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Red recombinase assisted gene replacement in *Klebsiella pneumoniae*

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Abstract The Red recombinase system, the most convenient genetic tool applied in *Escherichia coli* and other bacteria, was introduced for gene replacement in *Klebsiella pneumoniae*. The novel *K. pneumoniae* gene replacement system comprised the Red and FLP recombinases expression vector pDK6-red and pDK6-flp, and linear DNA fragments which encompassed a selective marker gene with target gene flanking extensions; the latter were PCR amplified using a plasmid DNA template obtained by in vivo recombination in *E. coli*. In this study, *dhak1* gene, encoding a subunit of dihydroxyacetone kinase II, was deleted markerlessly at a transformation ratio of 260 CFU/μg DNA, i.e., 1,000-fold higher than that achieved in the native way. Our studies provide an efficient method with detailed protocol to perform gene replacement in *K. pneumoniae* and has great potential to be developed as a routine genetic approach for this important industrial microorganism.

Keywords *Klebsiella pneumoniae* · Gene replacement · Red recombinase

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Introduction

Klebsiella pneumoniae, a model species of the *Klebsiella* genus in the *Enterobacteriaceae* family, is ubiquitous in the natural environment and on mucosal surfaces of mammals [20]. It has been intensively studied because of its opportunistic pathogenesis. *K. pneumoniae* is also an important industrial microorganism in the production of many useful chemicals, such as 1,3-propanediol and 2,3-butanediol [1, 13].

Gene manipulation of the chromosome is an important experimental method for exploiting gene function and metabolic engineering investigations. Lots of work on gene replacement in *K. pneumoniae* has been done through various approaches, among which suicide vector conjugation is mostly used; this method was developed in *Klebsiella oxytoca* M5a1 for investigation of nitrogenase mutants [28]. This method was successfully applied in *K. pneumoniae* for deletion of D-lactate dehydrogenase gene and capsular polysaccharides synthesis operon [9, 25]. With a similar suicide vector system, glycerol dehydrogenase and dihydroxyacetone kinase encoding genes were inactivated in *K. pneumoniae* [12]. Another way to perform gene replacement in *K. pneumoniae* is linear DNA homologous recombination. In *K. pneumoniae* YMU2, aldehyde dehydrogenase gene was mutated after chemical transformation of a linear DNA with homologous extensions [27]. After electroporation of linear plasmid with homologous extensions to *K. pneumoniae* Cu, glycerol dehydrogenase and dihydroxyacetone kinase encoding genes and their regulatory gene *dhaR* were inactivated [21]. Since all those gene replacements occurred via the host cells' native homologous recombination system, the efficiency was low and long homologous extensions were required.

The Red recombinase system derived from bacteriophage Lambda is an efficient gene recombination tool and widely used in *Escherichia coli* strain modification. The Red recombinase system consists of three genes, *exo*, *bet*, and *gam*. Exo is a 5′–3′ exonuclease that act on double-stranded DNA, Bet is a single-stranded DNA binding protein capable of annealing complementary ssDNA strands, and Gam inhibits host RecBCD exonuclease and prevents RecBCD-promoted digestion of Lambda phage DNA [19]. Red recombinase enhances DNA recombination efficiency when acting on linear DNA fragments. The linear DNA can be prepared directly by PCR using primers with homology extensions of 36 nt (at least). The antibiotic resistance gene has often served as a selectable marker; when flanking the FRT sequence, the resistance gene can be eliminated by a helper plasmid expressing the FLP recombinase, which promotes the recombination between the directly repeated FRT sites [4]. The antibiotic resistance gene can also be eliminated by a second round of recombination with an unmarked linear DNA; this operation was easily done with the help of a counter-selectable marker *sacB*, which suppressed the cell's growth in medium supplemented with sucrose [18]. The Red recombinase system has been since modified as recombination tools suitable for other microbia, including various bacteria, fungi, and bacteriophages [22], such as *Streptomyces coelicolor* [10], *Salmonella enterica* [6], *Yersinia pseudotuberculosis* [5], *Pantoea ananatis* [15], *Pseudomonas aeruginosa* [17], *Burkholderia cepacia* [14], *Vibrio cholerae* [26], and *Aspergillus nidulans* [2]. But the Red recombinase system has not been successfully used in *K. pneumoniae* directly [23]. In this study we constructed a Red recombinase-based plasmid system and developed a new strategy for *K. pneumoniae* chromosome gene replacement.

Materials and methods

Strains, plasmids, medium, and primers

Bacteria strains and plasmids used in this paper are listed in Table 1. *K. pneumoniae* and *E. coli* were grown in LB medium at 37 °C. The antibiotics used in the selective medium were ampicillin (50 μg ml^{-1}), kanamycin (100 μg ml^{-1}), apramycin (50 μg ml^{-1}), or chloromycetin (25 μg ml^{-1}). Primers used for PCR in this study are listed in Table 2.

Construction of Red recombinase vector pDK6-red

The Red recombinase gene was amplified from plasmid pIJ790 with the primer pair red-s and red-a (Table 2), and the DNA fragment was inserted into pMD18-T simple with the TA cloning kit to produce pMD18-T-red. Both pMD18-T-red and pDK6 were treated with restriction enzyme *Kpn*

Table 1 Strains and plasmids

Strain or plasmid	Relevant genotype and description	Reference or source
Strains		
K. pneumoniae CGMCC 1.6366 (TUAC01)	Wild type, Amp^r	[11]
K. pneumoniae/red	*K. pneumoniae* CGMCC 1.6366, pDK6-red	This work
K. pneumoniae Δdhak1/red	*K. pneumoniae* CGMCC 1.6366, Δdhak1, *aac(3)IV*, pDK6-red	This work
K. pneumoniae Δdhak1	*K. pneumoniae* CGMCC 1.6366, Δdhak1, *aac(3)IV*	This work
K. pneumoniae Δdhak1F/flp	*K. pneumoniae* CGMCC 1.6366, Δdhak1, pDK6-flp	This work
K. pneumoniae Δdhak1F	*K. pneumoniae* CGMCC 1.6366, Δdhak1	This work
Plasmids		
pCP20	Amp^r, Cm^r, carries the yeast Flp recombinase gene	[3]
pDK6	Kan^r, $lacI^Q$, *tac*, 5.1 kb	[16]
pDK6-flp	Kan^r, carries the yeast FLP recombinase gene, 6.3 kb	This work
pDK6-red	Kan^r, carries λ-Red genes (*gam*, *bet*, *exo*), 7.1 kb	This work
pIJ773	$Apra^r$, *aac(3)IV* with FRT sites, 4,334 bp	[10]
pIJ790	Cm^r, encodes λ-Red genes (*gam*, *bet*, *exo*), 6,084 bp	[10]
pMD18-T simple	Amp^r, TA cloning vector, 2,692 bp	Takara
pMD18-T	Amp^r, TA cloning vector, 2,692 bp	Takara
pMD18-T-2028	Amp^r, carries *dhak1*, *dhak2*, and part of *dhak3*	This work
pMD18-T-2028/a	Amp^r, carries part of *dhak1*, *aac(3)IV*, *dhak2*, and part of *dhak3*	This work
pMD18-T-flp	Amp^r, carries the yeast Flp recombinase gene	This work
pMD18-T-red	Amp^r, carries λ-Red genes (*gam*, *bet*, *exo*), 4,649 bp	This work

Table 2 Oligonucleotides used for PCR

Primer name	Sequence (5′–3′)
red-s	GGTACCTACTGTTTCTCCATACCCGTT
red-a	AAGCTTGCGTCATCGCCATTGCTC
flp-s	ATGCCACAATTTGGTATATTATG
flp-a	TTATATGCGTCTATTTATGTAGGATG
dhak-s	AAGCTTCCGCTCAGCCAGAATCATCAT
dhak-a	TCTAGATTCGATCGCTTCCATGACCTT
FRT-s1	CCGCGGCGTCGCCAATACCGTACTGATCGA AAAACTGGTATTCCGGGGATCCGTCGACC
FRT-a1	GACGATTTGCGTTCTGTTCAGTGACATGTC GTGCTCCTTATGTAGGCTGGAGCTGCTTC
dhak1-s1	GCGGCATAAAAGTCACCA
dhak1-a1	CTTTATCGAGGCCGGTGA
dhak1-s2	GCGATGCAGATTGACGGC
dhak1-a2	ATCGGCAATCGACGGCAG
dhak1-s3	CCGATGCACTGCGGCTAT
dhak1-a3	GTGGGCCTGAGTGACCTG
dhak1-s4	CGCAGCTGACCCTGCACC
dhak1-a4	CCTGGCTCCGCTTTACCG
Test773	GCAAATACGGCATCAGTTACC

I and *Hind* III, and the recombinase fragment was ligated with linear pDK6 vector DNA to generate plasmid pDK6-red.

Construction of FLP recombinase vector pDK6-flp

The plasmid pDK6-flp was constructed similarly to pDK6-red. FLP recombinase gene was amplified from plasmid pCP20 with the primer pair flp-s and flp-a (Table 2). The PCR product was linked to pMD18-T to generate pMD18-T-flp. The pMD18-T-flp was digested with *Bam* HI and *Pst* I to produce the *flp*-containing fragment, which was then ligated using the same enzymes with pDK6 to generate pDK6-flp.

Construction of homologous extension DNA fragments

The gene *dhak1*, which encodes a subunit of dihydroxyacetone kinase II, was selected as the target gene in the chromosome. *dhak1* and the nearby *dhak2* and *dhak3* were amplified with chromosome DNA of *K. pneumoniae* CGMCC 1.6366 as the template, using the primer pair dhak-s and dhak-a. The PCR product was TA cloned into vector pMD18-T simple, generating plasmid pMD18-T-2028. *E. coli* DH5α containing plasmid pIJ790 was transformed with pMD18-T-2028. A disruption cassette with short homologous extensions and apramycin resistance gene *aac(3)IV* was amplified from plasmid pIJ773 using the primer pair FRT-s1 and FTR-a1. The disruption cassette was electroporated into 10 mM arabinose-induced *E. coli* DH5α competent cells harboring pMD18-T-2028 and pIJ790. Gene replacement between the linear DNA and pMD18-T-2028 generates plasmid pMD18-T-2028/a.

Plasmid pMD18T-2028/a was used as the template for amplification of DNA fragments containing different sizes of homologous extensions using primer pairs of FRT-s1 a1, dhak1-s1 a1, dhak1-s2 a2, dhak1-s3 a3, dhak1-s4 a4, and dhak-s a; the products were used as disruption cassettes for gene homologous recombination in *K. pneumoniae* (Fig. 1).

Red facilitated homologous recombination in *K. pneumoniae*

Transformation of K. pneumoniae with pDK6-red

The electrocompetent cells of *K. pneumoniae* CGMCC 1.6366 were prepared from culture grown in LB at 37 °C; after the cell density reached an OD_{600} of 0.7, the culture was immediately move onto ice for 30 min. After washing with cold water twice, the cells were resuspended with sterile water to a final OD_{600} of 30. Electrocompetent cells of *K. pneumoniae* CGMCC 1.6366 were transformed with purified plasmid pDK6-red using a Bio-Rad MicroPulser and 2-mm electroporation cuvette. The instrument was set to 2.0 kV, 200 Ω, and 25 μF; transformants of *K. pneumoniae*/red were screened from the LB plate supplemented with kanamycin at 37 °C.

Gene homologous recombination in K. pneumoniae/red transformant

Electrocompetent cells of *K. pneumoniae* transformant were prepared in a similar way to *K. pneumoniae* CGMCC 1.6366, but isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to the medium for Red recombinase expression. DNA fragments were electrotransformed into *K. pneumonia* as described above, and the recombined strain *K. pneumoniae* Δdhak1/red was screened from the LB plate at 37 °C, supplemented with apramycin (Fig. 1).

Elimination of antibiotic resistance genes

Elimination of plasmid pDK6-red from K. pneumoniae Δdhak1/red

Overnight culture of *K. pneumoniae* Δdhak1/red at 42 °C was diluted and plated onto solid LB to form single colonies which were screened for loss of resistance to kanamycin; colonies not growing on the plate supplemented with kanamycin were isolated and named *K. pneumoniae* Δdhak1.

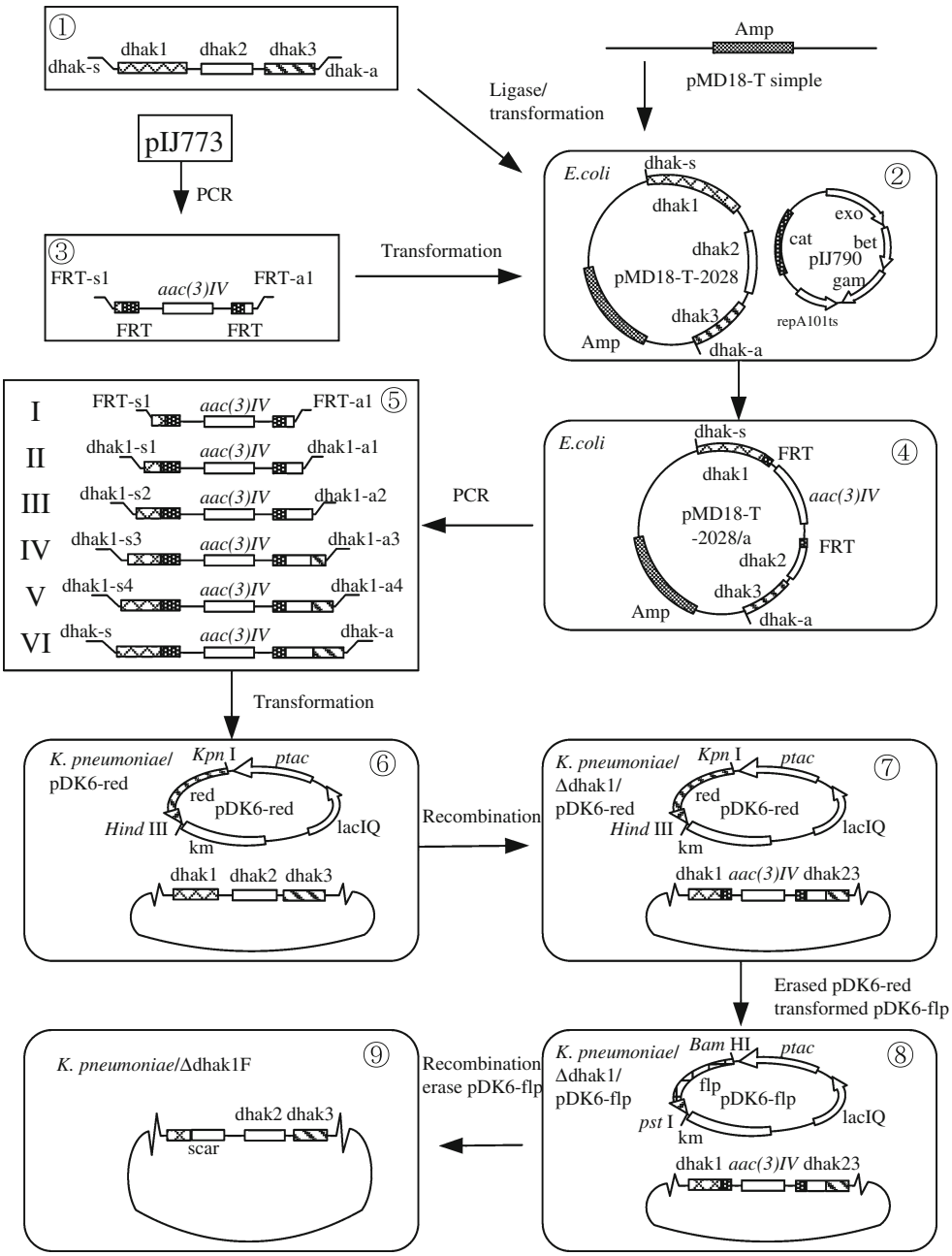

Fig. 1 Construction of homologous extension DNA fragments. *dhak123* was amplified from chromosome DNA of *K. pneumoniae* CGMCC 1.6366, and the PCR product was TA cloned into pMD18-T simple to form pMD18-T-2028. *E. coli* DH5α was transformed with pMD18-T-2028. A gene replacement was done on plasmid with the help of Red recombinase expressed by plasmid pIJ790 to generate pMD18-T-2028/a. The disruption cassette used was PCR amplified form pIJ773. Linear DNA fragments used for *K. pneumoniae* gene replacement were PCR amplified with pMD18-T-2028/a as template

FRT site recombination mediated by FLP recombinase

K. pneumoniae Δdhak1 was transformed with pDK6-flp after pDK6-red was lost through the same way described above to generate *K. pneumoniae* Δdhak1/flp. *K. pneumoniae* Δdhak1/flp was cultured overnight in liquid LB supplemented with IPTG. The culture was diluted and plated for single colony formation, and colonies sensitive to apramycin were isolated and designated *K. pneumoniae* Δdhak1F/flp. The FLP recombination ratio was obtained by the strains sensitive to apramycin divided by the total colonies. Elimination of plasmid pDK6-flp in *K. pneumoniae* Δdhak1F/flp gave the final markerless strain *K. pneumoniae* Δdhak1F; the experiments followed the same methods described previously.

Results and discussion

Construction of pDK6-red and pDK6-flp

pIJ790 and pCP20, a Red recombinase system successfully employed in *E. coli*, were tested in *K. pneumoniae*. Transformation of *K. pneumoniae* CGMCC 1.6366 with

both plasmids afforded antibiotic-resistant transformants; however, no recombinase activity was detected in the cells (data not shown). It has been reported that pIJ790 and pCP20 might be not able to replicate in *K. pneumoniae* [23]; therefore, a novel *K. pneumoniae* replicable vector system expressing Red/FLP recombinases has to be constructed. Therefore, pDK6, a stable multi-copy plasmid both in *E. coli* and in *K. pneumoniae* were chosen to produce recombinase expression plasmids pDK6-red and pDK6-flp, which were constructed as described in the "Materials and methods".

Construction of pMD18-T-2028/a via Red recombination in *E. coli*

In order to prepare linear DNA with different sizes of homologous extension, pMD18-T-2028/a, with a selective *aac(3)IV* gene flanking the full length of dhak2 and a truncated dhak1 (Fig. 1) was constructed with Red recombination between pMD18-T-2028 and *aac(3)IV* cassette. Linear DNA with 39 and 40 nt homologous extensions at both ends of apramycin resistance gene *aac(3)IV* was amplified from plasmid pIJ773. *E. coli* DH5α harboring plasmids pIJ790 and pMD18-T-2028 were transformed with 200 ng DNA fragments. In total about 2,500 colonies were obtained on selective LB plates, and the recombination efficiency was higher than 10^4 CFU/μg DNA. pMD18-T-2028 was constructed as described in the "Materials and methods" section.

Usually with a homologous extension of 40 nt at both ends, the Red recombination efficiency with *E. coli* chromosome DNA is about 500 CFU/μg DNA. The fact that the recombination efficiency (10^4 CFU/μg DNA) achieved on plasmid DNA was much higher might be caused by the high copy number of the plasmid pMD18-T-2028 in *E. coli* cells. However, the disadvantage of recombination on plasmids is that the plasmids extracted from *E. coli* transformants resistant to apramycin were a DNA mixture of pMD18T-2028 and pMD18T-2028/a (data not shown).

pMD18-T-2028/a was applied as the template for PCR preparation of linear DNA with flanking homologous regions, which conferred the Red-mediated recombination in *K. pneumoniae* via two-step technology that was previously adopted in *Streptomyces coelicolor* [10] and *Aspergillus nidulans* [2].

Red recombinase inducible expression in *K. pneumoniae*

The Red recombinase gene cloned in pDK6-red, a multi-copy plasmid in *K. pneumoniae*, was supposedly tightly regulated by the $lacI^Q$ allele of the *lac* repressor; therefore, the level of Red recombinase in the cell should correlate with the concentration of the inducer IPTG used in the medium. The correlation between the dosage of IPTG and the recombination efficiency was tested by a *dhak1* recombination experiment with a linear DNA fragment that has 478- and 663-nt homologous extensions at different concentrations of IPTG (Table 3).

As shown in Table 3, in the absence of plasmid pDK6-red, the native recombination system had a recombination ratio of 0.254, which was much lower than that of Red-mediated homologous recombination. Even without addition of IPTG, the recombination ratio in the presence of pDK6-red was still 20 times more than the native recombination ratio, which indicates the leaky expression of Red recombinase in *K. pneumoniae*. The maximal recombination ratio was obtained at 0.8 mM of IPTG (266.8 CFU/μg DNA). Interestingly, higher concentrations of IPTG reduced the recombination ratio significantly; this was probably caused by the toxic effect due to overexpression of Red recombinase in the cells, which is consistent with the report that the Red recombinase expressed in *Pantoea ananatis* was toxic to the cell [15]. In *E. coli* low copy number plasmid was used to express the Red recombinase system [10], and the recombination ratio was about 500 CFU/μg DNA, which means a low expression level of Red should be enough for efficient recombination.

Length of homologous extensions

To test the effect of the length of homologous extension on the recombination ratio, DNA fragments for *dhak1* recombination were amplified by PCR using pMD18T-2028/a as the template, and the lengths of homologous extensions were chosen from 39 to 663 nt. *K. pneumoniae*/red was transformed with linear DNA fragments, and the recombination ratio was calculated from the number of transformants on plates supplemented with apramycin (Table 4). All transformants were confirmed by PCR using the gene-specific primer pair dhak-s and Test773; Test773 was designed from the apramycin resistance gene *aac(3)IV*.

Table 3 Recombination ratio of different IPTG concentrations in competent cells

Strain	*K. pneumoniae*	*K. pneumoniae*/red				
IPTG concentration (mM)	0	0	0.2	0.4	0.6	0.8	1.0
Recombination ratio (CFU/μg)	0.254	6.61	62.04	94.08	174.76	266.80	11.57

Table 4 Recombination ratios of different length homologous extensions

	Primers used					
	FRT-s1 FRT-a1	dhak1-s1 dhak1-a1	dhak1-s2 dhak1-a2	dhak1-s3 dhak1-a3	dhak1-s4 dhak1-a4	dhak-s dhak-a
Length of homologous extensions (nt)	39	112	212	308	400	478
	40	116	196	316	409	663
Recombination ratio (CFU/μg)	0	0	3.46	13.21	25.94	266.80

As seen in Table 4, the recombination ratio correlated closely with the length of homologous extensions. The 478- and 663-nt homologous extension DNA fragment gave the highest recombination ratio of 267 CFU/μg DNA. The recombination ratios dropped sharply when shorter DNA fragments were applied, and no transformant was obtained when using DNA fragments with homologous extension of 116 nt or less (Table 4).

It is possible to use the Red recombinase system in *E. coli* with very short homologous extensions of 36–50 nt, and such short homologous extensions can be directly synthesized on primers. As mentioned previously, the recombination ratio achieved in *E. coil*, using 39- and 40-nt homologous extensions, was about 500 CFU/μg DNA, whereas the recombination ratio achieved in *K. pneumoniae* with a long homologous extension DNA fragment (478 and 663 nt) was slightly lower (267 CFU/μg DNA). It is therefore harder to achieve a high recombination ratio in *K. pneumoniae* than in *E. coil* when using the same recombinase system. The homologous extensions on linear DNA used in *Yersinia pseudotuberculosis* and *Burkholderia cepacia* for Red recombination were also about 500 nt [5, 14]. In *Pseudomonas aeruginosa* the minimal length of homology required for Red the recombination system was 100 nt [17]. In *Vibrio cholerae*, genes had been knocked out by Red recombination using 50- and 100-nt homologous extensions DNA; however, longer extensions of 1,000 nt increased the recombination ratio greatly [26]. Even in *E. coli* K-12 derivative strain DH5α, 280-bp homologous regions was suggested for optimal phage gene recombination [22]. Those studies indicated that Red recombinase exhibits different efficiency in different microorganisms.

Although the *recBCD* sequence of *K. pneumoniae* CGMCC 1.6366 was not known, the genome sequence of a similar subspecies MGH 78578 has been published. Comparison of the DNA sequence of *recBCD* in *K. pneumoniae* MGH 78578 and *E. coil* K12 DH10B revealed that the identity values of *recB*, *recC*, and *recD* between them were 72, 75, and 69 %, respectively [7]; however, *recBCD* was very conservative in the *K. pneumoniae* species, as seen in the genome sequence of *K. pneumoniae* 342 and *K. pneumoniae* NTUH-K2044 [8, 24]. Therefore, the reduced recombination ratio in *K. pneumoniae* may reflect the lower inhibition efficiency of Gam on *K. pneumoniae* RecBCD exonuclease than on its *E. coli* homologue.

Antibiotic resistance gene deletion by FLP recombinase

The plasmid pDK6-red was eliminated from *K. pneumoniae* mutant strain Δdhak1 as described in the "Materials and methods" section. Although pDK6 replicates in *K. pneumoniae* as a multi-copy plasmid, host cells presented a low plasmid-losing rate upon removal of selective pressure. Out of a total of 150 colonies cultured overnight and plated, only two colonies without pDK6-red were obtained. Typically in our experiments used to eliminate pDK6-derived plasmid, the elimination occurred at a frequency of about 10^{-2}.

The *K. pneumoniae* Δdhak1 mutant was transformed with pDK6-flp to remove the *aac(3)IV* cassette, which was done by induction with IPTG at different levels. Table 5 shows the FLP-conferred recombination ratio relative to IPTG concentration. The resultant apramycin-sensitive *K. pneumoniae* strain with plasmid pDK6-flp was designated Δdhak1F/flp.

As seen in Table 5, the recombination ratio was not correlated with the concentration of the inducer IPTG; leaky expression of *flp* recombinase in the absence of the inducer still conferred the same order of recombination ratio (6 %) as in the presence of the inducer (4–12 %).

The strain *K. pneumoniae*/flp was subsequently cultured in medium without kanamycin and screened for loss of plasmid pDK6-flp. The kanamycin-sensitive mutant was

Table 5 FLP recombination ratio at different levels of IPTG induction

IPTG concentration (mM)	0	0.2	0.4	0.6	0.8	1.0
Recombination ratio (%)	6	12	12	10	4	6

isolated and named Δdhak1F. This markerless strain can now be utilized for the next round of gene replacement.

pCP20, a plasmid with temperature-sensitive replication origin, was used to effect FLP recombinase-mediated recombination in *E. coli*. The plasmid replicates poorly at temperatures higher than 37 °C; moreover, the transcription of *flp* gene was governed by a temperature-sensitive repressor λ cI857. Thus, cultivation of transformants at higher temperature not only helps in the removal of the selective marker but also causes the instability of pCP20 from the cells [3, 4]. In the studies presented here, *flp* gene was put under the control of *tac* promoter in pDK6-flp, and induction by IPTG supposedly leads to higher levels of recombinase; however, *tac* promoter was not tightly controlled, and the high transformation ratio was achieved owing to sufficient amounts of leaky expression of the recombinase. And, because pDK6 used in this study was not temperature sensitive, it can not be deleted from host cells by shifting of the culture temperature. No curable plasmid for use in *K. pneumoniae* has been reported yet. As an alternative approach, a counter-selectable marker *sacB* was employed and screened for self-elimination in *K. pneumoniae*, but the transformants with the plasmid harboring *sacB* and *flp* were not able to grow on medium supplemented with sucrose (data not shown). In *Yersinia pseudotuberculosis*, the Red recombinase system with *sacB* was used for successful counter-selection in *Yersinia* colonies [5].

Conclusion

To facilitate markerless gene replacement in *K. pneumoniae*, the Red recombinase system was systematically investigated during gene deletion of *dhak1*. Recombinase gene *red* or *flp* was constructed in plasmid pDK6-red or pDK6-fp, both under the IPTG-inducible promoter *tac*. The novel recombinase system conferred a transformation efficiency that was almost 1,000 times more than that obtained using the conventional way; moreover, when combined with a direct plasmid elimination strategy, markerless gene replacement can be routinely performed. The effects of the length of homologous extension and dose of inducer IPTG on the recombination ratio were investigated during gene deletion of *dhak1*. Linear DNA with homologous extensions of about 500 nt or longer was required to achieve a good recombination ratio. Using this system, we have knocked out several other genes for metabolic research, and performed one cycle of gene recombination, which required about 3 weeks. In summary, the constructed Red/flp recombinase system strengthens the arsenal for gene manipulation in *K. pneumoniae*; nevertheless, optimization of the expression of recombinase and introduction of more self-curable genes based on this system would propel further development of biotechnology and exploitation of *K. pneumoniae* in industrial applications.

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