Biotechnol Lett (2023) 45:191–197
https://doi.org/10.1007/s10529-022-03333-z

ORIGINAL RESEARCH PAPER

Characterization of a novel *Escherichia coli* recombineering selection/counterselection cassette

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Received: 13 September 2022 / Revised: 31 October 2022 / Accepted: 23 November 2022 / Published online: 10 December 2022

Abstract Recombineering is a highly efficient DNA cloning and modification technique by using the recombinase-mediated homologous recombination. Selection/counterselection cassette is often used in chromosomal DNA or large episomal DNA manipulation, in which the selection marker is used for the first step cassette selection while deleting the target gene via allelic exchange, and the counterselection marker is used for the second step replacement of the cassette by the foreign DNA fragment. A variety of selection/counterselection cassettes are reported, however, the cassettes suffer from the shortcomings of the requirement of pre-engineered strain or specific culture medium. Herein, we report a novel *S-tetR-*P_{tetA}*-ccdB-aacC1-S* selection/counterselection cassette that sidesteps the disadvantages. As a proof-of-concept, one-step gene cloning (0.7, 1.7, and 4.2 kb) and two-step *Escherichia coli* chromosomal gene knock-in (0.7 and 4.2 kb) were performed. The gene cloning and gene knock-in efficiencies are high up to 90%. The novel selection/counterselection cassette adds a powerful tool to the recombineering repertoire.

Keywords *CcdB* · Cloning; *I-SceI* · Knock-in · Recombineering · Selection/counterselection cassette

Introduction

Recombineering (recombination-mediated genetic engineering), also called λ Red recombineering, is a homologous recombination-based technique (Murphy 2016). Due to its high efficiency and easy manipulation, recombineering has found a variety of applications including gene cloning and genome engineering (Lobanova et al. 2022; Ma et al. 2015; Martin-Pascual et al. 2021; Robertson et al. 2021; Tiwari et al. 2022). Firstly established in *Escherichia coli*, recombineering has extended to a large number of gram positive and gram negative bacteria. Selection/counterselection cassette is usually involved in a two-step modification of bacterial chromosome or large episomal DNA (like bacterial artificial chromosome). The first step is integration of the cassette into the target DNA via selection marker, while the second step is the replacement of the cassette with oligonucleotide or target gene, resulting in gene deletion, replacement or knock-in.

Numerous of counterselection markers were developed, such as *galK*, *thyA*, *rps*L, *pyrF*, *pheS*, *sacB*, *upp*, *ccdB*, *tetA-sacB*, and *tolC*. However, these

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10529-022-03333-z.

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counterselection markers normally require pre-generated strain or specific culture medium. For example, *rpsL*, thyA, *pyrF, upp*, and *tolC* need corresponding gene-deficient mutant; and *galK*, *pheS*, *sacB*, *tetA-sacB* need medium containing specific (sometimes expensive) chemicals (Baas-Thomas et al. 2022; Li et al. 2013; Wang et al. 2014). Herein, we constructed four selection/counterselection cassettes, and after transformation efficiency comparison, a new selection/counterselection cassette, *S-tetR-*P_{tetA}*-ccdB-aacC1-S,* was characterized for *E. coli* recombineering. The cassette contains the gentamicin resistance gene (*aacC1*) as selection maker and toxic gene *ccdB* and homing endonuclease encoding gene *I-SceI* as double counterselection markers. Phage F1 origin *ccdB* is part of dote-antidote pair of *ccdAB*. CcdB targets the bacterial DNA gyrase encoding gene *gyrA*, thus inhibiting DNA replication. *Saccharomyces cerevisiae* mitochondrial intron origin I-SceI cleaves a unique 18 bp sequence, creating double-strand break in the DNA sequence. Proof-of-concept characterization of the cassette was demonstrated by one-step gene cloning (0.7, 1.7, and 4.2 kb) and two-step *E. coli* chromosomal gene knock-in (0.7 and 4.2 kb). Application of the cassette is straightforward, requires no pre-engineered strain or specific medium. The highly efficient selection/counterselection cassette adds a powerful tool to the recombineering toolbox.

Materials and methods

Bacterial strains, culture conditions, and chemical regents

E. coli DH10B was used as general vector construction and chromosomal gene knock-in host. *E. coli* LS027 (Zhang et al. 2017) was used for R6K replicon plasmid cloning. LB (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl) medium and LB plate containing 1.5% agar were used for strain culture. When required, the antibiotics were supplemented as follows: Ampicillin (Ap), 50 μg/ml; chloramphenicol (Cm), 25μg/ml; gentamicin (Gm), 25 μg/ml; kanamycin (Km), 30 μg/ml; tetracycline (Tc), 25 μg/ml. Restriction enzymes and T4 ligase were purchased form NEB (USA). Plasmid extraction kit, gel extraction kit, chlortetracycline (cTc) and other chemicals were ordered from Sangon (China). Heat-treated cTc was prepared by dissolving cTc in fresh prepared LB and autoclaved, a final concentration of 25 μg/ml cTc (w/v) was used for the induced expression of toxic gene *ccdB* in *S-tetR-*P_{tetA}*-ccdB-aacC1-S*.

Vector construction

Molecular biology preformation was carried out as per standard protocols. The detailed vector construction procedure is provided in Supplementary material. Strains and plasmids used and generated in this research are listed in Supplementary Table 1. The PCR primers are listed in Supplementary Table 2.

Recombineering manipulation

pLS2358 (Chen et al. 2016), initially constructed for genome engineering of *Pseudomonas putida* KT2440, was used as Red helper and I-SceI expression plasmid in this study. pLS2358 harbors a broad-host range of *oriV* replicon with the Red operon and I-SceI were induced with 2 mM m-toluic acid and 0.2% L-rhamnose (w/v), respectively. *E. coli* DH10B harboring pLS2358 recombinase-proficient electrocompetent cells preparation and electroporation were carried out as reported (Chen et al. 2016). The recombineering primer was consisted of 50 bp homologous arm (HA) plus 20 bp priming nucleotides. For gene cloning, one μg of PCR amplified targeting DNA and 10 ng of the *S-araC-*P_{araB}*-ccdB-aacC1-S* cassette plasmid pLS4512 were co-electroplated into the electrocompetent cells using 1 mm cuvette and Gene Pulser electroporator (Bio-Rad; Hercules, CA, USA); electroporated cells were immediately suspended in 1 ml of LB and incubated at 37 °C for 1 h before diluted and plated on LB agar plate containing Km and cTc. Randomly picked Km^RcTc^R clones were duplicated on LB agar plate containing Gm. Km^RGm^S clones were then cultured for plasmid extraction, restriction enzyme digestions and nucleotide sequencing. For chromosomal gene knock-in, the HA-flanked *S-araC-*P_{araB}*-ccdB-aacC1-S* cassette was amplified from pLS4520 and electroporated into the electrocompetent cells. Under Gm selection, the cassette knock-in strain was confirmed via PCR in which the primers were designed around 100 bp away from the recombination junctions; and further validated via sequencing. The second, target gene knock-in step was conducted by electroporating the PCR

amplified target gene into the recombinase-proficient intermediate strain under the cTc selection. The randomly selected cTc^R clones were duplicated on LB agar plate with or without Gm, from which the Gm^S clones were genotyped by PCR and sequencing of the knock-in locus. Finally, pLS2358 was eliminated from the knock-in strain by plating the growth cells on LB (without NaCl) agar plate supplemented with 10% (w/v) surose and selection of Tc^S clones.

Results and discussion

Selection of the best selection/counterselection cassette

Both CcdB are SacB are powerful counterselecction markers; however, we still observed a large number clones under CcdB or SacB counterselection (data not shown). I-SceI is also an often used counterselecction marker, we thus assumed that double counterselection by combing I-SceI with either CcdB or SacB would greatly reduce or totally eliminate the background clones. Based on this speculation, we constructed four novel selection/counterselection cassettes: *S-araC-*P_{araB}*-ccdB-aacC1-S* (pLS4509), two types of *S-tetR-*P_{tetA}*-ccdB-aacC1-S* (pLS4512 and pLS4514) and *S-sacB-aacC1-S* (pLS4516). Besides *ccdB* or *sacB*, I-SceI acts as the counterselection marker by cleaving the two flanked I-SceI recognition sites. The pLS4509 L-arabinose-inducible *ccdB* expression system harbors a ribosome binding site (RBS) variant sequence that reduces the translation efficiency of the downstream gene (Chen and Zhao 2005).

We initially tried to clone the *ccdB* under the control of native *tetR-*P_{tetA} regulatory system (Posfai et al. 1999), expecting that transformed clones would survive as tight regulation of the tetracycline derivative inducible promoter would avoid the CcdB toxicity. However, only a few clones were obtained, and sequence analysis revealed that the *ccdB* gene was mutated in all five clones, indicating that the native

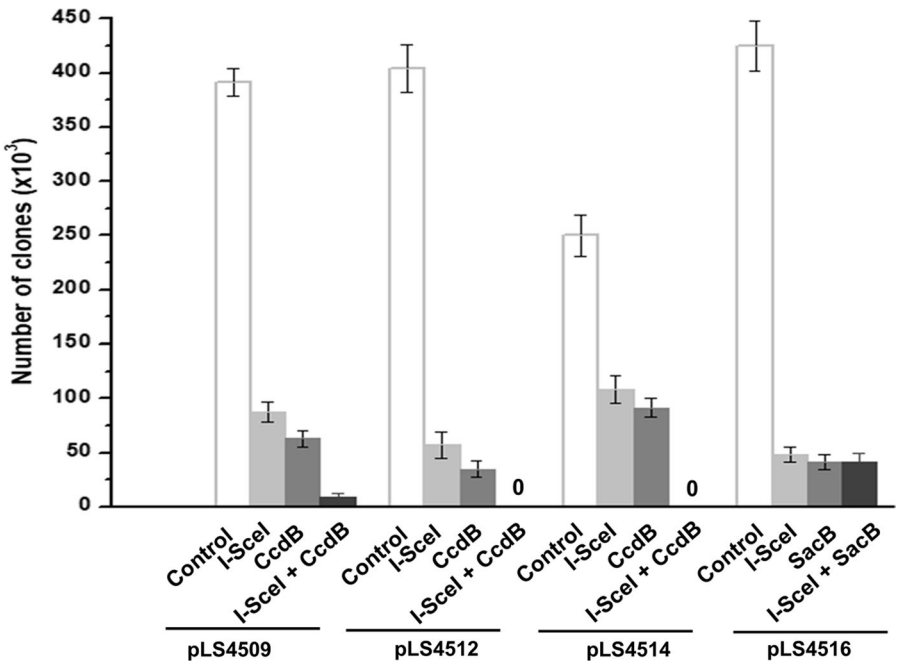

Fig. 1 Electroporation efficiency comparison among four cassettes. For all the selections, control was LB agar plate without inducer while 0.2% (w/v) L-rhamnose was used for single I-SceI counterselection. Strain and its counterselection were as follows. DH10B/pLS4509: 0.2% (w/v) L-arabinose for CcdB counterselection, 0.2% (w/v) L-arabinose and 0.2% (w/v) L-rhamnose for both CcdB and I-SceI counterselection. DH10B/pLS4512: 25 μg/ml (w/v) cTc for CcdB counterselection, 25 μg/ml (w/v) cTc and 0.2% (w/v) L-rhamnose for both CcdB and I-SceI counterselection. DH10B/pLS4514 was the same as that of DH10B/pLS4512. DH10B/pLS4516: 10% (w/v) sucrose for SacB counterselection, 10% (w/v) sucrose and 0.2% (w/v) L-rhamnose for both SacB and I-SceI counterselection

P_{tetA} promoter was not stringent. We then followed the first cassette paradigm by changing the original RBS containing sequence (before start codon ATG) from GAGAAAAGTGAA to GATTGAAAACG, resulting in the first *S-tetR-*P_{tetA}*-ccdB-aacC1-S* cassette plasmid pLS4512 with CcdB E87K mutation. We previously found that I-SceI was tightly regulated by the L-rhamnose inducible system in *P. putida* KT2440 (Chen et al. 2016), therefore we also cloned the AGGATCACATT sequence from *E. coli* L-rhamonose operon promoter before the start codon of *ccdB,* leading to the second *S-tetR-*P_{tetA}*-ccdB-aacC1-S* cassette plasmid pLS4514 with CcdB L36R S70N mutation.

Comparison of the four selection/counterselection cassettes was subsequently carried out by electroporation of 10 ng of each plasmid into *E coli* DH10B. The cells were $10^{-threefold}$ diluted and spread on various selection plates. Under counterselection conditions, only recombinant plasmid could be obtained and no or very few plasmids with intact cassette would survive. Therefore, the cassette with the least obtained clones would be the best candidate for recombineering experiments. As shown in.Figure 1, single I-SceI or CcdB counterselection still generated a large number of clones, and although combined counterselection of I-SceI and SacB showed no difference as single SacB counterselection, combined counterselection of I-SceI and CcdB resulted in much less transformants in P_{araB}-inducible *ccdB* system. Remarkably, no transformant was obtained for the two *S-tetR-*P_{tetA}*-ccdB-aacC1-S* cassettes under the combined counterselection of I-SceI and CcdB. As pLS4512 showed more clones (which means less background CcdB toxicity) in the control group than that of pLS4514, it was selected for further characterization.

Gene cloning

Gene cloning is a routine experiment in molecular biology. As a proof-of-concept, we first demonstrated the usage of *S-tetR-*P_{tetA}*-ccdB-aacC1-S cassette* via gene cloning with the scheme shown in Fig. 2.

Three genes were selected: 702 bp proteinase encoding gene *TEV*, 1700 bp levansucrase encoding gene *sacB*, and 4107 bp endonuclease encoding gene *cas9*. The *TEV* gene, *sacB* gene and *cas9* gene were PCR amplified from LS2416 (Luo et al. 2015), pKSacB (Luo et al. 2016), and pCas9 (Jiang et al. 2013), respectively. After co-electroporation of plasmid and HA-flanked targeting DNA as described in "Materials and methods" usually one electroporation generated hundreds of Km^R cTc^R clones. A total of 100 clones were randomly picked and duplicated on LB plate with Gm, from which 20 Km^RGm^S clones were subjected to culture, plasmid extraction and validation. From one typical experiment, 87, 84 and 79% clones were Km^RGm^S for *TEV* gene, *sacB* gene and *cas9* gene cloning, respectively. And all the 20 selected Km^RGm^S clones were the correct recombinant plasmids. The restriction enzyme map of each recombinant plasmid (*TEV* gene cloning plasmid pLS4517, *sacB* gene cloning plasmid pLS4518, and *cas9* gene cloning plasmid pLS4519) and its restrictive digestions are shown in Fig. 3A–C, respectively.

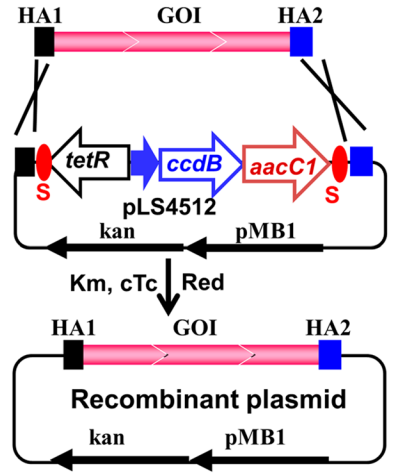

Fig. 2 Scheme shown the *S-tetR-*P_{tetA}*-ccdB-aacC1-S* cassette-mediated gene cloning. The homologous arms (HAs)-flanked gene-of-interest (GOI) and cassette harboring plasmid pLS4512 were co-electroporated into the recombinase-proficient electrocompetent cells. Recombinase-catalyzed recombination between the HAs in the targeting DNA and pLS4512 generated the recombinant plasmid by replacing the cassette region of pLS4512 with GOI. I-SceI cut the two S sequences (S indicates the I-SceI recognition and cleavage site), creating double-strand break; chlortetracycline (cTc) induces the expression of CcdB which was toxic to the cells. The combined counterselection of I-SceI and CcdB eliminates the residue pLS4512

Chromosomal gene knock-in

One main utility of recombineering is chromosomal gene knock-in. Introducing foreign gene(s) into the

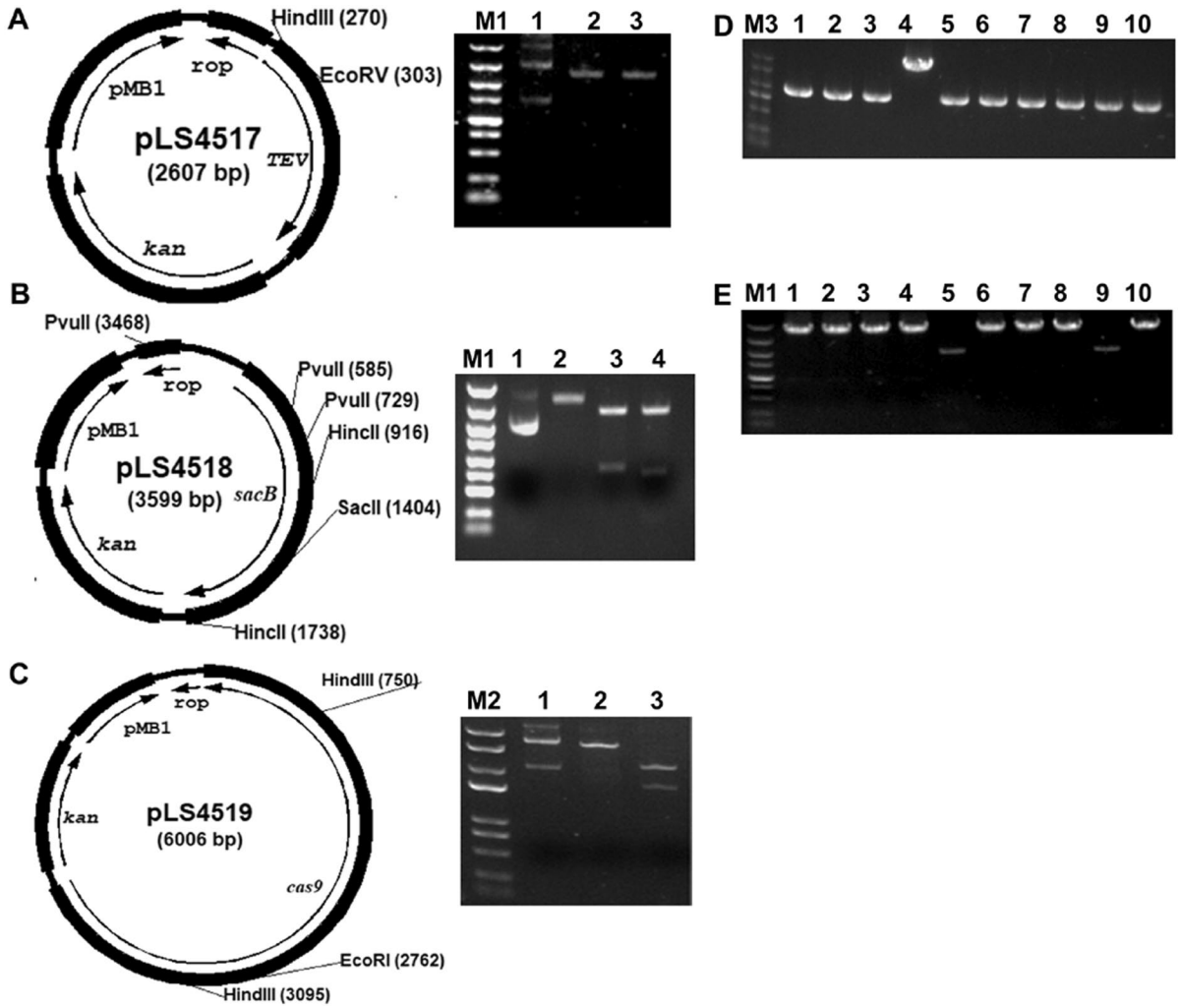

Fig. 3 Confirmation of recombinant plasmids and PCR screening of gene knock-in clones. **A** Confirmation of *TEV* gene cloning plasmid pLS4517. Left panel: plasmid map. Right panel: lanes 1, 2, and 3 are uncut, *Hin*dIII digestion (2.6 kb), and *Eco*RV digestion (2.6 kb), respectively. **B** Confirmation of *sacB* gene cloning plasmid pLS4518. Left panel: plasmid map. Right panel: lanes 1, 2, 3, and 4 are uncut, *Sac*II digestion (3.6 kb), *Hinc*II digestion (0.8 and 2.8 kb), and *Pvu*II digestion (0.7 kb and 2.9 kb), respectively. **C** Confirmation of *cas9* gene cloning plasmid pLS4519. Left panel: plasmid map. Right panel: lanes 1, 2, and are uncut, *Eco*RI digestion (6.0 kb) and *Hin*dIII digestion (2.3 and 3.7 kb), respectively. **D** PCR screening of *egfp* knock-in clones. Nine of 10 clones were knock-in clones (941 bp). **E** PCR screening of *cas9* knock-in clones. Eight of 10 clones were knock-in clones (4334 bp). The clone without target gene knock-in kept the cassette with PCR amplicon size of 2070 bp. M1, DL5000 DNA molecular marker, sizes in kb: 5.0, 3.0, 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, and 0.1; M2, Trans2K PlusII DNA molecular marker, sizes in kb: 8.0, 5.0, 3.0, 2.0, 1.0, 0.75, 0.5, 0.25, and 0.1 kb; M3, DL2000 DNA molecular marker, sizes in kb: 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, and 0.1 kb

chromosome is essential for the construction of value-added product producing strain. Compared with plasmid-based gene expression, chromosome-based gene expression shows the advantages of cells homogeneity, no use of antibiotics, and no metabolic burden for the plasmid maintenance. As stated in "Material and methods", we firstly knocked the *S-tetR-*P_{tetA}*-ccdB-aacC1-S* cassette into *E. coli* DH10B chromosomal *endA1* locus. Then HA-flanked 714 bp *egfp* gene which was amplified from pJOE4905.1 (Motejadded and Altenbuchner 2009), and HA-flanked 4107 bp *cas9* gene which was amplified form pCas9 (Jiang

et al. 2013), were each electroporated into the intermediate strain. After duplication, Gm^S clones were subjected to PCR screening. Nine of 10 Gm^S clones for *egfp* knock-in and eight of 10 Gm^S clones for *cas9* knock-in were correct knock-in clones. The PCR screening results are shown in Fig. 3D and E, respectively. The high knock-in efficiency makes the duplication step for selection of Gm^S clones unnecessary. Indeed, we directly PCR genotyped the transformants and found that for both *egfp* and *cas9* knock-in, each eight of randomly picked 10 clones were correct. The chromosomal gene knock-in scheme, PCR genotyping of the *egfp* and *cas9* knock-in strains, and fluorescent image of the *egfp* knock-in stain, are shown in Supplementary Fig. 1.

In summary, a highly efficient selection/counterselection cassette was developed for *E. coli* recombineering. The application of the cassette requires neither pre-generated strain nor specific medium; and unlike single CcdB counterselection, the cassette circumvents the CcdA-expression plasmid (Wang et al. 2014) or chromosomal *ccdB* inducible elements (Zhang et al. 2017). Besides gene cloning and chromosomal gene knock-in, the novel system might also be useful for gene mutation (Wang et al. 2022) and gene replacement (Robertson et al. 2021).

Acknowledgements We thank for Dr. Josef Altenbuchner and Dr. Michael Kahn for the plasmids used in this study.

Author contributions GS: conceived and designed the study. GZ, QZ, JW and JZ: performed the experiments. All authors contributed to data analysis, writing and editing of the manuscript. All authors read and approved the final manuscript.

Funding This work was generously funded by National Natural Science Foundation of China (NSFC 82073742).

Declarations

Conflict of interest The authors report no conflicts of interest.

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