

# Positive and negative selection using the *tetA-sacB* cassette: recombineering and P1 transduction in *Escherichia coli*

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## ABSTRACT

The two-step process of selection and counter-selection is a standard way to enable genetic modification and engineering of bacterial genomes using homologous recombination methods. The *tetA* and *sacB* genes are contained in a DNA cassette and confer a novel dual counter-selection system. Expression of *tetA* confers bacterial resistance to tetracycline (Tc<sup>R</sup>) and also causes sensitivity to the lipophilic chelator fusaric acid; *sacB* causes sensitivity to sucrose. These two genes are introduced as a joint DNA cassette into *Escherichia coli* by selection for Tc<sup>R</sup>. A medium containing both fusaric acid and sucrose has been developed, in which, coexpression of *tetA-sacB* is orders of magnitude more sensitive as a counter-selection agent than either gene alone. In conjunction with the homologous recombination methods of recombineering and P1 transduction, this powerful system has been used to select changes in the bacterial genome that cannot be directly detected by other counter-selection systems.

## INTRODUCTION

Genetic and functional genome analyses demand precise manipulation and modification of regulatory elements and the genes they control. To this end, recombineering has been shown to be a powerful method to modify genetic targets *in vivo* by the process of homologous recombination (1–4). This recombination is carried out by phage recombinases, like those of the Red system, which are functions that mediate precise targeted insertion of linear double-strand DNAs (dsDNAs) or single-strand DNA

oligonucleotides (oligos) into the DNA of the bacterial genome and its episomes (1,2,5,6). These Red-promoted recombination events require only limited, ~50 base segments of homology to the target. Exo binds dsDNA ends and removes bases from the 5'-end, and Beta binds single strands and enhances annealing to complementary target strands during the recombination event (3). Recombination with oligos requires only the Beta protein to bind the single-strand DNA and anneal it to the target (6). A third Red function, Gam, inhibits the host RecBCD and SbcCD nucleases and thereby enhances linear dsDNA recombination 10-fold (7).

Genetic modifications engineered by the process of recombineering often involve one or more selection steps. For example, replacing genes with drug markers generates gene knockouts, and adding genetic tags to genes by co-selection with a linked drug marker generates gene fusions. Creating point mutations or changes of a few bases can be accomplished with extremely high efficiency using Red Beta-mediated oligonucleotide recombination and screening for recombinants by a polymerase chain reaction (PCR) with primers specific for the mutation (8). However, a two-step process of selection and counter-selection is required when low efficiency events are expected, i.e. to construct seamless deletions or fusions in which large segments of DNA are removed or inserted without leaving a mark. First, a counter-selection cassette is inserted, and subsequently removed to generate the appropriate modification. Both steps use recombineering. Many such two-step systems exist. Here we describe the *tetA-sacB* cassette, a particularly useful two-step system, which provides unique advantages over other counter-selection methods.

The *tetA* gene product, located in the cytoplasmic membrane, prevents cellular accumulation of tetracycline, conferring resistance (Tc<sup>R</sup>). TetA also causes the cell to become sensitive to lipophilic chelators, like fusaric acid,

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and to have increased sensitivity to kanamycin and osmotic shock (9). Thus, the TetA protein has both positive and negative selection effects on cell growth (10,11). The *sacB* gene product converts sucrose to levan, which accumulates in the periplasm and is toxic to *E. coli* (12,13). Combining both of these genes into a *tetA-sacB* cassette provides Tc<sup>R</sup> as the selection for insertion, and each gene product exerts independent toxic effects that allow removal by counter-selection. We have developed a new medium that selects against both *tetA* and *sacB*. An additional benefit of this rich Tet/SacB medium is that incubation times are reduced relative to minimal media selections. This *tetA-sacB* cassette also has the advantage of not imposing any restraints on strain genotype, unlike most counter-selectable markers that require special mutant alleles such as *rpsL* (Str<sup>R</sup>), *galK*, *thyA* or *tolC* to be present in the genome (See the respective references 14–17).

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains are described in Table 1. Standard genetic methods including recombinering and P1 transduction (20–22) were used for strain construction. Three sequenced strains MG1655, W3110 and DH10B (23–25) have been used to test the efficiencies of counter-selection. The pSIM5 and pSIM18 plasmids, which provide Red recombination functions and confer either chloramphenicol or hygromycin resistance, respectively, have been transformed into these strains and their derivatives for recombinering purposes (26).

Materials

DNA oligos are listed in Table 2 and were purchased from Integrated DNA Technologies and used in the unpurified, salt-free form. The sequences of DNA primers to amplify

Table 1. Bacterial strains

Strain	Genotype	Reference
CC4231	W3110 <i>tilacU169 gal490 pgl/ti8 [ cl857 ti(cro-bio.A)] araD&lt;&gt;cat-sacB-amp</i>	This work
DH10B	<i>mcrA ti(mrr-hsdRMS-mcrBC) 80lacZtiM15 tilacX74 recA1 endA1 araD139 ti(ara-leu)7697 galU galK rpsL mupG</i>	(18)
DY330	W3110 <i>tilacU169 gal490 pgl/ti8 [ cl857 ti(cro-bio.A)]</i>	(2)
LT1594	MG1655 <i>tilacZ&lt;&gt;hyg [pSIM5]</i>	This work
LT1610	MG1655 <i>tilacZ&lt;&gt;hyg sfi-lacZ</i>	This work
LT1619	MG1655 <i>tilacZ&lt;&gt;hyg sfiA&lt;&gt;tetA-sacB [pSIM5]</i>	This work
MG1655		(19)
T-SACK	W3110 <i>araD&lt;&gt;tetA-sacB-amp fliC&lt;&gt;cat argG::Tn5</i>	This work
W3110	<i>IN(rrnD-rrnE)1 rph-1</i>	(19)
XTL298	CC4231 <i>araD&lt;&gt;tetA-sacB-amp</i>	This work
XTL425	DH10B <i>sfiA&lt;&gt;tetA-sacB [pSIM18]</i>	This work
XTL426	DH10B <i>galM&lt;tetA-sacB&gt;gpmA [pSIM18]</i>	This work
XTL621	W3110 <i>sfiA&lt;&gt;tetA-sacB</i>	This work
XTL622	W3110 <i>galM&lt;tetA-sacB&gt;gpmA</i>	This work
XTL623	MG1655 <i>sfiA&lt;&gt;tetA-sacB</i>	This work
XTL624	MG1655 <i>galM&lt;tetA-sacB&gt;gpmA</i>	This work
XTL629	W3110 <i>sfiA&lt;&gt;tetA-sacB [pSIM18]</i>	This work
XTL630	W3110 <i>galM&lt;tetA-sacB&gt;gpmA [pSIM18]</i>	This work
XTL631	MG1655 <i>sfiA&lt;&gt;tetA-sacB [pSIM18]</i>	This work
XTL632	MG1655 <i>galM&lt;tetA-sacB&gt;gpmA [pSIM18]</i>	This work
XTL634	W3110 <i>araD&lt;&gt;tetA-sacB-amp</i>	This work
XTL635	MG1655 <i>sfi&lt;&gt;luc</i>	This work
XTL671	DH10B <i>galM&lt;tetA-sacB&gt;gpmA</i>	This work

Table 2. Oligonucleotides for PCR amplification and oligo recombination<sup>a</sup>

<i>galM / tet</i>	<u>AAATCACCAGCAAACACCGACATATTTGCAACTCAATATTCACAACAACCTCCTAATTTTTGTTGACACTCTATC</u>
<i>gpmA / sacB</i>	<u>CAACAGCAATGCTTACGCATAACCATAGCGAAAAATAGTGGCGCAGTGTAATCAAAGGGAAAACTGTCCATATGC</u>
<i>sfiA / tetA</i>	<u>AACTCACAGGGGCTGGATTGATTatgTACACTTCAGGCTATGCACATCGTTCCTAATTTTTGTTGACACTCTATC</u>
<i>sfiA / sacB</i>	<u>CATTGGCTGGGCGACAAAAAAGTTCCAGGATTAATCCTAAAATTTACttaATCAAAGGGAAAACTGTCCATATGC</u>
Oligo A	<u>GATTGATTatgTACACTTCAGGCTATGCACATCGT//taaGTAATTTAGGATTAATCCTGGAACTTTTTT</u>
Oligo B	<u>ACCGACATATTTGCAACTCAATATTCACAACAACCTCCTAATTTTTGTTGACACTCTATC</u>
<i>galM / parS-1</i>	<u>AAATCACCAGCAAACACCGACATATTTGCAACTCAATATTCACAACAACCCGATAAAAAAGCCGAAGCCTTAAA</u>
<i>gpmA / parS-2</i>	<u>CAACAGCAATGCTTACGCATAACCATAGCGAAAAATAGTGGCGCAGTGTAATGTTGACTTTCTCGTGACAAGC</u>
<i>sfiA / luc atg</i>	<u>ACTGGATGTACTGTACATCCATACAGTAACACAGGGGCTGGATTGATTatgGAAGACGCCAAAAACATAAAG</u>
<i>sfiA / luc stop</i>	<u>AAGCATTGGCTGGGCGACAAAAAAGTTCCAGGATTAATCCTAAAATTTACttaCACGGCGATCTTTCCGCCCTTCT</u>
<i>sfiA / lacZ atg</i>	<u>AACTCACAGGGGCTGGATTGATTatgTACACTTCAGGCTATGCACATCGTgATTCACTGGCCGTCGTTTTAC</u>
<i>sfiA / lacZ stop</i>	<u>CATTGGCTGGGCGACAAAAAAGTTCCAGGATTAATCCTAAAATTTACttaTTTTGACACCAGACCAACTGG</u>

<sup>a</sup>Underlined sequence is the primer for PCR amplification. The lowercase sequences represent the start and stop codons within the sequence. The // represents the center point in the 70 base Oligos A and B with flanking homologies to delete *tetA-sacB*. Oligo A and B correspond to the lagging strand during DNA replication of the bacterial chromosome.

*tetA*, *cat*, *amp*, *cat-sacB* and *tet-sacB* cassettes are described (see FAQs in the <http://redrecombinering.ncifcrf.gov/> website). The High Fidelity Platinum® *Taq* DNA polymerase from Invitrogen was used for PCRs. Sequencing was done by SAIC-Frederick, Inc. and results were analyzed with Sequencher version 4.8.

Standard cultures were grown with LB broth or agar (1.5%) containing per liter: 10 g tryptone, 5 g yeast extract and 5 g NaCl. Counter-selection against *sacB*-carrying strains is done on L sucrose agar (1.5%) containing per liter 10 g tryptone, 5 g yeast extract and 60 g sucrose (no NaCl). The sucrose (60% solution) is autoclaved separately and added to the molten agar after its sterilization. Tetracycline (12.5 mg/ml), hygromycin (200 mg/ml), chloramphenicol (10 mg/ml) and ampicillin (30 mg/ml) are used as indicated. Bochner/Maloy agar was prepared as described previously (11).

#### Tet/SacB counter-selection medium

The Tet/SacB counter-selection agar that was developed here contains, per liter, 15 g of Difco agar, 4 g of tryptone, 4 g of yeast extract, 8 g of NaCl, 8 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.11 g  $\text{ZnCl}_2$ , 24 mg fusaric acid and 60 g sucrose. The agar, tryptone and yeast extract were autoclaved in a 400-ml volume with water. The NaCl and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  were mixed and autoclaved in a 400-ml volume with water. Sucrose at 60 g in 100 ml was autoclaved. The molten agar mix, salt mix and sucrose were combined together after autoclaving. The fusaric acid (Sigma) was stored at 48 mg/ml in ethanol at 20 °C in a lightproof container. The  $\text{ZnCl}_2$  was stored in water at 25 mM after filter sterilizing; note that higher concentrations precipitate. Fusaric acid (0.5 ml) and  $\text{ZnCl}_2$  (32 ml) were added to the molten agar mixture after cooling to 55 °C. The final volume was brought to 1 liter with sterile  $\text{H}_2\text{O}$ .

Petri plates (100 mm) were poured so that each contained a 40-ml volume of the final molten agar mixture. The large volume ensures that the plates will remain hydrated over several days of incubation at 42 °C. After hardening, the plates were placed in their original plastic bags, wrapped in aluminum foil and stored at 4 °C. We emphasize the importance of precisely following these procedures as variations can dramatically affect the selection.

#### P1 Tet/SacB counter-selection agar

When Tet/SacB counter-selection agar is used during a P1 transduction (22), it is modified by adding 5 ml of a sterile 1 M sodium citrate solution to the agar for a final concentration of 5 mM. This is the P1 Tet/SacB counter-selection agar.

#### Creating the *tetA-sacB* counter-selection cassette

We have created a new dual counter-selection system, *tetA-sacB*, starting with strain CC4231, which is DY330 with the *cat-sacB* cassette inserted to replace *araD* with an *amp* cassette located just beyond *sacB* (Table 1). We note that this intermediate strain can be used to place genes under arabinose control in the chromosome using sucrose counter-selection. To broaden the application and usefulness of this construct, we replaced *cat* with a second counter-selectable cassette, *tetA*, as follows. The *tetA* gene was amplified from a *Tn10* transposon by PCR with primers that retain its promoter region but do not amplify the adjacent *tetR* gene encoding the repressor of *tetA*. Replacement of *cat* with *tetA* leaves *sacB* with its own promoter downstream of the *tetA* gene. This generates the *tetA-sacB* dual cassette with independent promoters for each gene and with *tetA* constitutively expressed (Figure 1). A  $\text{Tc}^R$  derivative, XTL298, contains *tetA-sacB* in the arabinose operon. It remains

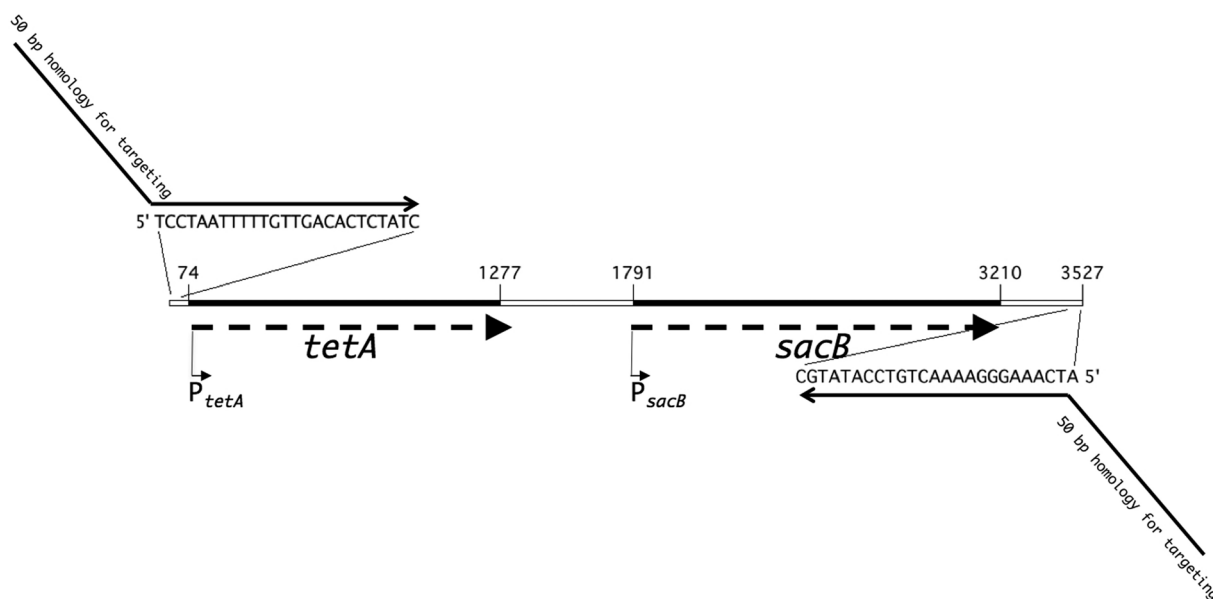


Figure 1. The *tetA-sacB* cassette. The diagram shows the relative positions of the two genes and their promoters. Dashed arrows indicate the direction of transcription through *tetA* and *sacB* coding sequences (black bars). Numbers indicate base pair positions. The sequences of primers used for PCR amplification of the cassette are indicated. These priming sequences are placed at the 3'-ends of chimeric primers that contain 50 nt of homology for targeting the dual cassette to a region of choice. With the exception of the chimeric primers, this diagram is to scale.

sensitive to sucrose and was confirmed by sequence analysis (see <http://redrecombineering.ncifcrf.gov/>).

#### Tet/SacB counter-selection and recombineering conditions

Counter-selections for *tetA* alone (11) and *sacB* alone (27) have been defined previously; we reproduced both selection conditions with specific modifications to combine them in one agar plate. The protocol for replacing *tetA-sacB* is similar to our general recombineering protocols (<http://redrecombineering.ncifcrf.gov/>, 20,21) with minor modifications. Cells are grown overnight at 30 in LB with either chloramphenicol or hygromycin to maintain pSIM5 or pSIM18, respectively. The overnight culture, diluted 80-fold in LB without drug, is grown at 32 to OD<sub>600</sub> ~0.4, induced for 15 min at 42 to express Red, and then made electro-competent. After DNA electroporation, the cells are transferred to 5 ml of LB at 32 and grown for >4 h with aeration before plating on pre-warmed (42 ) Tet/SacB counter-selection agar and incubated at 42 for 2 days. Total viable cells were determined on LB agar at 32. The same cells electroporated without added DNA were the control for spontaneous mutants that survive the double selection.

#### Insertion of *tetA-sacB* into host strains DH10B, W3110 and MG1655

The *tetA-sacB* DNA flanked by appropriate homology arms was amplified from XTL298 cells by colony touch PCR (21) using chimeric primers (Table 2) as illustrated in Figure 1. These *tetA-sacB* products were inserted into DH10B[pSIM18] either within *sfIA* or near *galM* selecting for Tc<sup>R</sup> by using previously described recombineering methods (20,21) to generate strains XTL425 and XTL426, respectively. These genomic regions, *sfIA*<>*tetA-sacB* and *galM*<*tetA-sacB*>*gpmA*, were then transferred by P1 transduction (22) from XTL425 and XTL426 into W3110 (generating strains XTL621 and XTL622, respectively) and MG1655 (generating strains XTL623 and XTL624, respectively). The <> designation indicates insertion of a construct made by recombineering within the designated gene, whereas the <*tetA-sacB*> flanking symbols indicate insertion of the construct in an intergenic region.

#### General considerations when constructing new *tetA-sacB* insertions by recombineering

In generating XTL425 and XTL426, 16 independent Tc<sup>R</sup> recombinant colonies of DH10B[pSIM18] were patched to LB tetracycline and L sucrose agar. The patches on L sucrose were compared to each other and to a XTL298 control patch to ensure that the most sucrose sensitive recombinants were chosen; this helps to avoid any PCR-generated mutations that reduce sucrose sensitivity. We have also noticed that in some genomic locations sucrose sensitivity of all recombinants is reduced. Where this occurs, we either insert the construct in the same site but in the opposite orientation and retest, or if necessary and where possible, change to a nearby insertion site. Once several of the most sucrose sensitive recombinants are isolated for a particular location, these are then

retested directly on Tet/SacB counter-selection agar and compared with XTL298 to ensure that the most sensitive candidate is used.

#### Modified P1 transduction method for replacement of *tetA-sacB* by counter-selection

P1 transductions were carried out as described previously (22) with the modification that recipient cells infected with P1vir were diluted and grown in 5 ml LB broth containing 200 mM sodium citrate for 8 h to ensure cellular segregation of the DNA markers, as well as the TetA and SacB proteins present in the starting cells. This culture was diluted and titered for total cells on LB. The remaining culture was concentrated into 100 ml of M9 salts. The concentrated cells were plated for recombinants on the selective P1 Tet/SacB counter-selection agar.

#### Creation of T-SACK, a versatile template strain for cassette amplification by PCR

The *araD*<>*tetA-sacB amp* region was moved from XTL298 to W3110 by P1 transduction, selecting for ampicillin resistance, creating XTL634. We also transferred the *argG::Tn5* transposon carrying the *kan* (Km<sup>R</sup>) cassette into this strain by P1. Finally, a *cat* (Cm<sup>R</sup>) cassette, *fliC*<>*cat* was introduced by P1. This new strain, T-SACK, has several drug resistance cassettes [*tetA-sacB*, *amp*, *cat* and *kan*]; all of which can be used as templates for PCR amplification to generate recombineering reagents by colony touch PCR. This strain is used as a template for colony touch PCR, instead of plasmid DNA. Even small amounts of plasmid DNA, which efficiently transform, show up as drug-resistant colonies, making true recombinants hard to find. Note that a *tetA* PCR product can be generated and used for Red-mediated homologous recombination when appropriate 50 base homology arms are included on the 5'-ends of the *tetA* primers (28).

## RESULTS AND DISCUSSION

### The presence of TetA enhances sucrose counter-selection

CC4231 and XTL298 differ by having *cat* or *tetA*, respectively, upstream of *sacB*. We tested whether those functions differentially affect the sensitivity caused by the *sacB* cassette on L sucrose agar. The two strains were grown to OD<sub>600</sub> ~0.4 and titered directly on L sucrose or LB agar at 32. In three independent trials, there was a 4-fold increase in sucrose sensitivity for XTL298. In a similar experiment after growth to OD<sub>600</sub> ~0.4, both cell types were made electro-competent, subjected to electroporation without DNA and plated on L sucrose and LB agar. Sucrose resistant colonies appeared with an average frequency of 1.8 × 10<sup>3</sup> per 10<sup>8</sup> colonies on LB agar for CC4231 and 4.4 × 10<sup>2</sup> per 10<sup>8</sup> colonies on LB agar for XTL298. These data indicate that sucrose counter-selection improves ~ 4-fold in cells expressing the TetA protein. We suspect that the presence of TetA in the membrane accentuates the toxicity of the periplasmic levan produced from the sucrose. It is also possible that *sacB* expression is somewhat higher in the *tetA-sacB* construct.

### Plating on Bochner/Maloy and Tet/SacB counter-selection media

We tested the inhibitory effect of *tetA-sacB* expression when challenged on Bochner/Maloy agar, which is designed to be counter-selective only for *tetA* expression (11). A log phase culture of XTL671 (DH10B *galM<tetA-sacB>gpmA*) was titered with a relative average survival of  $8.2 \times 10^3$  colonies on Bochner/Maloy versus  $10^8$  colonies on LB agar, but surviving colonies grew very poorly. When XTL622 and XTL624 (W3110 and MG1655 containing *galM<tetA-sacB>gpmA*, respectively) were tested on Bochner/Maloy agar, very little counter-selection occurred with  $\sim 4 \times 10^7$  survivors per  $10^8$  colonies on LB agar. Thus, as described previously, Bochner/Maloy agar is selective for the *recA* DH10B background but not for healthier genetic backgrounds like W3110 and MG1655 (11). As a first attempt to create a double selection plate, we added 6% sucrose to Bochner/Maloy agar; however, W3110 and MG1655 *tetA-sacB* derivatives still gave high background growth on this medium (data not shown). We therefore developed a new Tet/SacB counter-selection agar (see 'Materials and Methods' section), optimized for coselection against both gene products. It has been noted that the counter-selection growth condition against *tetA* on fusaric acid media is most efficient at 42 (29), and we have confirmed that our modified system is also most efficient at 42. Therefore, when removing the *tetA-sacB* cassette by recombineering methods, strains have been used that express the Red recombination functions from plasmids (26) rather than from the defective prophage, since bacteria with the prophage are unable to grow at 42 (2).

The three parental strains MG1655, W3110 and DH10B all form colonies on the Tet/SacB counter-selection agar at 42 with a final efficiency equal to that on LB agar. However, colonies are only barely visible after 1 day; 2 days are required for full colony formation on Tet/SacB counter-selection agar because these selective conditions reduce cell growth even without *tetA-sacB* present. We also note that MG1655 grows better than W3110, which grows better than DH10B on LB as well as on Tet/SacB counter-selection agar. We next tested survival of the

six MG1655, W3110 and DH10B derivatives containing *tetA-sacB* at either *sfi* or *galM* on the Tet/SacB counter-selection agar (Table 3). Here, when both gene products are present, they exert a synergistic toxicity, causing an extremely low survival of  $6 \times 10^7$ . We have not found reports of any counter-selection system this restrictive.

### Recombineering with the *tetA-sacB* dual counter-selection system

Single-stranded DNA recombination with 70 base long oligonucleotides was used to delete the *tetA-sacB* cassette located at *sfiA<>tetA-sacB* or *galM<tetA-sacB>gpmA* from all six derivatives of the host strains (Table 3). Oligo-generated recombinants were orders of magnitude more frequent than spontaneous survivors in the control electroporation without DNA. All colonies tested were recombinants and had lost the *tetA* and *sacB* markers.

To test the efficiency of counter-selection with a short (279 bp) PCR product, a dsDNA containing a protein binding site, P1 *parS* (30), was used to replace the *tetA-sacB* cassette at *galM<tetA-sacB>gpm* (Table 3). The frequency of removing *tetA-sacB* by recombination with this short PCR product was similar to that found using oligo B (Table 3). All 16 survivors, tested by PCR analysis, had lost the *tetA-sacB* and gained the *parS* site.

### Generation of *sfiA-luc* and *sfiA-lacZ* translational gene fusions by replacing *sfiA<>tetA-sacB*

We sought to test the efficacy of the *tetA-sacB* counter-selection with large inserts by replacing *tetA-sacB* with the luciferase gene, *luc*. DNA damage by agents like mitomycin C induces gene expression of *sfiA*, an SOS response gene (31). A fusion between the luciferase gene, *luc*, and *sfiA* was made in DH10B, MG1655 and W3110 derivatives carrying *sfiA<>tetA-sacB* [pSIM18]. The *luc* open reading frame starting with the initiation codon replaces just the open reading frame of *sfiA*. The *sfiA-luc* recombinants were generated at a frequency of  $\sim 2 \times 10^3 / 10^8$  viable cells (Table 3). Those derived from MG1655 were analyzed further. All 16 independent colonies tested were recombinant and carried *luc* as judged by PCR

Table 3. Counter-selection used to replace *tetA-sacB* cassettes by recombineering

Strain <sup>b</sup> (Parent)	Colonies on Tet/SacB Counter-Selection Agar <sup>a</sup>						
	<i>sfiA&lt;&gt;tetA-sacB</i>			Strain <sup>b</sup> (Parent)	<i>galM&lt;tetA-sacB&gt;gpmA</i>		
	No DNA <sup>c</sup>	Oligo A <sup>d</sup>	<i>sfiA-luc</i> <sup>e</sup>		No DNA <sup>c</sup>	Oligo B <sup>d</sup>	<i>parS</i> <sup>c</sup>
XTL425 (DH10B)	7	21 000	1500	XTL426 (DH10B)	53	50 000	61 000
XTL629 (W3110)	21	57 000	1600	XTL630 (W3110)	23	56 000	110 000
XTL631 (MG1655)	26	21 000	1500	XTL632 (MG1655)	42	25 000	44 000

<sup>a</sup>Survivors on Tet/SacB Counter-Selection Agar are normalized to  $10^8$  cells on LB agar.

<sup>b</sup>All strains carry the pSIM18 plasmid for Red recombination.

<sup>c</sup>Control without added DNA. The average survival of the same six strains on L sucrose was 28-fold higher than on Tet/SacB medium, with a range from 14- to 53-fold. Note that the sucrose counter-selection for cells containing *tetA* is already 4-fold more selective than for cells lacking *tetA*, as shown by comparing CC4231, the *cat-sacB* strain, to XTL298, the *tetA-sacB* strain.

<sup>d</sup>70 base oligos used to remove the *tetA-sacB* cassette by Red-mediated recombination.

<sup>e</sup>PCR products of either *sfiA-luc* ( $\sim 1.6$  kb) or *parS* (279 bp) to replace the respective *tetA-sacB* cassette in *sfiA* or the *tetA-sacB* cassette between *galM* and *gpmA*.

amplification. When assayed for luciferase, five out of eight recombinants had higher mitomycin C-inducible activity. The remaining three recombinants may have

PCR-generated mutations that lowered the luciferase activity. One of the MG1655 *sfiA-luc* fusions with the highest inducible luciferase activity was saved as XTL635.

We also created a *sfiA-lacZ* translational gene fusion by Red-mediated recombination that can be used to observe and quantitate SOS induction kinetics. LT1594, the *lacZ* deletion mutant (*tilacZ<>hyg*) of MG1655 carrying pSIM5 was used. Into this strain, the *sfiA<>tetA-sacB* counter-selection cassette was introduced by recombineering selecting for Tc<sup>R</sup>. To make the *sfiA-lacZ* gene fusion, we amplified *lacZ* by PCR using a primer with the first nine codons of *sfiA* fused in frame to the 6th codon of *lacZ*, and a primer containing the last eight codons of *lacZ*, including the TAA stop codon. These chimeric primers were used to

generate a PCR product containing from codon 6 to the stop codon of *lacZ* complete with flanking homologies.

The entire *sfiA-lacZ* gene fusion was used to replace the *sfiA<>tetA-sacB* counter-selection cassette by recombineering. LT1619 (MG1655 *tilacZ<>hyg sfiA<>tetA-sacB* [pSIM5]) expressing the Red recombination functions

was electroporated with the *sfiA-lacZ* PCR fragment and following outgrowth recombinant colonies were selected on Tet/SacB counter-selection agar. A low frequency of Tet/SacB resistant colonies ( $\sim 1 \cdot 10^2 / 10^8$  viable cells) was found, and this frequency was similar to that of a control electroporation without DNA. The lower level of *lacZ* recombinants likely reflects the greater difficulty in replacing the  $\sim 3.5$  kb *tetA-sacB* cassette with the similar sized  $\sim 3.2$  kb *lacZ* fragment rather than with the smaller  $\sim 1.6$  kb *lacZ* fragment. Plating the same recombinant cell mixture on L sucrose gave  $\sim 50$ -fold higher background than did the Tet/SacB plates, indicating the difficulty in identifying recombinants on L sucrose. Examination of 16 survivors on the Tet/SacB counter-selection agar revealed that five had become Tc<sup>S</sup> and now expressed functional LacZ, as evidenced by colonies that turned red on MacConkey lactose agar but only in the presence of mitomycin C. The other 11 colonies remained Lac and still carried some form of the *tetA-sacB* cassette as evidenced by PCR amplification and reduced Tc<sup>R</sup>.

All five recombinants expressed similar levels of  $\beta$ -galactosidase, and one was saved as LT1610. It is shown to increase LacZ expression as mitomycin C levels increase (Figure 2).

P1 transductants can be isolated by Tet/SacB counter-selection

P1 transduction was used to transfer the *sfiA-luc* fusion construct from the MG1655 derivative XTL635 into the recipient XTL621 (W3110 *sfiA<>tetA-sacB*). P1 transduction occurs by recombination between the recipient genome and the donor strain DNA, which P1 transfers to the recipient. Since only a few recombinants are generated in such crosses, transduction necessitates that there be a direct selection conferred on the recipient by the recombining donor DNA. Counter-selections are not normally stringent enough to enable selection of rare

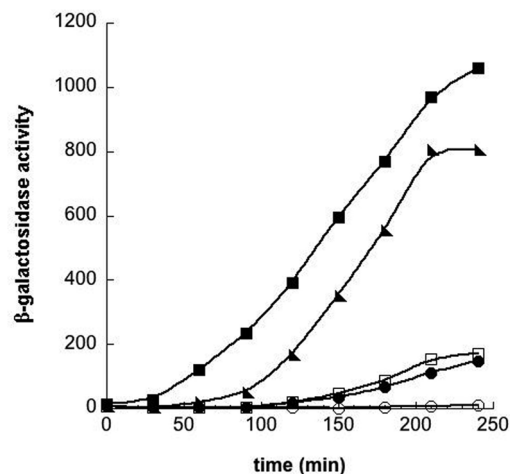


Figure 2. SOS induction of the *sfiA-lacZ* reporter construct. An overnight culture of LT1610 was diluted 1/500 into LB, distributed among five flasks, plated at 37 in a shaking water bath, and grown to an OD<sub>600</sub> of 0.1. Mitomycin C was then added ( $t = 0$ ) to the following concentrations (mg/ml): open circles, no drug; filled circles,  $3 \cdot 10^{-3}$ ; open squares,  $1 \cdot 10^{-2}$ ; filled triangles, 0.1; and filled squares, 1.0. Time points were taken every 30 min during growth and  $\beta$ -galactosidase activity in each sample was determined according to Miller (32), and plotted on the y-axis; time of exposure to Mitomycin C is plotted on the x-axis.

Table 4. Counter-selection used to replace *tetA-sacB* cassettes by P1 transduction

Donor Strain <sup>a</sup>	Recipient Strain	P1 lysate (ml) <sup>b</sup>	CFU <sup>c</sup>
XTL635	XTL621	0	31
MG1655 <i>sfiA&lt;&gt;luc</i>	W3110 <i>sfiA&lt;&gt;tetA-sacB</i>	1	185
		10	132

<sup>a</sup>A lysate of P1 vir was prepared by infection of XTL635.

<sup>b</sup>Volume of the donor P1 lysate used to infect the recipient XTL621.

<sup>c</sup>The raw number of colonies resistant to Tet/SacB counter-selection agar when plating 100 ml of concentrated cells where the total number of cells was about  $5 \cdot 10^9$ . The frequency of P1 transduced recombinants among resistant colonies tested by PCR analysis was 63%. In a second independent transduction (data not shown), the frequency of recombinants was 56%.

recombinants generated by P1 transduction. Here, *sfiA-luc* is the donor DNA, and the only selection is the Tet/SacB counter-selection against the *sfiA<>tetA-sacB* cassette present in the recipient. A control culture yielded fewer resistant colonies than cultures that had been infected with the P1 donor lysate (Table 4). Examination of the target region by PCR analysis demonstrated that the *sfiA-luc* fusion had been transferred into W3110 replacing the *tetA-sacB* cassette in 10 out of 16 resistant colonies tested (Table 4). Our ability to readily identify transductants using the P1 Tet/SacB counter-selection agar demonstrates the efficacy and general usefulness of this system.

## CONCLUSIONS

The Tet/SacB dual counter-selection system described here has unique and substantial advantages over other

selection/counter-selection systems. The *tetA* and *sacB* genes of the counter-selection cassettes each express a protein that is toxic to the bacterial cell growing on the newly developed Tet/SacB counter-selection agar. This toxic combination causes a stronger counter-selection than achieved by either gene product alone. This two-gene cassette can be used without regard to the genotype of the strain in which selection is imposed. Importantly, when combined with a homologous recombination method, like recombineering, this counter-selection system allows facile replacement of the *tetA-sacB* cassette in generating deletion, substitution or fusion mutants at the targeted region. This system is likely to be useful for genetic manipulations in other bacterial species and modification of genomic clones on bacterial artificial chromosomes.

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