account that different mini-Tn*5*–bearing plasmids need other antibiotics (e.g., Sm at 80 μg/mL or Gm at 10 μg/mL in the case of plasmids pBAMD1-4 and pBAMD1-6, respectively) to select for positive transconjugants.
12. Note that the specific ME primers have to be chosen depending on the mini-Tn*5* plasmid used for

insertions and on the selected ME-end.

13. Clean-up the PCR products obtained after the first round of arbitrarily primed PCR with a commercial kit to eliminate unbound primers in the case of experiencing problems (e.g., no amplification in the second round of arbitrarily primed PCR).
14. If the insertion of transposon cannot been unequivocally mapped using the protocol and primers suggested here, select other set of arbitrary primers, such as primer ARB1 (*23*) or even others as described by Das *et al*. (*31*). Alternatively, a new custom arbitrary primer could be designed by changing the five nucleotides at the 3'-end of the oligonucleotide sequence to match the G+C content of the recipient bacterial strain, thereby increasing the frequency of appearance of that motif in the target genome.
15. For other target bacterial species, use the BlastN tool against the genome of the desired recipient strain. If the complete genome sequence of your favorite microorganism is not available, perform a more general BlastN search in order to identify homologous genes or sequences in related species. Specific primers could then be designed on the basis of these results to sequence the exact locus in which the mini-Tn*5* insertion has occurred.
16. Other plasmids can be used for the ectopic expression of the FLP recombinase, such as plasmid pBBFLP (*41*). The procedure to be followed in this case is essentially the same as the one described in the main protocol, but using tetracycline (at 15 μg/mL) instead of Cb to select for the presence of the pBBFLP helper plasmid. If the insertion of pBELs or pBEXs vectors is carried out in *E*. *coli*, plasmid pCP20 (*38*) is recommended for the FLP-dependent removal of antibiotic-resistance determinants after transposition.
17. It is a good procedure to perform several independent matings in order to yield a representative random mutant library.

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Table 1. Bacterial strains used in this protocol.

Bacterial strain	Description and genotype	Relevant characteristics[a]	Reference
Escherichia coli			
CC118 λ*pir*	Cloning host for plasmids containing an R6K origin of replication; Δ(*ara-leu*) *araD* Δ*lac*X174 *galE galK phoA20 thi-1 rpsE rpoB argE*-(Am) *recA1,* λ*pir* lysogen	Sp^R, Rif^R, Thi^-, Leu^-	(*42*)
SM10 λ*pir*	Cloning and mobilizing host for plasmids containing an R6K origin of replication; F^- *thi-1 thr leu tonA lacY glnV recA*::RP4-2-Tc::Mu, λ*pir* lysogen	Km^R, Thi^-, Thr^-, Leu^-	(*43*)
S17-1 λ*pir*	Cloning and mobilizing host for plasmids containing an R6K origin of replication; F^- *recA1 endA1 thiE1 pro-82 creC510 hsdR17* RP4-2-Tc::Mu-Km::Tn*7*, λ*pir* lysogen	Sm^R/Sp^R, Tp^R, Thi^-, Pro^-	(*44*)
MFD λ*pir*	Cloning and mobilizing Mu-free host for plasmids containing an R6K origin of replication; F^- λ^- ilvG rfb-50 rph-1 RP4-2-Tc::[ΔMu1::*aac(3)IV* Δ*aphA* Δ*nic35* ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	$Apra^R$, Zeo^R, Erm^R, DAP^-	(*45*)
HB101	Mating helper strain; F^- λ^- *hsdS20*(r_B^- m_B^-) *recA13 leuB6*(Am) *araC14* Δ(*gpt-proA*)*62 lacY1 galK2*(Oc) *xyl-5 mtl-1 thiE1 rpsL20 glnX44*(AS)	Sm^R, Thi^-, Leu^-, Pro^-	(*46*)
Pseudomonas putida			
KT2440	Wild-type strain; derivative of strain mt-2 (*47*) cured of the TOL plasmid pWW0	Prototroph	(*48*)

[a] Antibiotic and auxotrophy markers: Apra, apramycin; Erm, erythromycin; Km, kanamycin; Rif,

rifampicin; Sp, spectinomycin; Sm, streptomycin; Tp, trimethropim; Zeo: zeocin; DAP, diaminopimelic acid; Leu, leucine; Thi, thiamine (vitamin B1); Thr, threonine; and Pro, proline. Please note that not all these features are used in the experiments described in the present protocol.

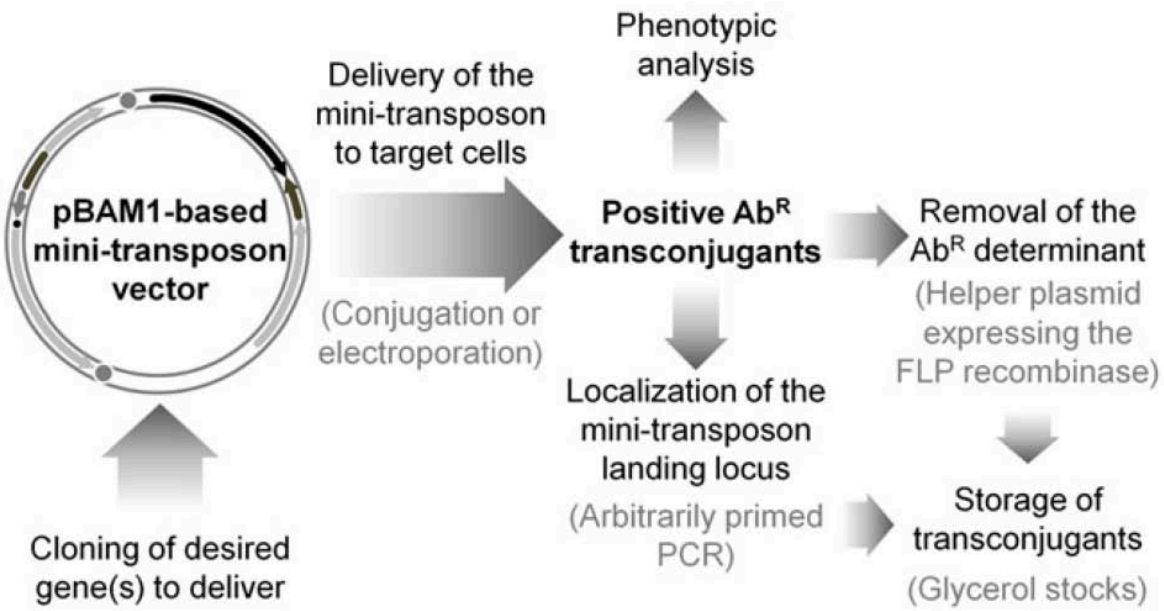
Table 2. Plasmids used in this protocol.

Plasmid	Description and relevant characteristics[a]	Reference
pRK600	Helper plasmid used for conjugation; *oriV*(ColE1), RK2(*mob*$^+$ *tra*$^+$); derivative of plasmid pRK2013 (*49*); Cm^R	(*50*)
pBAMD1-2	Mini-Tn*5* delivery plasmid; *oriV*(R6K), *oriT*; Ap^R, Km^R	(*9*)
pBAMD1-4	Mini-Tn*5* delivery plasmid; *oriV*(R6K), *oriT*; Ap^R, Sm^R/Sp^R	(*9*)
pBAMD1-6	Mini-Tn*5* delivery plasmid; *oriV*(R6K), *oriT*; Ap^R, Gm^R	(*9*)
pBAM1	Mini-Tn*5* delivery plasmid; *oriV*(R6K), *oriT*; Ap^R, Km^R	(*7*)
pBAM1-GFP	Mini-Tn*5* delivery plasmid to create random *gene*::*gfp* fusions by insertion; *oriV*(R6K), *oriT*; Ap^R, Km^R	(*7*)
pBELK	Mini-Tn*5* delivery plasmid for inserting a DNA cargo under the control of the LacI^Q/P_{trc} expression system; *oriV*(R6K), *oriT*; Ap^R, Km^R	(*10*)
pBELG	Mini-Tn*5* delivery plasmid for inserting a DNA cargo under the control of the LacI^Q/P_{trc} expression system; *oriV*(R6K), *oriT*; Ap^R, Gm^R	(*10*)
pBEXK	Mini-Tn*5* delivery plasmid for inserting a DNA cargo under the control of the XylS/*Pm* expression system; *oriV*(R6K), *oriT*; Ap^R, Km^R	(*10*)
pBEXG	Mini-Tn*5* delivery plasmid for inserting a DNA cargo under the control of the XylS/*Pm* expression system; *oriV*(R6K), *oriT*; Ap^R, Gm^R	(*10*)
pFLP2	Helper plasmid used to eliminate antibiotic markers flanked by *FRT* sequences; *oriV*(pRO1600), RK2(*mob*$^+$ *tra*$^+$), *oriT*, λP_R::*FLP*, λ*cI857*, *sacB*; Cb^R	(*39*)

[a] Antibiotic markers: Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Gm, gentamicin; Cb, carbenicillin.

Figure

FIG. 1. Outline of the procedure described in this protocol.



Mini-transposon vectors can be used for delivering gene(s) into a target chromosome in virtually any Gram-negative bacterium, as well as to obtain random mutant libraries. Ab^R, antibiotic resistance.