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

Xin-tian Li¹, Lynn C. Thomason¹,², James A. Sawitzke¹, Nina Costantino¹ and Donald L. Court¹,*

¹Molecular Control and Genetics Section, Gene Regulation and Chromosome Biology, National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD 21702, USA and ²Leidos Biomedical Research, Inc., Frederick National Laboratory, Frederick, MD 21702, USA

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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 (TcR) and also causes sensitivity to the lipophillic chelator fusaric acid; sacB causes sensitivity to sucrose. These two genes are introduced as a joint DNA cassette into Escherichia coli by selection for TcR. 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 (TcR). TetA also causes the cell to become sensitive to lipophilic chelators, like fusaric acid,
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 TetA 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, 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. General genetic methods including recombineering 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 recombineering 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 tilaC-U69 gal490 pg[h]8 | This work       |
| DH10B        | ncrA til(mrr-hsdRMS-merBC) gal80lacZ15 tilacX74 recA1 endA1 araD139 tara-1 (aro-leu)7697 galU galK rpsL mupG | (18)            |
| DT330        | W3110 tilaC-U69 gal490 pg[h]8 | This work       |
| LT1594       | MG1655 tilaC-Z<hyg p[SI5]   | This work       |
| LT1610       | MG1655 tilaC-Z<hyg sfi-lacZ | This work       |
| LT1619       | MG1655 tilaC-Z<hyg sfi4< tetA-sacB | This work   |
| MG1655       | T-SACK                     | This work       |
|             | W3110 araD< tetA-sacB-amp  | This work       |
|             | flIC<cat argG::Tn5         | This work       |
| W3110        | in(rrnD-rrnE1) rph-1        | This work       |
| XTL298       | CC4231 araD< tetA-sacB-amp | This work       |
| XTL425       | DH10B sfa< tetA-sacB [pSIM18] | This work     |
| XTL426       | DH10B galM< tetA-sacB [pSIM18] | This work |
| XTL621       | W3110 sfa< tetA-sacB       | This work       |
| XTL622       | W3110 galM< tetA-sacB [pSIM18] | This work |
| XTL623       | MG1655 galM< tetA-sacB     | This work       |
| XTL624       | MG1655 galM< tetA-sacB [pSIM18] | This work |
| XTL629       | W3110 sfa< tetA-sacB [pSIM18] | This work |
| XTL630       | W3110 galM< tetA-sacB [pSIM18] | This work |
| XTL631       | MG1655 sfa< tetA-sacB [pSIM18] | This work |
| XTL632       | MG1655 galM< tetA-sacB [pSIM18] | This work |
| XTL634       | W3110 araD< tetA-sacB-amp | This work       |
| XTL635       | MG1655 sfi< Luc            | This work       |
| XTL671       | DH10B galM< tetA-sacB [pSIM18] | This work |

### Table 2. Oligonucleotides for PCR amplification and oligo recombination

| Oligo | Description |
|-------|-------------|
| galM / tet | AATCCACCAGCAAAACCCGACATTTTGCACTCATAATTCACAAACCTCATAATTGGTGGACACTCTATC |
| gnaA / sacB | CAAACAGCAATGCTTACGATAATCATAATGAAATGGGCCAGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / tetA | AACTGACCGGCTGGTATGATTATGACATTCTGAGTACTCCAACTAACCCGTTGAGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / sacB | CATTGGCTTGCTGGCACAACAAAAGCTTCAGGCTTTATCTCAATATTTCAGCAATATTCACTGCTTACAGGAAACCCGAACTCTTAA |
| Oligo A | GATTGTTAGATGACATTCTGAGTACTCCAACTAACCCGTTGAGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| Oligo B | ACCGACATTTGGGACCTATTTCAAAACCCGCTATTTGGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| galM / parS-1 | AATCCACCCAAACCACTCAGCTCCGAGATATGGTGACACTACTCAATTCACAAACCCGTTGAGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / luc atg | ACTGGTGTAGATGACATTCTGAGTACTCCAACTAACCCGTTGAGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / luc stop | AAGTCAGGGTTACGAGGCTAAAGAAATGGTCGAAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / lacZ atg | AACTACCAACCCAGCTTTGGAATGGTCGAAATCACAGGAAACCCGAACTCTTAA |
| sfaA / lacZ stop | CATTGGCTTGCTGGCACAACAAAAGCTTCAGGCTTTATCTCAATATTTCAGCAATATTCACTGCTTACAGGAAACCCGAACTCTTAA |

*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://redrecombineering.ncifcrf.gov/ website). The High Fidelity Platinum® Taq DNA polymerase from Invitrogen was used for PCRs. Sequencing was done by SAIC Frederic, 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 1 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, 8g of NaCl, 8g of NaH2PO4•H2O, 0.11 g ZnCl2, 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 NaH2PO4•H2O 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 ZnCl2 was stored in water at 25 mM after filter sterilizing; note that higher concentrations precipitate. Fusaric acid (0.5 ml) and ZnCl2 (32 ml) were added to the molten agar mixture after cooling to 55°C.

The final volume was brought to 1 liter with sterile H2O.

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-selector 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°C 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°C to OD600~0.4, induced for 15 min at 42°C to express Red, and then made electro-competent. After DNA electroporation, the cells are transferred to 5 mL of LB at 32°C and grown for >4 h with aeration before plating on pre-warmed (42°C) Tet/SacB counter-selection agar and incubated at 42°C for 2 days. Total viable cells were determined on LB agar at 32°C. 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 TcR 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 TcR 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-TSACK, 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 (KmR) cassette into this strain by P1. Finally, a cat (CmR) cassette, fliC<>cat was introduced by P1. This new strain, T-TSACK, 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 in 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 OD600~0.4 and titered directly on L sucrose or LB agar at 32°C. In three independent trials, there was a 4-fold increase in sucrose sensitivity for XTL298. In a similar experiment after growth to OD600~0.4, 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 1.8·10^5 per 10^8 colony-forming units on LB agar for CC4231 and 4.4·10^5 per 10^8 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 × 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 cell growth even without tetA-sacB were 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>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.

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 ~2 × 10^1/10^8 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.

| Strain (Parent) | galM</teta-sacB>gpmA | Strain (Parent) | tetA-sacB | sfiA-luc |
|-----------------|----------------------|-----------------|------------|----------|
|                  | No DNA^c             | Oligo A^d       | sfiA-luc   | No DNA^c | Oligo B^d | parS^e |
| 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 |

^aSurvivors on Tet/SacB Counter-Selection Agar are normalized to 10^8 cells on LB agar.

^bAll strains carry the pSIM18 plasmid for Red recombination.

^cControl 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.

^dPCR products of either sfiA-luc (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.

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

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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 (tlacZ<>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>8</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 tlacZ<>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 (~1 × 10<sup>2</sup> /10<sup>8</sup> 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 ~3.5 kb tetA-sacB cassette with the similar sized ~3.2 kb lacZ fragment rather than with the smaller ~1.6 kb luc fragment. Plating the same recombinant cell mixture on L sucrose gave ~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>8</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>8</sup>. All five recombinants expressed similar levels of β-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 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 \textit{tet}A and \textit{sac}B 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 \textit{tet}A-\textit{sac}B 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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