Improved seamless mutagenesis by recombineering using ccdB for counterselection

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ABSTRACT

Recombineering, which is the use of homologous recombination for DNA engineering in Escherichia coli, usually uses antibiotic selection to identify the intended recombinant. When combined in a second step with counterselection using a small molecule toxin, seamless products can be obtained. Here, we report the advantages of a genetic strategy using CcdB as the counterselectable agent. Expression of CcdB is toxic to E. coli in the absence of the CcdA antidote so counterselection is initiated by the removal of CcdA expression. CcdB counterselection is robust and does not require titrations or experiment-to-experiment optimization. Because counterselection strategies necessarily differ according to the copy number of the target, we describe two variations. For multi-copy targets, we use two E. coli hosts so that counterselection is exerted by the transformation step that is needed to separate the recombined and unrecombined plasmids. For single copy targets, we put the ccdA gene onto the temperature-sensitive pSC101 Red expression plasmid so that counterselection is exerted by the standard temperature shift to remove the expression plasmid. To reduce unwanted intramolecular recombination, we also combined CcdB counterselection with Redx omission. These options improve the use of counterselection in recombineering with BACs, plasmids and the E. coli chromosome.

INTRODUCTION

DNA engineering methods are central to molecular biology. However, the original, revolutionary, ‘cut and paste’ methods based on restriction enzymes and DNA ligations are limited to engineering exercises for small DNA molecules. Recombineering using phage protein-mediated homologous recombination in Escherichia coli (1–10) was developed to engineer cloned DNA molecules of all sizes. Recombination occurs through homology regions, which are stretches of identical DNA sequence shared by the two molecules that recombine. Because the homology regions can be chosen freely, recombineering is not dependent on the location of restriction sites and any unique position on a target molecule can be specifically altered.

Recombineering is mediated by transient expression of the phage recombinase pairs, either RecE/RecT from the Rac prophage (1,3,11,12) or Redα/Redβ from λ phage (2–5,11–13). RecE and Redα are 5'-3' exonucleases (14,15), and RecT and Redβ are DNA annealing proteins (16–18). Linear DNAs, either double-stranded, usually in the form of polymerase chain reaction (PCR) products, or single-stranded synthetic oligonucleotides (19,20) are introduced by electroporation and provide the substrates to introduce genetic change adjacent to the region of homologous recombination. Interaction between RecE and RecT, or Redα and Redβ, facilitates double-stranded
homologous recombination (11). However, only the annealing protein is required for recombination promoted by single-stranded oligonucleotides (19,20). Recombineering is convenient because efficient recombination can be achieved with short lengths of perfect sequence identity, typically ≤50 bp, and the adjacent regions of nonhomology can range from 1 to >50 kb (1,12,13) so virtually any mutation or insertion can be achieved. Recombineering is now widely used to engineer cloned DNA in all commonly used vectors (e.g. BACs, fosmids, plasmids) and several prokaryotic chromosomes. Applications include subcloning of precisely defined sections by gap repair (3), oligonucleotide-directed mutagenesis (19,20), BAC engineering for gene targeting (21–23), high-throughput DNA engineering (24–27) and a variety of other precise applications.

Seemless mutagenesis refers to site-directed mutagenesis without any other nearby change, such as the presence of the selectable gene used to introduce the mutation. Mutagenesis in a protein coding region is a clear example of the need for seamless DNA engineering because any extraneous sequence introduced during the mutagenic step could interfere with protein expression. Seamless mutagenesis has been achieved using a two-step selection/counterselection strategy, which first involves insertion at the target site of a selectable cassette such as an antibiotic resistance gene accompanied by a counterselectable gene. The cassette is then subsequently replaced seamlessly with the desired sequence by selecting against the counterselectable gene usually involving the administration of a small molecule, such as streptomycin or a sugar (1,28–37). Popular options of counterselectable markers include sacB (1,28), rpsL (29–32), as well as markers that can, in the right host background, both be selected for and against including galK (33), thyA (34) and tolC (35; Supplementary Table 1 provides a summary of counterselectable systems).

Bacterial toxin–antitoxin (TA) systems are based on closely linked genes that together encode a protein poison and an antidote. The best characterized TAs operate to maintain plasmids. Typically the toxin is stable and the antitoxin is unstable so if the plasmid encoding the TA pair is lost, the host will die by a mechanism known as post-segregational killing (38–40). The encoding the TA pair is lost, the host will die by a mechanism known as post-segregational killing (38–40). The best characterized TAs closely linked genes that together encode a protein poison and an antidote. The best characterized TAs are the ccdB and ccdA genes, which together confer CcdB and CcdA resistance (Figure 1A bottom and Figure 1C). The concentration of L-rhamnose used for induction was 1.4 mg/ml.

Recombineering

Recombineering was performed as previously described (58). PCR was performed with Phusion polymerase (New England Biolabs, GmbH, Frankfurt am Main, Germany) according to the manufacturer’s protocol. Oligonucleotides that can act as Okazaki-like primers were preferred for the counterselection step to achieve higher efficiencies of mutagenesis (19,20).

Plasmids

pSC101-ccdA-gbaA (Figures 1A and 2C) carries the ccdA gene under the control of the arabinose-inducible P_BAD promoter, as well as the λ phage redA, redB and redY genes together with the E. coli recA gene (redYβfaA) in a polycistronic operon (60) under the control of the rhamnose-inducible P_RhaB promoter. Induction with l-rhamnose promotes homologous recombination, whereas induction with l-arabinose promotes CcdA expression to confer CcdB resistance (Figure 1A bottom and Figure 2D). pSC101-ccdA-gbaA is based on pSC101, which is a low copy number (∼5 per cell) and temperature sensitive plasmid that replicates at 30°C but not at 37°C (59). Consequently, it can be easily eliminated from the host by temperature shift in the absence of selection. pSC101-ccdA-gb was generated from pSC101-ccdA-gbA by recombineering to delete the redA and recA reading frames. p15A-ccdB-amp (Figure 2A) and p15A-ccdB-em were constructed by recombineering. p15A-rpsL-ccdB (Figure 2B) was derived from p15A-ccdB-amp by inserting a rpsL-genta cassette (32) between ccdB and amp.

Materials and Methods

Bacterial Strains, Plasmids and culturing conditions

Escherichia coli strains used in this work are listed in Table 1. Escherichia coli strains were maintained in LB medium at 30 or 37°C and selected with appropriate antibiotics [chloramphenicol (cm), 15 µg/ml; ampicillin (amp), 100 µg/ml; gentamycin (genta), 3 µg/ml and tetracycline (tet), 5 µg/ml]. The concentration of l-arabinose and l-rhamnose used for induction was 1.4 mg/ml.

Recombineering

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E. coli strains

| Strain       | Genotype or relevant features                           |
|--------------|--------------------------------------------------------|
| GB2005       | (HS996, ΔrecET, ΔybcC). The endogenous recET locus and the DLP12 prophage ybcC, which encodes a putative exonuclease similar to the Reda, were deleted |
| DB3:1        | gyrA462 (gyrA462, Invitrogen GmbH, Karlsruhe, Germany). Used for propagating plasmids containing ccdB |
| GB05-red     | (GB05-red, gyrA462) GyrA mutation of Arg462Cys |
| GBred-gyrA462| (GB2005, araC-BAD-γara) lambda red operon and recA under PBAD promoter were inserted at the ybcC locus |
| GB05-MtaA    | (GB2005, mtaA-genta) a Pant transferase coding gene (mtaA) from myxobacterium Stigmatella aurantiaca DW4/3-1 was randomly transposed into the chromosome. |

**Generation of CcdB-resistant E. coli GBred-gyrA462 strain**

Two single-stranded complementary oligonucleotides (88 mers, 50 pmol), containing the $gyrA_{Arg462Cys}$ mutation, gryA1 and gryA2 (Table 2), were electroporated into arabinose-induced E. coli GB05-red (58) and incubated at 37°C overnight. CcdB resistance was used to identify mutant cells by electroporation with p15A-rlspL-ccdB (Figure 2B). Surviving clones were then cultured in 500 μg/ml streptomycin to eliminate the plasmid.

**Using ccdB counterselection to modify pBeloBAC11**

The general strategy for BAC or chromosome recombineering using ccdB counterselection is depicted in Figure 1A. Briefly, the dual inducible expression plasmid pSC101-ccdB-gbaA was electroporated into E. coli DH10B harboring pBeloBAC11. The linear targeting molecule containing ccdB-amp was amplified from BcRI digested p15A-ccdB-amp by PCR using oligonucleotides BACccdB-amp1 and BACccdB-amp2. As shown in Figure 4A, the correct first recombinant product, pBeloBAC11-ccdB-amp, was obtained after plating on LB plates containing amp and L-arabinose. The resulting recombinants were analyzed by EcoR1 restriction digestion. In the second round of recombineering, 50 pmol of synthetic lagging oligonucleotide, BACccdB-res, was electroporated. After recovery, the cultures were diluted 100 times and 100 μl was plated on LB plates supplemented with cm, 0.1 mM IPTG and 40 μg/ml X-Gal. The plates were incubated at 37°C overnight and the number of colonies was counted.

**Point mutagenesis of plu3263**

Two rounds of recombineering were used to introduce a point mutation in plu3263 in a pBR322 vector (Figure 5A). Briefly, the target plasmid pGB-plu3263 together with a ccdB-cm PCR product, amplified from BcRI-digested p15A-ccdB-cm, were co-electroporated into arabinose-induced GBred-gyrA462 competent cells. The recombinant plasmid pGB-plu3263-PCP3-ccdB-cm was selected on LB plates containing cm and amp, confirmed by restriction analysis and transformed into GBred-gyrA462 to separate away the parental plasmid. The oligonucleotides used for the PCR amplification of the ccdB-cm cassette were 3263PCP3ccdBcm5 and 3263PCP3ccdBcm3. In the second round of recombineering, 50 pmol of synthetic single-stranded lagging oligonucleotide, 3263SPCP3A-B, and the pGB-plu3263-PCP3-ccdB-cm plasmid DNA were co-electroporated into arabinose-induced GB05-Red competent cells. The recombinant plasmid pGB-plu3263M was screened on LB plates containing amp and identified by restriction analysis and sequencing. Three correct pGB-plu3263M clones were subsequently electroporated into E. coli GB05-MtaA (12.61) to evaluate lumnimide production.

**Point mutagenesis of human Brd4 BAC**

A modified 170-kb BAC (RP11-106D4) containing human Brd4 gene was mutated to change amino acid 433 of the human Brd4 gene (ensembl Transcript ID: ENST00000263377) from asparagine (AAC) to phenylalanine (TTC). The oligonucleotides hBrd4-M2-5 and hBrd4-M2-3 were used to amplify the 1.4-kb ccdB-amp cassette from the template p15A-ccdB-amp. The oligonucleotide hBrd4-M2-Rescue, which hybridizes with the lagging strand DNA of the BAC, was used to replace the ccdB-amp cassette. Except for the use of pSC101-ccdB-gb and the E. coli host obtained from the CHORI genome resources carrying the hBrd4 BAC, the methods used were the same as described above for modification of pBeloBAC11. A detailed description of the method can be found in the Supplementary Material.

**Cultivation, extraction and HPLC-MS analysis**

*Escherichia coli* GB05-MtaA containing pGB-plu3263 or pGB-plu3263M was inoculated from overnight cultures (2%) into 5 ml of LB medium with amp in 15-ml glass tubes. The expression of Plu3263 was induced with anhydrotetracycline (0.5 μg/ml) at 4 h after inoculation, the cultures were incubated for another 4 h and 2% of XAD-16 absorber resin was added. After a further 24 h culture at 30°C, the cells and resin were harvested by centrifugation and extracted with acetone and methanol. The solvents were removed in vacuo by Genevac and the residue was dissolved in 500 μl methanol. An aliquot of 5 μl was analyzed by HPLC-MS using an Agilent 1100 series solvent delivery system coupled to Bruker HCTplus ion trap mass spectrometer. The chromatographic conditions were as follows: RP column Nucleodur C18, 125 by 2 mm, 2.5 μm particle size and precolumn C18, 8 x 3 mm, 5 μm. Solvent gradient [with solvents A (water and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid)] from 5 to 95% B within 20 min, followed by 3 min with 95% B at a flow rate of 0.4 ml/min. Detection was carried out in positive and negative ion models.
1. Introduce the dual expression plasmid pSC101-ccdA-gbaA.

2. Recombineering step 1
   — Electroporate PCR product.
   — Select for amp.
   — CcdB expression is permitted, because CcdA is present.

3. Recombineering step 2
   — Electroporate ssDNA/dsDNA.
   — Select for antibiotic resistance on BAC backbone (CmR).
   — Raise temperature to eliminate pSC101-ccdA-gbaA and CcdA. If CcdB remains, it is lethal.

Figure 1. Overview of ccdB counterselection strategies. (A) Strategy for seamless mutagenesis of BAC or chromosome using ccdB counterselection. First, pSC101-ccdA-gbaA is introduced into the host carrying the target BAC or chromosome then Red/Red/Red/RecA and CcdA expression are induced by L-rhamnose and by L-arabinose, respectively. Electro-competent cells are prepared and electroporated with a linear targeting molecule containing a ccdB-selectable marker (here amp) fusion gene. Correct recombinants are obtained by selection for amp resistance together with L-arabinose induction of CcdA expression and incubation at 30°C (to retain pSC101-ccdA-gbaA). In the next step, cells harboring pSC101-ccdA-gbaA and correctly integrated ccdB-amp are grown in LB medium plus amp and L-arabinose at 30°C. Shortly before electroporation with another linear targeting molecule or oligonucleotide for the counterselection step, L-rhamnose is added to the cultures to induce Red/Red/Red/RecA expression. After electroporation, the cells are incubated at 37°C without L-arabinose to eliminate the pSC101 plasmid and CcdA expression. The surviving cells must have eliminated CcdB expression. (B) Strategy for seamless mutagenesis in multi-copy plasmids using ccdB counterselection. GBred-gyrA462 is induced with L-arabinose to express Red/Red/Red/RecA before electro-competent cells are prepared. Then the target multi-copy plasmid and the linear ccdB-selectable marker (here cm) targeting molecule are co-electroporated. Recombinants are obtained by plating on LB plates containing cm. Then the plasmids containing the correctly targeted ccdB/cm cassette are purified and co-electroporated into GB05-red cells that have been cultured in L-arabinose to induce Red/Red/Red/RecA expression. After selection for the antibiotic resistance conveyed by the plasmid, only cells that carry the plasmid without CcdB expression will survive.
RESULTS

The ccd counterselection strategies are illustrated in Figure 1. For single copy targets, the ccdA gene is expressed from the same plasmid as the Red genes. In pSC101-ccdA-gbaA, ccdA is expressed from the arabinose-inducible P\textsubscript{BAD} promoter and the Red\textsubscript{y kaA} operon from the rhamnose-inducible P\textsubscript{RhaB} promoter. The plasmid is introduced into a host containing the target followed by arabinose and rhamnose induction and electroporation with a ccdB-antibiotic resistance gene cassette (here \textit{amp}) flanked by homology arms. After selection for amp resistance, the culture is induced only with rhamnose, and recombination is evaluated by co-electroporating a linear PCR product carrying the kanamycin resistance gene and a target plasmid into L-rhamnose induced (ii) or uninduced (i) GB2005 cells harboring pSC101-ccdA-gbaA. The linear PCR product had two 50bp homology arms to the plasmid. After recovery, 10\mu l of culture were plated on LB plates supplemented with kanamycin. The results show that expression of Red\textsubscript{y kaA} mediates recombination and the rhamnose-inducible P\textsubscript{RhaB} promoter is stringent. Expression of CcdA and CcdB from pSC101-ccdA-gbaA and p15A-ccdB-amp was tested by electroporating p15A-ccdB-amp into L-arabinose induced (iv) or uninduced (iii) GB2005 cells harboring pSC101-ccdA-gbaA. After recovery, 10\mu l of culture were plated on LB plates containing \textit{amp} and L-arabinose (iv) or not (iii). The results show that CcdB is toxic and counteracted by expression of CcdA, which is tightly regulated by the arabinose-inducible P\textsubscript{BAD} promoter.
electroporated with DNA containing the seamless mutation (either double-stranded DNA or a single-stranded oligonucleotide) and maintained at 37°C to eliminate the expression plasmid. Cells that have eliminated the ccdB gene will survive, whereas cells that have not will die from CcdB toxicity.

For multi-copy targets, there is an implicit limitation with counterselection. If a host cell contains both the intended product without the counterselectable gene and the parental plasmid with the counterselectable gene, it will die under counterselection pressure. In other words, the presence of the counterselectable gene is dominant and will occlude recovery of the intended product. Hence counterselection with plasmids is best exerted by including a transformation step to separate the parental and recombined plasmids. To incorporate the transformation step in an optimized counterselection strategy, we made a gyrA462 mutation in the recombineering strain, GB05-red, which has the arabinose-inducible PBad-Red/βaA operon integrated into the chromosome (58). In the first step, GBred-gyrA462 is induced with arabinose, then co-electroporated with the target plasmid and the ccdB-erm cassette flanked by homology arms for recombineering. After selection for cm and plasmid-mediated resistance, plasmids are isolated and co-electroporated with DNA containing the seamless mutation (either double-stranded DNA or a single-stranded oligonucleotide) into arabinose induced GB05-red and selected for plasmid-mediated resistance. Cells harboring plasmids that have eliminated the ccdB gene will survive.

To facilitate ccdB counterselection, we built pSC101-ccdA-gbaA, p15A-ccdB-amp (Figure 2) and p15A-ccdB-cm (not shown). These reagents were verified by functional tests (Figure 2D).

Generation of CcdB-resistant E. coli GBred-gyrA462

During our initial work with ccdB counterselection for recombineering, we introduced pSC101-BAD-gbaA into the gyrA462 strain, DB3.1, but found that recombination efficiencies were low. This raised the possibility that the gyrase mutation adversely affects Red recombination. To address this possibility, we introduced the gyrA462 arginine to cysteine mutation into GB05-red by oligonucleotide-directed mutagenesis (Figure 3A) and compared GBred-gyrR462 to the parental strain, GB05-red, as well as DB3.1 with pSC101-BAD-gbaA (Figure 3B). These data exclude an impact of the gyraseA mutation on Red recombination and establish GBred-gyrA462 as a useful host for CcdB counterselection.

Seamless BAC mutagenesis using ccdB counterselection

A functional test based on β-galactosidase expression from pBeloBAC11 was used to evaluate the efficiency and fidelity of ccdB counterselection for BAC recombineering (Figure 4A). The lacZ gene of pBeloBAC11 was first disrupted with the ccdB-amp cassette. Then the ccdB-amp cassette was replaced with an 80-nt oligonucleotide by counterselection against CcdB after termination of CcdA expression (by removing arabinose and culturing at 37°C to eliminate pSC101 replication).

After counterselection, restoration of the lacZ gene was scored by blue versus white colonies on LB plates supplemented with IPTG and X-Gal (Figure 4B). We used this test to evaluate the relationship between CcdA removal and CcdB toxicity using several recovery methods with different parameters (Figure 4C). All variations worked well, which indicates that the method is robust and is not sensitive to small variations of protocol, unlike other counterselection methods.

After counterselection, ~95% of the colonies were blue and 5% were white (Figure 4C). We picked 16 white colonies and 4 blue colonies for restriction analysis (Figure 4D). Unexpectedly, all 16 white clones had the same digestion pattern as the 4 blue clones, suggesting that the white clones were also successful recombinations but other mutations had been introduced into lacZ during the procedure. Four of these white clones were sequenced to find that all the additional mutations occurred in the homology arms, indicating that the errors came from defective oligonucleotide synthesis.

Point mutation in a multi-copy plasmid carrying repeated sequences

The candidate secondary metabolite operon, plu3263, was identified in the genome sequence of Photorhabdus luminescens TT01 DSM15139 (62). It encodes an
nonribosomal peptide synthetase (NRPS) composed of five modules and was directly cloned into the pBR322 vector, pASK3 under a tet inducible promoter by linear plus linear homologous recombination (12). The luminmides were identified after tet induction and expression in E. coli GB05-MtaA (12). The invariant serine 2742 residue in the signature sequence (G-G-D/H-S-L) of the PCP3 domain in Plu3263 is likely to be the active site for attachment of the 40-phosphopantetheine cofactor (63), catalyzing the conversion of apo-PCP to holo-PCP. When this serine residue is mutated, the function of PCP3 domain should be completely lost, thereby preventing luminmide production. To change serine 2742 to alanine, TCG was changed to GCG using CcdB counterselection (Figure 5A). In the first recombineering round, a 1-bp deletion was introduced into codon 2742 in addition to insertion of the ccdB-cm cassette. In this step, 10 out of 12 random checked colonies on the selection plates were shown to be mixtures of pGB-plu3263 and pGB-plu3263-PCP3-ccdB-cm as expected (data not shown). The recombinant and unmutated plasmids were separated by retransformation into E. coli GBred-gyrA462. We checked 12 random colonies to find six clones of pGB-plu3263-PCP3-ccdB-cm. These were electroporated into E. coli GB2005 to check the function of ccdB gene and all six clones killed the host, indicating that CcdB was expressed as expected.

pGB-plu3263-PCP3-ccdB-cm was then co-electroporated into GB-05red with an oligo to replace the ccdB-cm cassette. We checked 24 colonies and found 15 to be correct (Figure 5B and data not shown). Like most NRPS genes, plu3263 contains many repeated sequences. We found that the remaining nine incorrect clones were all owing to intramolecular recombination between the repeated sequences. Background from intramolecular recombination is inherent to counterselection because any mutation that ablates expression of the counterselectable gene will be selected. Three correct pGB-plu3263M clones were subsequently electroporated into E. coli GB05-MtaA to check for the production of luminmides (the products of Plu3263). HPLC-MS analysis showed that the mutation abolished luminmide production (Figure 5C).

CcdB counterselection in difficult cases
As illustrated by the above plu3263 example, intramolecular recombination to eliminate the counterselection cassette can be a significant problem. Previously we showed that unwanted intramolecular recombination events during counterselection can be reduced by omitting expression of Reda and using single-stranded oligonucleotides (32). To apply Reda omission to ccdB
counterselection, we deleted RedE from pSC101-ccdA-gbaA to generate pSC101-ccdA-gb (Figure 6A). For an application, we chose point mutagenesis of a 170-kb human Brd4 BAC because previous attempts using rpsL counterselection had failed owing to intramolecular recombination between repeated sequences. In our experience, the point mutation to change Brd4 amino acid 433 from asparagine to phenylalanine was a challenging counterselection exercise. In the first step, ccdB-amp integration was more efficiently promoted by pSC101-ccdA-gbaA than pSC101-ccdA-gb as expected (Figure 6B). Although less efficient, double-stranded DNA recombination mediated by RedE is still sufficient to be useful (32). For the oligonucleotide counterselection step, both pSC101-ccdA-gbaA and pSC101-ccdA-gb promoted similar numbers of surviving colonies (Figure 6C; Rha+/oligo). However for pSC101-ccdA-gbaA, about half of these colonies were correct and half were intramolecular deletions (Figure 5C and D; compare Rha+/oligo to Rha+/H2O), whereas most surviving colonies were correct in the pSC101-ccdA-gb case (Figure 6C and E). A detailed protocol for this point mutation exercise is available in the Supplementary Data.

**DISCUSSION**

The development of recombineering has enabled a wide variety of DNA engineering applications (6–10). Among these, methods based on consecutive steps of insertion by selection and replacement by counterselection are prominent because they permit seamless mutagenesis, which is an ideal DNA engineering goal. However, seamless mutagenesis by counterselection remains challenging in many applications. Here we report an improved counterselection system based on the ccdA/ccdB toxin/antitoxin system. We first used sacB for counterselection in recombineering (1,28), which is based on addition of a high concentration of sucrose. However, sacB counterselection is often inefficient with false positives commonly surviving. Furthermore, the sacB gene is present in the vector backbones of pBACe3.6 and pTARBAC series, making it unsuitable for use with many BAC constructs. To find a better counterselection system, we then used rpsL, which conveys sensitivity to streptomycin (20). Among other counterselection systems, galK/2-deoxy-galactose + glycerol (33), thyA/tri-methoprim + thymine (34) and tolC/colicin E1 (35) have been used (Supplementary Table 1). However, these systems require specifically mutated hosts and consequently are not portable. Transformation into the required host is not a problem for small plasmids but is a concern for larger clones like BACs because they can rearrange. Furthermore, all counterselection systems so far described often require experiment-to-experiment titration to find the optimal conditions for the counterselection window, which can be small. In contrast, genetic counterselection based on the ccdA/ccdB toxin/antitoxin system appears to be effective across a wide range of conditions (Figure 4). Thus, the need to optimize the counterselection step for each different application is reduced. We suggest that counterselection with the
The ccdA/ccdB system will be not only easier but also more routinely successful than any other system yet described. In addition to the intended recombination event, any mutation that ablates expression of the counterselectable gene will also be counterselected. The greatest source of unwanted mutagenesis during counterselection comes from intramolecular recombination between repeated sequences to delete the counterselectable gene. Because intramolecular recombination is inherently based on double-stranded DNA, it can be minimized during counterselection by omission of Red/ and use of single-stranded oligonucleotides (32). Previously we implemented the advantages of this strategy using rpsL and streptomycin counterselection. Here we applied it to the ccdA/ccdB system. We chose a point mutation exercise that had been notably problematic with rpsL due to intramolecular recombination between repeated sequences in the human Brd4 gene. Not only did we find that Red omission again improved the yield of correct recombinants after counterselection but also that the ccdA/ccdB system delivered a better outcome than rpsL (data not shown). We think that this improvement reflects the robustness of the ccdA/ccdB system. Consequently we are confident that counterselection with ccdA/ccdB adds to the recombinase repertoire and will be a helpful improvement. As a final note, the advantage of Red omission during counterselection can also be applied to multi-copy plasmids simply by using a strain containing pSC101-ccdA-gb rather than GB-05red as the second host (Figure 1B).

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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