Positive and negative selection using the \textit{tetA-sacB} cassette: recombineering and P1 transduction in \textit{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 \textit{tetA} and \textit{sacB} genes are contained in a DNA cassette and confer a novel dual counter-selection system. Expression of \textit{tetA} confers bacterial resistance to tetracycline (TcR) and also causes sensitivity to the lipophillic chelator fusaric acid; \textit{sacB} causes sensitivity to sucrose. These two genes are introduced as a joint DNA cassette into \textit{Escherichia coli} by selection for TcR. A medium containing both fusaric acid and sucrose has been developed, in which, coexpression of \textit{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 \textit{in vivo} by the process of homologous recombination (1–4). This recombination is carried out by phage recombinases, like those of the \textit{\lambda}\ 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, \textit{\sim}50 base segments of homology to the target. \textit{\lambda}\ Exo binds dsDNA ends and removes bases from the 5'-end, and \textit{\lambda}\ 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 \textit{\lambda}\ 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 \textit{tetA-sacB} cassette, a particularly useful two-step system, which provides unique advantages over other counter-selection methods.

The \textit{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 levans, which accumulates in the periplasm and is toxic to E. coli (12,13). Combining both of these genes into a tet-A-sacB cassette provides Te" 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 tet-A-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 (StrR), galK, thyA or tolC to be present in the genome (See the respective references 14–17).

**Table 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC4231</td>
<td>W3110 ΔluxU169 gal490 pgIΔ8 [lacI857 Δ(cro-hisA)] araD-&lt;tet-sacB-amp</td>
<td>This work</td>
</tr>
<tr>
<td>DH10B</td>
<td>mcrA Δ(mcrA::rpsL M15 lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara-leu)7697 galU galK rpsL mupG</td>
<td>(18)</td>
</tr>
<tr>
<td>DY330</td>
<td>W3110 ΔluxU169 gal490 pgIΔ8 [lacI857 Δ(cro-hisA)]</td>
<td>(2)</td>
</tr>
<tr>
<td>LT1594</td>
<td>MG1655 ΔlacZ&lt;--&gt;hyg [pSIM5]</td>
<td>This work</td>
</tr>
<tr>
<td>LT1610</td>
<td>MG1655 ΔlacZ&lt;--&gt;hyg sfiA&lt;--&gt;tetA-sacB</td>
<td>This work</td>
</tr>
<tr>
<td>LT1619</td>
<td>MG1655 ΔlacZ&lt;--&gt;hyg sfiA&lt;--&gt;tetA-sacB [pSIM5]</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655</td>
<td>T-SACK W3110</td>
<td>This work</td>
</tr>
<tr>
<td>XTL425</td>
<td>DH10B sfiA&lt;--&gt;tetA-sacB [pSIM18]</td>
<td>This work</td>
</tr>
<tr>
<td>XTL426</td>
<td>DH10B galM&lt;tetA-sacB&gt;gpmA [pSIM18]</td>
<td>This work</td>
</tr>
<tr>
<td>XTL621</td>
<td>W3110 sfiA&lt;--&gt;tetA-sacB</td>
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<td>XTL622</td>
<td>W3110 galM&lt;tetA-sacB&gt;gpmA</td>
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<td>XTL623</td>
<td>MG1655 sfiA&lt;--&gt;tetA-sacB</td>
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<td>XTL624</td>
<td>MG1655 galM&lt;tetA-sacB&gt;gpmA</td>
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<td>XTL629</td>
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<td>MG1655 sfiA&lt;--&gt;tetA-sacB [pSIM18]</td>
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<td>XTL632</td>
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<td>XTL671</td>
<td>DH10B galM&lt;tetA-sacB&gt;gpmA</td>
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**Table 2. Oligonucleotides for PCR amplification and oligo recombination**

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>galM / tet</td>
<td>ACAACAGCTGCGACACTATTTGTGCAACTCAATTTGTGACACTATTC</td>
</tr>
<tr>
<td>gpmA / sacB</td>
<td>CAATCCCGGGCGATAAAAGGGAAACTGCTCATGCA</td>
</tr>
<tr>
<td>sbfA / luc stop</td>
<td>AAGCAATTTTTGTGCAACTACATTTGTGCAACTCAATTTGTGACACTATTC</td>
</tr>
<tr>
<td>Oligo B</td>
<td>AAATCCCGTGCTGCGACACTATTTGTGCAACTCAATTTGTGACACTATTC</td>
</tr>
<tr>
<td>galM / parS-1</td>
<td>ACAACCGATGCTGCGACACTATTTGTGCAACTCAATTTGTGACACTATTC</td>
</tr>
<tr>
<td>gpmA / parS-2</td>
<td>CAATCCCGGGCGATAAAAGGGAAACTGCTCATGCA</td>
</tr>
<tr>
<td>sbfA / luc stop</td>
<td>AAGCAATTTTTGTGCAACTACATTTGTGCAACTCAATTTGTGACACTATTC</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Bacterial strains and plasmids**

Bacterial strains are described in Table 1. Standard genetic methods including recombination 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

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[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 tet-A-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-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 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 µg/ml), hygromycin (200 µg/ml), chloramphenicol (10 µg/ml) and ampicillin (30 µg/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 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 was then autoclaved. The final volume was brought to 1 liter with sterile H2O.

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 TcR derivative, XTL298, contains tetA-sacB in the arabinose operon. It remains

![Figure 1](http://nar.oxfordjournals.org/)

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 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°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 used 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 restested 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 µl 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::Th5 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-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 λ Red-mediated homologous recombineering 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 OD600 ~0.4 and tittered 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 x 10^4 per 10^8 colonies on LB agar for CC4231 and 4.4 x 10^4 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 sucrase. 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>galM) was titered with a relative average survival of 8.2×10^2 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 ~4×10^6 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 grew 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°C (29), and we have confirmed that our modified system is also most efficient at 42°C. Therefore, when removing the tetA-sacB cassette by recombinase 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°C (2).

The three parental strains MG1655, W3110 and DH10B all form colonies on the Tet/SacB counter-selection agar at 42°C 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 ≤6×10^-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>gpmA (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 starts with the initiation codon replaces just the open reading frame of sfiA. The sfiA-luc recombinants were generated at a frequency of ~2×10^3/10^8 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>
<thead>
<tr>
<th>Strainb (Parent)</th>
<th>Colonies on Tet/SacB Counter-Selection Agara</th>
<th>Strainb (Parent)</th>
<th>Colonies on Tet/SacB Counter-Selection Agara</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sfiA&lt;&gt;tetA-sacB</td>
<td>galM&lt;tetA-sacB&gt;gpmA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No DNAc</td>
<td>Oligo A^d</td>
<td>sfiA-lue^e</td>
</tr>
<tr>
<td>XTL425 (DH10B)</td>
<td>7</td>
<td>21 000</td>
<td>1500</td>
</tr>
<tr>
<td>XTL629 (W3110)</td>
<td>21</td>
<td>57 000</td>
<td>1600</td>
</tr>
<tr>
<td>XTL631 (MG1655)</td>
<td>26</td>
<td>21 000</td>
<td>1500</td>
</tr>
</tbody>
</table>

\(^a\)Survivors on Tet/SacB Counter-Selection Agar are normalized to 10^8 cells on LB agar.
\(^b\)All strains carry the pSIM18 plasmid for Red recombination.
\(^c\)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.
\(^d\)70 base oligos used to remove the tetA-sacB cassette by Red-mediated recombination.
\(^e\)PCR 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.
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 (ΔlacZ::hyg) of MG1655 carrying pSIM5 was used. Into this strain, the sfiA<->tetA-sacB counter-selection cassette was introduced by recombination selecting for TeC. 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 lacZ complete with flanking homologies. The entire sfiA-lacZ gene fusion was used to replace the sfiA<->tetA-sacB counter-selection cassette by recombination. LT1619 (MG1655 ΔlacZ::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 x 10^7 /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 ~3.5 kb tetA-sacB cassette with the similar sized ~3.2 kb lacZ fragment rather than with the smaller ~1.6 kb lac 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 TeC 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 TeC. 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 recombinating 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). The raw number of colonies resistant to Tet/SacB counter-selection agar when plating 100 μl of concentrated cells where the total number of cells was about 5 x 10^7. 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%.

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

<table>
<thead>
<tr>
<th>Donor Strain</th>
<th>Recipient Strain</th>
<th>P1 lysate (μl)</th>
<th>CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>XTL635</td>
<td>XTL621</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>MG1655</td>
<td>W3110 sfiA&lt;-&gt;tetA-sacB</td>
<td>1</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>132</td>
</tr>
</tbody>
</table>

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

**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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Conflict of interest statement. The National Institutes of Health has a patent covering methods used for recombinase. Authors Court, Costantino, Li and Sawitzke are inventors on these patents and employees of NIH.

REFERENCES

24. Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K.,
    Choi, S., Obitsubo, E., Baba, T., Wanner, B. L., Mori, H. et al. (2006)
    Highly accurate genome sequences of Escherichia coli K-12 strains
25. Durfee, T., Nelson, R., Baldwin, S., Plunkett, G. 3rd, Burland, V.,
    (2008) The complete genome sequence of Escherichia coli DH10B:
    insights into the biology of a laboratory workhorse. *J. Bacteriol.*, 190,
    2597–2606.
    recombineering plasmids for gram-negative bacteria. *Gene*, 379,
    109–115.
    Allelic exchange in Escherichia coli using the Bacillus subtilis sacB
    Microbiol.*, 5, 1447–1457.
    Rapid oligonucleotide-based recombineering of the chromosome of
    Salmonella enterica. *Appl. Environ. Microbiol.*, 75,
    1575–1580.
    Dynamics of Escherichia coli chromosome segregation during
    (umuC) by 4-quinolone antibacterial drugs. *J. Med. Microbiol.*, 36,
    78–82.