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Gene replacement with linear DNA in electroporated wild-type *Escherichia coli*

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ABSTRACT

Gene replacement using linear double-stranded DNA fragments in wild-type *Escherichia coli* transformation is generally inefficient due to exonucleolytic degradation of incoming DNA. Recombination-proficient strains, in which the exonucleolytic activity of RecBCD is inactivated, have been used as transformation recipients to overcome this difficulty. Here we report that gene replacements using linear double-stranded donor DNA can be achieved in wild-type *E.coli* if electrocompetent cells are used. Using a plasmid target, we obtained 10^2–10^3 gene replacement events/μg linear DNA. Using an independent chromosomal target, ~60 gene replacement events/μg linear DNA were obtained. The presence of Chi sites on the linear DNA, which are known to block DNA degradation and stimulate recombination in *E.coli*, had no effect on gene replacement efficiency in either case. RecBCD-mediated exonucleolytic activity was found to be diminished in electroporated cells. Electrotransformation thus provides a simple way to perform gene replacements in many *E.coli* strains.

INTRODUCTION

Gene targeting using linear double-stranded (ds)DNA fragments in wild-type *Escherichia coli* transformation is generally inefficient due to exonucleolytic degradation of incoming DNA. Recombination-proficient strains in which the exonucleolytic activity of RecBCD is inactivated (such as *recD*, *recB recC sbcA* and *recB recC sbcB sbcCD* mutants or strains which express bacteriophage λ recombination functions) have been used as transformation recipients to overcome this difficulty (1–4). Recently, an approach was developed to obtain gene replacement in wild-type cells, in which the transforming linear DNA contained Chi sequences (5′-GCTGGTGG-3′) at both ends flanking the homologies (3). These sequences are known to attenuate RecBCD exonuclease activity and stimulate its recombination activity (5–7). Here we report that gene replacements using linear DNA without Chi sequences can be achieved in wild-type *E.coli*, on a plasmid as well as a chromosomal target, if electrocompetent cells are used. Electrotransformation seems to reduce the exonucleolytic activity of RecBCD in *E.coli*, thus allowing gene replacement to occur. This method provides a simple way to perform gene replacement in many *E.coli* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strains and plasmids used in these experiments are described in Tables 1 and 2.

Media

LB broth and agar plates, TB, BBL agar plates, minimal medium and phage suspension medium (SM) have been described (8). Ampicillin (Amp) was used at 100 μg/ml, kanamycin (Km) at 35 μg/ml and chloramphenicol (Cm) at 15 μg/ml.

Gene replacement with a plasmid target

The plasmid target (named pΔBla) is a pBR322 derivative with a 111 bp deletion in the β-lactamase gene (*bla*). The intact *bla* gene is restored via a double exchange event with a linear DNA fragment (Fig. 1; see 9 for details of construction). In brief, primers were designed to PCR amplify a *bla* gene internal fragment covering the DNA deleted from pΔBla, plus an additional 360 bp flanking homology with *bla*. One primer couple contained double Chi sites while the other did not (9). To avoid having Chi sites at extremities (since recognition of Chi may require a minimal distance from the end), we added heterologous DNA at the ends of the linear fragment. For this purpose, the PCR fragments were cloned into a pBS derivative containing a Km-resistant (Km^R) marker (kindly provided by P. Renault, INRA, Jouy en Josas, France); the final linear fragment containing DNA internal to the *bla* gene flanked or not by Chi sites and surrounded by heterologous DNA was excised on a *Pvu*II fragment and its structure was confirmed by sequencing. Electrocompetent cells of strain TG1 carrying pΔBla were prepared and electrotransformed (resistance used 250 Ω) with the linear DNA as described (10). Cells were incubated for 90 min after electrotransformation and colony counts were performed after a 2 day incubation. Linear DNA samples were quantitated on ethidium bromide-stained agarose gels using marker DNAs of known quantities. Electrocompetence was determined by transforming cells with known amounts of supercoiled pACYC184 DNA and selecting for Cm resistance.

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Table 1. Strain list

Strain	Genotype	Source/reference
TG1	(F′ *traD36 LacI*^{q} Δ(*lacZ*)*M15*) *proA+B+*/ *supE* Δ(*hsdM-mcrB*)5($r_k^-m_k^-McrB^-$) *thi* Δ(*lac-proAB*)	
V66	*argA21 recF143 hisG4 met rpsL31 galK2 xyl-5 rac–* λ– F–	(15)
V1904	as V66 but *his+*	(3)
AC113	Δ(*argA-thyA*)*232* IN(*rrnD-rrnE*)1 λ– F–	(16)
JC9387	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 proA2 hisG4 argE3 rpsL31 tsx-33 mtl-1 recB21 recC22 sbcB* λ– F–	(15)

Table 2. Plasmid list

Plasmid	Description	Source/reference
pΔBla	pBR322 derivative with an internal deletion (*Sca*I–*Pvu*I) in the *bla* gene	(9)
pDA15	pBR322 derivative containing the *his*::*kan* insertion without Chi sites	(3)
pDA16	as pDA15 with Chi sites on both ends of the *his*::*kan* insertion	(3)
pDWS2	pBR322 derivative containing cloned *recBCD* genes of *E.coli*	(17)

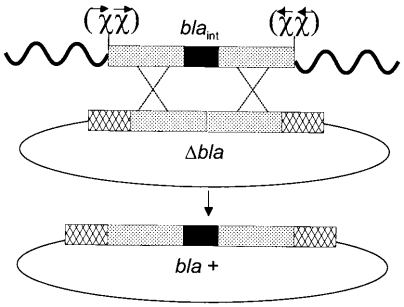

Figure 1. Gene replacement strategy (plasmid target). The gene replacement target is plasmid pΔBla, which bears an internal deletion of *bla* (Δ*bla*). Linear transforming DNA contains an internal fragment of *bla* (*bla*$_{int}$, black rectangle) which spans the *bla* deletion and has an additional 360 bp flanking homology with *bla* (gray rectangles). For the fragment Chi$^+$, double Chi sites (shown as χχ in parentheses) are present adjacent to the homologous region. Wavy lines represent heterologous dsDNA tails. Double exchange homologous recombination would be required to convert cells to Amp^R (*bla*$^+$). Hatched rectangles on pΔBla represent *bla* DNA outside homologous regions (the figure is as in ref. 9; with permission from the National Academy of Sciences USA, © 1998).

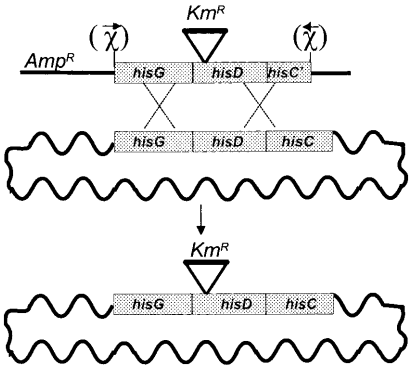

Figure 2. Gene replacement strategy (chromosomal target). The gene replacement target is the *his* operon on the *E.coli* chromosome. Linear transforming DNA contains *hisGDC*' (grey rectangle), interrupted by the Km^R gene (*Km^R*, black line). For the Chi$^+$ fragment, single Chi sites (shown as χ in parentheses) are present adjacent to the homologous regions. Wavy lines represent the chromosome. Double exchange homologous recombination produces Km^R His$^-$ Amp^S cells (3).

Gene replacement with a chromosomal target

The chromosomal target is the *E.coli* histidine synthesis (*his*) operon. Gene replacement results in the interruption of this operon by a Km^R determinant. The construction of linear DNA used for targeting is as described (Fig. 2; see 3 for details). In brief, two pBR322 derivatives were constructed with a 3 kb fragment (*hisGDC*′) of the *his* operon interrupted approximately in the middle by a Km^R determinant (*his*::*kan*) with or without Chi sites flanking the *his* fragment. These plasmids (pDA15 and pDA16) were then linearized by *Eco*RI digestion and the 6.5 kb fragment was purified (3). Electrocompetent cells of V1904 were prepared and electrotransformed (resistance used 250 Ω) with the linear DNA fragments as described (11). Cells were incubated for 1 h after electrotransformation and colony counts were performed after 24 h incubation. Km^R colonies were patched on fresh LB-Km plates and replicated onto minimal medium lacking histidine and onto LB-Amp plates. Gene replacement events were scored as Km^R, His$^-$, Amp^S transformants. Linear DNA samples were quantitated by determining the UV absorption spectrum from 220 to 340 nm using a Shimadzu UV-1201 spectrophotometer. Electrocompetence was determined by transforming cells with known amounts of supercoiled pDA15 or pDA16 DNA, selecting for Km resistance.

Assay for ATP-dependent double-strand DNA degradation

An overproducing RecBCD strain (AC113 carrying the plasmid pDWS2; 17) was prepared for electroporation using standard procedures (10) and aliquots of ~5 × 10^9 cells were frozen. Electroporation was performed in triplicate on thawed aliquots using varying resistance (0, 200, 600 or 800 Ω). Following electroporation, 1 ml of LB was added to each sample and the cells incubated at 37°C for 10 min. The cells from each electroporation condition were pooled, pelleted and crude extracts prepared as described previously (12). Extracts were assayed for ATP-dependent DNA solubilization of ^{3}H-labeled phage T7 dsDNA as described (8).

Table 3. Gene replacement frequencies in electrocompetent *E.coli* host using linear donor DNA and plasmid or chromosomal target

Strain	Supercoiled DNA	Linear DNA			
	Transformants/μg DNA	Plasmid gene replacements/μg DNA[a]		Chromosomal gene replacements/μg DNA[b]	
		Chi^0 [c]	Chi^+	Chi^0	Chi^+
TG1 (pΔBla)	3×10^9	1012	482	–	–
V1904	5×10^8	–	–	60	65

, not done.

[a]Experiments were performed with 2, 6, 12, 50, 100 or 500 ng of linear DNA. The number of transformants is proportional to the amount of DNA used within these concentrations and values shown are extrapolated to 1 μg. In total, 670 ng of either Chi^+ or Chi^0 DNA was used.

[b]Experiments were performed with 100 ng of linear DNA. Values shown are extrapolated to 1 μg. Results are means of five experiments on 3 days for the Chi^0 fragment and two experiments for the Chi^+ fragment.

[c]Chi^0 indicates DNA has *no* Chi sites. Chi^+ indicates DNA fragment contains a single or double Chi site near each end (see Figs 1 and 2 and Materials and Methods).

Bacteriophage T4 and T4 *gene2*$^-$ *in vivo* test for exonuclease activity

Strain V66 (*recBCD*$^+$) was electroporated at 0, 200 or 600 Ω as described (see above). After electroporation 1 ml of TB was added and cells were incubated at 37°C for 20 min. Aliquots of 5×10^8 cells were mixed with 2.5×10^7 particles of T4 or T4 *gene2*$^-$ phage (as assayed on strain JC9387; *recBC*) and incubated at 37°C for 10 min. The bacteria–phage mixtures were serially diluted in SM and 0.1 ml of the dilution added to 0.2 ml of *E.coli* strain JC9387 as indicator bacteria. To this, 2.5 ml of soft top agar was added and the mixture was poured onto BBL plates. After overnight incubation at 37°C the number of plaque-forming units was determined.

RESULTS

Electrotransformation allows efficient gene replacement on a plasmid target

We designed a model system to examine gene replacement on a plasmid target using linear DNA in transformed electrocompetent wild-type *E.coli*. The gene replacement plasmid target is an internally deleted β-lactamase gene (*bla*) which is present on pΔBla, a pBR322 derivative (9; Fig. 1; Materials and Methods). Restoration of *bla* on pΔBla by gene replacement with a linear molecule requires a double exchange event (Fig. 1). The linear DNA fragments used contain the internal region missing from pΔBla plus ~360 bp of adjacent *bla* DNA (Materials and Methods). Cells which have undergone gene replacement are selected as Amp^R. As Chi is known to attenuate RecBCD exonuclease activity and stimulate its recombination activity (5–7) we tested linear fragments with no Chi sites (referred as Chi^0) as well as identical fragments containing Chi sites (referred as Chi^+). On the Chi^0 fragment, heterologous DNA flanked the regions of homology; on the Chi^+ fragment, double Chi sites flanked the homology on either side, followed by the same heterologous DNA (Fig. 1). Note that no Chi sites were present on the linear DNA fragments other than those added in Chi^+ fragment. To determine the efficiency of gene replacement, we transformed electrocompetent TG1 cells containing pΔBla with the linear DNA fragments and counted the number of Amp^R transformants obtained with each DNA (Table 3).

For both fragments Chi^+ and Chi^0, 10^2–10^3 gene replacement events were obtained per μg linear DNA and we observed no significant difference in the number of gene replacement events using either fragment within a single experiment. Amp^R transformants restored the plasmid-carried *bla* gene, as confirmed by PCR (data not shown). Electrotransformation using PCR-amplified linear DNA fragments (rather than plasmid-derived linear DNA) gave similar results (data not shown). Thus, efficient gene replacement was obtained by electrotransformation with linear DNA fragments and a plasmid target. The efficiency was not altered by the presence of Chi sites.

Gene replacement on a chromosomal target by electrotransformation

To test whether *E.coli* electrotransformation also allows efficient gene replacement on a chromosomal target, we made use of a second model system in which the target was the chromosomal *his* operon. To generate the linear DNA fragment, a pBR322 derivative, containing the *his*::*kan* fragment (a 3 kb segment of the *his* operon interrupted by a Km^R determinant), was linearized by *Eco*RI restriction (Materials and Methods; 3). Homologous gene replacement of the chromosomal *his* locus with this fragment results in His^- Km^R cells. The Amp^R determinant of pBR322 is lost during gene replacement (3). The linear DNA fragments Chi^+ and Chi^0 were designed such that single Chi sites or no Chi flanked the *hisG* and *hisC′* genes (Fig. 2). Note that no Chi sites were present on the linear DNA fragments other than those added in Chi^+ fragment. To determine the efficiency of gene replacement, we transformed electrocompetent V1904 cells with the linear DNA fragments and counted the number of Km^R transformants which were His^- and Amp^S (Materials and Methods; Table 3). For both Chi^+ and Chi^0 fragments ~60 gene replacement events were obtained per μg linear DNA. These results show that gene replacement on a chromosomal target can be obtained by electrotransformation. The efficiency is not altered by the presence of Chi sites.

Table 4. Exonuclease activity of RecBCD is reduced after electroporation

Resistance used for electroporation (Ω) AC113(pDWS2)[b]	ATP-dependent dsDNA exonuclease activity (U/mg protein) V66[c]		Phage forming an infection center[a] (%)	
			T4 *gene2*–	T4
0	700	50	<4	73
200	40	7	11	79
600	12	5	58	64
800	<3	<3	–	–

–, not done.
[a]The strain used is V66. Results are means of two experiments. Total phage titers were determined on strain JC9387.
[b]Strain contains cloned *recBCD* genes. Results are means of two experiments; individual values differ by <10%.
[c]Results are from one experiment.

The exonuclease activity of RecBCD is reduced after electroporation

Our results show that the frequencies of gene replacements with linear DNA are not affected by the presence of Chi sequences on the linear fragments. This could be due to an inactivation of RecBCD nucleolytic activity during electroporation. After electroporation we measured the ATP-dependent dsDNA exonuclease activity in crude extracts of a strain overproducing RecBCD enzyme (AC113, containing the plasmid pDWS2, with the cloned *recBCD* genes). We observed a dramatic decrease in *in vitro* exonuclease activity when cells were electroporated at 200, 400 and 600 Ω (cells are electroporated at 250 Ω in routine electrotransformation protocols) (Table 4). Comparable results were obtained in an *E.coli* strain containing the chromosomal copy of RecBCD (V66). This result was confirmed *in vivo* by examining sensitivity to bacteriophage T4 *gene2*$^-$ infection. Bacteriophage T4 *gene2*$^-$ DNA is sensitive to exonuclease degradation (13) and its plaque-forming ability provides a simple test to evaluate host nuclease activity (14). A wild-type strain, which is normally resistant to bacteriophage T4 *gene2*$^-$ infection, became very sensitive upon electroporation. The infection capacity of the T4$^+$ bacteriophage, which is not sensitive to exonuclease degradation, was not altered by electroporation. Taken together, these results show that the exonuclease activity of RecBCD is diminished after electroporation.

DISCUSSION

Our results show that gene replacement events in wild-type *E.coli* can be readily selected using linear donor DNA when introduced into electrocompetent cells. This could be due to partial inactivation of RecBCD exonuclease activity: reduced degradation of the linear DNA fragment could allow the gene replacement event to occur.

Two other approaches have been recently developed to obtain gene replacement with linear DNA. The first method uses the property of Chi sites to regulate RecBCD exonuclease activity and stimulate recombination. Chi sites present near the ends of linear DNA fragments stimulate the frequency of gene replacement events when wild-type *E.coli* cells are made competent by treatment with $CaCl_2$ (3). One drawback of this method is that it requires DNA constructions that add Chi sites at the fragment extremities. The second method uses the bacteriophage λ recombination functions to stimulate gene replacement (4). Although extremely efficient, this system requires the use of a particular *E.coli* strain and thus limits its range of use.

In contrast, the method described here to obtain gene replacement can be used in many different *E.coli* strains and does not necessitate special DNA constructions. The frequencies of gene replacement events obtained (with a chromosomal target) are comparable to those obtained in the Chi-stimulated recom-bination method (3). Electrotransformation may thus constitute a straightforward method to obtain gene replacements with linear DNA in wild-type *E.coli* on plasmid and chromosomal targets. It may also be used to make gene disruptions on plasmid-carried targets which can then be transferred to the organism of interest.

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