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Direct cloning and transplanting of large DNA fragments from *Escherichia coli* chromosome

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We applied a resistance split-fusion strategy to increase the *in vivo* direct cloning efficiency mediated by Red recombination. The *cat* cassette was divided into two parts: *cma* (which has a homologous sequence with *cmb*) and *cmb*, each of which has no resistance separately unless the two parts are fused together. The *cmb* sequence was integrated into one flank of a target cloning region in the chromosome, and a linear vector containing the *cma* sequence was electroporated into the cells to directly capture the target region. Based on this strategy, we successfully cloned an approximately 48 kb DNA fragment from the *E. coli* DH1-Z chromosome with a positive frequency of approximately 80%. Combined with double-strand breakage-stimulated homologous recombination, we applied this strategy to successfully replace the corresponding region of the *E. coli* DH36 chromosome and knock out four non-essential genomic regions in one step. This strategy could provide a powerful tool for the heterologous expression of microbial natural product biosynthetic pathways for genome assembly and for the functional study of DNA sequences dozens of kilobases in length.

Red homologous recombination, resistance split-fusion, target cloning, transferring

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INTRODUCTION

Red homologous recombination has been widely used in gene knockout, mutation and replacement strategies in molecular biology research (Datsenko and Wanner, 2000; Ellis et al., 2001; Murphy, 1998; Muyrers et al., 2000; Yu et al., 2000). Target DNA can be directly cloned into the cloning vector mediated by the Red recombinant technique based on the interaction between homologous sequences *in vivo* (Bian et al., 2012; Cobb and Zhao, 2012; Fu et al., 2012; Zhang et al., 2000). Depending on whether the target DNA is circular or linear, the direct cloning method can be divided into two categories (Fu et al., 2012): LCHR (linear plus circular homologous recombination) and LLHR (linear plus linear homologous recombination). Target DNA regions of approximately 30 kb, 3 kb fragments from plasmid BACs (bacterial artificial chromosomes) and genomic DNA fragments can be easily cloned by LCHR assay (Zhang et al., 2000). Due to the complexity of the DNA sequence, it is almost impossible to obtain positive clones using conventional methods when the length of the target DNA is more than 10 kb.

In order to directly clone a target DNA sequence of dozens of kilobases in length from the bacterial chromosome, a resistance split-fusion strategy was applied (Figure 1). The

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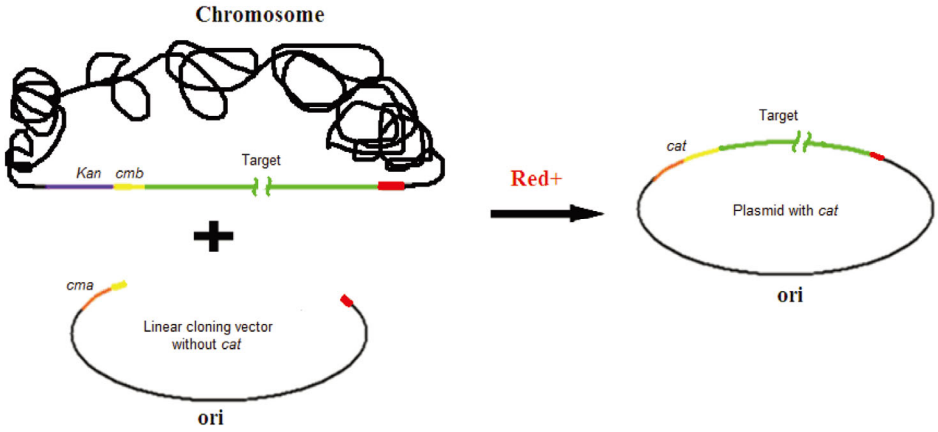

Figure 1 The principle of the resistance split-fusion strategy for direct cloning DNA sequence.

cat cassette harbored in linear plasmid was divided into two parts: *cma* (which shares a homologous sequence, 135 bp in length, with *cmb*) and *cmb*, each of which has no resistance separately unless the two parts are fused together. First, we directionally integrated the *cmb* sequence and a full-length kanamycin-resistance gene (as the marker for this step) into a flanking region adjacent to the target region on the chromosome. Next, a linear vector containing the *cma* sequence was electroporated into the cells; this vector also contained a 50 bp sequence with homology to the other flank of the target region. The *cmb* sequence adjacent to the target region of the chromosome and the *cma* sequence in the linear cloning vector were fused to form a complete *cat* gene. With the aid of ampicillin resistance screening, the direct cloning efficiency was greatly improved. Based on this strategy, we successfully cloned an approximately 48.2 kb DNA fragment named "unit Z" from the *E. coli* DH1-Z chromosome (unit Z has four markerless-deleted nonessential genomic regions). Then, the recombinant plasmid containing unit Z was introduced into DH36 cells. Combined with double-strand breakage-stimulated homologous recombination, the corresponding region of the *E. coli* DH36 chromosome was successfully replaced by unit Z to produce an *E. coli* strain DH40 with four nonessential genomic regions knocked out in one step. This strategy can be used in genome assembly and the functional study of DNA sequences dozens of kilobases in length.

RESULTS

Direct cloning and identification of unit Z

To further reduce the *E. coli* DH36 chromosome by replacing the corresponding region with unit Z after deleting four nonessential genomic regions, the deleted unit Z (hereinafter referred to as unit Z) should be directly captured first. Unit Z is approximately 48 kb in length and could not be captured by general Red reconstruction method (Zhang et al., 2000), but was successfully cloned from *E. coli* DH1-Z chromosome by resistance split-fusion strategy (Figure 2). When screened by PCR with the primer pairs 38-0 and AD-1, and 35-1 and M13F, the frequency of positive clones was as high as 89% (17/19) (Figure 3A and B). The recombinant plasmid p15AD2IC-Z was transferred to *E. coli* DH1 and then extracted, for the *E. coli* DH1-Z genomic DNA may influence the PCR identification result. To ensure the four non-essential genomic regions were deleted, PCR identification of the recombinant plasmid was performed with the primer pairs ZL-R5 and 35-1, 36-0 and 36-1, 37-0 and 37-1 and 38-0 and 38-1. In addition, three restriction enzymes, *Apa* I, *Sac* I, and *Xba* I were chosen for the restriction digestion analysis of p15AD2IC-Z. Consistent with the predicted pattern, digested p15AD2IC-Z plasmids generated DNA fragments of approximately approximate 17.2,

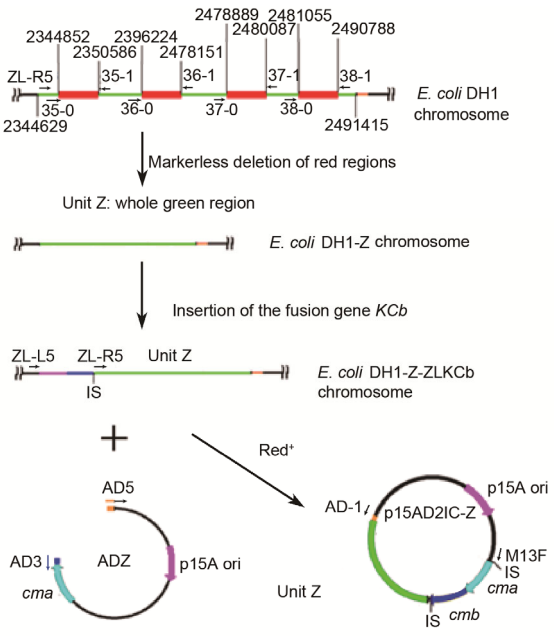

Figure 2 Schematic diagram for cloning unit Z. IS, I-*Sce* I restriction site.

14.7, 14.4, 3.3, and 1.4 kb (Figure 4A). The restriction digest showed that the first three bands were too close in size to be separated, although there was a bright and wide band near 15 kb, and bands were visible at 3.3 and 1.4 kb (Figure 4B). The two identification results above showed that p15AD2IC-Z was the positive recombinant plasmid.

Knocking out four non-essential regions of the receptor chromosome by the replacement of unit Z

The schematic diagram for the co-transformation of the donor plasmid p15AD2IC-Z and the helper plasmid pAAIS-GBE into *E. coli* DH36-35LRIK-38LRIT is shown in Figure 5. The Red recombinase system and I-*Sce* I endonuclease were induced by arabinose, and after recombination, the cells were plated onto LB plates and cultivated. The positive clone, without kanamycin and ampicillin resistance, was obtained by resistance loss screening.

Further PCR identification of the recombinant strain was performed with primer pairs ZL-L5 and 35-1, 36-0 and 36-1, 37-0 and 37-1, and 38-0 and ZR-R3, and showed that four regions of the positive clone were all ~1.2 kb in length, as predicted (Figure 6). High fidelity PCR amplification of the sequences 35LR, 36LR, 37LR and 38LR was performed with primer pairs 35-1 and 35-0, 36-1 and 36-0, 37-1 and 37-0 and 38-0 and 38-1, and the products were sequenced; the sequences obtained were as expected. In addition to the identification of 36 other deleted regions, the recombinant strain *E. coli* DH40 was confirmed to contain the markerless deletion of four non-essential chromosomal regions.

Furthermore, re-sequencing of genomic DNA was performed on *E. coli* DH36 and *E. coli* DH40 strains to confirm the internal sequence of unit Z had no unexpected recombination; the alignment of the relevant chromosome regions obtained using CLC Genomics Workbench is shown in Figure 7. The blank region of the *E. coli* DH40 chromosome compared with *E. coli* DH36 were the knocked out regions 35, 36, 37, 38, and the remainder of the sequence did not recombine unexpectedly. As a result, 40 nonessential regions of the positive recombinant *E. coli* strain DH40 have been knocked out tracelessly, and the genome was reduced by 20.4% for a total deletion of 945 kb.

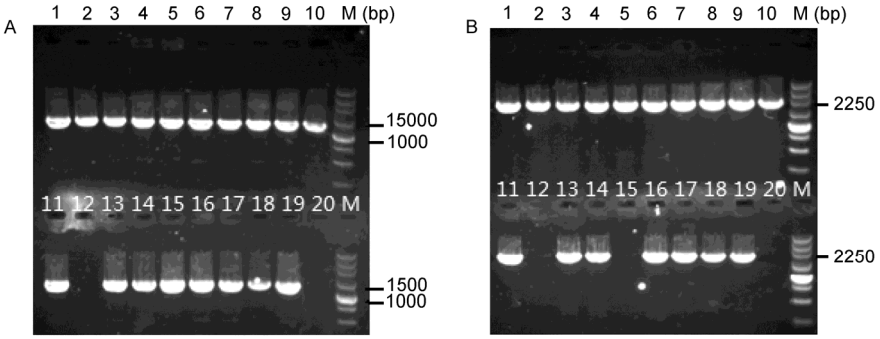

Figure 3 PCR screening for cloning unit Z. A, Primers 38-0 and AD-1 (the positive product is approximately 1.5 kb). B, Primers M13F and 35-1 (the positive product is about 2.5 kb). 1–19, sample; 20, p15AD2IC-SacB; M, 250 bp DNA ladder marker.

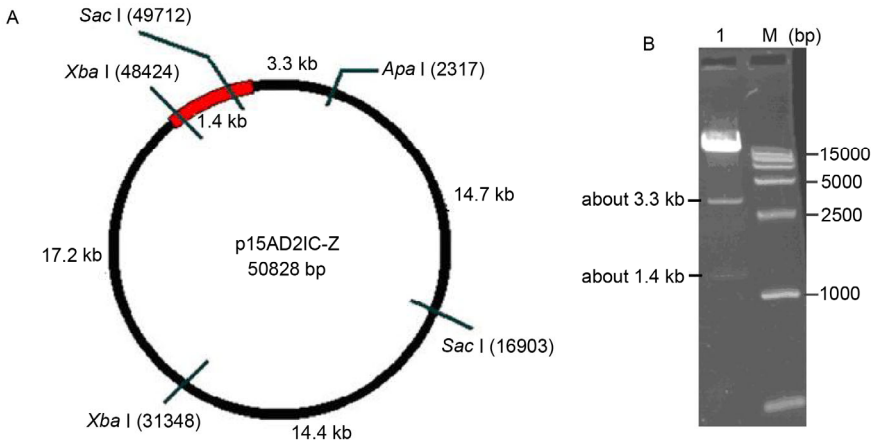

Figure 4 The restriction site of p15AD2IC-Z. A, The restriction site of p15AD2IC-Z (the red region is the cloning vector). B, The digestion analysis of p15AD2IC-Z by restriction enzymes *Apa* I, *Sac* I, and *Xba* I. M, DL 15000 DNA Marker; 1, p15AD2IC-Z.

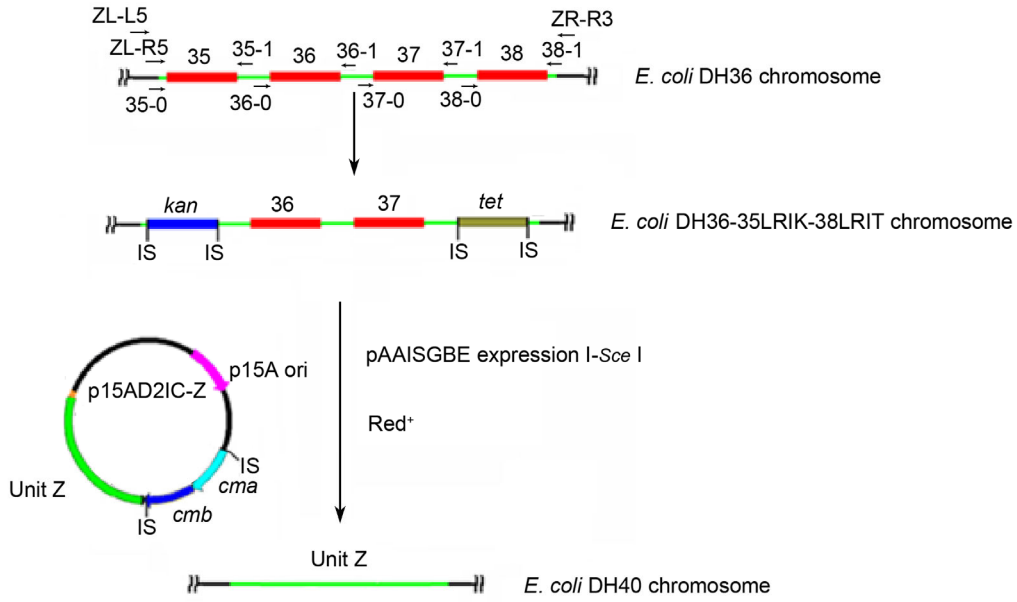

Figure 5 Schematic diagram of transplanting unit Z.

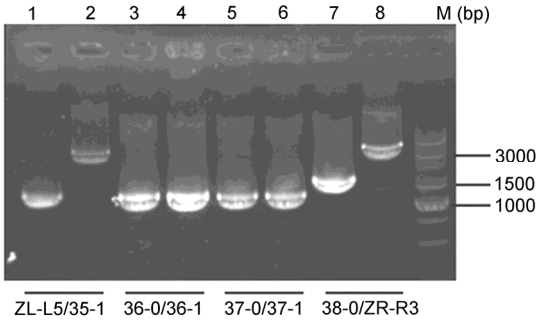

Figure 6 PCR verification of transplantation of unit Z. 1 and 2, Primers ZL-L5/35-1; 3 and 4, Primers 36-0/36-1; 5 and 6, Primers 37-0/37-1; 7 and 8, Primers 38-0/ZR-R3. 1,3,5,7, *E. coli* DH40; 2,4,6,8, *E. coli* DH36-35LRK-38LRT/p15AD2IC-Z/pAAISGBE. M, 250 bp DNA ladder marker.

DISCUSSION

Target DNA, dozens of kilobases in length, can be directly cloned from BAC vectors, mediated by the Red recombinant system. However, the positive rate of direct cloning from chromosomes is very low because the sequence and structure of chromosomes are much more complex than that of the BAC vectors (Fu et al., 2012; Zhang et al., 2000). There are two strategies for direct cloning of large DNA sequences: LCHR and LLHR (Fu et al., 2012). Target DNA sequences from genomes or BAC vectors can be directly cloned by LCHR, and can also be digested to fragments containing the target DNA sequence using restriction enzymes and then co-electroporated with linear vectors into *E. coli* Red/ET$^+$ cells to allow direct cloning of the target sequence via LLHR. The LCHR method can also be applied to the reformation of recombinant plasmids, such as the replacement wild type promoters with synthetic promoters, adding modifier genes and so on; these recombinant plasmids may acquire changes in copy number, expression direction and host range of heterologous expression (Sharan et al., 2009; Venken et al., 2006; Wenzel et al., 2005). However, both of these strategies will produce a large number of empty vectors and recombinants generated by the linear vectors and other nonspecific regions. Therefore, the PCR screening of recombinants can be time-consuming, for false positive clones may also contain the antibiotic resistance marker present in the original vector.

In this study, we improved the conventional Red homologous recombination method using a resistance split-fusion strategy, and combined with the LCHR method, we successfully directly cloned a target DNA sequence, 48.2 kb in length, from the *E. coli* DH36-35LRIK-38LRIT chromosome. The positive rate reached 80%, yet the target fragment could not be directly cloned using Red homologous recombination alone.

For practical applications, the different plasmids should be selected according to the size of the target DNA fragments. Studies have found that using high-copy plasmids and medium-copy vectors as the acceptor such as pUC, pBluescript (copy number 500–700), pBR322, p15A, (copy number 15–20), the upper limit length of the target DNA sequence was about 25–80 kb (Venken et al., 2006). When length of the target DNA sequence was hundreds of kilobases, single copy plasmids such as BAC plasmids were required, for BAC plasmids can carry 200–250 kb exogenous DNA fragments, and single-copy plasmids are more stable in host cells (Kotzamanis and Huxley, 2004).

In this study, the resistance split-fusion strategy was designed to increase the direct cloning efficiency based on the Red recombination system. The *cat* cassette in p15A plas-

mid was divided into two parts: *cma* and *cmb*, and the *cmb* fragment was integrated into chromosome while the *cma* fragment was retained in a linear plasmid. Each of these two parts has no resistance separately unless they are fused together. Based on this strategy, a target DNA fragment of approximately 48 kb in length was directly cloned from the *E. coli* DH1-Z chromosome. *E. coli* strain DH1-Z was constructed from *E. coli* DH1 by constructing a markerless knockout of four non-essential genomic regions in unit Z; thus, unit Z of *E. coli* DH1-Z was reduced from 146.8 to 48.2 kb, and the size of the recombinant plasmid p15AD2IC-Z (which replicated stably in cells) was about 50 kb. Then, combined with double-strand breakage-stimulated homologous recombination, the corresponding region of *E. coli* DH36 chromosome was replaced with unit Z of *E. coli* DH1-Z, and the resulting strain was *E. coli* DH40. In short, the chromosome was decreased in size by about 100 kb as four non-essential genomic regions were knocked out in one step.

This strategy provides a powerful tool for the heterologous expression of microbial natural product biosynthetic pathways, genome assembly, and the functional study of DNA sequences dozen of kilobases in length. Gene clusters can be directly cloned from unsequenced genomes into expression vectors, or integrated into heterologous chromosomes for heterologous expression to characterize their functions (Ongley et al., 2013a). Based on this, through homologous recombination, the function and regulation of gene clusters can be reconstructed to improve metabolic flux and then improve the expression of natural products. Traditional *de novo* assembly is not conducive to the assembly of disparate biosynthetic pathways, which are encoded by dozens or even hundreds of kilobases of DNA, but methods of direct cloning can not only obtain target gene clusters and assemble multiple large fragments into large gene cluster families (Kotzamanis and Huxley, 2004), but can also reform them by site-directed mutagenesis, insertion, and markerless deletion. Then, the reformed gene clusters may synthesize derivatives of natural products. Furthermore, direct cloning and heterologous expression of corresponding pathways for the synthesis of natural products from some microorganisms that are difficult to cultivate in the laboratory may have high-yield products with safety and efficiency and save on costs (Ongley et al., 2013b). In conclusion, this cloning method could be used to discover potential large gene clusters and find valuable natural products; on the other hand, it can also be used in reconstructing pathways for heterologous expression and the synthesis of novel derivatives of natural products.

MATERIALS AND METHODS

Strains, plasmids, primers, enzymes and reagents

Bacterial strains, plasmids and primers used in this study are shown in Tables 1 and 2.

Dpn I was purchased from NEB (Ipswich, USA) and other enzymes and DNA markers were purchased from TaKaRa Biotechnology (Dalian). Mini plasmid kits were purchased from TIANGEN BIOTECH (Beijing). PurePlasmid mini kits were purchased from CWBIOTECH (USA). Ampicillin, chloramphenicol, kanamycin, tetracycline were purchased from GENVIEW (USA).

LB medium: peptone (Oxoid, UK) 10 g L^{-1}, yeast extract (Oxoid) 5 g L^{-1}, NaCl 10 g L^{-1}, pH 7.0. LB plates also contained 20 g L^{-1} agar and sucrose LB plates also contained 60 g L^{-1} sucrose, without NaCl. When appropriate, antibiotics were added to the culture medium at the following concentrations: ampicillin 100 mg L^{-1}, chloramphenicol 50 mg L^{-1}, kanamycin 50 mg L^{-1}, tetracycline 10 mg L^{-1}.

Construction of fragment *KCb* containing fused *kan* and *cmb*

Fragment *KCb* was produced using PCR amplification as follows. PCR amplification of *kan* was performed with primers K-F (which created a *Eco*R I restriction site at the 5′ end of the amplicon) and K-R, using plasmid pKD4 as the template. PCR amplification of *cmb* was performed with primers ICB-F and ICB-R (which created a *Bam*H I restriction site in the 3′ end of the amplicon), using plasmid p15AD2IC-SacB as the template. Primers K-F and ICB-R were then used to amplify the fusion PCR product, *KCb*, using the PCR fragments mentioned before as the template. *KCb* was digested with *Eco*R I and *Bam*H I and inserted into the *Eco*R I and *Bam*H I sites of vector pUC19, and the resulting plasmid was named pUC19-KCb.

Direct cloning unit Z from *E. coli* DH1-Z chromosome by the resistance split-fusion strategy

Unit Z was a region of the *E. coli* DH1 chromosome (2344629…2491415), and *E. coli* DH1-Z was constructed by knocking out four non-essential genomic regions, region 35 (2344852…2350586), region 36 (2396224…2478151), region 37 (2478889…2480087) and region 38 (2481055…2490788) (Figure 2). Unit Z of the *E. coli* DH1-Z chromosome was 48.2 kb in length with a reduction of about 100 kb compared to the *E. coli* DH1 chromosome. The process of direct cloning unit Z via the Red homologous recombination system was achieved in two steps:

(i) Fragment *KCb* was integrated into the left flank of unit Z on the *E. coli* DH1-Z chromosome (2344628...2344629) using a method similar to Gene-Doctoring (Lee et al., 2009); the resulting strain was *E. coli* DH1-Z-ZLRKCb. PCR amplification of the upstream homologous region ZLL was performed using the primer pair ZL-L5 and ZL-L3, using DH1 chromosome as the template. PCR amplification of the downstream homologous region ZLR was performed using the primer pair ZL-R5 and ZL-R3, using DH1 chromosome as the template. We digested ZLL with *Eco*R I and

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Source or reference
E. coli Strain		
BL21(AI)	*F- ompT hsdSB* (r_B- m_B-) *gal dcm araB*::T7RNAP-tet*A*. To supply Tet^r gene	Invitrogen
DH5α	*supE44 lacZ*ΔM15 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Transgen
DH1	The genomic sequence is listed in Accession no. CP001637	ATCC 33849
DH36	Derivative of *E. coli* DH1 with 36 non-essential regions knocked out	This study
DH1-Z	Derivative of *E. coli* DH1 with region 35, region 36, region 37, region 38 in unit Z knocked out, shown in Figure 2	This study
DH40	Derivative of *E. coli* DH36 with 4 non-essential regions knocked out	This study
plasmid		
pKD4	Conferred *kan* cassette	(Datsenko and Wanner, 2000)
pAAGBE	Derivative of pKOBEG (Chaveroche et al., 2000) with *cat* replaced by *amp*. A temperature-sensitive helper plasmid expresses Red recombinase by adding L-arabinose	This study
pAAISGBE	A temperature-sensitive helper plasmid expresses I-*Sce* I endonuclease and Red recombinase by adding L-arabinose, *amp*	This study
pAAICDGBE	A temperature-sensitive helper plasmid expresses I-*Cre* I endonuclease and Red recombinase by adding L-arabinose, *amp*	This study
p15AD2IC-SacB	The donor plasmid containing p15A ori, *cat* resistance gene flanked by I-*Cre* I cleavage site (named as IC) and the counter-selection marker *sacB* (a plasmid having *sacB* cannot grow in media containing sucrose) (Li et al., 2013)	This study

Table 2 Primers used in this study

Primers	Sequence 5′→3′
K-F	GGAATTCGTGTAGGCTGGAGCTGCT
K-R	GTGGTATTCACTCCAGAGCATGGGAATTAGCCATGGTCC
ICB-F	GCTCTGGAGTGAATACCAC
ICB-R	CGGGATCCTACGACTCACTATAGGGAGA
35-55	GCCAAGCTTAACAGTAACCGCCACGAC
35-53	GCCGAATTCATCATCACATAACCATGACATTTTTCTGATATTCCCCGGTAACGCCAGATAACGATGCCAAATTTACCGT
35-35	GCCGGATCCATCTGGCGTTACCGGGGA
35-33	GCCCTCGAGGTGGTGGTGCAGTGCTTT
35-0	GGCGGATTTGATAACACTAC
35-1	GAGAGCTACACATTATTGGA
AD3	GGATTGGCTGAGACGAAAA
AD5-Z	AGCATAATTTAATATGCCTGATGGTGTTGCACCATCAGGCATATTCGCGCCCTGCCGCTGAGCAATAA
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGACC
AD-1	GACGCTAGTTATTGCTCAG
36-0	GCATCGCCCATTCAAACAT
36-1	GTGAACAAGCTGGCACAAAT
37-0	TGTGGGTGATTTGCGATGA
37-1	GACGGGACAGGTTGTAGAT
38-0	CCTGGTGGGATTTGACGC
38-1	GCCAGAGCTGCTTCGTAAT
ZL-L5	GCCAAGCTTCCTCTTCCAGTTCCA CCA
ZL-L3	GCCGAATTCATGGTATCGCCTCGCGAA
ZL-R5	GCCGGATCCGAGCCGATTCATCCCCATA
ZL-R3	GCCCTCGAGAACGATGCCAAATTTACCGT
ZL-0	CCAATCATCACCCTCCTG
ZL-1	CCCATCCGTTGCGTTTGT
35-L5	AACA GTAACCGCCACGAC
35-L3	AGAGACCTCGTGTGCTACAACGATGCCAAATTTACCGT
35-R5	TCTCCCTATAGTGAGTCGTAATCTGGCGTTACCGGGGA
35-R3	GTGGTGGTGCAGTGCTTT
38-L5	TGA TGCGTCTGATGAGCG
38-L3	AGAGACCTCGTGTGCTACACAGGGTCTCCTTACAACAA
38-R5	TCTCCCTATAGTGAGTCGTAAATAGCGTTTGCTGTGTTATTG
38-R3	TTTCAGTGGGCCATCAGC
BIT-5	GTAGCACACGAGGTCTCTTAGGGATAACAGGGTAATTAAGCAGCTCTAATGCGCA
BIT-3	TACGACTCACTATAGGGAGAATTACCCTGTTATCCCTAGCGAATTGGGCCCTCTAG
BIK-5	GTAGCACACGAGGTCTCTTAGGGATAACAGGGTAATGTGTAGGCTGGAGCTGCT
BIK-3	TACGACTCACTATAGGGAGAATTACCCTGTTATCCCTAATGGGAATTAGCCATGGTCC
ZR-R3	GCCAAGCTTCTGTTCGTCGCCCGCTTT

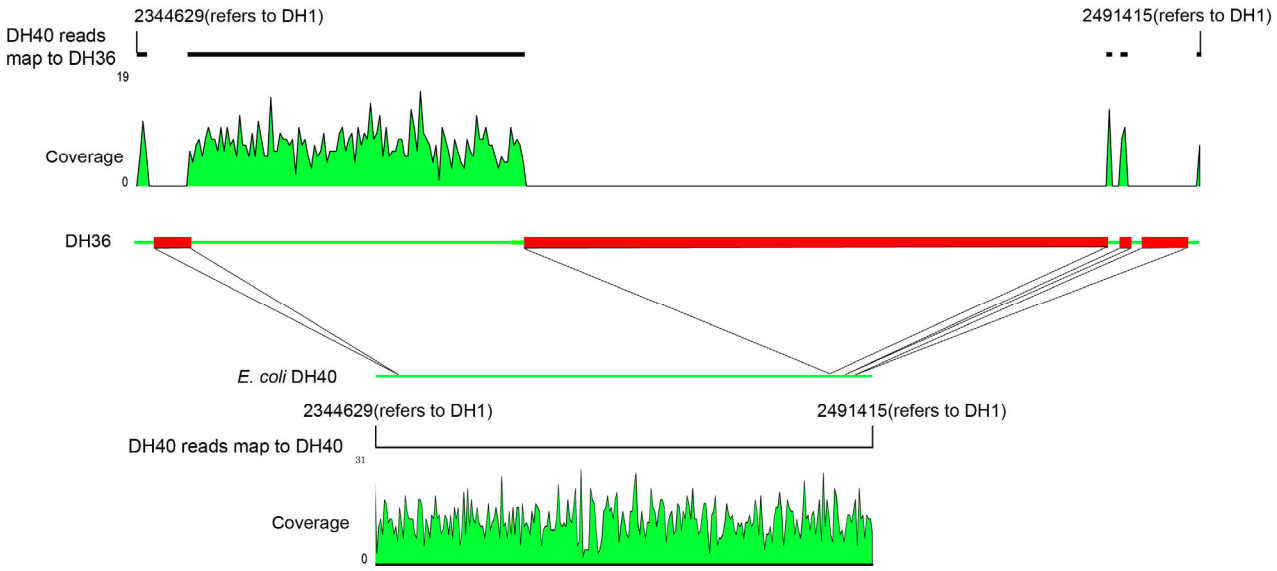

Figure 7 Genome DNA re-sequencing alignment of *E. coli* DH36 and DH40. Sequence 2344629…2491415 refers to *E. coli* DH1; red regions were deleted regions.

*Hin*d III, ZLR with *Bam*H I and *Xho* I, *KCb* with *Eco*R I and *Bam*H I and inserted them into *Hin*d III and *Xho* I sites of vector p15AD2IC-SacB; the resulting plasmid was named p15AD2-ZLLR-KCb-SacB. After double resistance screening (using ampicillin and kanamycin), a single clone of strain *E. coli* DH1-Z harboring p15AD2-ZLLR-KCb-SacB and pAAICDGBE was inoculated into the LB medium with ampicillin and then cultivated at 30°C for 4 h with 0.2% L-arabinose in medium. 10-fold dilutions of the cells were plated onto LB plates containing kanamycin and sucrose and cultured overnight. The resulting strain *E. coli* DH1-Z-ZLRKCb was verified by PCR identification using the primer pair ZL-0 and ZL-1.

(ii) PCR amplification of the linear plasmid ADZ was carried out using p15AD2IC-SacB (after *Bam*H I digestion) as the template and using the primer pair AD5-Z and AD3. As designed, the ends of ADZ had 135 and 50 bp homologous sequences with *cmb* and the chromosome, respectively. The PCR fragment ADZ was electroporated into *E. coli* DH1-Z-ZLKCb harboring pAAGBE, and the recombinant strain was selected for on LB plates containing kanamycin and sucrose. After PCR identification using the primer pairs M13F and 35-1 and 38-0 and AD-1, the positive vector was transferred into *E. coli* DH1, and the extracted plasmids were analyzed and identified by restriction digest and PCR; the resulting plasmid was p15AD2IC-Z.

Transplanting of unit Z to *E. coli* DH36 chromosome

It was difficult to achieve a complete replacement of the target region, for sequence of unit Z in p15AD2IC-Z was highly homologous with the corresponding region on the *E. coli* DH36 chromosome. Two I-*Sce* I restriction sites were inserted on both sides of the replacement region of DH36 chromosome, and the corresponding region of approximately 146.8 kb in length was replaced by unit Z (48.2 kb in length) via a double-strand breakage-stimulated homologous recombination strategy. The schematic diagram of transplanting unit Z is shown in Figure 5. Fragments 35LR-IK *and* 38LR-IT were integrated into region 35 and region 38, respectively, then I-*Sce* I homing endonucleases were used to digest the DNA at the I-*Sce* I restriction sites in the chromosome. As a result, the double-stranded DNA was cleaved and then repaired by homologous recombination between unit Z and over 500 bp of homologous sequence of double strand breaks due to the action of Red recombinant enzyme.

Fragments 35LR-IK and 38LR-IT containing the markers *IS-kan* and *IS-tet* were obtained by overlap extension PCR. PCR amplification of 35L and 35R was performed using the primer pairs 35L-5 and 35L-3 and 35R-5 and 35R-3, and using the DH1 chromosome as the template. PCR amplification of *IS-kan* was performed using the primer pairs BIK-5 and BIK-3 and pKD4 as the template, and then using the primer pairs 35L-5 and 35R-3 and the PCR fragments mentioned before as template; the resulting PCR fragment was 35LR-IK. Using the same method, fragment 38LR-IT was obtained. Both of these fragments were electroporated into *E. coli* DH36 and integrated into region 35 and region 38, and the resulting strain was *E. coli* DH36-35LRIK-38LRIT.

Plasmid p15AD2IC-Z was co-transformed with pAAISGBE into *E. coli* DH36-35LRIK-38LRIT, and then a single clone was inoculated into LB medium containing chloramphenicol and ampicillin and cultured overnight. After that, the cells were cultivated at 30°C for 4 h with 0.2% L-arabinose in the medium. Ten-fold dilutions of the

cells were plated onto LB plates and cultured overnight. *E. coli* strain DH40 was confirmed by screening by resistance loss and PCR identification using the primer pairs ZL-R5 and 35-1, 36-0 and 36-1, 37-0 and 37-1 and 38-0, and ZR-R3.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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