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A versatile element for gene addition in bacterial chromosomes

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ABSTRACT

The increasing interest in genetic manipulation of bacterial host metabolic pathways for protein or small molecule production has led to a need to add new genes to a chromosome quickly and easily without leaving behind a selectable marker. The present report describes a vector and four-day procedure that enable site-specific chromosomal insertion of cloned genes in a context insulated from external transcription, usable once in a construction series. The use of rhamnose-inducible transcription from *rhaBp* allows regulation of the inserted genes independently of the commonly used IPTG and arabinose strategies. Using *lacZ* as a reporter, we first show that expression from the rhamnose promoter is tightly regulatable, exhibiting very low leakage of background expression compared with background, and moderate rhamnose-induced expression compared with IPTG-induced expression from *lacp*. Second, the expression of a DNA methyltransferase was used to show that rhamnose regulation yielded on-off expression of this enzyme, such that a resident high-copy plasmid was either fully sensitive or fully resistant to isoschizomer restriction enzyme cleavage. In both cases, growth medium manipulation allows intermediate levels of expression. The vehicle can also be adapted as an ORF-cloning vector.

INTRODUCTION

Genetic engineering of plasmids provides a rapid and flexible method of adding individual genes to a bacterial gene complement. However, multiple stable changes to the metabolic architecture of a strain are often desirable, for example providing new metabolic pathways, novel chaperone suites to allow proper folding of exotic target proteins, or exotic tRNA genes to allow smooth translation of foreign codons. Strain-building strategies thus quickly exhaust the repertoire of compatible replicons and drug-selection options. Direct manipulation of the genome itself then becomes an attractive strategy.

Genome manipulation strategies in bacteria at present range from whole-genome synthesis (1) to selective deletion or gene addition via homologous recombination using PCR products (2,3) and in favorable cases oligonucleotide mutagenesis *in vivo* (4). The first, while a technical tour-de-force, is not yet of routine utility. The second is very useful, especially due to generality, but a scar remains at the site of manipulation. The third is a scarless method useful for making small changes, but not for adding functions (4–6). A method less widely used (7) requires two cloning steps, additional steps for drug resistance removal and also leaves a scar. Older approaches that rely on homologous recombination are still in use but are more cumbersome (8).

Methods that focus on gene addition at defined neutral positions are useful complements to the replacement methods. These rely on site-specific recombinase functions. Lambdoid bacteriophages were the first site-specific systems used for adding genes to a bacterial chromosome (9–11). The utility of the site-specific approach has been expanded with a series of different phage integrase vectors, but each system leaves behind a drug-resistance marker, limiting its utility for strain building (12,13).

Transposon Tn7 has a site-specific mode of insertion, with a unique attachment site (*attTn7*) downstream of the highly conserved *glmS* gene. This system has the advantage that it inserts at very high frequency (14) into a single conserved site distributed through many taxa (15). A basic mini-Tn7 (mTn7) donor plasmid vector, pGRG36, was previously designed to deliver cargo to the specific site, *attTn7* (16). The insertion (mobilization) procedure is straightforward: the desired DNA is placed in a multiple cloning site located between the Tn7R and Tn7L end sequences, at which the transposition proteins act to move the element. The donor thus constructed has (i) transposition genes separated from the region to be mobilized; (ii) a selectable marker for the donor plasmid, also separate; and (iii) a thermosensitive replicon, so that the donor is lost from the cell when grown at 42°C. When the donor is present in the cell at 30°C, chromosomal

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insertions accumulate due to the highly efficient action of the transposition proteins. Subsequent growth at 42°C and resulting plasmid curing allows recovery of strains carrying these chromosomal insertions at *attTn7*. In the model situation, 50–100% of colonies obtained at 42°C carry the mobilized element inserted at *attTn7*. It should be noted that the Tn7 system exhibits transposition immunity, so that insertion of a second element into an occupied site occurs at about a 60-fold lower frequency than the first insertion. With suitable transposase mutants the frequency of second insertions is only 3-fold lower than with wild type first insertions (17), although the stability of such tandem elements has not been investigated.

Some complications were anticipated in the use of the basic Tn7 donor plasmid pGRG36, stemming from expression signals in adjacent DNA both on the donor and in the chromosomal destination. Tn7R permits transcription to enter the element from the outside; insertion into chromosomal *attTn7* disrupts the *glmS* transcription terminator, allowing readthrough into the element (17). This is an undesirable situation for the introduction of alien genes, particularly for those encoding toxic products. The expression context on the donor plasmid could also be problematic for the intermediate construct: adjacent to Tn7R is *araC*, reading into the element without a terminator; adjacent to Tn7L is *ori-pSC101*, with transcription also reading toward the element.

We based the design of the new vector, pMS26, on the backbone of pGRG36, with a new cloning region cassette between the sites of transposition protein action, Tn7L and Tn7R. To insulate expression of cloned genes from external signals, transcription and translation terminators are placed immediately inside the Tn7 ends. The design includes *rhaBp,* a rhamnose-regulatable promoter; convenient cloning sites and rapid protocol; and adaptability for use as a vector to clone open reading frames (ORFS) stripped of their native expression signals. ORF clones can then be expressed at the experimenter's direction using tightly regulated expression signals provided by the design for the cargo ORF. Due to the high efficiency and easy cloning strategy adopted, the present method has significant advantages in rapidity of construction. Performance was compared with familiar examples in *E. coli* using *lacZ* expression with or without its native promoter. In addition, complete plasmid protection via expression of the foreign methyltransferase, M.FnuDII (m5CGCG) was demonstrated using this cassette.

MATERIAL AND METHODS

Nomenclature

Genetic elements and fusions are designated according to the recommendations of ref. (18) and the American Society for Microbiology. For brevity, 'mini-Tn7' is abbreviated to '*mTn7*'; it signifies a genetic element mobilizable by the Tn7 transposition proteins. Mobilization results from action of those proteins on the two ends of the element and moves the element with all DNA between the ends to a new location. Each *mTn7* (Table 1) is named here for the cargo that it carries, with an allele number distinguishing different variants of a similar construction. For example, *mTn7Φ(rhaBp-lacZ)1* carries a fusion of the rhamnose promoter to the *lacZ* open reading frame (with one configuration of translation signal) while *mTn7Φ(rhaBp-lacZ)2* carries a different such fusion (a different translation signal). *mTn7Φ(rhaBp-lacZp-lacZ)* carries the *lacZ* transcription unit downstream of the rhamnose promoter. USER: Uracil Specific Excision Reagent (NEB #M5505). USERBstBI: refers to the combination of Nt.BbvCI and BstBI that creates long extensions for efficient USER-enabled cloning described below. These extensions are not the same as those created by the combination of Nt.BbvCI and XbaI described for the original USER-enabled vector (19).

Mini-Tn7 (*mTn7*) elements

The elements listed in Table 1 were constructed by plasmid digestion and ligation with high concentration ligase (for synthetic segment received from Genscript), or processing of PCR products obtained using primers listed in Table 2 followed by USER assembly as described below. Junction sequences were verified for all constructions. Sequences were assembled and analyzed using the LaserGene suite from DNAStar. pMS26, pMS33 and pMS34 and sequences of all plasmids and mTn7 elements are available at Addgene (http://www.addgene.org/Elisabeth_Raleigh).

Plasmids and Synthetic DNA

pGRG36 (16) was obtained from N. Craig (Johns Hopkins University School of Medicine). Oligonucleotides were obtained from IDT. The *T7Et-rhaBp-rrnB1t* cassette was obtained from Genscript. This cassette was cloned between the PacI and AscI sites of pGRG36 to create pMS26.

Enzymes and size standards

All molecular biology reagents [restriction enzymes, OneTaq Hot Start DNA polymerase, ligase (NEB202M),

Table 1. Plasmids and *mTn7* donors

Vector	Source	*mTn7* cargo	Description
pMC63	NEB	Not relevant	Substrate for modification by M.FnuDII (26)
pGRG36	N.Craig	*MCS GRG36*	Tn7 delivery vector; contains *tnsABCD* and Tn7 left and right ends (16)
pMS26	This work	*MCS MS26*	Multiple cloning site of pGRG36 replaced with insulated cloning and expression cassette.
pMS33	This work	*Φ(rhaBp-lacZ)2*	*rhaBp*-regulated *lacZ* ORF with short translation signal (STS)
pMS34	This work	*Φ(rhaBp-lacZ)1*	*rhaBp*-regulated *lacZ* ORF with long translation signal (LTS)
pMS36	This work	*Φ(rhaBp-lacZp-lacZ)*	*lacZ* transcription unit with *lacZp* downstream of *rhaBp*
pMS37	This work	*Φ(fnuDIIM)1*	*rhaBp*-regulated *fnuDIIM* ORF

Table 2. Oligonucleotide primers used in this work

	Primer and description	Sequence
1	*lacZp* upstream	5′-GGAGACATUCGGGCAGTGAGCGCAACG-3′
2	*LacZ* downstream	5′-GGGAAAGUGAAAAAGAATAAACCGAACATCCA-3′
3	*lacZ* upstream (*short translation signal*)	5′-GGGAAAGU*CACAGGAGGGACGTC*ATGACCATGATTACGGATTCAC-3′
4	*lacZ* upstream with (*long translation signal*)	5′-GGAGACATU*CATAACAATTTCACACAGGAGGGACGTC*ATG ACCATGATTACGGATTCAC-3′
5	*fnuDIIM* upstream with (*long translation signal*)	5′-GGAGACATU*CATAACAATTTCACACAGGAGGGACGTC*ATG AAAATTAAAGAAAAACGAG-3′
6	*fnuDIIM* downstream	5′-GGGAAAGUTTAATCTGTTTCTTTTTTAAAATATAG-3′
7	*attTn7* upstream	5′-AATCTGTAACGTTCCGGGTTC-3′
8	*attTn7* downstream	5′-CATTAATAACGAAGAGATGAC-3′

Note. Primers 1–6 include upstream (5′-GGAGACATU) or downstream (5′-GGGAAAGU) sequences needed for subsequent USER cloning. The 3′-end of primer 1 anneals 133 bp upstream of *lacZ* ATG; the 3′-end of primer 2 anneals 80 bp downstream of *lacZ* stop codon TAA; primers 3 and 4 to 17 nucleotides of *lacZ* open reading frame beginning with ATG; primer 5 anneals to 22 nucleotides of *fnuDIIM* gene starting at ATG; primer 6 anneals to 26 nucleotides downstream of *fnuDIIM* gene ending at stop codon TAA. Translation initiation signals are italicised. Primers 7 and 8 flank the *glmS* terminator, into which the mini-Tn7 (*mTn7*) inserts.

Table 3. Strains used

Strain	Genotype	Source, reference
ER2566	F⁻ *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*–TetS*)2 [dcm] R(zgb-210::Tn10*–TetS*) endA1 Δ(mcrC-mrr)114::IS10*	NEB C2566, T7 Express
ER3019	F⁻ *araD139 Δ(ara-leu)7697 fhuA lacX74 galK [ϕ80 Δ(lacZ)M15] mcrA galU recA1 endA1 nupG rpsL* (StrR) *Δ(mrr-hsdRMS-mcrBC)*	NEB C3019, NEB 10-beta
ER2683	F′*proAB lacI*^{q} *Δ(lacZ)M15 zzf::miniTn10* (KanR)/ *fhuA2 Δ(lacI-lacA)200 glnV44 e14*⁻ *rfbD1? relA1? endA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10*	(32)
ER3169	F⁻ *endA1 thi-1 glnV44 mcrB1 hsdR2 relA1? spoT1? rfbD1?*/pBR322*fnuDIIM*	(33)
ER3228	NEB10-beta *attTn7::mTn7Φ(rhaBp-lacZp-lacZ)1*	This work
ER3233	ER2683 *attTn7::mTn7Φ(rhaBp-lacZ)1*	This work
ER3235	ER2683 *attTn7::mTn7Φ(rhaBp-lacZ)2*	This work
ER3237	ER2566 *attTn7::mTn7Φ(rhaBp-lacZ)1*	This work
ER3238	ER2566 *attTn7::mTn7Φ(rhaBp-lacZ)1*	This work
ER3239	ER2566/*pMC63 cat*	This work
ER3240	ER2566 *attTn7::mTn7Φ(fnuDIIM)1*/*pMC63 cat*	This work

USER, and 2-log DNA ladder] were from New England Biolabs except Pfu Turbo C_x Hotstart DNA polymerase (Agilent Genomics).

Bacterial strains Derivatives of K-12 (NEB 10-beta and ER2683) and B (ER2566) are listed in Table 3.

Media were Luria-Bertani [Rich Broth (RB) (20)], LB (21) or M9 minimal salts (22). Minimal media were supplemented with glucose or rhamnose to 0.2%, or both glycerol and rhamnose to 0.2%. Drugs were ampicillin (100 μg/ml) and chloramphenicol (5 μg/ml for ER2566 derivatives, 15 μg/ml for NEB 10-beta derivatives). Colonies were picked with capillary pipets into 0.85% NaCl for temporary storage before colony PCR or growth for testing of properties.

Constructions using pMS26

pMS26 was prepared for USERBstBI cloning according to the protocol below with the following additions and changes. Plasmid was purified with Qiaprep Spin miniprep kit (Qiagen); BstBI digestion was for 6 h; the digested DNA was resuspended in 30 μl 10 mM Tris–HCl pH 8.5. The final concentration of linearized DNA was about 40 ng/μl. The USER assembly reaction was carried out as described in Validation, below.

Step 1: USERBstBI vector preparation protocol. Complete digestion of the sites revealing long extensions is important. The processed vector can be stored at least for several months at −20°C. Any standard plasmid DNA purification can be used as long as a phenol/chloroform extraction and subsequent ethanol precipitation step are employed. We have found that Qiagen DNA column purifications may not be completely digested unless the DNA is phenol/chloroform extracted and ethanol precipitated. This protocol is for 10 μg DNA but can, of course, be scaled up.

Protocol

1. Digest plasmid with BstBI restriction endonuclease (NEB #R0519):

10 μg	pMS26 DNA (at least 640 ng/μl)
10 μl	NEBuffer 4
1 μl	BSA (10 mg/ml)
2 μl	BstBI (40 U)
to 100 μl	H_2O

Incubate 6 h to overnight at 65°C.

2. Nick the BstBI-linearized vector with Nt.BbvCI (NEB #R0632) by adding 2 µl (20 U) of Nt.BbvCI (10 000 U/ml) to the above reaction and incubating for 1 h at 37°C.
3. Purify the linearized, nicked vector by phenol–chloroform extraction and ethanol precipitation. Resuspend in 30 µl of Tris–HCl pH 8.5 buffer.
4. Determine vector concentration. Dilute to 20 ng/µl final concentration Final vector concentration should be 20–40 ng/µl.

Step 2: PCR for USERBstBI cloning. Fragments (100 µl volume each PCR) to be cloned in pMS26 were prepared using 5 U Pfu Turbo C_x Hotstart DNA polymerase, 0.25 mM each dNTP, and the appropriate primers listed in Table 2 (0.5 µM). Template for the PCR was either ~10 ng MG1655 genomic DNA (for *lacZ* constructs) or ~1 ng Qiagen plasmid miniprep DNA from ER3169 (for *fnuDIIM* chromosomal insertions). Plasmid PCR template was first linearized with EcoRI to minimize plasmid carryover during subsequent USER cloning. PCR cycling parameters were as recommended by Agilent. Following PCR, samples were purified using the QIAquick PCR purification kit (Qiagen) prior to the USER assembly reaction.

Steps 3–5: USER assembly, transformation and rapid chromosomal insertion. An expedited protocol was developed that can be used to rapidly isolate multiple independent constructs and chromosomal insertions from them. Recovery of chromosomal insertions is lower (~10%) with this element and protocol than the original procedure (50–70%), but faster (4 days from PCR to identified insertion) and more automatable.

- General materials:
  - USERBstBI-compatible digested pMS26 (from step 1)
  - PfuCx_TurboC_x_Hotstart_DNA_polymerase (Agilent Genomics)
  - USER enzyme (NEB M5505)
  - PCR purification columns
  - Universal flanking primers (*glmS, ptsS*) to monitor chromosomal insertion
  - RB ampicillin plates
  - RB no drug plates
  - Incubators at 30°C and 42°C
  - SOC or other outgrowth medium
- Experiment-specific materials:
  - competent host cells
  - DNA template
  - gene-specific primers with 5′ sequences suitable to generate USERBstBI-compatible extensions.

Procedure

- Day 1:
  - Step 2 (described above). PCR from template (20 cycles)
  - Step 3. Add USER, mix with precleaved vector pMS26 of step 1.
  - Step 4. Transform, plate on ampicillin at 30°C. Incubate transformation plates at least 18 h.
- Day 2: Step 5. Restreak 10 *well-isolated* colonies per cloning without drug at 42°C; patch the same colonies on Amp at 30°C for temporary archiving to access plasmid later if desired. Patches should be indexed to the streaks in some way. 1/6 plate per streak should be enough to obtain two *well-isolated* colonies.
- Day 3: Patch two *well-isolated* colonies per Day 2 streak to storage (no drug) for temporary archiving and test for chromosomal insertions by colony PCR, run gel (20 per cloning). About 10% of tested colonies will have chromosomal insertions.
- Day 4: Retrieve strains with plasmid and chromosomal insertions from patch plates for permanent archiving and to test properties of chromosomal insertions. This procedure can yield up to 10 independent constructions.

Colony PCR: reactions for colony PCR screening of the 42°C colonies for chromosomal insertions contained 2.5 U OneTaq Hot Start DNA polymerase (NEB) in 50 µl with primers 7 and 8 (Table 2) (0.5 µM each). 100 µl aliquots of 0.85% saline colony suspensions were heated to 99°C for 5 min to prepare extracts; 5 µl of each extract was used for PCR. PCR cycling parameters were as recommended by NEB.

Assays for β-galactosidase were a modification of the Miller protocol (22). Briefly, cultures were grown from three single colonies per strain in the medium specified; A600 was assessed directly on mid-log cultures or on a 1:5 or 1:10 dilution of late-log or stationary phase cultures to obtain readings between 0.2 and 0.8; samples were collected by centrifugation and resuspended in the same volume of lysis buffer (recipe); debris was clarified by centrifugation, and 1–50 µl of the extract was used in 1 ml reactions with ortho-nitrophenyl-β-D-galactoside substrate as described in (22). Miller units were calculated as $1000 \times A420/(\text{volume} \times \text{time} \times A600)$.

Restriction digests for determination of M.FnuDII activity: ER3239 (negative control) and ER3240 were tested for the ability to modify DNA *in vivo* using as reporter a co-resident high-copy plasmid with an easily diagnosed banding pattern. A total of 10 µl of miniprep DNA prepared from cells grown under various induction regimens was digested with 3 U of AclI in 1×NEBuffer 4 supplemented with 100 µg/ml BSA at 37°C for 90 min to linearize the plasmid at a convenient location; 10 U of BstUI was added and incubation continued at 60°C for one h to produce fragments diagnostic of unmodified sites.

RESULTS

pMS26 vector design

Overview. The vector pMS26 receives a desired gene within a mobile element (Figure 1, Steps 1–3) that inserts with high efficiency at a unique site in the genome of *E. coli* (Figure 1, middle). The donor thus assembled is introduced into cells by transformation (Step 4) and selected at low temperature for ampicillin resistance encoded by the vector. Transformants are purified at high temperature (Step 5), leading to loss of

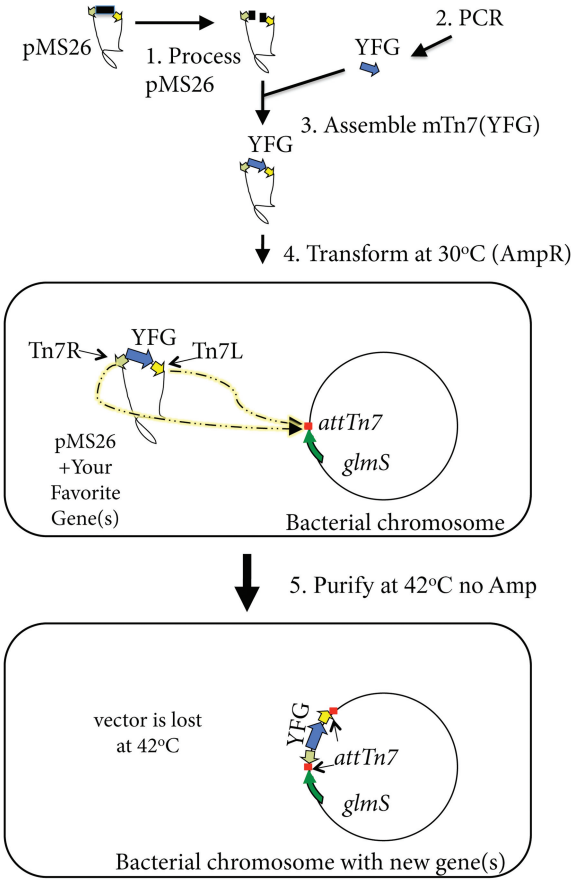

Figure 1. pMS26 mediates efficient insertion of a gene into a unique chromosomal location. The squiggly black line is pMS26 backbone, encoding transposition proteins, thermosensitive replication and ampicillin resistance. The mobile element *mTn7(MCS MS26)* is indicated by filled yellow and green arrows (Tn7 ends required for transposition protein to act) flanking the the cloning region (black box). The desired cargo (Your Favorite Gene, YFG) is represented by the blue arrow. Ovals represent bacterial cells; circles are chromosomes. The dashed yellow line represents the action of transposition proteins at the mobile element ends (Tn7R and Tn7L) and at the unique attachment site (red square on the chromosome). Action results in insertion of the element into the chromosome downstream of of *glmS*. pMS26 (and the assembled donor carrying YFG) can't replicate at 42°C. All survivors of 42°C growth have lost the donor; 10% have gained an insertion of *mTn7(YFG)*. Numbers are steps of the strain construction process, described in the text.

the temperature-sensitive replicon along with drug resistance and transposition protein genes. 10% of the survivors carry the mobile element downstream of *glmS* (Figure 1, bottom), disrupting its terminator, *glmSt* (Figure 2). The cloned segment is protected from expression driven by external signals with transcription and translation terminators flanking the cloning region (Figure 3A). Inducible expression is provided by the rhamnose-regulated promoter *rhaBp* (Figure 3A) characterized by very tight control. Uninduced expression is undetectable; maximal expression [activated by RhaS and CRP (23)] is good, around 10% of fully induced *lacp*. A cloning strategy that relies on long single-stranded extensions (Figure 1, Step 1, USERBstBI; 'Materials and Methods' section) allows this to be accomplished in four days, without isolation of the vector intermediate to verify its structure. The orientation of expression has been designed to minimize potential conflict with adjacent essential gene *glmS* (Figure 2), in case transcription emanating from the element does not terminate completely. Restriction enzyme sites within the element enable construction of alternative expression environments as desired (Figure 3B) while retaining protection from external transcription and translation.

Rationale and uses of the cloning region. A preliminary trial using pGRG36 revealed problems with transcription entering the mobile element from vector or chromosome (data not shown). Transcription from *glmS* on the chromosome and from *araC* on the vector will read into the element from the right (Figure 2 showing the *E. coli* chromosomal region; vector not shown). Transcription from *repA* on the vector will read in from the left (not shown); but *ptsS* reads away on the chromosome (Figure 2). Accordingly, design of pMS26 provides insulation from outside promoters and from translational readthrough both in the plasmid vector and in the chromosomal location. This is accomplished with three-frame stop codons inside each border, and transcription terminators (*rrnBt* inside Tn7R, the T7 early terminator *T7Et* inside Tn7L) as displayed in Figure 3A. The *T7Et* terminator is NOT adapted to terminate T7 RNAP; rather it functions in termination of early transcripts read by the *E. coli* RNAP (24). Here, it is oriented to terminate incoming transcription. The *rrnBt* terminator is reported to act bidirectionally (25). Providing regulated

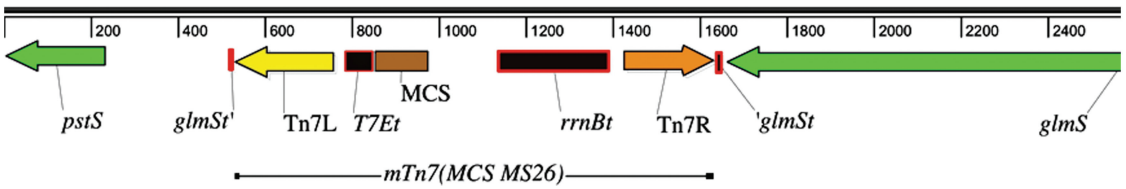

Figure 2. Gene organization surrounding *mTn7(MCS MS26)* in the chromosomal site. For legibility, the 'empty' transposon *mTn7(MCS MS26)* with no gene cloned is shown inserted in the chromosomal *attTn7*. The double bar shows distance (bp) from the end of *pstS*. Green arrows are genes (oriented with translation) coding for PstS (component of phosphate ABC transporter) and GlmS (glucosamine-6 phosphate synthase, essential for cell wall synthesis). Yellow and ochre arrows are the imperfect inverted repeats at which Tn7 transposition proteins act. Tn7L and Tn7R are not identical, and insertion is always in the orientation shown. Brown box is the multiple cloning site of pMS26; black boxes outlined in red are terminators: the *glmS* transcription terminator is interrupted by the insertion (as shown with apostrophe; *glmSt'*, *'glmSt*); transcription terminators (*rrnBt*, *T7Et*) insulate the MCS in *mTn7(MCS MS26)* from readthrough of external transcription.

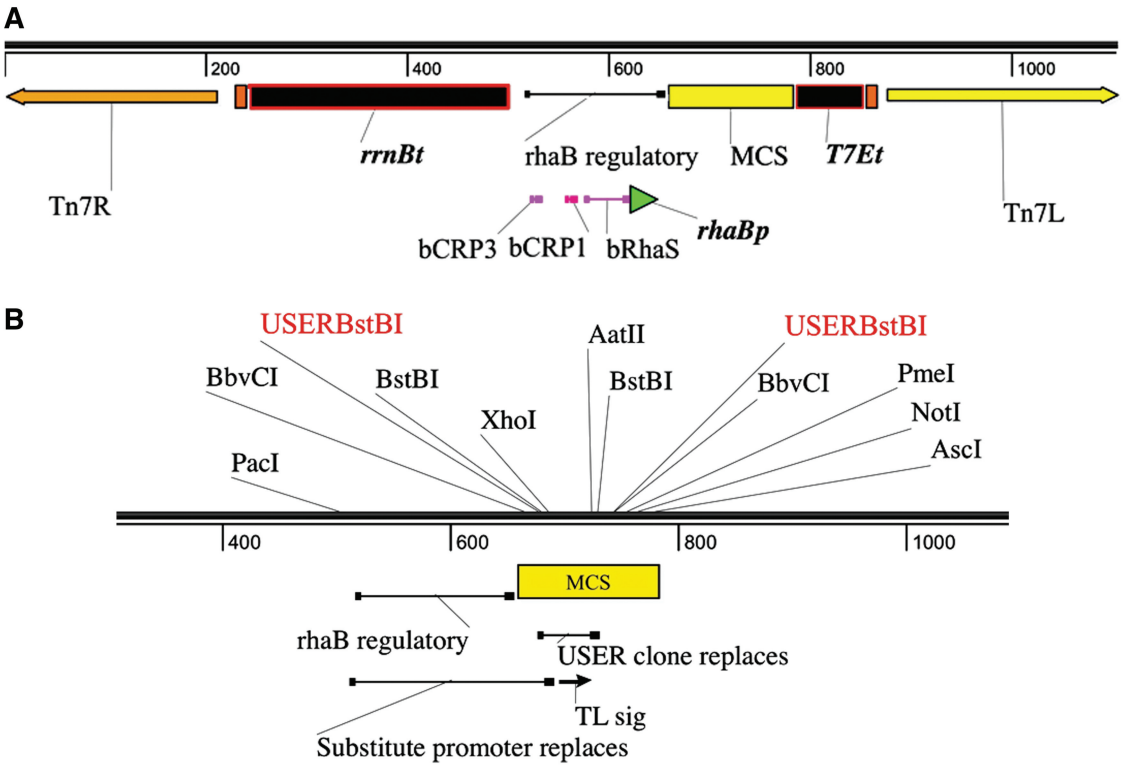

Figure 3. Components of *mTn7(MCS MS26)*. (**A**) Functional components of the transposon. Orientation has been reversed from that shown in Figure 2 to more easily envision expression. Distance from the end of Tn7R is shown (basepairs) below the double bar. Filled arrows: the ends of Tn7, at which the transposition proteins act to mobilize the element. The ends differ, so that the element always inserts with Tn7R (shown on the left here) adjacent to *glmS* (Figure 2). Boxes: insulating elements and cloning region–orange, three-frame translation stops (reading in); black/red, transcription terminators (reading in); yellow, multiple cloning site. Capped black bar shows the extent of the *rhaB* regulatory region. Below this are shown the regulatory sites involved: green arrow, promoter; pink bars, binding sites for the transcription activators RhaS and CRP. RhaS activates in the presence of rhamnose; CRP further activates in the absence of glucose (23). (**B**) Cloning options. Cloning sites discussed in the text are indicated above the double bar; distance from the outside end of Tn7R is marked below it. Alternative segments discussed in the text are shown with capped lines: '*rhaB* regulatory region' is the same as that shown in Figure 3A; 'USER clone replaces' indicates the segment replaced during USER cloning as described in the text; 'Substitute promoter replaces'—a desired substitute promoter may cloned between XhoI and PacI. Black arrow: 'TLsig' provides 5′-UTR translation initiation signals for substitute promoters, directing translation of ORFs cloned at the AatII site.

expression with a very low background was an additional goal; the sequence of the *rhaB* regulatory region was designed based on reference (23) and corresponds to bases −289 to −99 relative to the transcription start of *rhaBAD* in Fig. 1 of that reference. Use of cloning and expression options provided is discussed further below.

USER-facilitated cloning protocol. Efficient USER-facilitated cloning can be used to clone downstream of *rhaBp*. See 'Materials and Methods' section for a description of how to process the vector for this purpose (Step 1. USERBstBI Vector Preparation).

(1) Design of USER-facilitated cloning for *rhaBp* expression and evaluation.

Cloning with 8–12 basepair single-stranded extensions substantially improves the efficient recovery of inserts (19). Long single-strand extensions on insert DNA fragments can be created by employing uracil-containing PCR primers, followed by excision of uracil, and cleavage at the abasic site thus created. The excision and cleavage reactions are accomplished with the USER enzyme mixture (19). In the original report, ≥95% of transformants contain the designed clone; this efficiency has been reproduced here.

USERBstBI extensions: Creation of long extensions on the vector requires use of suitably disposed sites for a type II restriction enzyme and a nicking enzyme; in the original configuration, this was achieved with XbaI and Nt.BbvCI, each of which must be unique in the cloning vector. In the present instance, the starting vector pGRG36 had no BbvCI sites, but numerous XbaI sites were present. The type II enzyme must have a T 5′ to its cleavage position for the strategy to work; we identified BstBI (TT|CGAA) as a substitute for XbaI (T|CTAGA) that would be unique in the vector. For convenience, we call the resulting extensions USERBstBI extensions. Since BstBI cleaves after the second base of its site instead of after the first, use of this enzyme for vector preparation instead of XbaI necessitates adding an extra T before the target U to the PCR primer when amplifying the desired insert (see below).

(2) Design of primer for USER cloning with pMS26.

To use this vector processed as described in 'Materials and Methods' section, design PCR primers for your

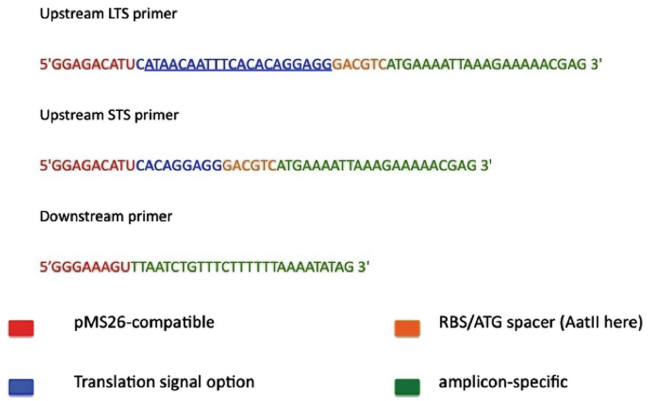

Figure 4. Design of primers for USER cloning with pMS26. Two choices of translation signal in the 5′-UTR are shown. The gene-specific sequence illustrated is of *fnuDIIM*. The underlined 21 bp of longer translation signal (LTS) is from the *tacp* regulatory region (34); the short signal (STS) is a truncation of it. The LTS and downstream primers illustrated are the same as primers 5 and 6 of Table 4; the STS construct was made but not used in this report. Fusion of *lacZ* to the signal as shown creates an RBS/ATG spacing of six, within the usual range of spacing (35); *lacZ* native spacing is seven (36).

favorite gene according to the example in Figure 4. (Note that these primers cannot be used with pNEB206A due to the extra base.)

Upstream forward primer (N-terminal side, for transcriptional fusion):

5′GGAGACATU-(optionally translation signals)-your-upstream-primer-binding-site3′

Downstream reverse complement (C-terminal side):

5′GGGAAAGTU-your-downstream-primer-binding-site3′

Transcription unit cloning: If the goal of the experiment is to use the native promoter and regulatory sites for expression, the primer binding sites should be upstream of that transcription unit. UserBstBI cloning will result in *rhaBp* upstream in tandem with the native promoter. In the absence of rhamnose, *rhaBp* is essentially silent.

Translation unit cloning: If *rhaBp*-directed expression is desired, the primer-binding sites should be upstream of translation signals (essentially the 5′ untranslated region or 5′-UTR) for your gene, or else translation signals should be designed into the primers. Two options for translation signals are described in the results below and displayed in Figure 4.

(3) Insert preparation, USER assembly and transformation

Efficient insertion results from PCR with primers described above and in Figure 4. These contain strategically located uracil, which can be acted on by the USER reagent to reveal 3′ single-strand extensions compatible with the processed vector (step 3 in Figure 1). For complex assemblies of multiple fragments, ligation and gel purification is recommended (19). However, if a single amplicon contains all the genes of interest, ligation is not needed and the assembly mixture can be transformed directly, plated on ampicillin and incubated at low temperature to allow vector replication. Expression of transposition proteins during colony growth results in mobilization of the *mTn7* to its chromosomal target, *attTn7* (Figure 1, middle). Repurification of transformant colonies at 42°C in the absence of ampicillin (Step 5 of Figure 1) results in efficient plasmid loss. The survivors all lack the plasmid, and about 10% carry *mTn7* inserted at *attTn7*.

Restriction enzyme cloning options. Alternatives to the USER cloning protocol and *rhaBp* expression are also enabled in pMS26. At least three expression configurations can be achieved using restriction enzyme sites in the vectors described here:

(1) Transcription unit cloning. For cloning of a gene or operon with its own expression signals, standard restriction enzyme cloning can be carried out between PacI on one side and AscI, NotI, PmeI, BbvCI, BstBI, AatII (ZraI) or XhoI (PspXI) on the other (Figure 3B). PacI, AscI, NotI and PmeI recognize 8 bp sites, and BbvCI recognizes 7 bp; thus, the likelihood of interfering sites occurring in the cloned fragment is much lower than if using common 6-base sites. Using this approach, no promoter or translation signals are provided; thus, expression must be directed by the insert. Transcription unit cloning can also employ the USERBstBI-facilitated procedure (see above).
(2) Creating an ORF-expression vehicle with a different promoter. The expression region is set up to allow replacement of *rhaBp* with an alternative promoter and provides translation signals for this (Figure 3A and B). To replace *rhaBp* and convert to a constitutive ORF-cloning vector, digest with XhoI and PacI to remove the *rhaBp* regulatory region and insert an oligonucleotide or fragment specifying a constitutive promoter such that transcription is directed to begin at or before the translation signal (5′-UTR) region ('TL sig'). (This signal is the same as 'LTS' described above and evaluated below in the context of *rhaBp* expression validation.) This will create a vector that can subsequently be used for ORF cloning: insert the ORF of interest at the AatII site, with an ATG immediately following the AatII site.
(3) Rhamnose-regulated expression vector. Standard restriction enzyme cloning for example between the two BbvCI sites, between the two BstBI sites, or between one of these and the AscI, NotI or PmeI sites will allow use of *rhaBp* (a rhamnose-activated promoter) as an inducible promoter (Figure 3). Translation signals should be designed into the insert to use this option.

Alternatively, plasmids that already have translation signals (described below, pMS33 or pMS34) can be adapted as ORF-cloning vectors with *lac* stuffer fragment, allowing *rhaBp*-directed expression. The *lacZ* fragment can be replaced with an alternative by digesting with AatII at the N-terminal end of the coding sequence and PmeI, NotI or AscI at the C-terminal end. Blue-white

screening with Xgal in the presence of rhamnose should identify replacement clones.

Method Validation

Validation of cloning protocol. In constructing ER3228 containing *mTn7Φ(rhaBp-lacZp-lacZ)1,* 0.06 pmole PCR product obtained using primers 1 and 2 of Table 2 (10 µl of 0.006 ng/µl) and 0.005 pmole (1 µl, 40 ng) processed pMS26 were incubated with 1 unit of USER enzyme in a total volume of 12 µl for 15 min at 37°C, and then incubated 15 min at room temperature. 3 µl of the assembly reaction (10 ng of pMS26) was mixed immediately with 50 µl commercial competent ER3019 host cells, incubated on ice for 30 min, at 37°C for 2 min, and finally on ice for 5 min. Cells were gently mixed with 1 ml LB and outgrown at 30°C for 1 h. 100 µl of undiluted, 10-fold diluted and 100-fold diluted culture was plated on LB with 100 µg/ml ampicillin and incubated 24 h at 30°C. 7300 transformants/ml were obtained (7×10^5 per µg processed vector). When the transformation mix was replated at 30°C with IPTG-Xgal present, ~3/5000 colonies were white, indicating a very low frequency of vector without insert.

With lesser insert-vector ratios and cells of lower competence, 20–200 colonies typically resulted from plating 10% of the transformation mix (~10^3–0^4 transformants per µg processed vector).

Validation of chromosome insertion protocol. Table 4 shows five trials in two strains employing the rapid insertion protocol. On the day following transformation of each USER assembly reaction, 7–20 transformants were streaked on to LB plates without drug and incubated overnight at 42°C to cure the plasmid.

Colonies picked from the 42°C plates were analyzed by colony PCR. Primers 7 and 8 (Table 2) yielded a product of 841 bp for an empty *attTn7* site, while colonies with *lacZ* or *fnuDIIM* inserts display products of 5129 and 3201 bp, respectively. The observed ratio # inserts/# tested colonies are given in Table 4, lines 1–5. Trials with two configurations of *lacZ* and one of *fnuDIIM* as cargo using the pMS26 vector in two hosts consistently yielded an insertion frequency of ~10% (Table 4).

We considered the possibility that additional generations of growth in the presence of the donor, or simply longer time, might yield a higher frequency of chromosomal insertion. To test this, in one experiment transformants (~10^7 cells/colony) were patched onto LB Amp, incubated overnight at 30°C, then a plug (~10^7 cells/plug) from the patch was grown in 1 ml RB at 30°C overnight (~10^9 total cells) then diluted and plated at 42°C overnight to cure the plasmid. This longer protocol had no effect on the fraction of descendants with insertions (Line 6 of Table 4).

Validation of expression parameters using the gene for β-galactosidase, lacZ.

(1) A transplanted *lac* promoter displays expected IPTG induction.

When the entire *lacZ* expression region is included in the cloned fragment, an appropriate 100-fold IPTG-dependent induction ratio is seen (compare lines 4 and 6 of Table 5). Rhamnose addition did not significantly increase β-galactosidase expression above the background from uninduced *lacp* (compare line 4, Table 5 with line 5, Table 5), suggesting that LacI binding may block RNAP transcription from *rhaBp* in this configuration. The nearest LacI binding site is 31 bp from the *rhaBp* transcription initiation site. The background (level in the absence of any element, 100–200 U, lines 1–3) is indistinguishable from zero; note that the detection limit can be reduced by using more cells and longer incubation times (increasing the denominator in calculating Miller units).

(2) Maximal *rhaBp* expression and translation signal comparison.

Expression of β-galactosidase in the *mTn7(MCS MS26)* context was evaluated in two experiments using *rhaBp*, USER cloning and the two different 5′-UTRs illustrated in Figure 4, with results shown in Figure 5. The longer version [*mTn7Φ(rhaBp-lacZ)1*; LTS] of the 5′-UTR consistently yielded 2- to 3-fold more activity than the short version [*mTn7Φ(rhaBp-lacZ)2*; STS]. Expression driven by *rhaBp* in this context (in minimal

Table 4. High-efficiency chromosomal insertion at *attTn7*

Trial	Host strain	Cargo	Frequency	Resulting strain(s)
1	ER2683	*mTn7Φ(rhaBp-lacZ)1*	1/10	ER3233
2	ER2683	*mTn7Φ(rhaBp-lacZ)1*	1/7	NA
3	ER2683	*mTn7Φ(rhaBp-lacZ)2*	1/10	ER3235
4	ER2566	*mTn7Φ(rhaBp-lacZ)1*	2/10	ER3237, ER3238
5	ER2566	*mTn7Φ(fnuDIIM)1*	3/20	
6	ER2683	*mTn7Φ(rhaBp-lacZ)1*	1/10	NA

Note. The rapid chromosomal insertion protocol was used except in trial 6, for which a longer protocol was used as described in the text. Donor plasmids pMS33 [*mTn7Φ(rhaBp-lacZ)2*] and pMS34 [*mTn7Φ(rhaBp-lacZ)1*] resulted from trials 2 and 4 (see Table 1) and were recovered from patches saved at low temperature as described in 'Materials and Methods' section, constructions with pMS26, Day 2. Insertion was verified with PCR primers 7 and 8 of Table 2. Trials 1 and 2 were carried out on different days with different PCR reactions.

Table 5. IPTG-inducible *lacZ* expression from *lacp*

	Element	Medium	Avg MU	StDev
1	None	RB	175	87
2	None	RB Rha	127	98
3	None	RB IPTG	225	279
4	*mTn7Φ(rhaBp-lacZp-lacZ)1*	RB	3274	976
5	*mTn7Φ(rhaBp-lacZp-lacZ)1*	RB Rha	4168	590
6	*mTn7Φ(rhaBp-lacZp-lacZ)1*	RB IPTG	312 726	76 343

Note. Data averaged from two experiments on different days. Miller units (MU) were determined as described in 'Materials and Methods' section, with 1 µl of overnight culture and assay time of 7–45 min. Strains used were ER3019 and ER3228. SD is standard deviation of the two triplicate determinations.

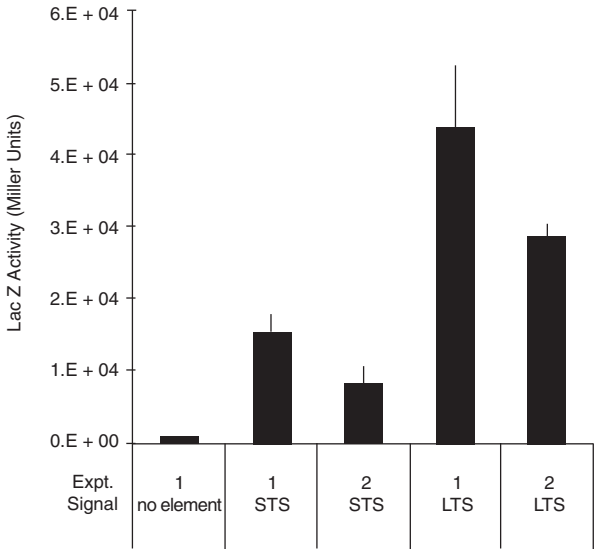

Figure 5. Rhamnose-driven expression with two translation initiation signals. Two experiments measured β-galactosidase expression driven by *rhaBp* and two variant translation signals (MU, a measure of specific activity). Cultures of ER3233 [LTS; *mTn7Φ(rhaBp-lacZ)1*] and ER3235 [STS; *mTn7Φ(rhaBp-lacZ)2*] were grown in M9 with 0.2% rhamnose; ER2683 (no element) was grown with 0.2% glucose. Volume of extract used was 1 or 50 µl of undiluted culture; time of incubation was 15–128 min. Using this approach, the empty site background was 5.4 MU. Average and standard deviation of three replicate cultures is shown.

Table 6. Effect of carbon source

	C-source	Element	MU	Mean MU	Induction
1	Glu	None-1	4	5	
2	Glu	None-2	1		
3	Glu	None-3	11		
4	Glu	LTS-1	17	18	(1)
5	Glu	LTS-2	7		
6	Glu	LTS-3	29		
7	Glu	STS-1	8	5	(1)
8	Glu	STS-2	5		
9	Glu	STS-3	1		
10	Rha	LTS-1	27 912	28 993	1639
11	Rha	LTS-2	30 912		
12	Rha	LTS-3	28 155		
13	Rha	STS-1	7126	8527	1764
14	Rha	STS-2	7194		
15	Rha	STS-3	11 261		
16	GlyRha	LTS-1	13 671	18 167	1027
17	GlyRha	LTS-2	15 112		
18	GlyRha	LTS-3	25 717		

Note. Effect of carbon source.
Triplicate colonies of ER2683 (no element), ER3233 (LTS) and ER3235 (STS) were suspended in 300µl saline, and 100µl each was inoculated into 2 ml M9 with glucose (Glu) or rhamnose (Rha) at 0.2% or with rhamnose and glycerol (GlyRha) at 0.2% each as carbon source, grown to saturation, and then assayed as in Figure 5. Each culture is reported separately; mean MU is the average of the triplicate cultures;'induction' is the ratio of mean MU, for each element and carbon source, to the glucose result for that element. Cultures with the same number in the 'element' column originated from the same colony.

medium) is maximally about 10-fold lower than fully induced *lacZ* (in RB; compare with Table 5).

(3) 1000-fold rhamnose induction of *rhaBp*.

Choice of growth media allows modulation of expression by rhamnose over a 1000-fold range, from the combined effect of catabolite activation and rhamnose activation. Table 6 shows that uninduced and glucose-repressed expression using STS (lines 7–9) is indistinguishable from the background with no *lacZ* (lines 1–3), while LTS may be slightly higher (line 4–6). Rhamnose results (lines 10–15) are the same data as experiment 2 of Figure 4, showing improved expression mediated by the LTS. Growth supported by glycerol together with rhamnose yields somewhat lower specific activity than rhamnose alone (line 16–18 of Table 6; Miller units are normalized for A600, a proxy for protein content). This mixture of carbon sources both subject to catabolite activation, in which both the rhamnose and glycerol regulons are sharing the positive activator CRP, may lead to lower expression of each regulon due to competition for activator. Despite the lower specific activity, the growth properties of all tested strains are considerably better when glycerol is present with or without rhamnose (shorter lag, faster growth and higher density; growth curves not shown).

Validation of rhaBp expression using a methyltransferase gene, fnuDIIM. Regulated expression is not limited to the reporter *lacZ* that was used for literature comparison. A strain constructed to drive expression of the FnuDII DNA methyltransferase from the *rhaB* promoter also showed large sugar-dependent expression changes. The substrate pMC63 is a high-copy plasmid with two CGCG sites, which can be modified by M.FnuDII or cleaved by BstUI (26). Plasmid-encoded CGCG sites modified by the methyltransferase (during cellular propagation in this experiment) are protected from BstUI cleavage (27). Figure 6 illustrates the protective action *in vivo*, mediated by induction of the methyltransferase in ER3240, containing pMC63 as a reporter and *mTn7-Φ(fnuDIIM)1* as an expression construct. When this plasmid was grown in the presence of *fnuDIIM* in M9 rhamnose, the *in vivo* expression levels were sufficient to fully modify these CGCG sites [no cleavage of the AclI-linearized extracted plasmid, lanes (minimal, R, +)]. On the other hand, the *in vivo* expression levels only partially modified the CGCG sites within pMC63 when grown in rich medium with rhamnose (rich, R, +). Glucose-grown cultures did not protect at all (minimal, G, +; rich G, +): the digest patterns match those from cultures entirely lacking the *mTn7Φ(fnuDIIM)1* element (minimal, G, −; minimal, R, −; rich, G, −, rich, R, −).

DISCUSSION

The complex transposon Tn7 has several features that can be adapted for genetic tool development. In the present study, the high-specificity, high-efficiency pathway targeting *attTn7* is the aspect of interest. This pathway, directed by the target selector protein TnsD together with the TnsABC catalytic assembly, mediates insertion into one

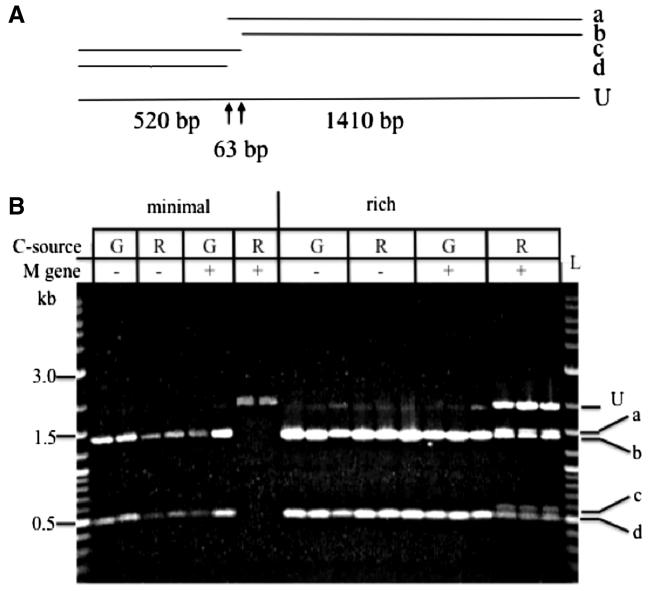

Figure 6. Complete *in vivo* modification of pMC63 by *rhaBp*-driven *fnuDIIM*. (**A**) Methylated (protected, uncut) substrate (U) and products (a–d) expected from digestion of AclI-linearized pMC63 with BstUI. The BstUI sites (CGCG; arrows) will be protected from digestion when modified by M.FnuDII (CGCG → mCGCG). Fragments (b) and (d) are expected from a complete digest; fragments (a) and (c) may be expected in partial digests, as may occur when the plasmid is partially modified. A 63-bp central fragment is also expected from complete digestion but runs off the gel in (**B**). (B) pMC63 was prepared from cultures of ER3239 (M gene −) or ER3240 (M gene +). Cultures were grown in M9 (minimal, duplicates grown) or RB (rich, triplicates grown) supplemented with 0.2% sugar: glucose (G) or rhamnose (R) to repress or induce expression of *fnuDIIM*. The extent of *in vivo* modification was tested by BstUI digestion of AclI-linearized plasmid DNA prepared from the cultures and interpreted according to the diagram in panel A. Size standards on each side are the 2-log ladder (L); selected bands are labeled with size in kb. The gel system was 1% agarose with Tris–borate buffer containing ethidium bromide. Higher cell density in RB accounts for higher yields in plasmid preps. The low-intensity band at the position of 'U' in the rich broth cultures G−, R− and G+ (which all should be unmodified at BstUI sites) presumably are linears that result from BstUI cleavage of plasmid that escaped AclI digestion.

(*E. coli, Yersinia, Acinetobacter, Francisella*) or a small number (*Burkholderia*) of sites in a broad array of bacteria (28). Even human DNA has a high-efficiency site, also near a gene encoding *N*-acetyl glucosamine synthase (29). This highly conserved gene (*glmS* in bacteria) provides the widely distributed recognition site, but since the site of actual insertion is outside the coding region, downstream of the recognition site, insertion is well-tolerated.

The arrangement presented here mitigates some of the problems associated with using the original pGRG36 system. Genes within the mobile element are insulated from expression signals originating outside of it, and the high-efficiency cloning approach reduces problems associated with manipulation of large plasmids.

High efficiency insertion allows screening (examination one-by-one) rather than selection (using drug selection to eliminate unproductive background) for the construct desired. The mobile element *mTn7(MCS MS26)*, carried on pMS26, still retains an insertion efficiency high enough to allow screening rather than selection for chromosomal insertions (10% of 42°C survivors; Table 4). Although ampicillin is used to select for the presence of the initial construct, that construct is efficiently lost at 42°C, and drug-sensitivity is restored in the final strain. The streamlined insertion protocol was effective in two host strains of very different pedigrees, with three constructs of distinct function. Possibly the insertion efficiency might be further increased by adding arabinose to the ampicillin plates used to select transformants (Step 4 of Figure 1).

The mobile element *mTn7(MCS MS26)* presumably could also be mobilized via the TnsE-dependent random insertion pathway that targets conjugal plasmids. We have not tested this lower efficiency transposition pathway. TnsE is not encoded on pMS26 but could be provided in trans.

We compared the *rhaBp* expression system to the well-characterized *lac* expression system in several ways. One trial (Table 5) examined *lacI*-regulated expression of the *lacZ* transcription unit, in the context of the *mTn7(MCS MS26)* element. In this situation *lacZp* directed transcription and was regulated by LacI encoded on the *ϕ80 Δ(lacZ)M15* prophage resident in the host. The *mTn7Φ(rhaBp-lacZp-lacZ)1* element contains, downstream of *rhaBp,* a segment of wild-type *lac* operon sequence extending from the end of *lacI* to the beginning of *lacY,* including all annotated regulatory elements. In the absence of rhamnose, IPTG mediates a 100-fold induction, similar to induction seen in the wild-type *lac* operon (not counting glucose repression, not measured here) and in other *lacZ* reporter controls [see, e.g. (30); *lacUV5p* was examined in that case].

Also notable was the lack of rhamnose induction above the background expression from uninduced *lacp*. The *rhaBp* transcription start is 31 bp from the closest (furthest upstream of *lacp*) LacI binding site. Several mechanisms by which bound regulators could interfere with each other or with RNAP can be imagined.

The provision of a tightly controlled inducible *rhaBp* promoter together with a strong translation initiation region adds flexibility. Expression is very low in the presence of glucose [Table 6, lines 4–9 and Figure 6 lanes (G, +)], indistinguishable from the negative control (Table 6, lines 1–3; Figure 6, lanes (G, –; R, –)]. The highest expression was achieved by combining the strong translation signal (LTS) in M9 medium with rhamnose as sole carbon source (Table 6, lines 10–12; Figure 5, LTS; Figure 6, M9, R, +). The level of β-galactosidase activity was ~10% of fully induced *lacZ* from its own promoter (compare Table 5, IPTG with Table 6, lines 10–12). For reasons we have not explored, growth under these conditions is slow, and final cell yields are half that with glucose as carbon source. Induction of the rhamnose operon is known to be slow (31), possibly accounting for the long growth lag.

Intermediate expression levels can be achieved by combining rhamnose with other nutrients (M9 glycerol + rhamnose, Table 6, lines 16–18; rich + rhamnose, Figure 6, lanes rich, R, +).

The donor plasmid pMS26 also was designed to accommodate alternative cloning and expression strategies, as described in the section on vector design. Such modified protocols will still enjoy the insulation from external expression and the high insertion specificity of the mobile element described here.

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