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Excision of selectable markers from the *Escherichia coli* genome without counterselection using an optimized λRed recombineering procedure

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ABSTRACT

The introduction of chromosomal mutations into the *E. coli* genome using λRed-mediated recombineering includes two consecutive steps—the insertion of an antibiotic resistance gene and the subsequent excision of the marker. The second step usually requires a counterselection method, because the efficiency of recombination is not high enough to find recombinants among non-recombinant cells. Most counterselection methods require the introduction of additional mutations into the genome or the use of expensive chemicals. In this paper, we describe the development of a reliable procedure for the removal of an antibiotic resistance marker from the *E. coli* genome without the need for counterselection. For this purpose, we used dsDNA cassettes consisting of two regions homologous to the sequences that flank the marker on the chromosome. We optimized the length of the homologous regions, the electroporation conditions, and the duration of recovery for the electroporated cells in order to maximize the recombination efficiency. Using the optimal parameters identified, we obtained a rate of 4–6% recombinants among the transformed cells. This high efficiency allowed us to find marker-less, antibiotic-sensitive recombinants by replica plating without the need for selection.

1. Introduction

Currently, λRed-mediated recombineering using synthetic oligonucleotides, or double stranded integrative cassettes with short homology regions, is the most powerful method for the precise introduction of mutations into the *Escherichia coli* chromosome (Datsenko and Wanner, 2000; Murphy et al., 2000; Yu et al., 2000; Ellis et al., 2001; Costantino and Court, 2003), as well as for *in vivo* plasmid DNA engineering (Thomason et al., 2007). Therefore, λRed-dependent homologous recombination has become a versatile tool in bacterial genetics and functional genome analyses.

The multistep construction of recombinant *E. coli* strains using the λRed system requires consecutive insertion and excision of selectable markers. The use of antibiotic resistance cassettes for insertion allows for the direct selection of recombinant clones. This procedure is simple and robust. Its variations lie in the choice of a suitable resistance gene and drug, most of which are similar in terms of their selection stringency and convenience of handling. This is not the case with counterselection (negative selection) methods, that are intended for marker excision and the generation of marker-less mutations without "scars."

The diversity of counterselection markers and methods is in itself evidence of their inferiority. The obvious limitation is a low stringency of recombinant selection due to the high frequency of spontaneous mutants that survive on the selective plates. Application of some markers, such as *galK* (Warming et al., 2005), *tolC* (DeVito, 2008), and *rpsL* (Bird et al., 2011), requires the preliminary introduction of a chromosomal mutation. These methods are applicable only for a specific genetic background. For others, selection of recombinants on minimal agar is required (Warming et al., 2005; Khetrapal et al., 2015), resulting in a prolonged cultivation step in comparison with selection on a rich medium.

One of the major drawbacks of using counterselection is the greater

Abbreviations: bp, base pair; PCR, polymerase chain reaction; ds, double-stranded; Cm^R, chloramphenicol resistant; *cat*, chloramphenicol acetyltransferase; Gal^-, inability to utilize D-galactose; Lac^-, inability to utilize lactose; Ara^-, inability to utilize L-arabinose

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length required for the integrative cassettes that allows them to contain markers for both positive and negative selection. It has been shown that the efficiency of the λRed-mediated linear DNA fragment insertion into the chromosome is highly dependent of fragment length (Kuhlman and Cox, 2010). As a result, the length of the integrative cassette is the limiting factor in experiments where a high recombination efficiency is required. These include, among others, promoter randomization or the introduction of a mutation that reduces cells viability. Thus, the use of a counter-selectable marker together with an antibiotic resistance gene imposes a substantial constraint on the procedure of integrative cassette insertion into the bacterial chromosome, which is the first step of the two-step recombineering procedure using dsDNA for the construction of marker-less mutations.

Methods for negative selection are required because the efficiency of dsDNA recombineering is usually too low to find recombinants without selection (Yu et al., 2000). Therefore, we have optimized recombineering parameters to increase efficiency. Using these parameters, we have developed a reliable procedure for antibiotic resistance gene removal without use of any counter-selectable marker.

2. Materials and methods

2.1. Media and reagents

For general manipulation, the *E. coli* cells were grown in Lysogeny Broth (LB) (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per 1 L of medium). To prepare LB plates, 20 g of agar was added per 1 L of the medium. SOB medium (20 g of tryptone, 5 g of yeast extract, 0.584 g of sodium chloride, 0.186 g of potassium chloride, and 2.4 g of magnesium sulfate per 1 L of water) was used for the preparation of electrocompetent cells. A concentrated solution of magnesium sulfate (1 M) was autoclaved separately and then added to the sterile SOB medium. When required, the medium was supplemented with antibiotics: 8 mg/L chloramphenicol and 100 mg/L ampicillin. M9 minimal agar (1 g of ammonium chloride, 0.5 g of sodium chloride, 6 g of disodium phosphate, 3 g of monopotassium phosphate, 0.5 g of magnesium sulfate heptahydrate, 0.01 g of calcium chloride, 20 g of agar-agar per 1 L of medium) with 4 g/L of appropriate carbohydrates was used for the direct selection of the sugar-utilizing recombinants. Concentrated (×10) M9 salt solution and solution of agar-agar with magnesium sulfate and calcium chloride were autoclaved separately and mixed before plate preparation. MacConkey agar (#M1024-500G, HiMedia Laboratories Pvt. Ltd., Mumbai, India) with 10 g/L of appropriate carbohydrates was used for non-selective screening of the recombinants. Both M9 agar and MacConkey indicator agar with lactose were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Stock solutions of carbohydrates and IPTG were sterilized by filtration through 0.22-μm pore-sized membranes. Sterile 0.9% sodium chloride solution was used for dilution of the cells before plating.

2.2. Bacterial strains and plasmids

All strains (Table 1) were constructed using λRed-mediated recombineering and MG1655 as the parental strain. pDL17 (Bubnov et al., 2018) was used as a helper plasmid to provide λRed functions. The strains B1415 and B1421 were constructed by *cat* cassette (encoding chloramphenicol acetyltransferase and conferring chloramphenicol resistance) insertion into the β-galactosidase (*lacZ*) and ribulokinase (*araB*) gene, respectively. In both cases an internal region of the coding sequence 42 bp in length was deleted. The integrative cassettes *lacZ::cat* and *araB::cat* were amplified from pACYC184 (Chang and Cohen, 1978) using the primer pairs 3234–3235 and 3236–3237, respectively. The B1411 strain contains a deletion of 410 bp in length in the galactokinase (*galK*) gene.

Table 1
Bacterial strains used in this work.

Strain	Genotype	Phenotype	Source
MG1655	F$^-$ λ$^-$*rph-1*	WT	Laboratory collection
B1411	F$^-$ λ$^-$*rph-1galK::cat*	Gal$^-$, Cm^R	(Bubnov et al., 2018)
B1415	F$^-$ λ$^-$*rph-1lacZ::cat*	Lac$^-$, Cm^R	This study
B1421	F$^-$ λ$^-$*rph-1araB::cat*	Ara$^-$, Cm^R	This study

2.3. Oligonucleotides

The oligonucleotides used in this study are listed in Supplementary Table S1. The oligonucleotides were purchased from the Evrogen Joint Stock Company (Moscow, Russia) and had undergone standard purification and desalting.

2.4. Preparation of electrocompetent cells with induced λRed function and electroporation

Electrocompetent cells carrying pDL17 were grown, induced, prepared, and transformed by electroporation largely as described in our previous work (Bubnov et al., 2018). Differences were that the low salt LB medium was replaced with SOB for growth of the competent cells, and the cells were washed with ice-cold deionized water in a 50-mL tube not once but twice. Electroporated cells were diluted in 5 mL of fresh LB medium and grown with shaking at 200 rpm and 37 °C. The duration of the recovery varied, with the exact value of this parameter being described in Sections 3.2 and 3.3 of the Results and Discussion.

2.5. Preparation of integrative cassettes

The DNA fragments used for integrative transformation into λRed-induced cells were amplified using KAPA HiFi DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA) in accordance with the manufacturer's instructions. The primers used for amplification of each DNA fragment are listed in Table 2. All fragments were amplified from the genomic DNA of the MG1655 strain. DNA was precipitated with ethanol and then purified using a GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania) skipping the gel-purification step. Before dissolving the DNA in deionized water, the columns were additionally washed using 500 μL of ice-cold 80% ethanol to remove any residual salts. Measurement of DNA concentration was performed using gel-electrophoresis with a GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) as a standard. Aliquots were stored at −20 °C.

2.6. Measurement of recombination efficiency

Measurement of the recombination efficiency was based on the restoration of the ability of the strains B1411, B1415, and B1421 (Table 1) to utilize D-galactose, lactose, and L-arabinose, respectively. In

Table 2
Primers used for the amplification of integrative cassettes.

Target locus	Length of homologous regions	Primer pairs
lacZ::cat	50	3425–3426
	100	3427–3428
	200	3429–3430
	350	3431–3432
	500	3433–3434
	650	3435–3436
	800	3437–3438
	1000	3439–3440
galK::cat	350	3611–3612
araB::cat	350	3609–3610

these strains the sequences encoding *galK*, *lacZ*, and *araB*, respectively, were disrupted by replacement of an internal segment of the gene (*galK* – 410 bp in length, *lacZ* and *araB* – 42 bp in length) with a *cat* cassette. Thus, each of these strains is unable to growth on a specific sugar. λRed-induced cells from these strains were electroporated with the integrative cassettes. The sequences of these cassettes correspond to the sequence of the wild type genes. The construct consists of two regions of flanking homology and an internal gene segment which is deleted in the appropriate strain by integration of the *cat* cassette. Sugar-utilizing recombinants were scored on MacConkey indicator agar with 10 g/L of the appropriate sugar. M9 minimal medium supplemented with 4 g/L of carbohydrate was used for direct selection if < 1% of the cells were recombinant. In this case, the total number of viable cells was determined on M9 agar with 4 g/L of D-glucose.

In all experiments at least 50 recombinant colonies were scored. The recombination efficiency was calculated by dividing the number of recombinants by the total number of viable cells and is expressed as a percentage. For all experiments three independent repeats were performed. Results are expressed as the mean and the standard deviation.

3. Results and discussion

3.1. Rationale of the method

The excision of antibiotic resistance markers from bacterial genomes using λRed-mediated homologous recombination with a DNA fragment transformed into cells leads to the formation of a mixture of marker-less recombinants and non-recombinants. Negative selection methods are required because the efficiency of recombineering usually does not exceed 10^5 recombinants per 10^8 cells (0.1%) (Yu et al., 2000), which is too low to find recombinants without selection. In such experiments, recombinants can be found by screening several thousand colonies. This procedure is highly laborious and time consuming. In this case, the use of counterselection is preferable. However, if the recombination efficiency could be increased to much higher levels this would allow for the identification of marker-less recombinants without counterselection because such clones have easily recognizable phenotypes, namely sensitivity to an antibiotic. Replica plating of 100 colonies on medium supplemented with a specific antibiotic is a routine and rapid procedure. To find at least one recombinant colony with probability > 95% using this method, the percentage of the latter among non-recombinants should be more than ~3% (Fig. 1) in correspondence with the Bernoulli equation (Eq. (1)).

$$P = \sum_{k=1}^{10} \binom{n}{k} p^k (1-p)^{n-k} \quad (1)$$

where

P – the probability of finding at least 1 (from 1 to 10) recombinant colony among n randomly picked colonies
n – number of tested colonies
k – number of identified recombinant colonies
$\binom{n}{k}$ – binomial coefficient
p – recombination efficiency expressed as a decimal

3.2. Optimization of the length of the homologous regions of the integrative cassette

We hypothesized that such a level of recombination efficiency could be reached by optimization of the recombineering parameters. First of all, we had a choice between the types of recombineering substrate. Synthetic oligonucleotides have the highest recombination efficiency when used to introduce point mutations, short insertions, and deletions of several nucleotides in length (Wang et al., 2009). However, in the case of deletions longer that 1000 bp, their recombination efficiency is generally < 1% (Ellis et al., 2001; Wang et al., 2009), which is insufficient for our purpose. Therefore, we chose dsDNA as a substrate for recombineering. DNA fragments used for marker excision must contain two regions homologous to the segments that flank the marker in the chromosome. These integrative cassettes can be easily generated by joining the left and right flanks by overlap-extension PCR.

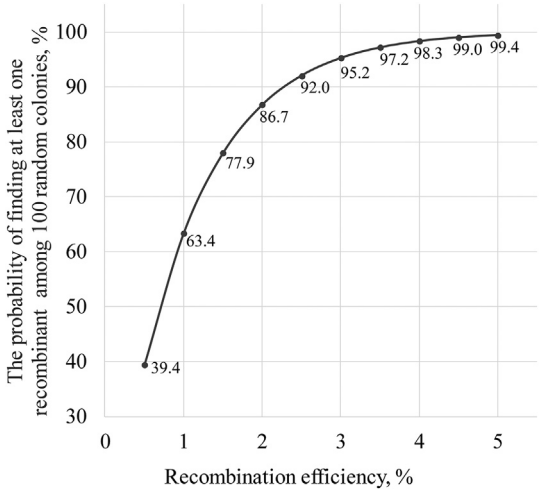

Fig. 1. Dependence of the probability of finding at least one recombinant colony by testing 100 randomly picked colonies. The recombination efficiency and the probability are expressed as percentages. The graph was plotted according to Eq. (1) at $n = 100$. The sum of the probabilities of finding from 1 to 10 recombinant colonies (k) are indicated.

The recombination efficiency of dsDNA is highly dependent on the length of the homology regions that flank the integrative cassette (Yu et al., 2000). Therefore, we first examined how homologous region length affects the efficiency of the excision of an antibiotic resistance marker from the chromosome. For this purpose, we constructed strain B1415, in which 42 bp of the *lacZ* coding sequence had been removed by insertion of the *cat* gene which confers chloramphenicol resistance. Because *lacZ* encodes β-galactosidase and controls lactose utilization, this strain is unable to growth on lactose as a single carbon and energy source. Strain B1415 was transformed with the previously described helper plasmid pDL17 (Bubnov et al., 2018) used for the inducible expression of λRed genes. The λRed-induced cells were transformed with 100 ng of each of eight integrative cassettes carrying 42-bp of the wild-type *lacZ* gene and homologous regions of 50–1000 bp in length (Fig. 2). Recombination of the chromosome with these cassettes led to the restoration of the *lacZ* coding sequence and the ability to grow on lactose. The electroporated cells were grown overnight and then plated on a selective M9 medium supplemented with lactose and IPTG, as well as on a non-selective M9 medium supplemented with D-glucose to count the Lac^+ recombinants and the total number of viable cells, respectively. IPTG was added to the medium to avoid the excessive repression of the P_{lac} promoter by the product of $lacI^q$ which is present on the multicopy plasmid pDL17. The dependency of the recombination efficiency (expressed as a percentage of the recombinants in culture) on the length of the homology region is shown on Fig. 3. As shown, the recombination efficiency increased 32-fold when the length of the homologous region increased from 50 to 350 bp. Further elongation of the flanking homology region did not enhance recombination efficiency. One possible reason for this result is the strong dependence of λRed-mediated recombination efficiency on the length of the DNA fragment to be integrated (Kuhlman and Cox, 2010; Maresca et al., 2010). For example, the recombination efficiency of the 1.1-kb *cat* cassette (which confers chloramphenicol resistance) is up to 45-fold higher than the 3.3-kb *cat-sacB* cassette (which confers chloramphenicol resistance and sucrose sensitivity) at equal molar amounts of DNA per transformation (Bubnov et al., 2018). A second obvious

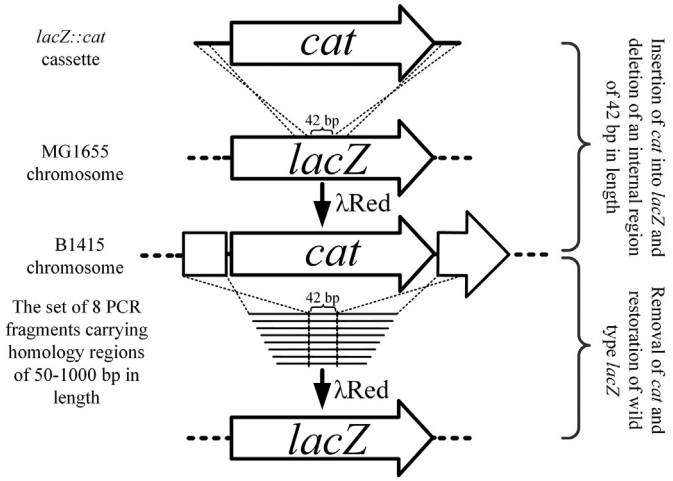

Fig. 2. Schematic representation of the experiment performed to optimize the length of the homologous regions. The lengths of the fragments depicted in this scheme are not meant to accurately reflect their actual lengths. See details in section 3.2 of the Results and Discussion.

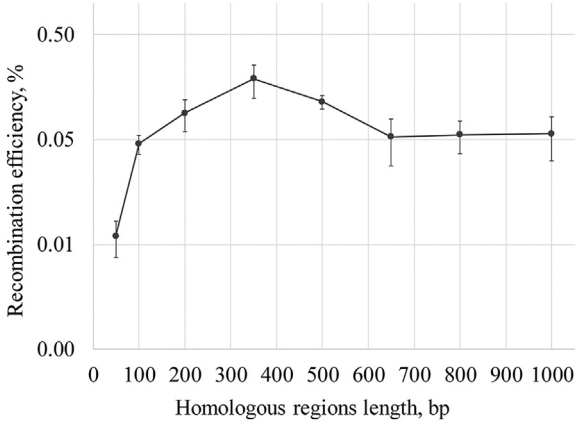

Fig. 3. Effect of homologous region length on the efficiency of recombination. Cultures of strain B1415 carrying pDL17 were induced with L-rhamnose for 20 min. The cells were made electrocompetent and electroporated with 100 ng of one of the eight cassettes carrying 42-bp of wild-type *lacZ* and homologous regions 50–1000 bp in length. Recombinants were selected on M9 minimal agar containing 4 g/L of lactose and 1 mM IPTG. The values shown are the average of three independent experiments; the error bars represent standard deviations.

reason is a reduction in the molar amounts of DNA because of the increase in total fragment length while maintaining a constant mass amount. Nevertheless, if the recombination efficiency is calculated as a percentage of the Lac$^+$ recombinants per mole of DNA, homologous regions of 500–1000 bp in length did not provide more efficient recombination than the region of 350 bp in length. Moreover, long homologous regions could affect gene sequences adjacent to the recombination sites on the chromosome. As a result, this often requires sequencing of the recombinant locus. Based on this reasoning, we chose 350 bp as the optimal length of the flanking homologous region for further studies.

3.3. The effect of DNA concentration and recovery duration on recombination efficiency

Next, we tried to increase of the amount per electroporation of the cassette containing the 350-bp homologous region. However, we were unable to reach a recombination efficiency > 1% at any DNA concentration ranging from 100 ng to 4 μg per 50 μL of electrocompetent cells (data not shown). In these experiments, as in the previous ones, the electroporated cells were diluted in fresh LB medium, and allowed to recover for 18–24 h. Therefore, we studied how the percentage of recombinants depends on the duration of the recovery. In these experiments, we used 750 ng of DNA per electroporation. We observed that a maximum recombination efficiency of 8.5–9% remained relatively constant after 1 h of recovery. In cultures recovered for 2 and 4 h, the percentage of Lac$^+$ recombinants decreased to 6.1% and 3.7%, respectively. However, further cultivation in liquid medium for 24 h before plating led to a drastic decrease in recombination efficiency to < 1% (Fig. 4A). We speculate, that this could be explained in terms of the cellular response to the introduction into the cytoplasm of the linear DNA fragment and subsequent recombination. Because the efficiency of the λRed-mediated recombination of both λ phage chromosomes (Stahl et al., 1974) and linear PCR-generated substrates (Yu et al., 2000) depend on the RecA protein, it could play a direct role in strand invasion and exchange processes. It is known that formation of the RecA-ssDNA filament leads to the autocleavage of LexA (Little, 1991), which represses the transcription of SOS response genes. Among these, the *sulA* gene encodes a protein that inhibits the polymerization of FtsZ and leads to cell filamentation and growth inhibition (Mukherjee et al., 1998). Thus, recombination with electroporated DNA may lead to a retardation in the growth of recombinant cells and a decrease in the apparent recombination efficiency due to an induction of the SOS response.

Immediate plating of the electroporated cells on MacConkey indicator agar with 1% lactose and IPTG showed that most of the recombinant colonies obtained this way are mixed and contain both recombinant and non-recombinant sectors (Fig. 4B). Most of the identified recombinant colonies were half-white. Such a phenotype appears due to the presence of several copies of the chromosome in a single electroporated cell. As a result, the cell could contain both recombinant and non-recombinant chromosomes. If such a cell is plated on solid medium, the daughter cells cannot be segregated and form mixed colonies. The same phenomenon takes place in particular when λRed-induced cells are transformed by synthetic oligonucleotides, which recombine with only one strand of the DNA duplex (Sawitzke et al., 2011).

Because testing for the loss of the antibiotic resistance marker by replica-plating requires homogeneous antibiotic-sensitive recombinant colonies, we examined the effect of recovery time on the percentage mixed colonies. As can be seen on Fig. 4A, the percentage of homogeneous recombinant colonies is < 25% in the absence of recovery and reaches 89% after 2 h of cultivation in liquid media before plating on the MacConkey indicator agar. The delay in cultivation led to a complete segregation of the recombinant and wild-type chromosomes. As a result, mixed colonies were not found if the electroporated cells were recovered in LB for > 2 h. However, under such conditions, the recombination efficiency was significantly reduced compared with the shorter recovery period. In the following experiments, the electroporated cells were cultivated for 2 h before plating.

We also determined the amount of DNA per electroporation that was saturating for the generation of recombinants. We tested amounts of DNA ranging from 250 ng to 2 μg and found that the recombination efficiency reached a maximum level of 5.7% when 750 ng of the integrative cassette was electroporated (Fig. 5). This amount coincides with that used in the previous experiment. Further increase led to a large drop in the percentage of recombinants obtained. This could be as a result of the observed reduction in the time constant (data not shown) due to an increased concentration of ions in the cell and DNA mixture.

3.4. Testing the optimal recombineering parameters for marker excision from other genomic loci

Finally, we tested our method on two other genomic loci. For this purpose, we used the strains B1411 (MG1655 *galK::cat*) and B1421 (MG1655 *araB::cat*) (Table 1), which are unable to utilize D-galactose or

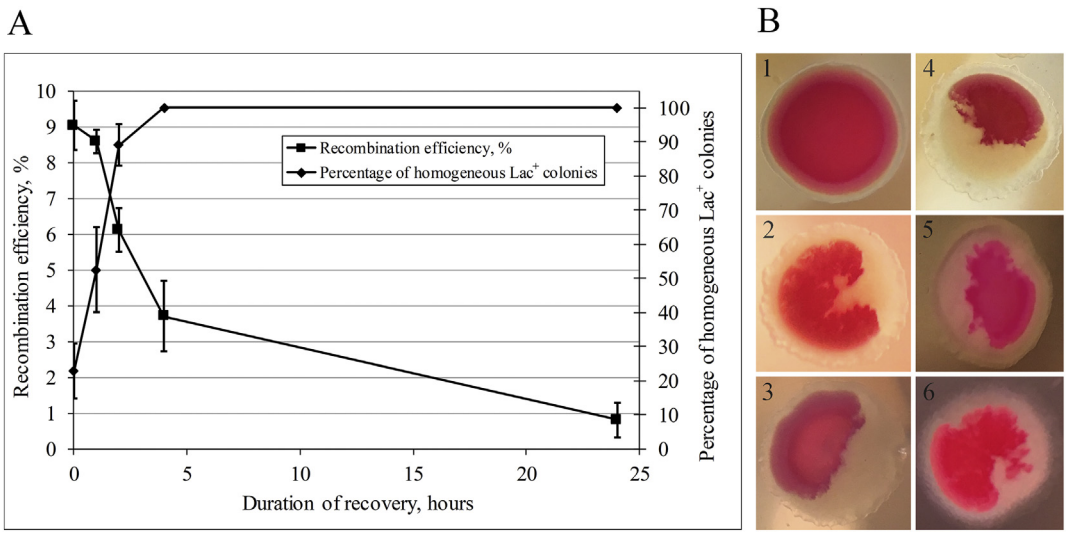

Fig. 4. Effect of recovery time on the recombination efficiency following electroporation. (A) Dependence of the recombination efficiency on the duration of recovery. Cultures of strain B1415 carrying pDL17 were induced with L-rhamnose for 20 min. The cells were made electrocompetent and electroporated with 750 ng of an integrative cassette carrying 42-bp of the wild-type *lacZ* gene and homologous regions 350 bp in length. The electroporated cells were diluted in 5 mL of liquid LB medium and grown for the indicated periods of time before plating onto MacConkey agar in the presence of 1% lactose and 1 mM IPTG. The values shown are the average of three transformations; the error bars represent standard deviations. (B) Examples of Lac^+ recombinant colonies scored as homogeneous (1) or mixed (2–6).

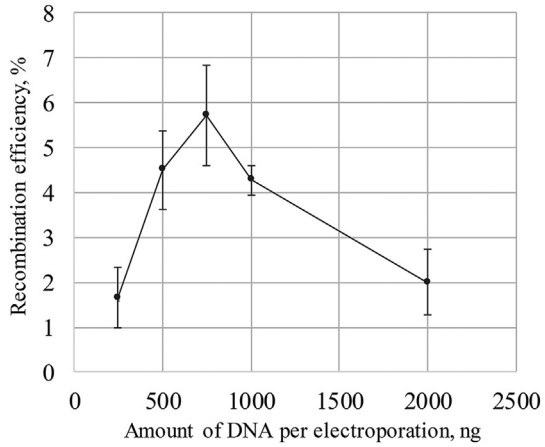

Fig. 5. Effect of the amount of DNA on the efficiency of recombination. Cultures of the B1415 strain carrying pDL17 were induced with L-rhamnose for 20 min. The cells were made electrocompetent and electroporated with varying amounts of the integrative cassette carrying homologous regions 350 bp in length. The cells were recovered for 2 h in 5 mL LB before plating. Lac^+ recombinants were scored on MacConkey agar supplemented with 1% lactose and 1 mM IPTG. The values shown are the average of three transformations; the error bars represent standard deviations.

Table 3
Testing of the optimized recombineering parameters for marker excision.

Strain	Target locus	Recombination efficiency, %[a]	Number of Cm^S carbohydrate-utilizing recombinants found by replica plating		
			Repeat 1	Repeat 2	Repeat 3
B1415	*lacZ::cat*	5.1 ± 1.3	3/96	5/96	2/96
B1421	*araB::cat*	5.8 ± 2.1	4/96	5/96	2/96
B1411	*galK::cat*	4.4 ± 1.0	2/96	4/96	3/96

[a] Values shown are the average and standard deviation of three independent experiments.

L-arabinose due to disruption of the *galK* (encodes galactokinase) or *araB* (encodes ribulokinase), respectively. The *cat* cassette was inserted into both genes and an internal part of the coding sequences (*galK*–410 bp and *araB*–42 bp) was deleted. This experimental design was the same as the one described previously. The λRed-induced cells of the indicated strains were electroporated with 750 ng of the DNA fragment carrying the deleted coding sequence and the 350 bp homologous region. The electroporated cells were diluted in 5 mL of fresh LB medium and grown for 2 h. The cells were then plated on MacConkey agar with 1% of the appropriate carbohydrate. In this experiment, only homogeneous carbohydrate-utilizing colonies were scored as recombinants. Table 3 shows that the recombination efficiency for all three loci were similar and exceeded the 3% threshold. Even in the case of the B1411 strain, which harbors a relatively long (410 bp) deletion in *galK* compared with 42-bp deletions in *lacZ* and *araB*, we obtained an efficiency of 4.4%, sufficient for the non-selective screening of Cm^S recombinants.

Next, we tested the possibility of finding marker-less recombinants by replica plating on selective and non-selective media. We plated electroporated cells of each strain onto LB agar from the same tubes, which were then used to determine the recombination efficiency. Following this, for each of the three repeats we randomly picked 96 colonies and tested them for their chloramphenicol sensitivity and carbohydrate utilization ability by replica plating on both LB agar supplemented with chloramphenicol and MacConkey agar supplemented with carbohydrate. For all the strains we found at least two recombinant Cm^S clones, which were red on MacConkey agar (Table 3, Fig. 6). Some sets of the 96 clones contained up to five recombinants, and there were no plates without recombinants.

This result was entirely consistent with our expectations (Fig. 1). The recombination efficiencies for all the tested loci were high enough to find at least one recombinant with a $> 98\%$ probability by testing 100 randomly picked colonies. This result shows that it is possible to routinely remove antibiotic resistance genes from the chromosomal DNA of *E. coli* without counterselection. Additionally, our results indicate that any segment ≤ 1000 bp in length can be deleted from the genome and short DNA fragments (such as promoters or affinity tags) ≤ 410 bp in length can be inserted into the chromosome without the need for the direct selection of recombinants. However, PCR screening

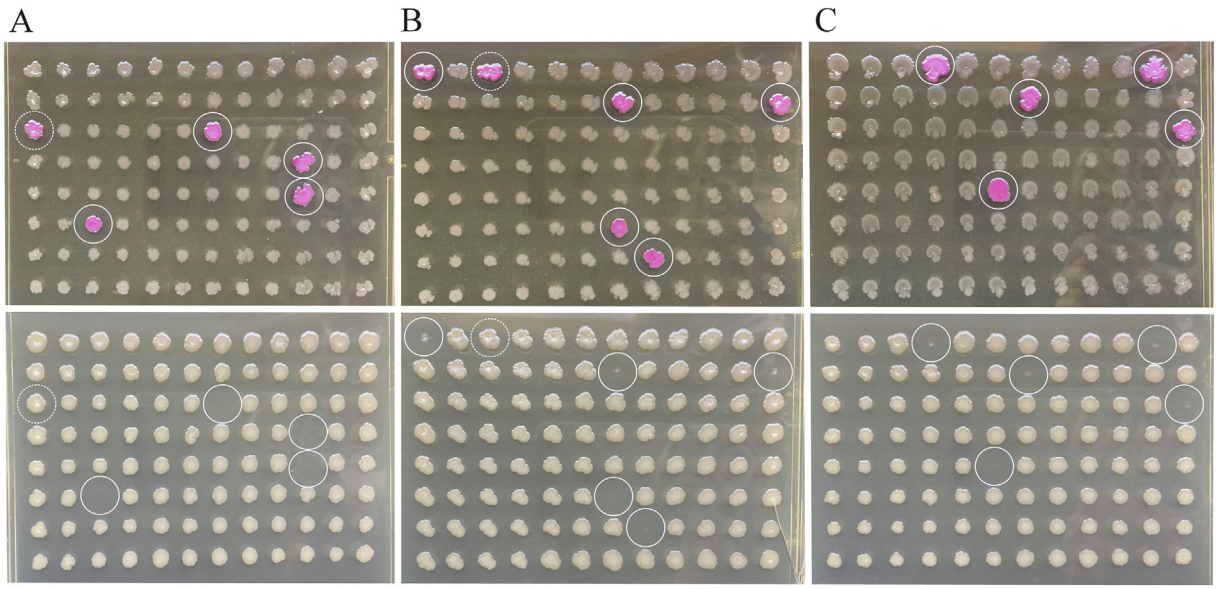

Fig. 6. Screening of marker-less recombinants by replica plating. Cultures of strains B1411 (A), B1421 (B), and B1415 (C) carrying pDL17 were induced with L-rhamnose for 20 min. The cells were made electrocompetent and electroporated with 750 ng of the appropriate integrative cassette carrying a homologous region 350 bp in length. The electroporated cells were diluted in 5 mL of liquid LB medium and grown for 2 h before plating onto LB agar. Following this, 96 colonies were randomly picked and replica-plated onto carbohydrate-supplemented MacConkey agar (upper row) and LB agar supplemented with chloramphenicol (bottom row). The solid-line circles indicate spots that correspond to recombinants that have lost the *cat* marker and have restored the ability to utilize the appropriate carbohydrate. The dotted-line circles indicate spots that are derived from mixed colonies.

of at least 100 colonies is required, which is highly labor intensive compared with the testing of colonies by replica plating.

The procedure described here eliminates most of the drawbacks of counterselection methods. First, it is not necessary to construct a special template, where an antibiotic resistance gene is joined to a second counter-selectable marker. An integrative cassette can be amplified from any available construct, such as plasmids, transposons, or genomic DNA, where the marker of interest is present. Secondly, when the excision of the marker from a bacterial chromosome is performed without counterselection, it is unnecessary to introduce chromosomal mutations that are required for some counterselection methods (Warming et al., 2005; DeVito, 2008; Barkan et al., 2011; Bird et al., 2011) or to use expensive (2-deoxy-galactose, fusaric acid) and commercially unavailable (colicin E1) compounds. Finally, in the absence of counter-selectable markers, the length of the integrative cassettes could be reduced by 2–3 fold, especially in the case of the *sacB*–sucrose sensitivity marker. In fact, all the integrative cassettes carrying the widely used antibiotic resistance genes, *cat* (660 bp), *aadA* (792 bp, conferring spectinomycin and streptomycin resistance), and *aph* (795 bp, conferring kanamycin resistance), could be shorter than 1000 bp. It has been shown, that the efficiency of λRed-mediated integration using short cassettes is much higher than that using longer cassettes (Wang et al., 2009; Maresca et al., 2010). The high efficiency of integration is helpful, because it generally increases the probability of success and reduces the quality required for the transformed DNA and other materials. Moreover, a high recombination efficiency allows the observation and study of very rare events in the *E. coli* genome, such as gene duplication (Yu et al., 2000). In summary, this procedure simplifies the routine construction of chromosomal mutations and could serve as a reliable and convenient tool for genome engineering of *E. coli* strains.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.022.

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