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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 Redα 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

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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 $\leq$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.

Seamless 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 *ccdA* and *ccdB* TA pair was found in the *ccd* (control of cell death) operon of the *E. coli* F plasmid where it serves to maintain this low copy plasmid (41,42). Binding of CcdB to the GyrA subunit of DNA Gyrase blocks the passage of DNA and RNA polymerases and leads to double-strand DNA breakage and cell death (43–47). The CcdA antidote (72 amino acids) prevents the CcdB toxin (101 amino acids) from inhibiting Gyrase by forming a tight CcdA–CcdB complex. On losing the F plasmid, CcdA is quickly degraded by Lon protease so that the concentration of CcdA decreases faster than CcdB, leaving free CcdB to bind gyrase and kill the cells (48–50).

TA systems have been implemented in several biotechnology applications. Most directly, they have been used to maintain plasmids in industrial culture (51,52) and to report ligation efficiency through loss of function. The *ccdB* gene has been used as a counterselectable marker in a number of commonly used applications (53–57).

Here we use *ccdB* counterselection in combination with recombineering for seamless DNA engineering. The two-step procedure first involves insertion at the target site of a *ccdB-antibiotic resistance gene* cassette by selection for antibiotic resistance, which needs to be performed either with CcdA coexpression or in a CcdB-resistant *E. coli* $gyrA462_{\mathrm{Arg}\rightarrow\mathrm{Cys}}$ strain. Because counterselection protocols must be different for single or multiple copy situations, we describe two versions of the strategy. For single copy targets such as BACs or the *E. coli* chromosome, the *ccdA* gene is expressed from a pSC101 recombineering expression plasmid. For multi-copy plasmids we use two *E. coli* strains.

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

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 *redα*, *redβ* and *redγ* genes together with the *E. coli recA* gene (*redγβαA*) 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-gbaA by recombineering to delete the *redα* and *recA* reading frames. p15A-ccdB-amp (Figure 2A) and p15A-ccdB-cm 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*.

Table 1. Strains

E. coli strains	Genotype or relevant features
GB2005	(HS996, Δ*recET*, Δ*ybcC*). The endogenous *recET* locus and the DLP12 prophage *ybcC*, which encodes a putative exonuclease similar to the Redα, were deleted
DB3.1	(*gyrA462*$_{\mathrm{Arg}\rightarrow\mathrm{Cys}}$, Invitrogen GmbH, Karlsruhe, Germany). Used for propagating plasmids containing *ccdB*
GB05-red	(GB2005, *araC*-BAD-*γβα*A) lambda *red* operon and *recA* under P_{BAD} promoter were inserted at the *ybcC* locus
GBred-gyrA462	(GB05-red, *gyrA462*) GyrA mutation of Arg462Cys
GB05-MtaA	(GB2005, *mtaA-genta*) a pPant 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*$_{\mathrm{Arg462Cys}}$ mutation, gryA1 and gyrA2 (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-rpsL-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-ccdA-gbaA was electroporated into *E. coli* DH10B harboring pBeloBAC11. The linear targeting molecule containing *ccdB-amp* was amplified from BseRI 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 BseRI-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 luminmide production.

Point mutagenesis of human Brd4 BAC

A modified 170-kb BAC (RP11-106J4) containing human Brd4 gene was mutated to change amino acid 433 of the human Brd4 gene (ensembl Transcript ID: ENST 00000263377) 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 × 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.

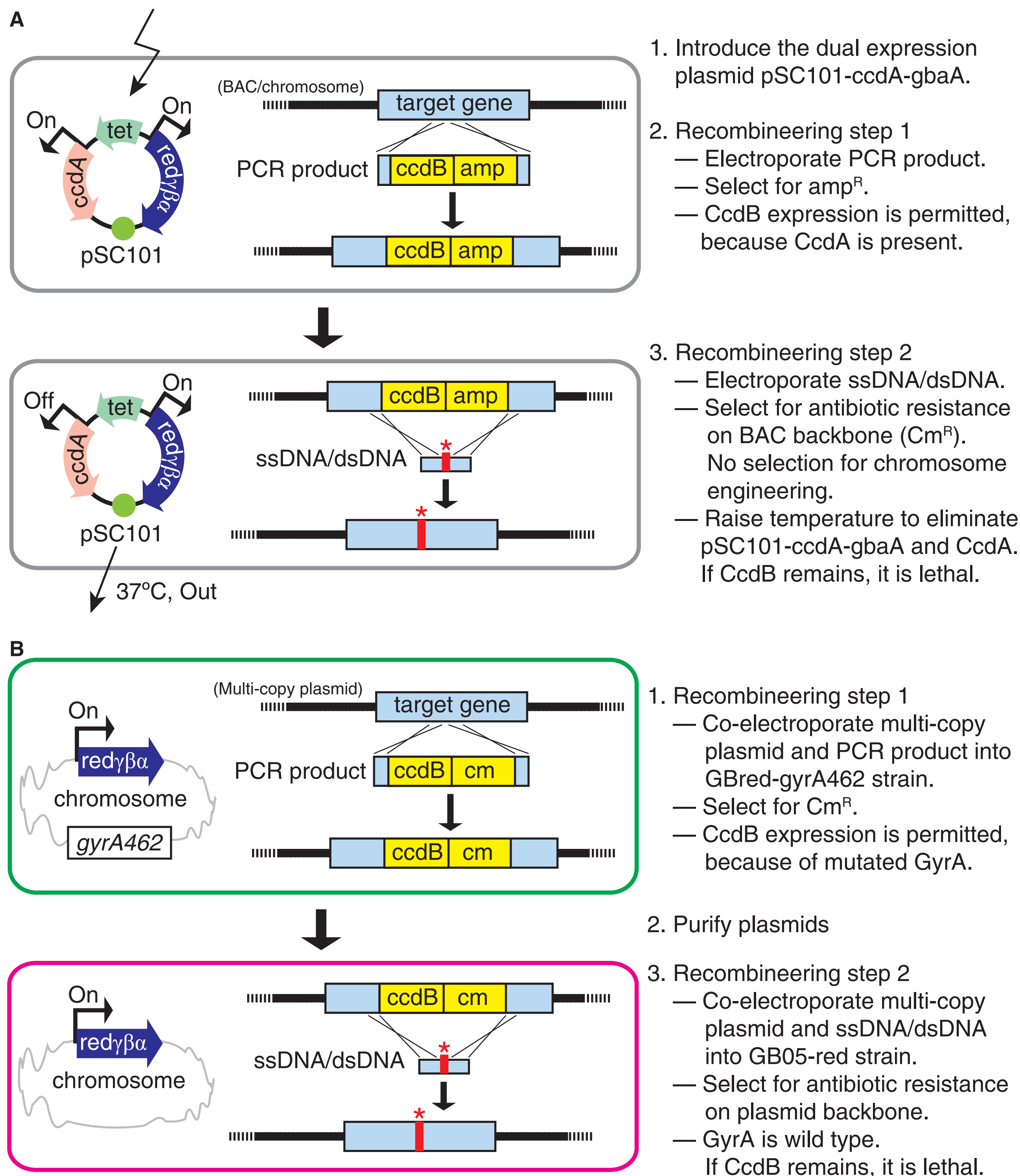

Figure 1. Overview of *ccdB* counterselection strategies. (**A**) Strategy for seamless mutagenesis of BAC or chromosome using *ccdB* counterselection. First, pSC101-ccdA-gbaA is introduced into the host carrying the target BAC or chromosome then Redα/Redβ/Redγ/RecA and CcdA expression are induced by 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(/Redγ/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 Redα/Redβ/Redγ/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 Redα/Redβ/Redγ/RecA expression. After selection for the antibiotic resistance conveyed by the plasmid, only cells that carry the plasmid without CcdB expression will survive.

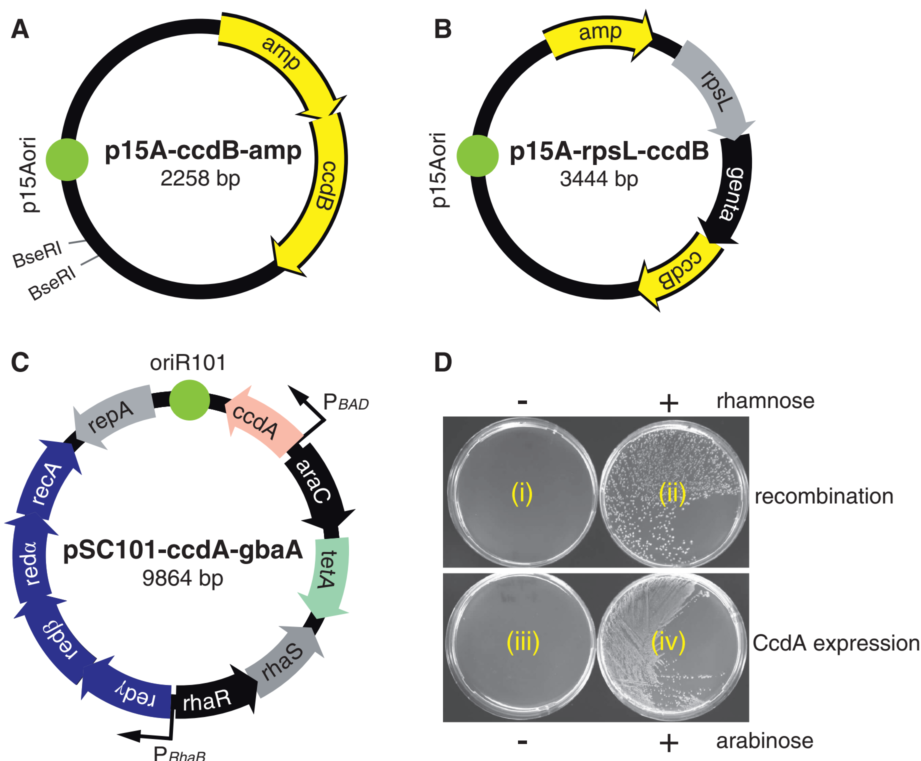

Figure 2. Plasmids and functional tests. (**A**) p15A-ccdB-amp contains the *ccdB-amp* fusion gene and is used as a PCR template after BseR1 digestion; p15A-ccdB-cm (not shown) is essentially the same except that the *amp* resistance gene (*amp*) has been exchanged with the *cm* resistance gene. (**B**) p15A-rpsL-ccdB contains the *amp* resistance genes as well as *rpsL/genta* resistance/*ccdB* operon. (**C**) pSC101-ccdA-gbaA, which conveys *tet* resistance, was constructed from pRedFlp (39). *ccdA* is expressed from the arabinose-inducible P_{BAD} promoter and *redγβαA* is expressed from the rhamnose-inducible P_{RhaB} promoter. (**D**) Recombination was 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 50 bp homology arms to the plasmid. After recovery, 100 µl of culture were plated on LB plates supplemented with kanamycin. The results show that expression of RedγβαA mediates recombination and the rhamnose-inducible P_{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 µl of culture were plated on LB plates containing *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_{BAD} promoter.

Table 2. Oligonucleotide sequences

Name	5′–3′
gryA1	gtcgagcagtttttcgtgctcaagaccggtcagtttctgca**GGCA**ca**A**atccagaatcgcctgagcttgctgttcggtcaggtagtac
gyrA2	gtactacctgaccgaacagcaagctcaggcgattctggat**T**tg**TGCC**tgcagaaactgaccggtcttgagcacgaaaaactgctcgac
BACccdB -amp1	aatacgactcactatagggcgaattcgagctcggtacccgtttgtttatttttctaaatac
BACccdB-amp2	ctcaagcttgcatgcctgcaggtcgactctagaggatcccagccccatacgatataagttg
BACccdB-res	ctcaagcttgcatgcctgcaggtcgactctagaggatccccgggtaccgagctcgaattcgccctatagtgagtcgtatt
3263PCP ccdBcm5	tttgaacaggttggccgacatgacagtttctttgccttgggcggtcactgtgacggaagatcacttcgcag
3263PCP3 ccdBcm3	ccaatcctatacgccgtaaacgttcgatcatcctgactgccaacagcgtttgttcaaaaaaaagcccgctc
3263SPCP3A-B	caggcccaatcctatacgccgtaaacgttcgatcatcctgactgccaacag**CGC**gtgaccgcccaaggcaaagaaactgtcatgtcggccaacctgttcaaca
hBrd4-M2-5	aggagtttggtgctgacgtccgattgatgttctccaactgctataagtactttgtttatttttctaaatac
hBrd4-M2-3	agcctcacctggagcttgcgggccatggccaccacctcatggtcaggagggagccccatacgatataagttgt
hBrd4-M2-Rescue	agcctcacctggagcttgcgggccatggccaccacctcatggtcaggaggg**AA**gtacttatagcagttggagaacatcaatcggacgtcagcaccaaactcctg

The PCR primers are underlined, the mutations are bold.

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_{BAD} promoter and the RedγβαA operon from the rhamnose-inducible P_{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 *amp*) flanked by homology arms. After selection for *amp* resistance, the culture is induced only with rhamnose,

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 P_{BAD}-Red$\gamma\beta\alpha$A 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-cm* 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

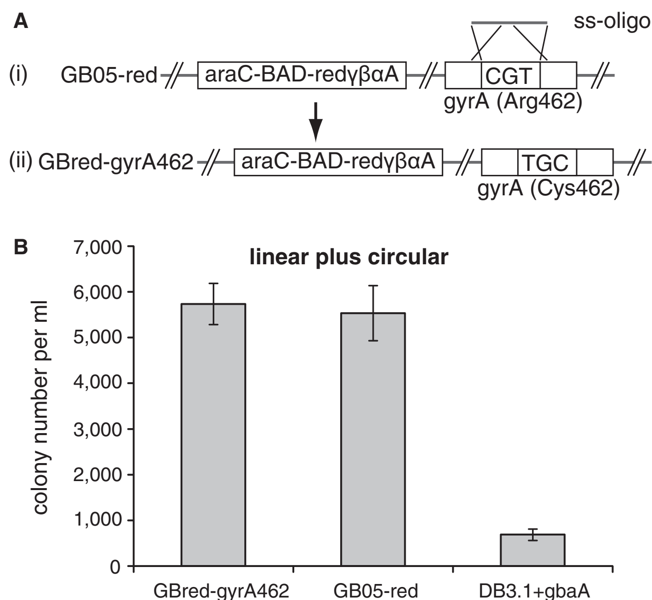

Figure 3. Generation and testing of *E. coli* GBred-gyrA462. (**A**) GB05-red, which carries the arabinose inducible red$\gamma\beta\alpha$A operon integrated at ybcC, was mutated with an oligonucleotide directed to change the Arg462 codon of gyrase into the cysteine codon TGC. Mutants were identified by resistance to CcdB expression from p15A-rpsL-ccdB. (**B**) A comparison of the recombination frequencies in GBred-gyrA462 with GB05-red and DB3.1 harboring pSC101-BAD-gbaA. The same test as used in Figure 2D was applied and colonies were counted after plating on LB kanamycin LB plates supplemented with appropriate antibiotics. Results are from three independent experiments, and the error bars represent the standard deviation.

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

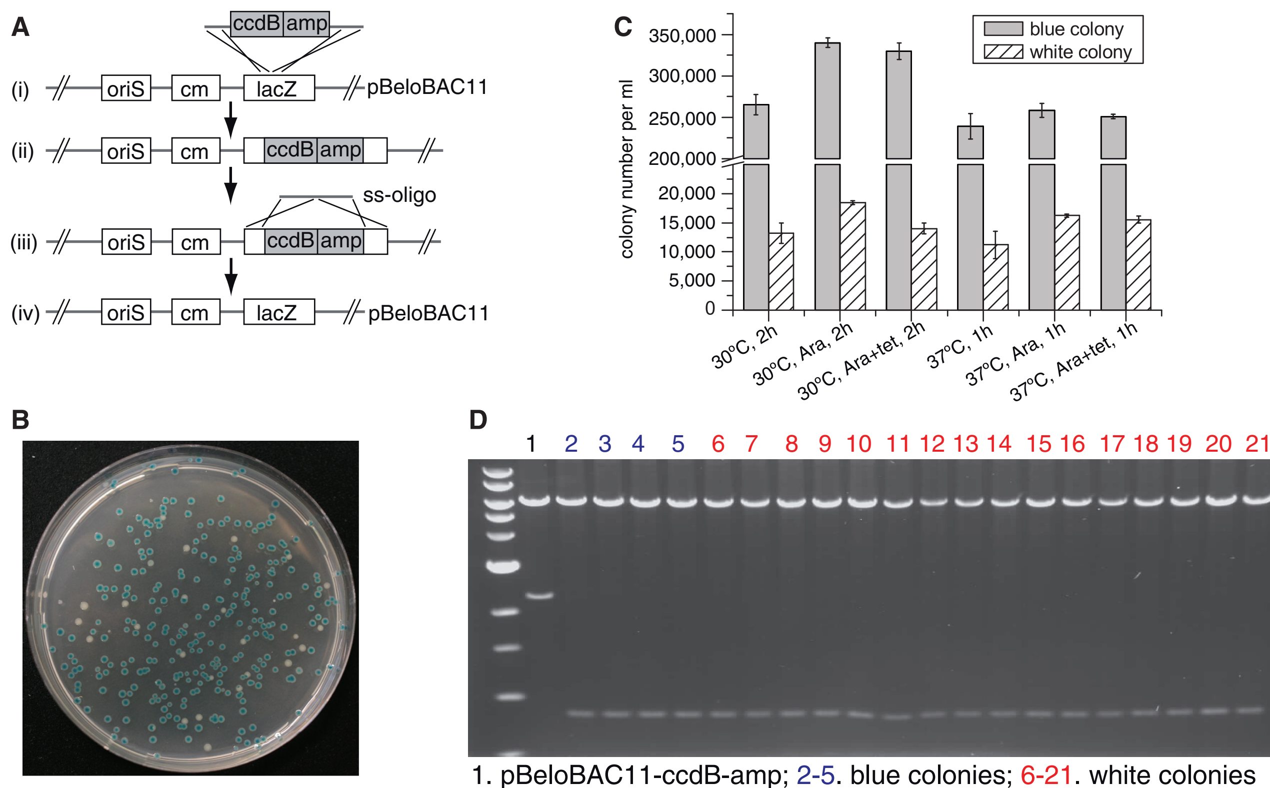

Figure 4. Point mutagenesis in a BAC using *ccdB* counterselection. (**A**) Scheme *ccdB* counterselection to modify pBeloBAC11. (**B**) A picture of the colonies on an LB plate supplemented with *cm*, IPTG and X-Gal after *ccdB* counterselection. (**C**) The recombination efficiencies of different recovery protocols after the *ccdB* counterselection step. The efficiencies are indicated by the number of blue and white colonies on LB plates. After electroporation, cells were incubated for 1–2 h in LB containing or not L-arabinose and/or *tet*, as indicated. Results are from three independent experiments and the error bars represent the standard deviation. (**D**) EcoRI restriction analysis of BAC DNAs from the *ccdB-amp* modified BAC (lane 1), four blue (lanes 2–5) or 16 white (lanes 6–21) colonies.

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 4′-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 Redα and using single-stranded oligonucleotides (32). To apply Redα omission to *ccdB*

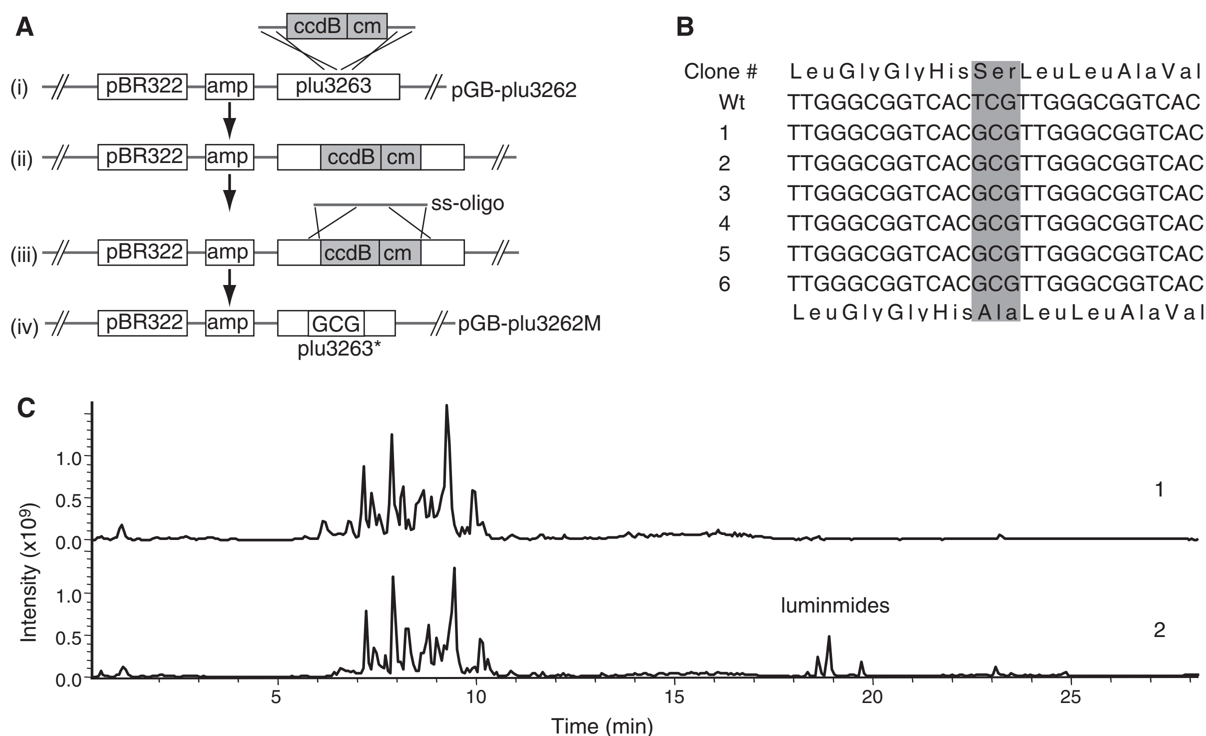

Figure 5. Mutation and expression of the *plu3263* biosynthetic operon. (**A**) Scheme of the single base pair substitution in *plu3263*. The serine 2742 TCG codon was changed into an alanine GCG codon. (**B**) Sequence analysis of the point mutated region. All six clones sequenced had only the intended substitution (shadow). (**C**) HPLC-MS analysis (base peak chromatogram (BPC), m/z = 200–1100) of *E. coli* GB05-MtaA containing pGB-plu3263M (line 1) and pGB-plu3263 (line 2). The luminmide products of *plu3263* (retention time = 18–20 min) were abolished.

counterselection, we deleted Redα 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 Redβ/Redγ 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+/H20), 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, sucrose 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*/trimethoprim+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

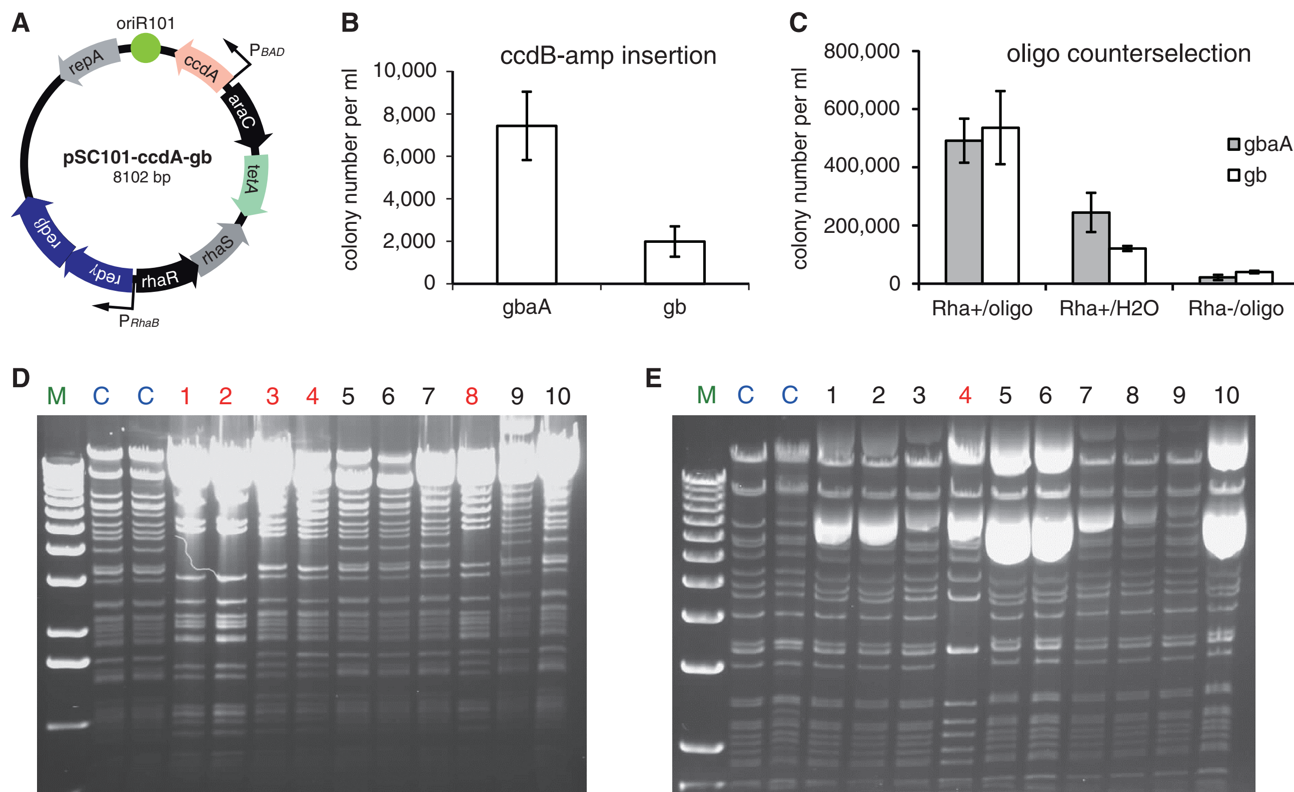

Figure 6. Omission of Red-alpha for point mutagenesis of a human BAC. (**A**) Diagram of the pSC101-ccdA-gb expression plasmid. The plasmid is a derivative of pSC101-ccdB-gbaA (Figure 2C). *ccdA* is expressed from the arabinose-inducible P_{BAD} promoter and *red* ((is expressed from the rhamnose-inducible PRhaB promoter. (B) Comparison of the recombination efficiency promoted by pSC101-ccdA-gbaA (gbaA) or pSC101-ccdA-gb (gb) for ccdB-amp cassette insertion scored by colony numbers on LB amp plates from three independent experiments. Error bars represent the standard deviation. (C) Comparison of the recombination efficiency promoted by pSC101-ccdA-gbaA (gbaA) or pSC101-ccdA-gb (gb) using a single-stranded oligonucleotide and CcdB counterselection scored by colony numbers on LB plates with amp. Rha+/oligo indicates rhamnose induction before oligonucleotide electroporation and Rha+/H2O indicates rhamnose induction with water only electroporation. The Rha-/oligo is a negative control without rhamnose induction but with oligonucleotide electroporation. Results are from three independent experiments and the error bars represent the standard deviation. (**D**) BAC restriction analysis after oligonucleotide counterselection. M: 1 kb DNA Ladder (Invitrogen); C: NcoI digestion of the original BAC as control; 1–10: NcoI digestion of BACs after counterselection with oligonucleotide recombination promoted by pSC101-ccdA-gbaA. Clones 1, 2, 3, 4 and 8 are from intramolecular recombination, whereas 5, 6, 7, 9 and 10 show the correct pattern. Correct oligonucleotide recombination will restore the original BAC pattern. The extra, heavy staining bands come from the persistence of the pSC101-ccdA-gbaA expression plasmid. (**E**) As for (D) except recombination was promoted by pSC101-ccdA-gb. Clone 4 is from intramolecular recombination, whereas the other 9 clones show oligonucleotide recombination.

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 Redα, which promotes dsDNA but not ssDNA recombination, 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 Redα 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 recombineering repertoire and will be a helpful improvement. As a final note, the advantage of Redα 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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REFERENCES

1. Zhang,Y., Buchholz,F., Muyrers,J.P.P. and Stewart,A.F. (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.*, **20**, 123–128.
2. Muyrers,J.P.P., Zhang,Y., Testa,G. and Stewart,A.F. (1999) Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.*, **27**, 1555–1557.
3. Zhang,Y., Muyrers,J.P.P., Testa,G. and Stewart,A.F. (2000) DNA cloning by homologous recombination in *Escherichia coli*. *Nat. Biotechnol.*, **18**, 1314–1317.
4. Yu,D., Ellis,H.M. and Lee,E. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **97**, 5978–5983.
5. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
6. Muyrers,J.P.P., Zhang,Y. and Stewart,A.F. (2001) Techniques: recombinogenic engineering-new options for cloning and manipulating DNA. *Trends Biochem. Sci.*, **26**, 325–331.
7. Copeland,N.G. and Jenkins,N.A. (2001) Recombineering: a powerful new tool for mouse functional genomics. *Nat. Rev. Genet.*, **2**, 769–779.
8. Court,D.L., Sawitzke,J.A. and Thomason,L.C. (2002) Genetic engineering using homologous recombination. *Annu. Rev. Genet.*, **36**, 361–388.
9. Sawitzke,J.A., Thomason,L.C., Costantino,N., Bubunenko,M. and Datta,S. (2007) Recombineering: *in vivo* genetic engineering in *E. coli*, *S. enterica*, and beyond. *Methods Enzymol.*, **421**, 171–199.
10. Sharan,S.K., Thomason,L.C. and Kuznetsov,S.G. (2009) Recombineering: a homologous recombination-based method of genetic engineering. *Nat. Protoc.*, **4**, 206–223.
11. Muyrers,J.P.P., Zhang,Y., Buchholz,F. and Stewart,A.F. (2000) RecE/RecT and Redα/Redβ initiate double-stranded break repair by specifically interacting with their respective partners. *Genes Dev.*, **14**, 1971–1982.
12. Fu,J., Bian,X., Hu,S., Wang,H., Huang,F., Seibert,P.M., Plaza,A., Xia,L., Muller,R., Stewart,A.F. *et al.* (2012) Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. *Nat. Biotechnol.*, **30**, 440–446.
13. Maresca,M., Erler,A., Fu,J., Friedrich,A., Zhang,Y. and Stewart,A.F. (2010) Single-stranded heteroduplex intermediates in lambda Red homologous recombination. *BMC Mol. Biol.*, **11**, 54.
14. Kovall,R. and Matthews,B.W. (1997) Toroidal structure of lambda-exonuclease. *Science*, **277**, 1824–1827.
15. Zhang,J., Xing,X., Herr,A.B. and Bell,C.E. (2009) Crystal structure of *E. coli* RecE protein reveals a toroidal tetramer for processing double-stranded DNA breaks. *Structure*, **17**, 690–702.
16. Thresher,R.J., Makhov,A.M., Hall,S.D., Kolodner,R. and Griffith,J.D. (1995) Electron microscopic visualization of RecT protein and its complexes with DNA. *J. Mol. Biol.*, **254**, 364–371.
17. Passy,S.I., Yu,X., Li,Z., Radding,C.M. and Egelman,E.H. (1999) Rings and filaments of β protein from bacteriophage λ suggest a superfamily of recombination proteins. *Proc. Natl Acad. Sci. USA*, **96**, 4279–4284.
18. Erler,A., Wegmann,S., Elie-Caille,C., Bradshaw,C.R., Maresca,M., Seidel,R., Habermann,B., Muller,D.J. and Stewart,A.F. (2009) Conformational adaptability of Redβ during DNA annealing and implications for its structural relationship with Rad52. *J. Mol. Biol.*, **391**, 586–598.
19. Ellis,H.M., Yu,D., DiTizio,T. and Court,D.L. (2001) High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc. Natl Acad. Sci. USA*, **98**, 6742–6746.
20. Zhang,Y., Muyrers,J., Rientjes,J. and Stewart,A.F. (2003) Phage annealing proteins promote oligonucleotide-directed mutagenesis in *Escherichia coli* and mouse ES cells. *BMC Mol. Biol.*, **4**, 1.
21. Valenzuela,D.M., Murphy,A.J., Frendewey,D., Gale,N.W., Economides,A.N., Auerbach,W., Poueymirou,W.T., Adams,N.C., Rojas,J., Yasenchak,J. *et al.* (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat. Biotechnol.*, **21**, 652–659.
22. Yang,Y. and Seed,B. (2003) Site-specific gene targeting in mouse embryonic stem cells with intact bacterial artificial chromosomes. *Nat. Biotechnol.*, **21**, 447–451.
23. Testa,G., Zhang,Y., Vintersten,K., Benes,V., Pijnappel,W.W., Chambers,I., Smith,A.J., Smith,A.G. and Stewart,A.F. (2003) Engineering the mouse genome with bacterial artificial chromosomes to create multipurpose alleles. *Nat. Biotechnol.*, **21**, 443–447.
24. Sarov,M., Schneider,S., Pozniakovski,A., Roguev,A., Ernst,S., Zhang,Y., Hyman,A.A. and Stewart,A.F. (2006) A recombineering pipeline for functional genomics applied to *Caenorhabditis elegans*. *Nat. Methods*, **3**, 839–844.
25. Poser,I., Sarov,M., Hutchins,J.R.A., Hériché,J.K., Toyoda,Y., Pozniakovsky,A., Weigl,D., Nitzsche,A., Hegemann,B. and Bird,A.W. (2008) BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. *Nat. Methods*, **5**, 409–415.
26. Skarnes,W.C., Rosen,B., West,A.P., Koutsourakis,M., Bushell,W., Iyer,V., Mujica,A.O., Thomas,M., Harrow,J., Cox,T. *et al.* (2011) A conditional knockout resource for the genome-wide study of mouse gene function. *Nature*, **474**, 337–342.
27. Sarov,M., Murray,J.I., Schanze,K., Pozniakovski,A., Niu,W., Angermann,K., Hasse,S., Rupprecht,M., Vinis,E., Tinney,M. *et al.* (2012) A genome-scale resource for *in vivo* tag-based protein function exploration in *C. elegans*. *Cell*, **150**, 855–866.
28. Muyrers,J.P.P., Zhang,Y., Benes,V., Testa,G., Ansorge,W. and Stewart,A.F. (2000) Point mutation of bacterial artificial chromosomes by ET recombination. *EMBO Rep.*, **1**, 239–243.
29. Imam,A.M., Patrinos,G.P., de Krom,M., Bottardi,S., Janssens,R.J., Katsantoni,E., Wai,A.W., Sherratt,D.J. and Grosveld,F.G. (2000) Modification of human β-globin locus PAC clones by homologous recombination in *Escherichia coli*. *Nucleic Acids Res.*, **28**, e65.
30. Wang,S., Zhao,Y., Leiby,M. and Zhu,J. (2009) A new positive/negative selection scheme for precise BAC recombineering. *Mol. Biotechnol.*, **42**, 110–116.
31. Westenberg,M., Soedling,H.M., Mann,D.A., Nicholson,L.J. and Dolphin,C.T. (2010) Counter-selection recombineering of the baculovirus genome: a strategy for seamless modification of repeat-containing BACs. *Nucleic Acids Res.*, **38**, e166.
32. Bird,A.W., Erler,A., Fu,J., Heriche,J.K., Maresca,M., Zhang,Y., Hyman,A.A. and Stewart,A.F. (2012) High-efficiency counterselection recombineering for site-directed mutagenesis in bacterial artificial chromosomes. *Nat. Methods*, **9**, 103–109.
33. Warming,S., Costantino,N., Court,D.L., Jenkins,N.A. and Copeland,N.G. (2005) Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.*, **33**, e36.
34. Wong,Q.N.Y., Ng,V.C.W., Lin,M., Kung,H., Chan,D. and Huang,J.D. (2005) Efficient and seamless DNA recombineering using a thymidylate synthase A selection system in *Escherichia coli*. *Nucleic Acids Res.*, **33**, e59.
35. DeVito,J.A. (2008) Recombineering with tolC as a Selectable/Counter-selectable Marker: remodeling the rRNA Operons of *Escherichia coli*. *Nucleic Acids Res.*, **36**, e4.

36. Jamsai,D., Orford,M., Nefedov,M., Fucharoen,S., Williamson,R. and Ioannou,P.A. (2003) Targeted modification of a human β-globin locus BAC clone using GET recombination and an I-SceI counterselection cassette. *Genomics*, **82**, 68–77.
37. Tischer,B.K., von Einem,J., Kaufer,B. and Osterrieder,N. (2006) Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. *Biotechniques*, **40**, 191–197.
38. Engelberg-Kulka,H. and Glaser,G. (1999) Addiction modules and programmed cell death and antideath in bacterial cultures. *Annu. Rev. Microbiol.*, **53**, 43–70.
39. Gerdes,K. (2000) Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. *J. Bacteriol.*, **182**, 561–572.
40. Hayes,F. (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science*, **301**, 1496–1499.
41. Ogura,T. and Hiraga,S. (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc. Natl Acad. Sci. USA*, **80**, 4784–4788.
42. Jaffe,A., Ogura,T. and Hiraga,S. (1985) Effects of the ccd function of the F plasmid on bacterial growth. *J. Bacteriol.*, **163**, 841–849.
43. Bernard,P. and Couturier,M. (1992) Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J. Mol. Biol.*, **226**, 735–745.
44. Couturier,M., Bahassi,E.M. and Van Melderen,L. (1998) Bacterial death by DNA gyrase poisoning. *Trends Microbiol.*, **6**, 269–275.
45. Van Melderen,L. (2002) Molecular interactions of the CcdB poison with its bacterial target, the DNA gyrase. *Int. J. Med. Microbiol.*, **291**, 537–544.
46. Dao-Thi,M.H., Van Melderen,L., De Genst,E., Afif,H., Buts,L., Wyns,L. and Loris,R. (2005) Molecular basis of gyrase poisoning by the addiction toxin CcdB. *J. Mol. Biol.*, **348**, 1091–1102.
47. De Jonge,N., Garcia-Pino,A., Buts,L., Haesaerts,S., Charlier,D., Zangger,K., Wyns,L., De Greve,H. and Loris,R. (2009) Rejuvenation of CcdB-poisoned gyrase by an intrinsically disordered protein domain. *Mol. Cell*, **35**, 154–163.
48. Van Melderen,L., Bernard,P. and Couturier,M. (1994) Lon dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid free segregant bacteria. *Mol. Microbiol.*, **11**, 1151–1157.
49. Van Melderen,L., Thi,M.H.D., Lecchi,P., Gottesman,S., Couturier,M. and Maurizi,M.R. (1996) ATP-dependent degradation of CcdA by Lon protease. *J. Biol. Chem.*, **271**, 27730–27738.
50. Afif,H., Allali,N., Couturier,M. and Van Melderen,L. (2001) The ratio between CcdA and CcdB modulates the transcriptional repression of the ccd poison–antidote system. *Mol. Microbiol.*, **41**, 73–82.
51. Szpirer,C.Y. and Milinkovitch,M.C. (2005) Separate-component-stabilization system for protein and DNA production without the use of antibiotics. *Biotechniques*, **38**, 775–781.
52. Peubez,I., Chaudet,N., Mignon,C., Hild,G., Husson,S., Courtois,V., De Luca,K., Speck,D. and Sodoyer,R. (2010) Antibiotic-free selection in *E. coli*: new considerations for optimal design and improved production. *Microb. Cell Fact.*, **9**, 65.
53. Bernard,P., Gabarit,P., Bahassi,E.M. and Couturier,M. (1994) Positive-selection vectors using the F plasmid ccdB killer gene. *Gene*, **148**, 71–74.
54. Betton,J.M. (2004) Cloning vectors for expression-PCR products. *Biotechniques*, **37**, 346–347.
55. Le Roux,F., Binesse,J., Saulnier,D. and Mazel,D. (2007) Construction of a Vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol.*, **73**, 777–784.
56. Chen,S., Songkumarn,P., Liu,J. and Wang,G.L. (2009) A versatile zero background T-vector system for gene cloning and functional genomics. *Plant Physiol.*, **150**, 1111–1121.
57. Mandi,N., Kotwal,P. and Padmanabhan,S. (2009) Construction of a novel zero background prokaryotic expression vector: potential advantages. *Biotechnol. Lett.*, **31**, 1905–1910.
58. Fu,J., Teucher,M., Anastassiadis,K., Skarnes,W. and Stewart,A.F. (2010) A recombineering pipeline to make conditional targeting constructs. *Methods Enzymol.*, **477**, 125–144.
59. Hashimoto-Gotoh,T. and Sekiguchi,M. (1977) Mutations of temperature sensitivity in R plasmid pSC101. *J. Bacteriol.*, **131**, 405–412.
60. Wang,J., Sarov,M., Rientjes,J., Hu,J., Hollak,H., Kranz,H., Xie,Y., Stewart,A.F. and Zhang,Y. (2006) An improved recombineering approach by adding RecA to λ red recombination. *Mol. Biotechnol.*, **32**, 43–53.
61. Bian,X., Huang,F., Stewart,F.A., Xia,L., Zhang,Y. and Müller,R. (2012) Direct cloning, genetic engineering, and heterologous expression of the syringolin biosynthetic gene cluster in *E. coli* through Red/ET recombineering. *Chembiochem*, **13**, 1946–1952.
62. Duchaud,E., Rusniok,C., Frangeul,L., Buchrieser,C., Givaudan,A., Taourit,S., Bocs,S., Boursaux-Eude,C., Chandler,M. and Charles,J.F. (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol.*, **21**, 1307–1313.
63. Schlumbohm,W., Stein,T., Ullrich,C., Vater,J., Krause,M., Marahiel,M., Kruft,V. and Wittmann-Liebold,B. (1991) An active serine is involved in covalent substrate amino acid binding at each reaction center of gramicidin S synthetase. *J. Biol. Chem.*, **266**, 23135–23141.