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SHORT COMMUNICATION

New strategy to improve efficiency for gene replacement in *Klebsiella pneumoniae*

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Abstract We previously reported the method for introducing gene replacement into *Klebsiella pneumoniae* through Red-assisted homologous recombination; and it demonstrated that a higher transformation efficiency required long flanking arms at both ends of the linear DNA. The assembly job of the linear DNA is usually time-consuming and laborious. We report here an innovative method for DNA exchange in *K. pneumoniae* based on PCR-mediated Red recombination. The novel procedure enables rapid gene replacement in *K. pneumoniae* without prior cloning of the gene of interest; the key modification is to perform PCR reaction to generate linear DNA with extra non-homologous fragments on both ends as mercenary sequences which come from a TA-cloning plasmid. We give a demonstration by deleting the gene *dhak1* in *K. pneumoniae* with high efficiency of about 20 CFU/μg DNA using the new technique.

Keywords *Klebsiella pneumoniae* · Gene replacement · PCR · Mercenary sequence

Introduction

Transgenic technology is an essential component in the genetic modification of bacteria. Among the numerous molecular biology techniques, the Red recombinase system is one of the most effective tools for gene replacement ever exploited in *Escherichia coli*. Linear DNA substrates generated by PCR are introduced into strains that harbor the plasmid expressing Red recombinase. This technique allows for gene replacement in *E. coli* without prior cloning of the gene of interest [9]; the utilization of the counter-selectable marker *sacB* or FLP recombinase facilitates elimination of the drug-resistant marker, therefore making simultaneous multi-gene replacement possible in the cell [2]. Red recombinase originates from λ bacteriophage and consists of three proteins encoded by *exo*, *bet* and *gam*. Exo is a 5′–3′ exonuclease that acts on double-stranded DNA and generates dsDNA with 3′ single-stranded DNA tails. Bet is a single-stranded DNA binding protein covering a fragment of ssDNA over 35 nucleotides and is capable of annealing the complementary ssDNA strand. Gam protects extraneous DNA by inhibiting host RecBCD exonuclease which recognizes and digests foreign linear DNAs. Through synergized action, Red recombinase enormously enhances the transformation efficiency promoted by linear DNA, which can be prepared directly by PCR using primers with homologous 36-nt as minimal extensions.

The Red recombinase system has been modified to be applicable for many other microorganisms, such as *Aspergillus nidulans* [1], *Streptomyces coelicolor* [5], *Yersinia pseudotuberculosis* [3], *Salmonella enterica* [4], *Pseudomonas aeruginosa* [8], *Pantoea ananatis* [7], *Vibrio cholerae* [12] and *Burkholderia cepacia* [6]. We successfully introduced this recombination system into *Klebsiella pneumoniae* to ease the DNA manipulation job in this bacterium [11]. It has been reported that the Red-facilitated transformation efficiency varies greatly in these microorganisms, and the requirement for a minimal length of homologous extension is also different. In *Salmonella enterica* and *Pantoea ananatis*, an extension of 50 or 40 nt is

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sufficient for mediating a good homologous recombination, which is quite similar in *E. coli*. However, to achieve a high transformation efficiency in all the other above-mentioned bacteria, the minimal size of homologous extensions are all larger than 100 bp, ranging between 100 and 1,000 bp. The longer the homologous extensions are, the more costly the primers will be. Therefore, those linear DNA with long flanking arms are usually prepared by time-consuming DNA cloning procedures preceded by PCR reactions. This may need weeks to finish the construction job, whereas the direct PCR preparation of linear DNA can be done in hours.

Klebsiella pneumoniae is an important microbial species in studies of both medical and industrial microbiology. Hereby we present our new finding that the long extensions used for increasing recombination efficiency can be constituted by flanking short homologous DNAs with mercenary backbone vector DNAs, and we put forward a novel strategy to prepare linear DNA for Red-assisted gene replacement in *K. pneumoniae* based on rapid PCR preparation to circumvent the lengthy bench work of DNA assembly.

Materials and methods

Strains, plasmids, medium and primers

The acteria strains and plasmids used in this paper are listed in Table 1. *K. pneumoniae* and *E. coli* was grown in LB medium at 37 °C. The concentration of antibiotics used in selective medium were ampicillin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}) and apramycin (50 μg mL^{-1}). Primers used for PCR reaction are listed in Table 2.

Construction of plasmids, linear DNA preparation

Plasmids pMD18T-k40, pMD18T-k100 and pMD18T-k200 were constructed for linear DNA preparation (Fig. 1) to perform a gene deletion of *dhak1*, which encodes one subunit of the dihydroxyacetone kinase II. pMD18T-k40 contains the selective marker *aac(3)IV* (apramycin resistance gene) flanked by 39 and 40 bp homologous extensions. *aac(3)IV* was amplified with plasmid pIJ773 as a template with the primer pair FRT-s1 and FRT-a1 (Table 2); the homologous extensions was designed at the primers, and the PCR product was TA-cloned into pMD18-T simple® to generate pMD18T-k40.

Both pMD18T-k100 and pMD18T-k200 are highly similar to pMD18T-k40, differing only in their extension lengths. The primer pair dhak1-s1/a1 was used to produce linear DNA comprising *aac(3)IV* flanked by 112 and 107 bp homologous extensions with plasmid pMD18-T-2028/a as the template [11]. pMD18T-k100 was generated after TA cloning of the PCR product into pMD18-T simple. Similarly, pMD18T-k200 was also constructed by PCR and a TA cloning kit, containing a fragment with flanking arms at 212 and 187 bp, respectively.

These plasmids were employed as the templates for the generation of DNA fragments comprising *aac(3)IV* and homologous extensions of 40, 100 and 200 bp each, plus 400 bp of extra DNA coming from pMD18-T simple at both ends. Such linear DNAs were tested to evaluate whether or how the extra mercenary DNA can affect the recombination efficiency of *K. pneumoniae*.

In addition, plasmid pMD18T-k40 was also used as a template for making linear DNAs containing different sizes of non-homologous flanking sequences coming from pMD18-T simple. The primer pairs pMD18t-s1/a1, pMD18t-s2/a2, pMD18t-s3/a3, pMD18t-s4/a4, pMD18t-s5/a5 and pMD18t-s7/a7 were designed to produce linear DNAs with only 39 or 40 bp of homologous extensions, surrounded by 100, 200, 300, 400, 500 and 700 bp of mercenary sequences, respectively.

Table 1 Strains and plasmids

Strain or plasmid	Relevant genotype and description	Reference or source
Strains		
E. coli DH5α	Host of plasmid	Laboratory stock
K. pneumoniae/red	*K. pneumoniae* CGMCC 1.6366, pDK6-red	[11]
Plasmids		
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo), 7.1 kb	[11]
pMD18-T-2028/a	Amp^r, carries part of *dhak1*, *aac(3)IV*, *dhak2* and part of *dhak3*	[11]
pMD18-T simple	Amp^r, TA cloning vector, 2,692 bp	Takara
pMD18T-k40	Amp^r, carries *aac(3)IV* with 39 and 40 bp homologous extensions, 4,150 bp	This work
pMD18T-k100	Amp^r, carries *aac(3)IV* with 112 and 107 bp homologous extensions, 4,280 bp	This work
pMD18T-k200	Amp^r, carries *aac(3)IV* with 212 and 187 bp homologous extensions, 4,460 bp	This work

Table 2 Oligonucleotides used for PCR

Primer name	Sequence (5′–3′)
FRT-s1	CCGCGGCGTCGCCAATACCGTACTGATCG AAAAACTGGTATTCCGGGGATCCGTCGACC
FRT-a1	GACGATTTGCGTTCTGTTCAGTGACATGTC GTGCTCCTTATGTAGGCTGGAGCTGCTTC
dhak-s	AAGCTTCCGCTCAGCCAGAATCATCAT
dhak1-s1	GCGGCATAAAAGTCACCA
dhak1-a1	CTTTATCGAGGCCGGTGA
dhak1-s2	GCGATGCAGATTGACGGC
dhak1-a2	ATCGGCAATCGACGGCAG
Test773	GCAAATACGGCATCAGTTACC
pMD18t-s1	GAAAGGGGGATGTGCTGCAAG
pMD18t-a1	CTTCCGGCTCGTATGTTGTGTG
pMD18t-s2	AGGAGAAAATACCGCATCAGG
pMD18t-a2	CACGACAGGTTTCCCGACTG
pMD18t-s3	GGGTGTCGGGGCTGGCTTAACTATG
pMD18t-a3	GAGCGCAGCGAGTCAGTGAGCGAG
pMD18t-s4	GACGGTGAAAACCTCTGACACATGC
pMD18t-a4	TGCTGGCCTTTTGCTCACATGTTC
pMD18t-s5	TGCCACCTGACGTCTAAGAAAC
pMD18t-a5	CACCTCTGACTTGAGCGTCGA
pMD18t-s7	TGGGTGAGCAAAAACAGGAAGGC
pMD18t-a7	ACAGCCCAGCTTGGAGCGAAC

Red recombinase promoted DNA recombination in *K. pneumoniae*

Electro-competent cells were prepared as following. Briefly, *K. pneumoniae*/red (see Table 1) was cultured in LB supplemented with IPTG until the optical density at a wavelength of 600 nm (OD_{600}) reached 0.7 at 37 °C. Cells were collected at 4 °C and washed with cold water twice, then resuspended in cold water to OD_{600} of around 30. DNA fragments were electro-transformed into *K. pneumoniae*/red using a Bio-Rad MicroPulser with a 2-mm electroporation cuvette at 2.0 kV, 200 Ω and 25 μF. Transformants were isolated with a LB plate supplemented with apramycin and were confirmed by colony PCR using the primers Test773 and dhak-s.

Results

The recombination efficiency with non-homologous extension DNA

As reported in our previous studies, the minimal size of an homologous extension required for gene replacement in *K. pneumoniae*/red was around 200 bp, although the recombination ratio was only 3.46 CFU/μg DNA. By

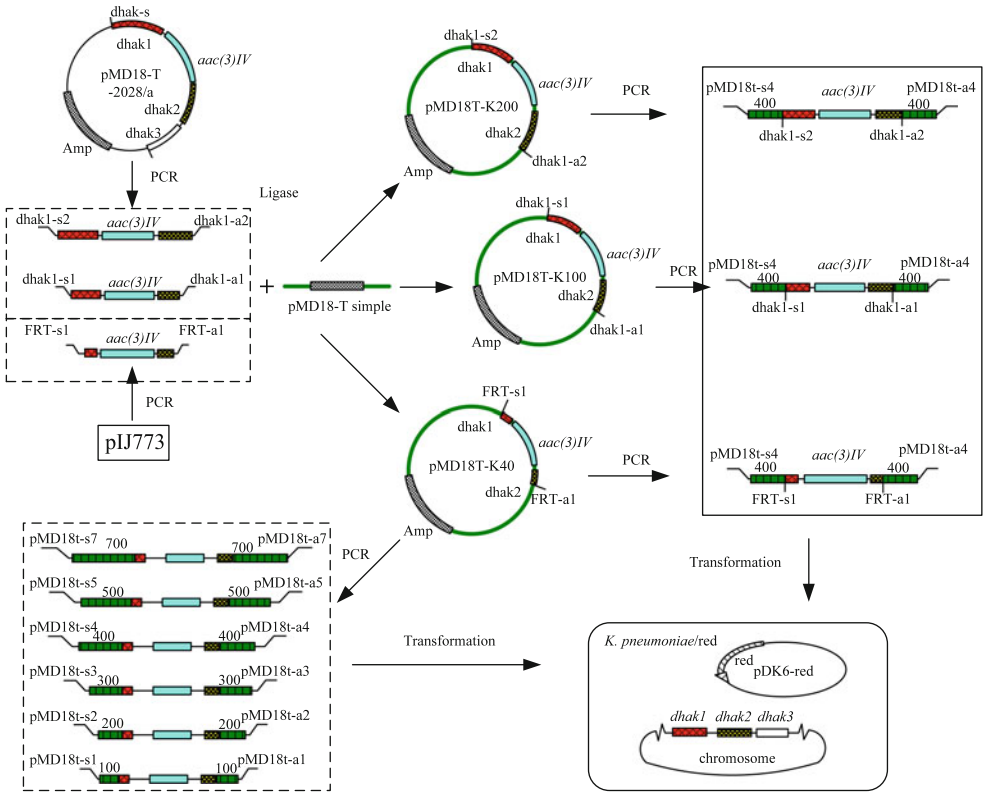

Fig. 1 Linear DNA preparation for Red-assisted gene deletion manipulation in *K. pneumoniae*

Table 3 Recombination ratios of safeguard linear DNA with different lengths of homologous extensions

Primers used	FRT-s1 FRT-a1	dhak1-s1 dhak1-a1	dhak1-s2 dhak1-a2
Length of homologous extensions	39 nt 40 nt	112 nt 107 nt	212 nt 187 nt
Recombination ratio (CFU/μg DNA)	26.96	24.82	23.59

increasing the length of the homologous arms, the recombination ratio improved significantly to around 266 CFU/μg DNA [11]. However, preparation of linear DNA with long extensions is time-costly in most standard laboratories. We therefore attempted to test the DNAs with 400 bp of foreign DNA as extra extensions besides 40, 100 or 200 bp of homologous fragments. As shown in Table 3, all three kinds of linear DNA achieved a good recombination efficiency in *K. pneumoniae* with similar recombination ratios (around 25 CFU/μg DNA), which is parallel to the efficiency obtained with DNA containing 400 bps of homologous fragments, as reported previously [11].

Minimal size of mercenary DNA in linear DNA

Although we knew that 400 bp of non-homologous extensions was sufficient for a good recombination ratio, the question of how long the minimal sequences should be still remained, as well as that of the length to achieve the best transformation ratio. Therefore, we prepared linear DNA with 40 bp of homologous core sequences plus different sizes of non-homologous arms, of 100, 200, 300, 400, 500 and 700 bp, respectively (Fig. 1). All the DNAs were electro-transformed into *K. pneumoniae*/red for calculation of recombination ratio after formation of apramycin-resistant transformants.

As shown in Table 4, no transformants were obtained with DNA having a mercenary sequence of 112 bp at each side; the minimal length of mercenary sequence to generate mutants is around 200 bp in our experiments. The recombination ratio improved sharply as the length increased from 200 to 400 bp; no further significant improvement was observed when longer mercenary DNAs were employed (500–700 bp).

Discussion

In *E. coli* the host RecBCD is inhibited by the Gam component of the Red recombinase system; however, the inhibitor might be not as efficient in *K. pneumoniae* as in *E. coli*. We speculate that extra DNAs are required to protect the homologous extensions, and such protective DNAs are not necessarily homologous to the host genomic DNA. Here we prove that mercenary DNAs surrounded by less than 40 bp of homologous extensions can achieve recombination with an efficiency as high as that obtained

Table 4 Recombination ratios of linear DNA with different lengths of mercenary sequences

Primers used	pMD18t-s1 pMD18t-a1	pMD18t-s2 pMD18t-a2	pMD18t-s3 pMD18t-a3	pMD18t-s4 pMD18t-a4	pMD18t-s5 pMD18t-a5	pMD18t-s7 pMD18t-a7
Length of mercenary sequence	112 nt	210 nt	300 nt	408 nt	503 nt	700 nt
	112 nt	208 nt	301 nt	409 nt	508 nt	701 nt
Recombination ratio (CFU/μg DNA)	0	0.41	6.32	16.03	18.70	16.01

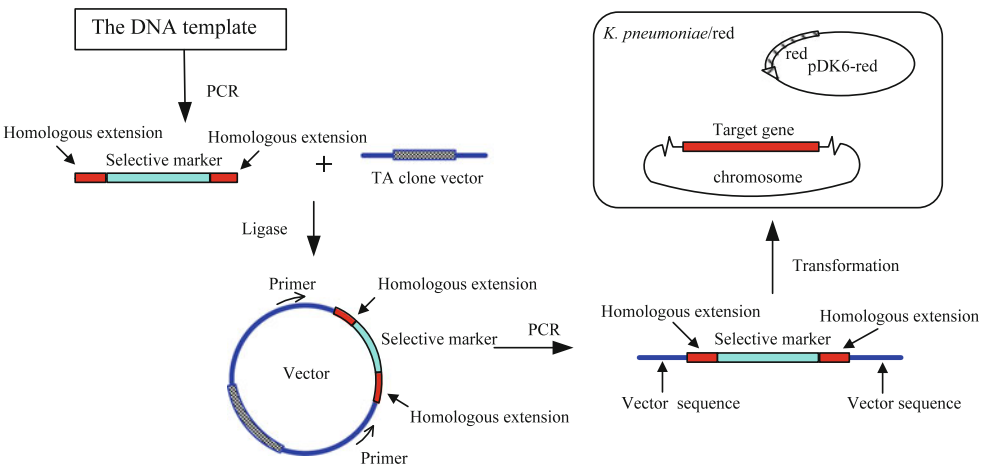

Fig. 2 Flow sheet of the novel strategy of PCR-mediated gene replacement in *K. pneumoniae*

with a homologous extension of 400 bp [11]. The successful recombination using mercenary DNAs implies that the minimal length of homologous extension needed in *K. pneumoniae* is as short as in the case of *E. coli* [2].

The results shown in Table 4 may reflect the fact that the effective recombination in *K. pneumoniae* is a dynamic process which involves Red recombinase racing the time clock with the DNA digestion system from the host cells. The recombination is possible only when the minimal homologous extensions remain intact for an effective strand invasion. For a successful DNA exchange to happen, the extension arms of the linear DNAs in *K. pneumoniae* might have to be longer than their *E. coli* counterpart, no matter whether the extra DNA is homologous or not. It is therefore plausible to speculate that the problem originates from the impotence of Gam in repressing *K. pneumoniae* RecBCD, due to the mismatch between *E. coli* Gam and its *K. pneumoniae* homolog.

For the same reason, even in *E. coli*, without the introduction of Red recombinase, gene replacement is also limited to some RecBCD nuclease-deficient strains [10]. Therefore, it could be a possible way to increase recombination efficiency if we perform gene deletion in RecBCD-deficient *K. pneumoniae*. However, disruption of RecBCD in the host strain may disturb the long-term stability of the cell's genetic system. Thus, our novel strategy of adding non-homologous extensions seems to be the easiest way to perform gene replacement with high efficiency in *K. pneumoniae*. The complete flow chart of this new method is demonstrated in Fig. 2.

Conclusion

A renovated strategy of Red recombinase-assisted gene replacement in *K. pneumoniae* is presented here. Briefly, mercenary sequences of 400 bp of each side from the backbone cloning vector were added to the linear DNA by PCR, and the existence of this non-homologous DNA was proved to significantly increase the recombination efficiency incurred by the 40 bp of homologous extensions. This novel strategy may be applied universally to other gene manipulation work in microorganisms which suffer low transformation efficiency or lengthy DNA cloning jobs.

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