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A Modified PCR-Directed Gene Replacements Method Using λ-Red Recombination Functions in *Escherichia coli*

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Abstract We have developed a modified gene replacement method using PCR products containing short homologous sequences of 40- to 50-nt. The method required λ-Red recombination functions provided under the control of a temperature-sensitive CI857 repressor expressed from the P_{lac} promoter in the presence of IPTG on an easily curable helper plasmid. The method promoted the targeted gene replacements in the *Escherichia coli* chromosome after shifting cultures of the recombinogenic host, which carries the helper plasmid, to 42°C for 15 min. Since this method employs λ-Red recombination functions expressed from the easily curable helper plasmid, multiple rounds of gene replacements in the *E. coli* chromosome would be possible. The procedures described herein are expected to be widely used for metabolic engineering of *E. coli* and other bacteria.

Key words: Gene replacement, λ-Red recombination, *cI857*, helper plasmid, *Escherichia coli*

Driven by the needs of functional genomics and metabolic engineering, a number of new methods to engineer bacterial chromosome by homologous recombination have emerged in recent years. The availability of an efficient chromosomal engineering technology is important in metabolic engineering, because of interest to introduce genetic modifications in order to easily develop new and useful cellular traits [1, 6]. The two main recently established methods are the ET [26] and λ-Red recombination system [2, 3, 11–14, 25]. The former is based on homologous recombination functions mediated by the *Rac* phage-derived protein pairs, the RecE and RecT proteins [26], and the latter utilizes the recombination functions encoded by bacteriophage λ genes *gam*, *bet*, and *exo*, which operate on linear DNA substrates [11]. The ET and λ-Red recombination systems can be employed for both efficient gene replacement and gap repair using short homologous sequences [21, 27]. In a comparative mechanistic study, it has been shown that the ET and λ-Red recombination systems are functionally and operationally equivalent [14]. Successful applications of such systems to perform oligo-directed gene replacements in *E. coli* hosts have been demonstrated with a prophage [25], a low-copy number plasmid [2, 12, 15, 26], or chromosomal substitution [3, 13]. The high efficiency of ET- and λ-Red-promoted recombination with such short homologous sequences has been achieved by regulated expression of the components of an ET or λ-Red protein pair (RecE and RecT or Gam, Bet, and Exo) from an inducible promoter in order to limit the recombinogenic window; that is, the time-span, in which recombination can take place, to a smaller interval for generating gene replacements [2, 11–13, 15, 25, 26].

In this study, we constructed a plasmid that contained λ-Red recombination functions expressed under the control of a temperature-sensitive CI857-repressor. With this helper plasmid, linear DNA substrates carrying 40- to 50-nt homologous sequences and the directly repeated two *loxP* sites flanking a selectable gene could efficiently replace chromosomal genes in *E. coli* K-12 W3110. After gene replacements, the helper plasmid could simply be cured from the host by nonselective growth. Thus, this presents a simple and efficient way to repeatedly generate oligo-directed gene replacements on the *E. coli* chromosome.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Media

Escherichia coli strain DH5α (*F- Φ80lac ZΔM15 [lacZYA-argF] U169 endA1 recA1 hsdR17 [rk⁻mk⁻] deoR thi1 supE44 λ⁻ gyrA96 relA1*) was used for the construction and propagation of plasmids. *E. coli* strain K-12 W3110 was used for gene replacement studies. Plasmid pBluescript II

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KS+ (Stratagene, La Jolla, CA, U.S.A.) was used to construct the plasmids described here. Cells were grown in LB broth or LB agar [19] and supplemented with ampicillin, chloramphenicol, and kanamycin at 100, 15, and 50 μg/mL, respectively, for drug resistant selection.

Recombinant DNA Techniques

Recombinant DNA techniques, including restriction enzyme digests, ligations, and bacterial transformations, were performed as described previously [19]. Standard PCR conditions were used to amplify linear DNA fragments with the *Top-Pfu* DNA polymerase (Solgent, Daejoen, Korea) as described elsewhere [7, 24]. All of the primer sequences used in this work are listed in Table 1. The plasmid containing λ-Red recombination functions was constructed in multiple steps. The bacteriophage λ *cI857* and P_L operon genes were amplified using primer pairs #1-#2, #3-#4, and #5-#6, respectively, and the genomic DNA of the lysogenic strain DY330 [25] (kindly provided by D. L. Court) as the template. The resulting 1,384-, 3,067-, and 2,351-bp products contained the sequence of bacteriophage λ 38,043–36,659, 36,659–33,592, and 33,592–31,241, respectively. The full nucleotide sequence of bacteriophage λ is available on-line (see accession number NC_001416). The 1,384-bp products were digested with *Sac*II and *Eag*I, and subcloned into gel-purified pBluescript II KS+ that had been digested with *Sac*II and *Eag*I. The resulting plasmid was digested with *Eag*I and *Kpn*I, and ligated with 3,067- and 2,351-bp products digested with *Eag*I and *Eco*RV, and *Eco*RV and *Kpn*I, respectively. The resulting plasmid was subsequently digested with *Kpn*I and *Sac*II, and the ends were blunted with T4 DNA polymerase. The 6,802-bp blunt-ended DNA containing the bacteriophage λ *cI857* and P_L operon genes was subcloned into the blunt-ended *Xba*I site of pBluescript II KS+ containing the *TEF2* transcriptional terminator [23] to generate the plasmid pSJ343 (Fig. 1). To construct the plasmid containing the *TEF2* transcriptional terminator, pBluescript II KS+ was digested with *Eag*I, blunted with Klenow fragment, and ligated with blunt-ended *Xba*I-*Xba*I PCR fragments containing the *TEF2* transcriptional terminator. Primer pairs #7-#8 were used to amplify the 401-bp *TEF2* transcriptional terminator region from the plasmid pUG6 [4].

Table 1. PCR primers and sequences. Restriction enzyme sites are underlined and lower case letters refer to the bacterial chromosomal sequences.

Name	**Primer sequence (5 → 3)**	Used for
	Plasmid construction	
#1	AATCCCCGCGGCATACAACCTCCTTAGTACATGC	λ 38043–36659
#2	AATCCCGGCCGATGAAATGCATATGC	λ 38043–36659
#3	AATCCCGGCCGAACAACGGTGAAGGTAGAAGCC	λ 36659–33592
#4	AATCCGATATCGGTAATTCTTATTCCTTCGC	λ 36659–33592
#5	AATCGGATATCGATACATCAGGAATATTTGATTCAG	λ 33592–31241
#6	CGGGGTACCTTTCAGTGGATTTCGGATAACAGAAAGGCC	λ 33592–31241
#7	CTAGCTAGTCTAGAATCTTGCCATCCTATGGAAC	*TEF2* terminator
#8	CTAGCTAGTCTAGATATTAAGGGTTCTCGAGAGC	*TEF2* terminator
	Gene replacements	
#9	atgcgaagctcggctaagcaagaagaactagttaaagcatttaaagcattGAGGTCGACGGTATCGATAA	*argR* 50-nt homologous substrate
#10	ttaaagctcctggtcgaacagctctaaaatcgcttcgtacaggtctttgaTAGGCCTAGGATGCATATGG	*argR* 50-nt homologous substrate
#11	atgcgaagctcggctaagcaagaagaactagttaaagcatGAGGTCGACGGTATCGATA	*argR* 40-nt homologous substrate
#12	ttaaagctcctggtcgaacagctctaaaatcgcttcgtacTAGGCCTAGGA TGCATATGG	*argR* 40-nt homologous substrate
#13	tattgagattgatcccattgggcgaatctacctcaattttgctgaactggCAGCTGAAGCTTCGTACGC	*tyrR* 50-nt homologous substrate
#14	aatatcctgtggacgcagctcataaccgtccagttgtgtcagtgcgcgatATAGGCCACTAGTGGATCTG	*tyrR* 50-nt homologous substrate
#15	tattgagattgatcccattgggcgaatctacctcaattttCAGCTGAAGCTTCGTACGC	*tyrR* 40-nt homologous substrate
#16	ggacgcagctcataaccgtccagttgtgtcagtgcgcgatATAGGCCACTAGTGGATCTG	*tyrR* 40-nt homologous substrate
#17	gctaacaatggcgacatattatggcccaacaatcaccctattcagcagcgCAGCTGAAGCTTCGTACGC	*trpR* 50-nt homologous substrate
#18	tccagccactggcgcagctcgacgggcgcggctttcaggctgttagatccATAGGCCACTAGTGGATCTG	*trpR* 50-nt homologous substrate
#19	gcgacatattatggcccaacaatcaccctattcagcagcgCAGCTGAAGCTTCGTACGC	*trpR* 40-nt homologous substrate
#20	ggcgcagctcgacgggcgcggctttcaggctgttagatccATAGGCCACTAGTGGATCTG	*trpR* 40-nt homologous substrate
	Diagnostic PCR	
#21	CGCGGATCCGCGAACCTGAAGGCAGTTGG	Verification of *argR* gene replacement
#22	CGCGGATCCGCCACACCACTTACGGAT	Verification of *argR* gene replacement
#23	AAGAAACGCTGCAAAAAGGC	Verification of *tyrR* gene replacement
#24	TTACGGTGTGGCAATTGCT	Verification of *tyrR* gene replacement
#25	TGTGAAGAACGTGCTGGCTTA	Verification of *trpR* gene replacement
#26	TATCACCAGGCGGTGATCCT	Verification of *trpR* gene replacement

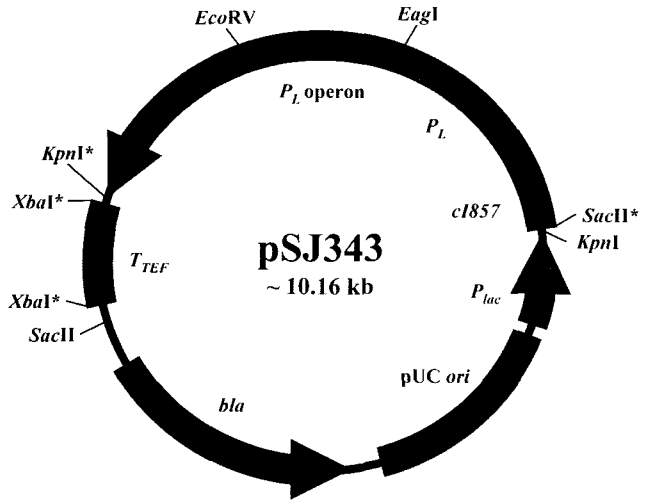

Fig. 1. Physical map of the pSJ343 used for targeted gene replacements with short homologous substrates.
It contains pUC replication origin (pUC *ori*) and an ampicillin resistant gene (*bla*) for propagation and selection, respectively, in *E. coli*. For λ-Red recombination activities, it harbors intact P_L operon genes transcribed under the control of the CI857-repressor. Transcription of *cI857* is driven by the P_{lac} promoter in the presence of IPTG. T_{TEF} indicates transcription terminator sequences. Asterisks represent the blunted restriction sites and only selected restriction sites are shown.

Gene Replacements and Confirmation of Recombinants

E. coli W3110 strains carrying the pSJ343 plasmid were grown overnight in 5-ml LB cultures with ampicillin at 30°C. Overnight cultures were diluted 100-fold in LB medium containing 10 mM IPTG (isopropyl-1-thio-β-D-galactoside) (Biosesang INC., Sungnam-si, Kyonggi-do, Korea) and were grown at 30°C to an OD_{600} of 0.6. Induction of λ-Red recombination functions was performed at 42°C according to the method described by Yu *et al.* [25]. Cells were made electrocompetent by concentrating 100-fold and washing three times with ice-cold 10% glycerol. *E. coli* W3110 strains carrying pKD46 plasmid were also made electrocompetent for gene replacement studies as described by Datsenko and Wanner [2]. The *argR* gene disruption cassette containing short regions of homology was amplified from pLoxCat2 [16] with primer pairs #9–#10 and #11–#12. The *tyrR* gene disruption cassette containing short regions of homology was amplified from pUG6 with primer pairs #13–#14 and #15–#16. The *trpR* gene disruption cassette containing short regions of homology was also amplified from pUG6 with primer pairs #17–#18 and #19–#20. The primers contain two parts: 5'-ends homologous to flanking regions of the chromosomal target gene and 3'-ends priming to sequences at the template DNA for PCR amplification. PCR products were gel-purified by using AccuPrep® (Bioneer, Daejeon, Korea) gel purification kits and suspended in elution buffer (10 mM Tris, pH 8.0). Electroporation was performed using a MicroPulser™ and a 0.2-cm pre-cooled electroporation cuvette according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.) using 100 μl of cells and 100 ng of PCR products. The electroporated cells were immediately added to 1 ml of LB medium, incubated for 1 h at 30°C, and aliquots were then spread onto LB agar to select Cm^R or Km^R transformants. After primary selection, transformants were verified by diagnostic PCR analyses to confirm the altered genomic structure caused by replacement of a gene. *E. coli* genomic DNA was isolated from overnight cultures as described elsewhere [7, 17], and used as templates for the diagnostic PCR analyses. The primer pairs used for the diagnostic PCR analyses were as follows: for the *argR* gene replacement, #21–#22; for the *tyrR* gene replacement, #23–#24; for the *trpR* gene replacement, #25–#26. Control colonies were always tested. PCR reaction mixtures contained 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM each of the primers, 250 μM deoxynucleoside triphosphates (dNTPs), 2.5 U of Taq DNA polymerase (Bioneer Co., Daedeok-gu, Daejeon, Korea), and 100 ng of genomic DNA in a volume of 50 μl. The samples were placed in PTC-100™ Programmable Thermal Controller (MJ Research, Waltham, MA, U.S.A.), and the thermal cycling program consisted of an initial step at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, and 5 min at 72°C. A final extension step of 7 min at 72°C was included. Samples from PCR reactions were run on 1% agarose gel along with a set of standards (1-kb DNA ladder; NEB, Beverly, MA, U.S.A.) and stained with ethidium bromide.

RESULTS AND DISCUSSION

Principle of the Method

The aim of this work was to improve the great advantages of the λ-Red recombination system for chromosomal engineering in *E. coli*. Previously, systems for gene replacements in the chromosome of *E. coli*, based on λ-Red recombination functions (Gam, Bet, and Exo), were reported [2, 25]. The method utilized a helper plasmid (pKD46) bearing the λ-Red recombination functions expressed under the control of the arabinose-inducible P_{araB} promoter [2]. We have noted that the helper plasmid (pKD46) efficiently promotes gene replacements with substrates containing long regions of homology to the target genes in *E. coli* W3110; however, oligo-directed gene replacements with the plasmid are not well suited for gene replacements in *E. coli* W3110 (Table 2), despite the reported success of the helper plasmid system. It is assumed that, since the arabinose-inducible P_{araB} promoter exhibits autocatalytic behavior [22], the intended use in fine modulation of gene expression from the P_{araB} promoter to limit recombinogenic window may be precluded. Furthermore, since expression levels of P_{araB} promoter can be varied with the carbon source used and genetic backgrounds of host strains [5, 10], the fine modulation of

Table 2. Oligo-directed gene replacements with short homologous substrates in *E. coli* W3110.

Targeted chromosomal gene	Helper plasmid	Length of homologous sequences	Km^R or Cm^R transformants[a]	Percent transformants harboring correct gene replacement[b]
argR	pSJ343	50-nt	>1,000	100 (20/20)
		40-nt	>1,000	100 (20/20)
tyrR	pSJ343	50-nt	18	33 (6/18)
		40-nt	23	35 (8/23)
	pKD46	50-nt	49	0 (0/20)
		40-nt	NT	NT
trpR	pSJ343	50-nt	104	70 (16/23)
		40-nt	78	70 (14/20)
	pKD46	50-nt	18	11 (2/18)
		40-nt	NT	NT

[a]Total transformants per electroporation are shown from one of three separate experiments.
[b]Number of positives as expected for correct gene replacement per number of transformants tested. The percentage of correct gene replacement for all cases was similar in each separate experiment.
NT, not tested.

gene expression from the P_{araB} promoter can be limited under certain conditions. The use of such λ-Red recombination system has also been demonstrated with either a modified prophage [25], *ΔrecBCD::*P_{tac}*-bet exo kan* [3, 11], or with a *ΔrecBCD::*P_{tac}*-gam bet exo* [12, 13] chromosomal substitution. However, the λ-Red recombination functions substituted in the chromosome would limit the metabolic engineering strategies; that is, the chromosomal region that harbors a modified λ prophage can be an important target to be engineered under certain conditions or loss of RecBCD function may interfere with the normal DNA repair function in *E. coli*. Thus, additional efforts would be necessary to overcome these problems.

To this end, the plasmid pSJ343 (Fig. 1) was constructed by inserting bacteriophage λ sequences between 38043 and 31241 to include the P_L operon, *cI857*, and *TEF2* transcription terminator sequences from the filamentous fungus *Ashbya gossypii* [23] into pBluescript II KS+, as described in Materials and Methods. In this helper plasmid pSJ343, the expression of P_L operon is controlled by the temperature-sensitive CI857-repressor. The *cI857* gene was fused to the P_{lac} promoter region present in pBluescript II KS+ such that it can efficiently be expressed in the presence of IPTG from the P_{lac} promoter, unless the CI857-repressor was inactivated at 42°C.

PCR-Directed Gene Replacements Using the Helper Plasmid

For the experiments to evaluate the efficiency of the helper plasmid, pSJ343, in PCR-directed gene replacements, the pUG6 or pLoxCat2 plasmid was used as a template for generating short homologous substrates. The *argR*, *tyrR*, and *trpR* genes were chosen for targeted gene replacements. PCR products were generated using 50-nt homologous sequences from the 5'- and 3'-end of the *argR* coding sequences and 20-nt priming sequences for pLoxCat2 containing Cm^R gene flanked by two *loxP* sites as direct repeats (Fig. 2). The 1.2-kbp PCR products were gel-purified and then electroporated into the *E. coli* W3110 strain carrying the helper plasmid pSJ343 that had been induced for P_L operon expression by growth at 42°C for 15 min, as described in Materials and Methods. The results are shown in Table 2. After electroporation, Cm^R transformants were selected at 30°C, and hundreds of transformants were routinely obtained. A diagnostic PCR using gene-specific primers outside the regions of the linear DNA substrates verified that all of the selected number of transformants had the predicted structures of *argR* gene replacements. Gene replacements with short homologous substrates, which included 50-nt homologous sequences from the 5'- and 3'-end of the *tyrR* or *trpR* coding sequences and 20-nt priming sequences for pUG6 containing the Km^R gene flanked by two *loxP* sites as direct repeats, were also performed in three separate experiments as described above. However, fewer Km^R transformants than that seen with the *argR*-homologous substrates was observed. From one of these experiments, only six out of eighteen (33%) or sixteen out of twenty three (70%) Km^R transformants selected were positive as expected for *tyrR* or *trpR* gene replacements, respectively, when verified by the diagnostic PCR. The number of transformants was varied, but percentage of correct gene replacements for *tyrR* or *trpR* was similar in each separate experiment, suggesting that the transformation frequency was not affected by the frequency of correct gene replacement. In the separate experiments, PCR products, containing 40-nt homologous sequences and 20-nt priming sequences for pLoxCat2 (in the case of *argR*) or pUG6 (in the case of *trpR* and *tyrR*), worked in a manner similar to that described above using 50-nt homologous substrates. The sequences used in 40-nt homologous substrates were all contained in the 50-nt homologous substrates of all three genes. It should be noted, however, that the frequency of

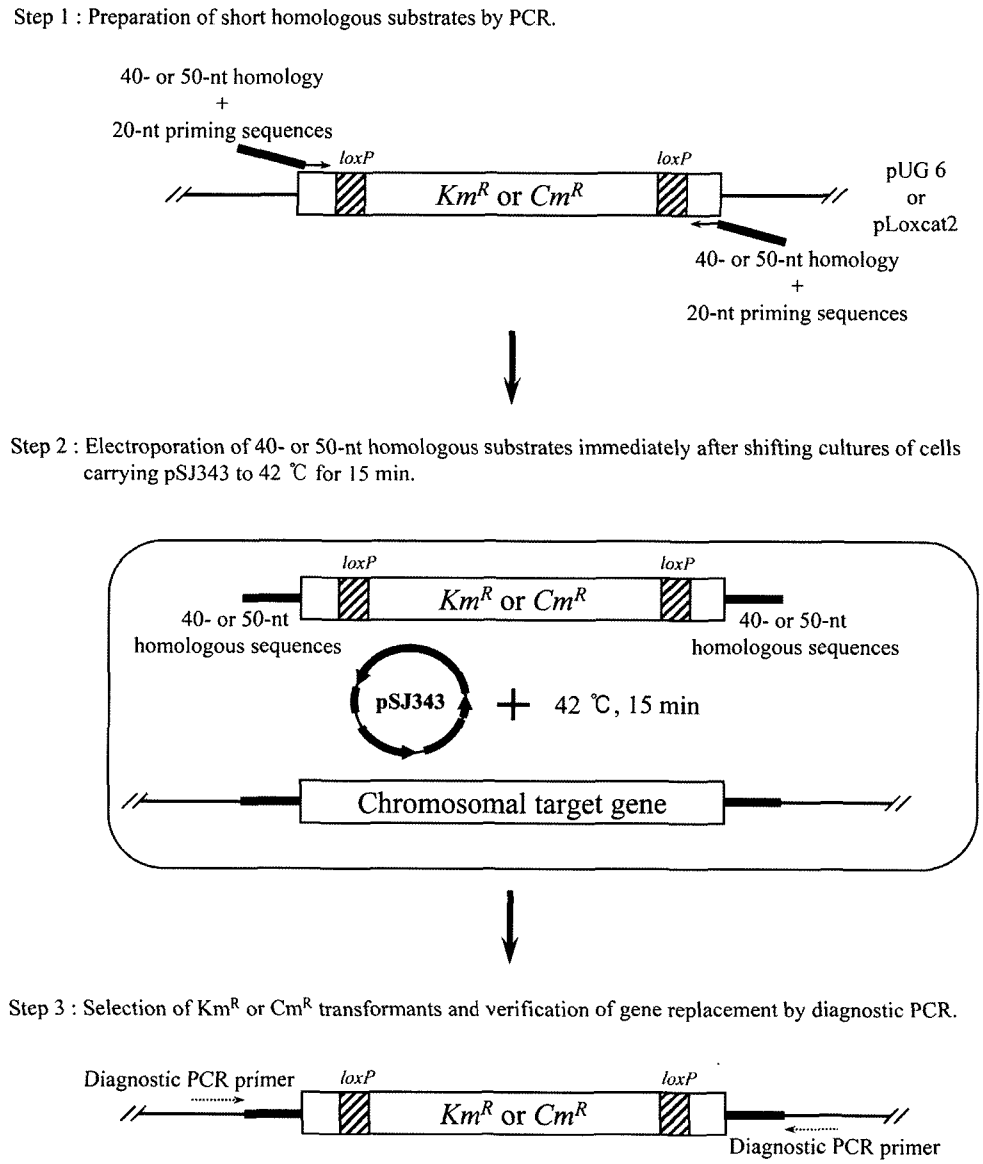

Fig. 2. A strategy employed in gene replacements with short homologous substrates.
Forty- or 50-nt homologous sequences (black bars) refer to the homologous sequences to the chromosomal target gene. Twenty-nt priming sequences (arrows) refer to the priming sequences for pUG6 or pLoxCat2 as template DNA. The direct repeats of two *loxP* sites (shaded box) are flanking the kanamycin resistance gene (*Km*^{R}) in pUG6 or chloramphenicol resistance gene (*Cm*^{R}) in pLoxCat2. Diagnostic PCR analyses were performed using gene-specific primers (dotted arrows) to confirm the correct gene replacements.

gene replacements for the *tyrR* or *trpR* gene appeared to be somewhat lower than that of the *argR* gene, when either 50- or 40-nt homologous substrates were used for gene replacements. The reason for the lower frequency of *tyrR* or *trpR* gene replacement is not clear, but the lower frequency of gene replacement with the same DNA substrates at the same loci was also observed in *E. coli* DY330 containing *cI857* and P_L operon genes in the chromosome (data not shown). Taken together, all three genes were successfully replaced, although there was variability in the frequency of gene replacements depending on the target gene.

To repeatedly use the *Km*^{R} or *Cm*^{R} gene for several gene replacements in one strain, it is necessary to eliminate the selection marker gene from the replaced chromosomal gene. To do this, the *argR*-mutant strain, in which the helper plasmid pSJ343 still remained, was grown nonselectively in LB medium. Nonselective growth of this strain for 24 h in LB medium at 30°C was sufficient to cure the helper plasmid in approximately 90% of the cell, as detected by plating cells on LB and replica-plating the colonies onto LB plus ampicillin. The absence of plasmids in ampicillin-sensitive cells was also confirmed by failure to isolate

plasmid DNA from some of these colonies by the method of plasmid rescue. The resulting *argR*-mutant strain could then be subsequently transformed with the *cre* expression plasmid such as pJW168 [16] to eliminate the chromosomally integrated Cm^R gene flanked by two *loxP* sites as direct repeats.

We have demonstrated a modified method of gene replacement between linear DNA substrates with 40- to 50-nt homologous sequences and the chromosomal target genes. This method depends on λ-Red recombination functions repressed at 30°C in the presence of IPTG and easily derepressed at 42°C. These recombination functions are provided from the helper plasmid bearing the pUC replication origin, but easily curable from the host by nonselective growth [9]. Several researchers reported that a λ Red-expressing multicopy plasmid efficiently promoted gene replacement with long homologous substrates to the target gene, but not with 40- to 60-nt homologous substrates [2, 11, 13]. Others also noted that expression of λ-Red recombination function from the chromosome or low-copy plasmid was more efficient for recombination activity [2, 11]. It has been suspected that λ-Red recombination functions expressed on multicopy plasmid induce the rolling circle mode of replication [18] that may interfere with λ-Red-promoted gene replacements with short homologous substrates. Thus, it has been assumed that λ-Red-promoted gene replacements with short homologous substrates require higher level expression of the λ-Red recombination functions relative to long homologous substrates [12]. It has also been noticed that, when the P_L operon is present on a multicopy plasmid to express λ-Red recombination functions, CI repression of P_L operon depends on high concentrations of repressor, thus making it more difficult to control P_L operon expression [8].

In our preliminary studies, a plasmid construct having bacteriophage λ *cI857*, P_L operon genes, and *TEF2* transcription terminator sequences inserted in the inverted orientation relative to that of pSJ343 was tested for recombination activity of these P_L operon-encoded proteins using 40- to 50-nt homologous substrates, but was unsuccessful; that is, the cells containing this plasmid construct failed to grow at 30°C when the culture was diluted 100-fold in LB medium following overnight growth at 30°C. It was considered that the expression level of the *cI857* gene was not sufficient to repress the P_L-operon expression in this construct to prevent cell killing activity of functions present in the P_L operon, since higher level expression of the P_L operon can cause cell death [20]. Thus, the orientation of the *cI857* gene in the helper plasmid, pSJ343, relative to the P_{lac} promoter appeared to be particularly important for the CI857 repressor to maintain repression of multiple copies of the P_L promoter, and the induced expression level of the *cI857* gene transcribed from the P_{lac} promoter in the presence of IPTG seemed to be necessary to prevent cell killing activity of functions present in the P_L operon.

Although others have reported that λ-Red recombination functions expressed from a multicopy plasmid are not suited for λ-Red-promoted gene replacements with short homologous substrates, other factors may exacerbate or moderate this effect. For example, the ability of λ-Red recombination functions to promote gene replacements with short homologous substrates might critically be dependent on the expression level of λ-Red recombination functions in a host. The expression level of λ-Red recombination functions from pSJ343 may be modulated by the presence of other P_L operon-encoded proteins that influence the expression of λ-Red recombination functions. The results presented here show that the helper plasmid pSJ343 can serve as a vehicle for providing λ-Red recombination functions to generate λ-Red-promoted gene replacements with short homologous substrates. We are currently attempting to adapt the helper plasmid expressing λ-Red recombination functions to other bacterial species, including *Corynebacterium glutamicum*.

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