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Characterization of Inducible *ccdB* Gene as a Counterselectable Marker in *Escherichia coli* Recombineering

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Abstract Recombineering is a homologous-based DNA cloning and modification technique. Recombineering-mediated chromosomal gene knock-in usually involves a selectable/counterselectable cassette. Though a variety of selectable/counterselectable cassettes were developed; however, a specifically designed gene deletion strain or minimal medium is often required. Herein, we describe a novel selectable/counterselectable cassette P_{lac}-*ccdB-aacC1* in which *aacC1* (gentamicin resistance gene) is used as the selectable marker for the homologous arm-flanked cassette knock-in, while the IPTG inducible *ccdB* gene is used as the counterselectable marker for chromosomal gene knock-in. The counterselection is achieved via supplementing 1 mM IPTG in the LB agar medium. An oligonucleotide designed to evade the mismatch repair system was utilized to engineer an *Escherichia coli* DH10B-derived *gyrA462* strain that was used to as the host for the plasmid harboring the P_{lac}-*ccdB-aacC1* cassette. By using the P_{lac}-*ccdB-aacC1* cassette, a linear–linear homologous recombination (LLHR) system was generated by knocking a 6.2 kb *araC*-P_{BAD}-*redγ-recET-recA* DNA fragment into the *E. coli* DH10B chromosome. The functional of the LLHR recombineering system was characterized by cloning of the target DNA from PCR product as well as from the genomic DNA mixture. The P_{lac}-*ccdB-aacC1* cassette will be a useful tool in *E. coli* recombineering.

Introduction

Recombineering is a homologous recombination-based technology that is widely used for bacterial genome editing, episomal DNA cloning, and modification [9, 10]. Selectable/counterselectable cassette is often used for recombineering-mediated *Escherichia coli* chromosomal knock-in. A variety of counterselectable markers were developed, including *sacB* [17], *galK* [15], *thyA* [16], *tolC* [4], *rpsL* [14], and *tetA-sacB* [8]. However, use of these counterselectable markers often involves a specific gene deletion strain which involves more steps or minimal medium which retards the strain's growth. Other shortcoming, such as accumulation of mutations in *E. coli* even when grown without sucrose in using *sacB*, is also worthy consideration.

Toxic gene *ccdB* in the toxin–antitoxin system [1] was recently developed as an efficient counterselectable marker [13]; yet the delicate, two plasmid-based toxin–antitoxin expression system complicated the screening.

To improve the process, herein a Plac-*ccdB-aacC1* cassette in which *ccdB* was driven by IPTG (isopropyl-β-D-1-thiogalactopyranoside) inducible Plac promoter was developed as a selectable/counterselectable marker. In the cassette, the *aacC1* (gentamicin resistance gene) was used for the first step recombinase-catalyzed homologous arm (HA) flanked Plac-*ccdB-aacC1* cassette knock-in, then the cassette was replaced by the same HA-flanked target gene though the second recombineering step via the counterselection by the agar medium with IPTG. Functional characterization of the selectable/counterselectable system was

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demonstrated by knocking a 6.2 kb fragment (*araC*-P_{BAD}-*redγ-recET-recA*) into the *E. coli* DH10B chromosome, generating a linear–linear homologous recombination (LLHR) recombineering system. The LLHR system was characterized by cloning of a 0.8 kb chloramphenicol resistance gene (*cat*) from linear PCR product as well as a 3.2 kb *lacZ* gene from *E. coli* MG1655 genomic DNA. Though oligonucleotide-mediated recombineering, a highly transformative *E. coli* strain was also engineered to host the Plac-*ccdB-aacC1* cassette template plasmid. The cassette has the potential to simplify the *E. coli* chromosomal gene knock-in as well as other recombineering applications.

Materials and Methods

Bacterial Strains, Culture, and Plasmids

Escherichia coli cells were grown in Luria–Bertani (LB) broth supplemented, when appropriate, with the following antibiotics: Ampicillin sodium (Ap, 50 μg/ml), Kanamycin sulfate (Km, 30 μg/ml), or Gentamicin sulfate (Gm, 25 μg/ml). *E. coli* DH10B was used as general cloning host; *E. coli* LS027 generated in this study was used as the cloning host of *ccdB* gene harboring plasmids. A final concentration of 25 μg/ml heat-inactivated cTc (chlortetracycline hydrochloride) was used for homing endonuclease I-SceI induction. Restriction enzymes were products of NEB, USA. Gel extraction kit was purchased from Qiagen, Germany. The strains and plasmids used in this study are listed in Table S1; oligonucleotides used in this study are listed in Table S2. Details of the vector construction are provided in Supplementary Material.

Molecular Biology and Recombineering Manipulations

Escherichia coli electrocompetent cells preparation, DNA transformation, plasmid extraction, restriction enzyme digestion, PCR, and *E. coli* MG1655 genomic DNA preparation with lysozyme treatment and phenol–chloroform extraction were carried out with standard procedures. Recombineering manipulation was carried out as described [3].

Results and Discussion

Construction of a Highly Transformative *ccdB* Host Strain

A highly transformative *E. coli* strain for harboring *ccdB* gene was firstly constructed by introducing a *gyrA462* mutation (arginine to cysteine mutation at the GyrA amino acid 462 position) via oligonucleotide-mediated recombineering. Co-transformation of 5 pmol of GYRA2 designed to evade mismatch repair by adding four mutations at wobble positions [11] and 100 ng of *ccdB*-harboring, kanamycin resistance plasmid pMK2010 [6] into the Red-competent cells of *E. coli* DH10β F'DOT [7] containing Red helper plasmid pLS3021 resulted in 65 Km^R clones. Genotype analysis revealed that seven of ten clones exhibited expected mutations. pMK2010 was eliminated from the engineered strain by transforming of a same pUC replicon-based, Ap resistance, self-cleavage plasmid pLS3050; pLS3050 was subsequently removed by culturing the cells with cTc induction. Three of 45 clones tested were Ap^S; one strain was named as *E. coli* LS027. Compared with that of DB3.1, *E. coli* LS027 exhibits approximately 100-fold of transformation efficiency (2.1×10^8 transformants/μg vs 3.2×10^6 transformants/μg) using pKD4 [4] as plasmid DNA. The strain construction scheme and genotype sequencing results are illustrated in Fig. 1.

Inducible Toxic Gene *ccdB* as a Novel Counterselectable Marker

A R6K replicon-based, selectable/counterselectable P_{lac}-*ccdB-aacC1* cassette template plasmid pLS3161 was constructed by fusing *ccdB* under the IPTG inducible Plac promoter with *aacC1*. The function was the 1165 bp cassette was characterized by construction of a chromosome-based LLHR recombineering system.

One microgram of 50 bp HA-flanked P_{lac}-*ccdB-aacC1* cassette amplified from pLS3161 was electroporated into the Red-induced electrocompetent cells of *E. coli* DH10B harboring pLS3021. The HAs were designed according to the truncated *lacZ* region of *E. coli* DH10B genome. A total of 218 clones were obtained under gentamicin selection; the clones showed no visible growth retardation, implying that uninduced CcdB exerts no observable deleterious effect. By contrast, 48 of 50 clones showed no growth when streaked on LB agar plate supplemented with 1 mM IPTG indicating the high toxicity of induced CcdB. The resulting strain, *E. coli* LS3125, was Red-induced and electroporated with 1 μg of targeting DNA released from pLS3185. Sixty-four IPTG resistant clones were generated, and eight out 10 clones analyzed were correct in which the P_{lac}-*ccdB-aacC1* cassette was replaced by the *araC*-P_{BAD}-*redγ-recET-recA* fragment. After pLS3021 elimination through non-selective passages, a LLHR recombineering strain *E. coli* LS3130 was obtained. The scheme for the inducible *ccdB* counterselection-based strain construction and genotype analysis are shown in Fig. 2.

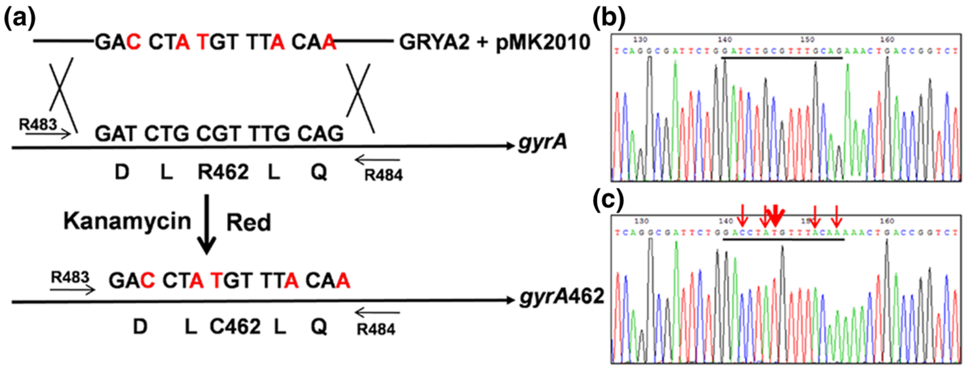

Fig. 1 Scheme shown a *gyrA462* mutation via oligo-mediated recombineering. Homologous recombination between GYRA2 and its chromosomal allele resulted in expected mutations (**a**). The triplets of the target region of the *gyrA* gene are indicated for the wild-type (**b**) and *gyrA462* mutant *E. coli* LS027 (**c**). Note that incorporation of GYRA2 changes a total change of 5 bases (*red* sequences that are indicated by *arrows* in (**c**) yielding a single amino acid change (R462C). Primers for screening (R483–R484) are indicated which generated a 341 bp amplicon (Color figure online)

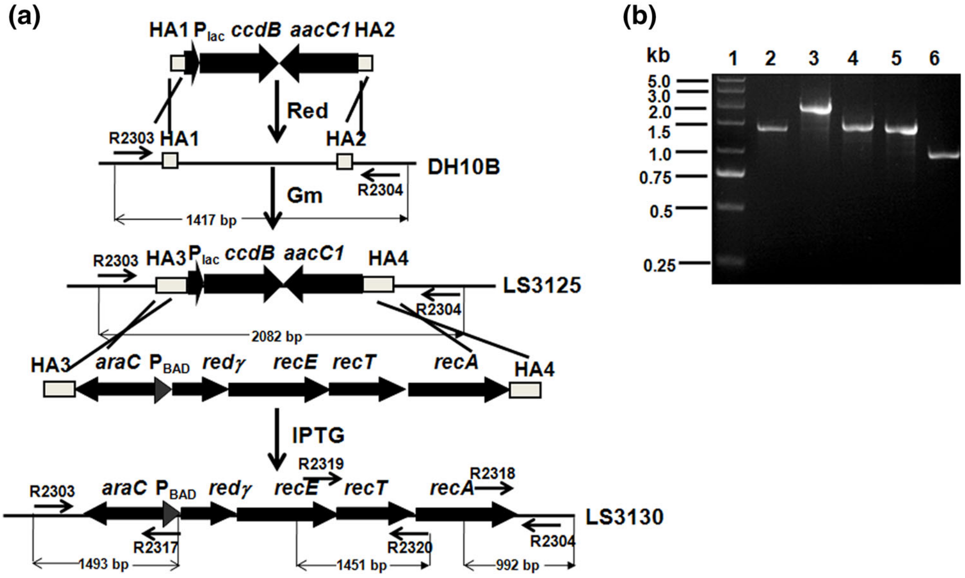

Fig. 2 Schematic representation of *ccdB* counterselection for the construction of a LLHR recombineering system. **a** Homologous recombination between the HAs in the targeting DNA and its chromosomal allele generated *E. coli* LS3125. The second recombineering step generated a LLHR strain *E. coli* LS3130 by replacing the P_{lac}-*ccdB-aacC1* cassette by a 6.2 kb *araC*-P_{BAD}-*redγ-recET-recA* DNA fragment under IPTG counterselection. **b** Genotype analysis. *Lane 1* DNA marker, *lane 2* and *3* are amplicons amplified from *E. coli* DH10B and *E. coli* LS3125 with primer set R2303–R2304, respectively, *lane 4*, *5*, and *6* are amplicons amplified from *E. coli* LS3130 with primer sets R2303–R2317, R2319–R2320, and R2318–R2304, respectively. The sizes of the amplicons are indicated at (**a**)

Recombineering System Characterization

Cloning performance of *E. coli* LS3130 and its comparison with other recombineering systems were carried by cloning of *cat* gene and *lacZ* gene. For *cat* gene cloning, 100 ng of HA-flanked *p15A-kan* amplified from pLS3163 with primers R2321–R2322 and 1 μg of HA-flanked *cat* amplified from pBAD322C [2] with primers R2323–R2324 were co-electroporated into each recombineering system. For *lacZ* gene cloning, 1 μg of HA-flanked *p15A-kan* amplified from pLS3163 with primers R2327–R2328 and 5 μg of sheared *E. coli* MG1655 genomic DNA mixture were co-electroporated into each recombineering system. For every recombineering, ten clones were subjected to plasmid extraction, restriction enzymes digestions, and DNA sequencing. As shown in Table 1, the decreasing order of cloning efficiency is plasmid-based LLHR system pLS3028, chromosome-based LLHR system *E. coli* LS3130, plasmid-based linear–circular homologous recombination (LCHR) system pLS3021, and chromosome-based LCHR system LS-GR [12]. This result consists with the finding of Fu et al. [5] that LLHR shows higher cloning efficiency than LCHR in cloning of linear DNA molecules. Although *E. coli* LS3130 was ~10-fold less

Table 1 Cloning efficiency comparison among four recombineering systems

System	*p15A-kan-cat*		*p15A-kan-lacZ*	
	Number of Km^R clones ($\times 10^2$)	Correct clones/tested	Number of Km^R clones ($\times 10^2$)	Correct clones/tested
pLS3028	341.3 ± 30.5	10/10	24.2 ± 2.5	9/10
E. coli LS3130	58.1 ± 1.0	10/10	3.36 ± 0.19	9/10
pLS3021	42.4 ± 1.67	9/10	3.16 ± 0.16	7/10
E. coli LS-GR	8.10 ± 0.21	8/10	0.50 ± 0.06	8/10

efficient than that of pLS3028, it circumvents the plasmid transformation and elimination steps and therefore is more convenient for DNA manipulation.

Inducible *ccdB* as a counterselectable marker circumvents the requirement of an antitoxin *ccdA* expression plasmid, making it a better choice for gene editing. Highly efficient counterselection can be realized by simply supplementing IPTG in the rich LB medium. The small size of the P_{lac}-*ccdB-aacC1* cassette is also easy for PCR amplification and genotyping. Besides *E. coli* LS3130 construction, we also knocked *mkan* fragment [16] and *I-SceI* gene into the chromosome of *E. coli* MG1665 via *ccdB* counterselection (data not shown). In summary, the inducible *ccdB* could be a powerful counterselectable marker for *E. coli* recombineering.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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