DNA oligonucleotide-assisted genetic manipulation increases transformation and homologous recombination efficiencies: evidence from gene targeting of *Dictyostelium discoideum*

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ABSTRACT

Artificial gene alteration by homologous recombination in living cells, termed gene targeting, presents fundamental and considerable knowledge of *in vivo* gene function. In principle, this method can possibly be applied to any type of genes and transformable cells. However, its success is limited due to a low frequency of homologous recombination between endogenous targeted gene and exogenous transgene. Here, we describe a general gene-targeting method in which co-transformation of DNA oligonucleotides (oligomers) could significantly increase the homologous recombination frequency and transformation efficiency. The oligomers were simply designed such that they were identical to both the ends of the homologous flanking regions of the targeting construct. Using this strategy, both targeted alleles of diploid cells were simultaneously replaced in a single transformation procedure. Thus, the simplicity and versatility of this method applicable to any type of cell may increase the application of gene targeting.

Key words: *Dictyostelium discoideum*, homologous recombination, gene targeting, transformation.

INTRODUCTION

As the number of completely sequenced organisms markedly increases, the need for elucidating gene function also increases. Targeted gene alteration and elimination, known as gene targeting, is the most direct way to analyze the function of genes. Furthermore, gene-targeting technology potentially enables the therapeutic replacement of an endogenous aberrant gene with a normal exogenous transgene. However, despite its significance, gene targeting is not yet versatile because its efficiency predominantly depends on the intrinsic homologous recombination frequency, which is generally not sufficiently high. Due to this low homologous recombination frequency, gene targeting can only be achieved when a massive amount of screening work is performed for a few homologous recombinants from a large pool of transformants (Sedivy et al., 1999).

Previous studies have reported some methods to increase the homologous recombination frequency, and these methods were based on the fact that double-strand break at the targeted genome region dramatically increases the homologous recombination efficiency (Urnov et al., 2005). This can be achieved by fusing a nuclease with engineered C_2H_2 zinc finger protein-based DNA binding domains (ZFN), which ensure the sequence-specific binding and site-directed double-strand break. Limitations of this method are imposed by the effort required to accurately and carefully design the zinc finger DNA binding domain fused nuclease, because binding specificity and affinity are the critical determinants of recombination efficiency. Furthermore, simultaneous expression of ZFN with a gene-targeting construct is indispensable.

Triplex-forming oligonucleotides (TFOs) can induce a DNA double-strand break and repair system and thus, increase the homologous recombination efficiency *in vivo* (Seidman and Glazer, 2003). However, TFOs that bind to double-stranded DNA are restricted to the polypurine or polypyrimidine tract; therefore, this technology is limited to segments with unique target sites.

A high concentration of gene-targeting construct generally provides considerably better transformation and homologous recombination efficiencies, although the amount of gene-targeting construct that can be used at transformation is limited. In this study, to overcome this limitation, we investigated whether only a part of the homologous region of a gene-targeting construct is effective. We report that simply designed oligomers homologous to both the ends of the gene-targeting construct can significantly improve the efficiencies of homologous recombination and transformation, regardless of their triplex-forming abilities and sequence specificities.

MATERIALS AND METHODS

Materials

Blasticidin S was purchased from Funakoshi Co., Japan. Salt-free deoxyoligonucleotides (oligomers) without the 5′-phosphate were synthesized by Nippon EGT (Toyama, Japan). The sequences of oligomers used for gene targeting and transformant verification are listed in Table 1. All the PCR procedures were performed using the Expand Long Template PCR system (Roche Diagnostics) or TAKARA LA Taq (TAKARA Bio Inc.).

Cell culture

Dictyostelium discoideum AX2 (a haploid strain) and dIR1 (a diploid strain; a gift from Dr. Jason King and Dr. Robert Insall) cells were cultured at 21°C in HL5 medium with 100 μg/ml streptomycin sulfate and 100 units/ml benzylpenicillin potassium, as previously described (Kuwayama et al., 2002). For selecting the transformants, the HL5 medium was supplemented with 10 μg/ml blasticidin S.

Gene-targeting constructs

All gene-targeting constructs were prepared using the drug selection system by involving the blasticidin S resistance gene (*bsr*) (Sutoh, 1993). Gene-targeting constructs for the cAMP-dependent protein kinase catalytic subunit *pkaC* (Gene ID in Entrez, 3390367) and the G-box-binding factor *gbfA* (Gene ID in Entrez, 3388141) were assembled, as described previously (Kuwayama et al., 2002); the resultant gene-targeting constructs had approximately 1-kb long 5′ and 3′ flanking regions.

Gene-targeting constructs for *D. discoideum* cortexillin A, i.e., *ctxA* (Gene ID in Entrez, 3387750) and cortexillin B, i.e., *ctxB* (Gene ID in Entrez, 3394130) were assembled, as described previously (Kuwayama and Nagasaki, 2007, in press).

In order to obtain the yield required for gene-targeting transformation, the constructs were amplified by large-scale PCR by using oligomer In and oligomer In′ (Fig. 1A and Table 1). The gene-targeting constructs (10 μg) were ethanol precipitated and dried. An electroporation buffer (400 μl; 10 mM $NaPO_4$ and 50 mM sucrose, pH 6.1) with or without oligomers was added to dissolve the dried construct, and the resultant suspension was used for transformation. The oligomer sequences and concentrations are indicated in Table 1 and in each figure legend, respectively.

Transformation and selection of transformants

The AX2 and dIR1 cells were transformed by electroporation, using a previously reported method with minor modifications (Kuwayama et al., 2002).

Immediately before electroporation, 10 μg of the precipitated gene-targeting construct was dissolved with the oligomers at the indicated oligomer concentration and 2.0×10^7 cells in 400 μl electroporation buffer. The sequences of the used oligomers are shown in Table 2. After the electroporated cells were adapted in 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at 21°C for 15 min, 40 ml HL5 medium was added, and 100 μl of this suspension was immediately added to each well of 4 flat-bottom 96-well plates. After 24 h, 100 μl HL5 medium containing 20 μg/ml blasticidin S was added to each well; this resulted in a final concentration of 10 μg/ml blasticidin S. All the clones obtained by drug selection were observed through a microscope to examine whether multiple colonies are formed in a single well. In the selection of the *pkaC* gene disruption transformants, we observed double or triple clones in a single well at the rate of 1–3 wells/96-well plate. In this case, we counted the number of clones as 2 or 3 in a well but excluded these clones from the calculation of gene-targeting efficiency because of possible heterogeneity of the clones.

Calculation of gene-targeting efficiency

Since both *pkaC* and *gbfA* null cells do not aggregate during development, gene targeting was assessed by analyzing the phenotype of the transformant clones. The aggregateless morphogenetic phenotype of each clone was assessed by plating the cells on a 5LP medium plate (0.5% lactose and 0.5% DIFCO peptone with 1.5% agar) with *Klebsiella aerogenes*. Gene-targeting efficiencies were calculated by dividing the number of aggregation-deficient clones by the total number of *bsr* clones, because cells lacking one of these genes were identified as

aggregation-deficient strains (Mann and Firtel, 1991; Schnitzler et al., 1994). Several aggregateless clones were selected and confirmed to be gene targeted clones by genomic PCR using the primer set ExIn and In′ for *pkaC* and primer set In and ExIn′ for *gbfA*, as previously reported (Table 1) (Kuwayama et al., 2002). PCR reactions were performed as follows: heating at 94°C for 2 min, 30 cycles at 94°C for 30 sec, 50°C for 20 sec, 65°C for 3 min, followed by a final extension for 3 min using approximately 10 ng genomic DNA.

ctxA and *ctxB* gene disruption was confirmed by genomic PCR (Table I). The primer sets used were designed such that they were homologous to the *bsr* gene (5′-AAGTAGCGACAGAGAAGATT-3′ for *ctxA* and 5′-GGGTATATTTGAGTGGAATG-3′ for *ctxB*) and downstream of the 3′ end of the *ctxA* (5′-AATTAGGTTATCATTGTACAAG-3′ for *ctxA*) or *ctxB* (5′-AATAATGGCCATTATTGAGGG-3′ for *ctxB*) clones (Kuwayama and Nagasaki, in press). The PCR reaction was followed by the method described above.

Results

The inward oligomers increase the efficiency of integration into the genome

The effect of the short DNA oligomers at both the ends of the gene-targeting constructs on the efficiency of integration into the genome (transformation efficiency) by electroporation was examined using cells of *D. discoideum*, a cellular slime mold. In this study, 4 genes were selected as the target genes, namely, *pkaC* (Mann and Firtel, 1991) and *gbfA* (Schnitzler et al., 1994) and the actin-bundling genes *ctxA* and *ctxB* (Faix et al., 1996). The transformation efficiency for each target gene varied considerably. With regard to the control experiments (no oligomers), an average of approximately 140 *bsr*-resistant clones were obtained by electroporation of 2.0×10^7 haploid AX2 cells with 10 μg *pkaC* gene-targeting construct. On the other hand, the *gbfA*, *ctxA*, and *ctxB* gene-targeting constructs provided only an average of approximately 8, 3, and 2 clones, respectively (Fig. 2A).

The oligomers used in this study were designed such that they had 20–24 monomers, and the sequences at both the ends were identical to those of the flanking regions, i.e., in the inward direction (oligomers In and In′; Fig. 1 and Table 1) or the outward direction (oligomers Out and Out′, complementary to the corresponding inward oligomers). When the AX2 cells were co-transformed with 100 μM of the 2 inward-directed oligomers In and In′, the transformation efficiency increased in all cases (Fig. 2A). These results indicate that the co-transformation of the designed homologous oligomers increases the transformation efficiency.

Next, the dose dependency of the effect of the added oligomers was examined using *pkaC* and *gbfA* because these lack the polyuridine or polypyrimidine tract (Table 1). For both the genes, oligomer concentrations up to 10 μM had little effect on the transformation efficiency. At 50 μM, a significant increase in efficiency was observed (Fig. 3A). Addition of the outward oligomers Out and Out′ had no effect on the transformation efficiency during the *pkaC* gene targeting, but they were effective during the *gbfA* gene targeting (Fig. 3A). Furthermore, a high concentration of only a single oligomer was not significantly effective in both the cases. These results indicate that the direction and the oligomer set both influence the transformation efficiency.

Oligomers also increase homologous recombination efficiency

We attempted to determine the number of clones among the isolated transformants examined in all the 4 cases that had undergone homologous recombination. When the inward oligomers In and In′ were co-transformed at a concentration of 100 μM, the homologous recombination efficiency increased significantly in all the 4 cases (Fig. 2B). For the low-efficiency homologous recombination genes *gbfA*, *ctxA*, and *ctxB*, the increase in the ratio of homologously recombined clones to transformed clones was particularly more significant than that FOR the high-efficiency homologous recombination gene *pkaC*. The dose dependency of the effect of the oligomers was examined for *pkaC* and *gbfA*. With regard to *pkaC*, an increase in the homologous recombination efficiency was observed at oligomer concentrations greater than 50 μM; however, with regard to *gbfA*, an oligomer concentration of 10 μM was also significantly

effective (Fig. 3B). In *pkaC* transformation, the addition of outward oligomers caused a slight increase in the homologous recombination efficiency. On the other hand, with regard to *gbfA* transformation, the outward oligomers did not exhibit any effect at a concentration of 100 μM. Furthermore, a high concentration of only a single In oligomer was not significantly effective in *pkaC* transformation, although the homologous recombination efficiency in *gbfA* with oligomer In′ increased as much as that with the inward oligomer set. These results suggest that the co-transformation of the inward oligomer set generally increases the homologous recombination efficiency, but the effect of only a single inward oligomer and the outward oligomers varies depending on the targeted gene.

Homozygous gene targeting in diploid cells

In principle, the use of gene-targeting technology for haploid strains is relatively easy due to the presence of only a single gene set in its genome. On the other hand, diploid cells require homozygous gene targeting. To achieve this, sequential gene targeting or genetic cross-linking of the heterozygous strain is necessary. We examined if oligomer addition is adequately effective in facilitating homozygous gene targeting in the diploid dIR1 cells obtained from *D. discoideum* (King and Insall, 2003). The *pkaC* and *gbfA* gene-targeting constructs were used to transform dIR1 cells. Homozygous gene disruption results in an aggregation-deficient phenotype because these 2 genes are essential during the early stages of development. When transformation was performed without oligomers, no aggregation-deficient clones were isolated (Table 2). However, when 100 μM of oligomers In or In′ was added, approximately 12% and 10% of the

transformants were aggregation-deficient clones, respectively, and were identified as homozygous gene-targeted clones by genomic PCR (Fig. 1B). The outward oligomers had no effect on the homozygous gene-targeting efficiency in either of the gene transformations. These results indicate that addition of the inward oligomers adequately increases the homologous recombination efficiency, thereby facilitating the isolation of homozygous gene-targeted clones of *D. discoideum* diploid cells.

Discussion

Our results indicate that the short DNA oligomers homologous to both the ends of the gene-targeting constructs can significantly increase transformation and homologous recombination efficiencies. Hence, a single transformation procedure yields simultaneous gene targeting in homozygous cells. The inward oligomers lead to an increase in transformation efficiency because they are probably used as “primers” for gene amplification by endogenous DNA polymerase. On the other hand, the reason underlying the increase in the homologous recombination efficiency after oligomer addition remains unknown, because oligomers lacking TFO signature sequences are also effective. This indicates that the mechanism by which our method results in improvements may be different from that of TFO-dependent homologous recombination. Furthermore, when genes with relatively low transformation and homologous recombination efficiencies are targeted, the co-transformation of oligomers appears to increase their homologous recombination efficiencies as much as that of a gene with a high homologous recombination efficiency, which is also difficult to explain. However, the high concentration of the oligomers may be effective in increasing the possibility of their binding to the open intrinsic chromosome, particularly in genes that are difficult to target, and in producing DNA double-strand breaks or increasing the accessibility of the donor DNA. Furthermore, the gene-targeting constructs may be amplified in the cell because of the simultaneous increase in the transformation efficiency.

When 10 μg of a gene-targeting construct is dissolved in 400 μl electroporation buffer, the concentration of the resultant suspension is approximately 10–13 nM. In this study, we added oligomers at concentrations up to 100 μM, which is approximately 7,700–10,000 times more than that of the gene-targeting constructs. Thus, using a substantially higher concentration of oligomers may produce better results. However, dissolving a higher concentration of oligomers in the electroporation buffer is difficult and may not be practical due to the high viscosity of the solution. On the other hand, another matter of concern was that the high concentration of oligomers might cause random integration of oligomers themselves into the genome, which might lead to potential second mutation. Methanol-resistant recessive mutation caused by disruption of *acrA* was a good marker to investigate this issue (Garcia et al., 2002). Approximately 500 clones isolated from each control and the co-transformation of inward oligomers at a high concentration with the *pkaC* gene-targeting construct did not produce a methanol-resistant phenotype. This indicates that a high concentration of oligomer is not likely to substantially increase the rate of potential second mutation.

In this study, we fixed the length of the oligomers between 19 and 24 monomers, because a longer oligomer usually increases the rate of secondary structure that interferes with its specific binding, and a short oligomer decreases its specificity. However, the optimal length might vary depending on genes and cells. Testing the variable length of oligomers may provide optimal efficiencies of transformation and homologous recombination.

In principle, the oligomer-aided homologous recombination presented in this study can be applied to any general transformation method, including microinjection and lipofection methods, and to various cell lines, including mammalian transformed and primary somatic cells. We observed a significant increase in the gene-targeting efficiency in the Hela cells (Kuwayama and Sako, unpublished results); therefore, we consider this method applicable for general procedures in gene targeting even for mammalian somatic cells. Further improvement in this method is expected to result in practical and clinically safe therapeutic modifications of human cells by using artificial nucleic acid analogues such as peptide nucleic acid (PNA) and 2′-O and 4′-C locked nucleic acid (LNA). The use of these analogues is expected to provide a higher homologous recombination frequency with a lower oligomer concentration because PNA and LNA have an increased affinity to native DNA and a high resistance to nucleases, thereby imparting higher biostability (Demidv, 2003).

ACKNOWLEDGEMENTS

We thank Dr. King, J. and Dr. Insall R.H. for providing us with the *Dictyostelium discoideum* diploid strain dIR1 and Dr. Hideko Urushihara for the helpful discussions. This work was supported by Grants-in-Aid for Scientific Research (S) (no. 15109003) from the Japan Society for the Promotion of Science and by a special fund for tenure-track faculty members of the Institute of Biological Sciences at the University of Tsukuba.

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Figure Legends

Fig. 1. Gene-targeting construct, homologous recombination, and genomic PCR of the transformants. (a) Diagrammatic representation of the gene disruption constructs and location of the DNA oligomers used in this study. The *bsr* cassette was inserted into the ORF region of the target genes with approximately 1 kbp 5′ and 3′ flanking regions for *pkaC* and *gbfA* and 0.7 kbp 5′ and 3′ flanking regions for *ctxA* and *ctxB*. The locations of the oligomers used for transformation are represented by solid arrows. (b) Genomic PCR of the transformants obtained from the AX2 (haploid) and dIR1 (diploid) strains by *gbfA* gene targeting. Rand and HR represent randomly inserted clone and homologously recombined clone, respectively. Solid arrows indicate the oligomers used for the gene-targeting experiments and genomic PCR. Dashed arrows indicate the primers used for the genomic PCR.

Fig. 2. Effect of the homologous oligomers on the transformation and homologous recombination efficiencies. (a) Transformation efficiencies were examined for the *pkaC*, *gbfA*, *ctxA*, and *ctxB* gene-targeting constructs. The data are represented as the number of primary transformants per transformation (2×10^7 cells). White bars represent transformation without oligomers. Hatched bars represent transformation with 100 μM inward oligomers In and In′. (b) Homologous

recombination efficiencies of the gene-targeting construct without the oligomers (white bar) and with 100 μM oligomers (hatched bar) are represented as an average percentage of the gene targeted transformants to the total number of transformants. Bars represent standard deviation (SD) of 3 independent transformations.

Fig. 3. Dose dependency of the transformation efficiencies and the effect of sequence direction of the homologous oligomers on transformation efficiencies. (a) Transformation efficiencies of *pkaC* and *gbfA* with the addition of inward oligomers at various concentrations, 100 μM outward oligomer set, and one of the inward oligomers (oligomer In for *pkaC* and oligomer In′ for *gbfA*) (b) Homologous recombination efficiencies of *pkaC* and *gbfA* with the addition of the inward oligomers at various concentrations, and 100 μM outward oligomers, and one of the inward oligomers (oligomer In for *pkaC* and oligomer In′ for *gbfA*). Open and hatched bars indicate *pkaC* and *gbfA* transformation, respectively. Bars represent SD of 3 independent transformations.

Figure 1a

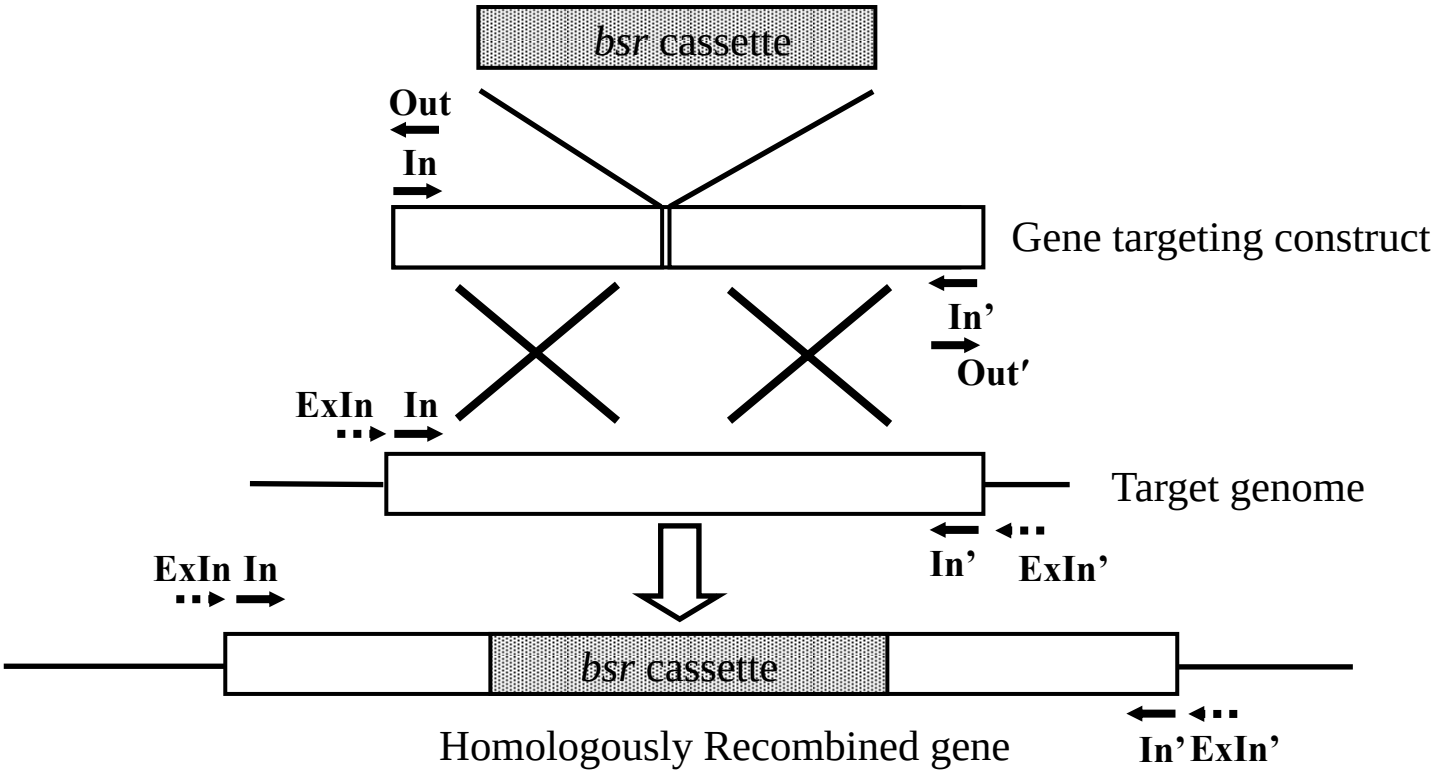
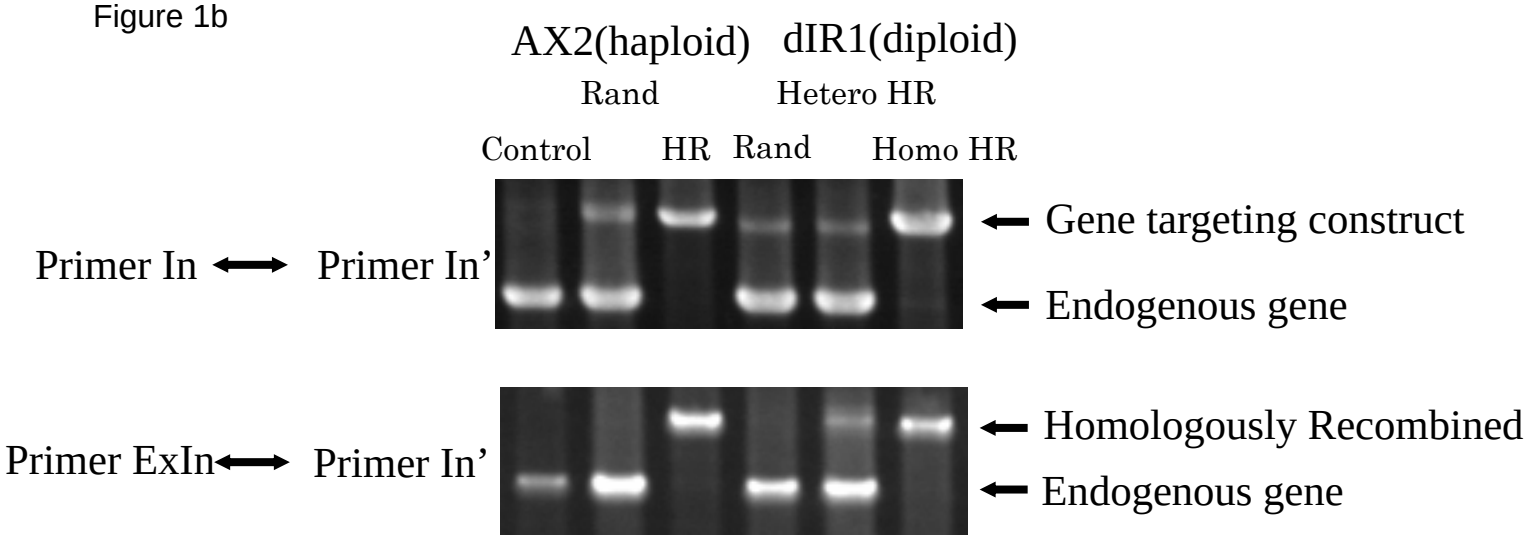


Figure 1b



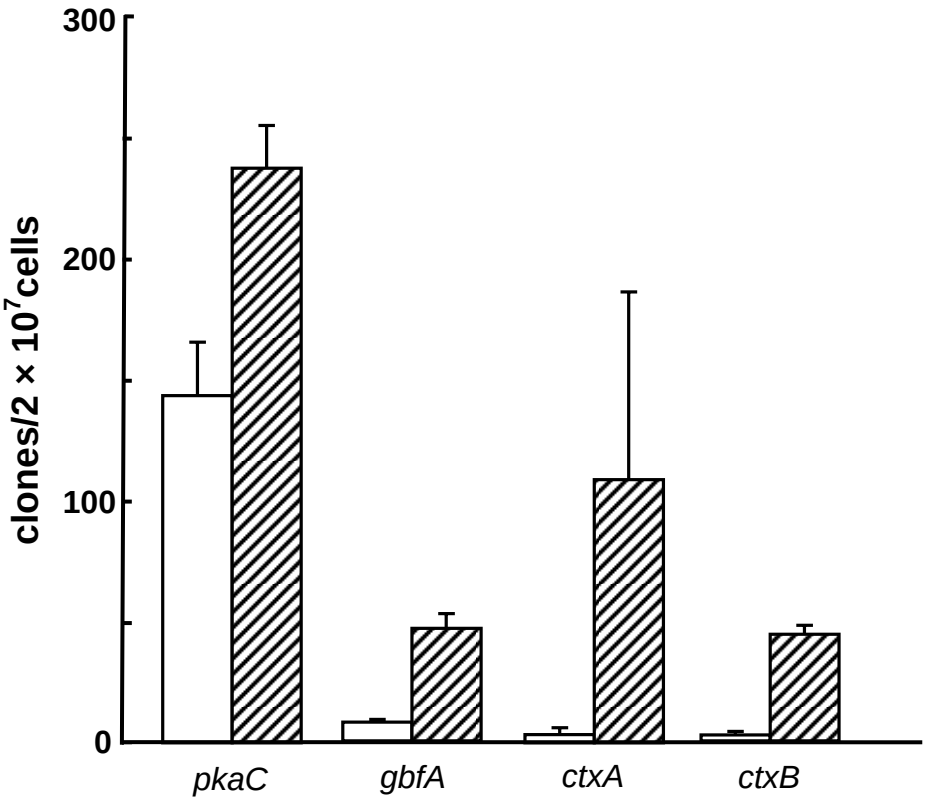


Figure 2a

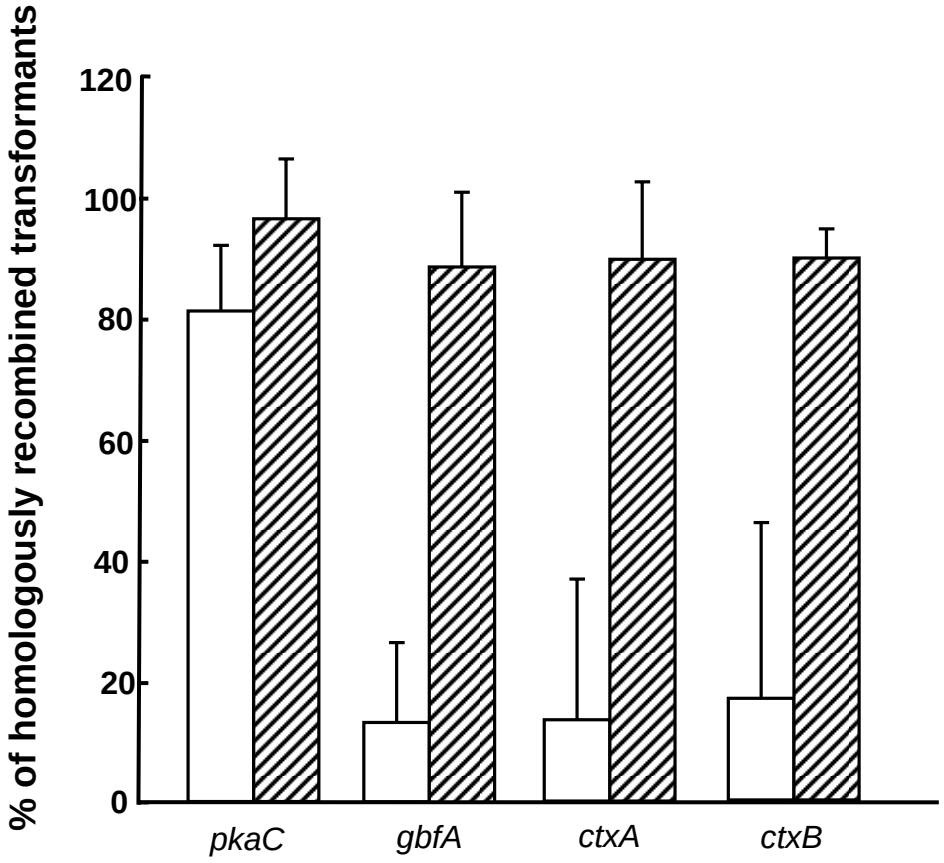

% of homologously recombined transformants
120
100
80
60
40
20
0
pkaC
gbfA
ctxA
ctxB

Figure 2b

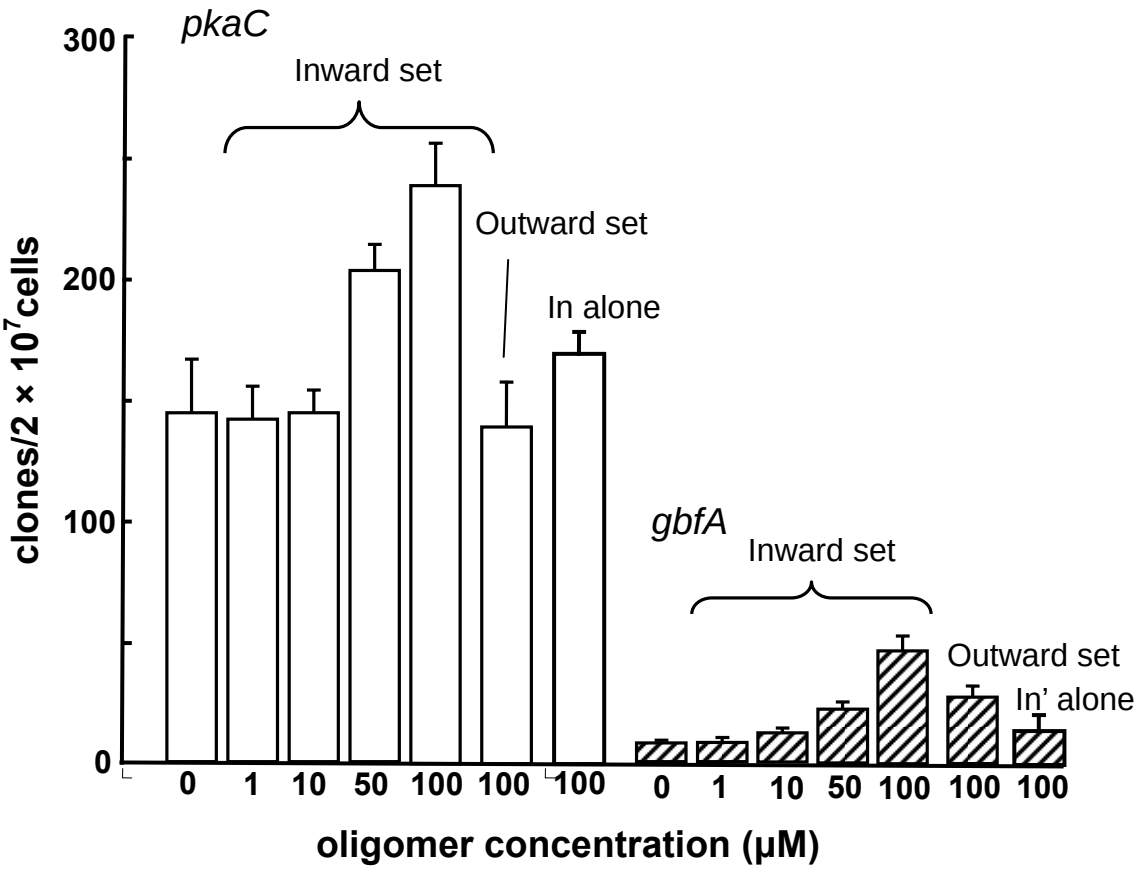

pkaC
Inward set
Outward set
In alone
gbfA
Inward set
Outward set
In' alone
clones/2 × 10^7 cells
300
200
100
0
0 1 10 50 100 100 100
0 1 10 50 100 100 100
oligomer concentration (μM)

Figure 3a

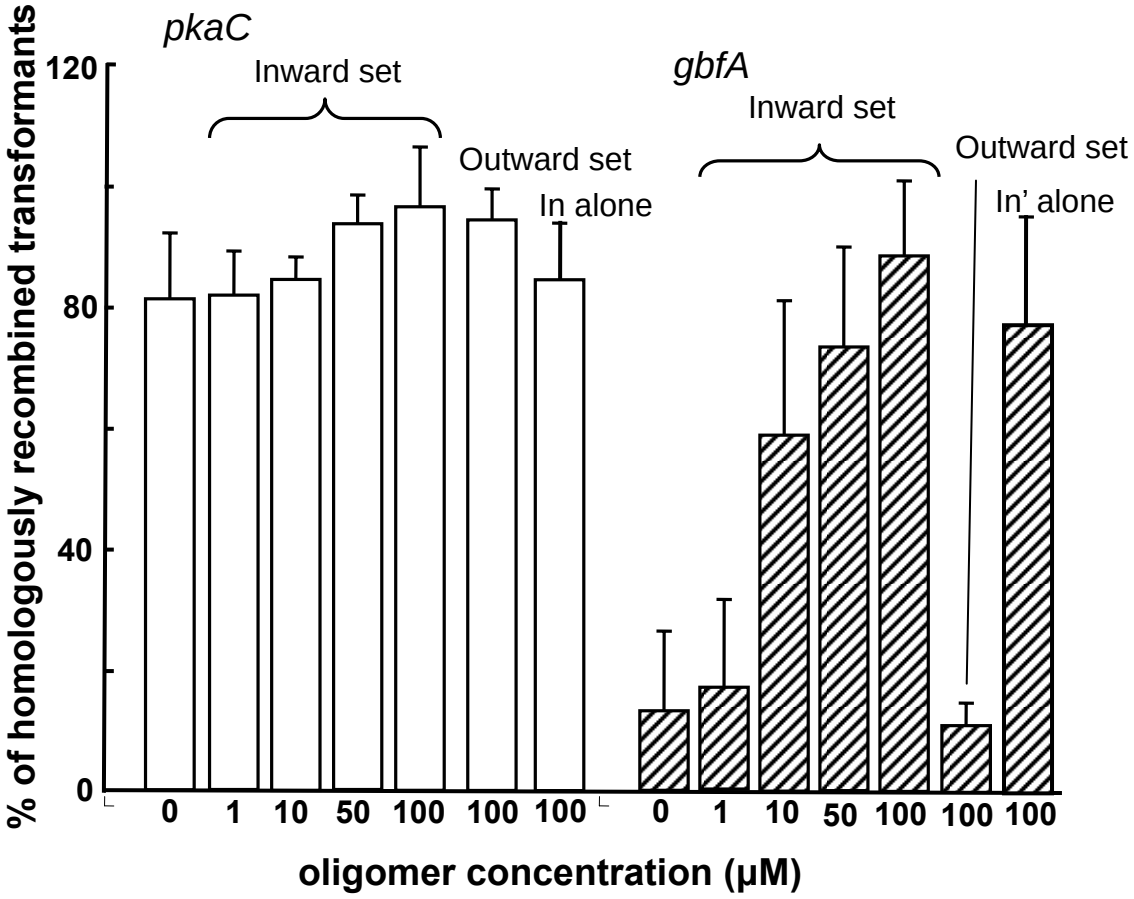

pkaC
gbfA
Inward set
Outward set
In alone
Inward set
Outward set
In' alone
% of homologously recombined transformants
120
80
40
0
0 1 10 50 100 100 100
0 1 10 50 100 100 100
oligomer concentration (μM)

Figure 3b

Table 1 Oligomers used in this study

Target gene	Oligonucleotide Oligomer	Sequence
pkaC	In	5′-CATGAGTAACTCAAATAATAATAG-3′
	In’	5′-CCTTGAAAAGATGTGCATATGGAT-3′
	ExIn’	5′-CATTAAACCCAACACATTTATTG-3′
gbfA	In	5′-CACAGACAATTGGCGGATCGG-3′
	In’	5′-CTATCACCATCAGGTATAATTG-3′
	ExIn	5′-GTTATCAACTCATCATCACC-3′
ctxA	In	5′-ATGGCAGGTAAAGATTGGG-3′
	In’	5′-TTATTTTTTTGATTTTGATG-3′
	ExIn’	5′-AATTAGGTTATCATTGTACAAG-3′
ctxB	In	5′-ATGGATTTAAATAAAGAATG-3′
	In’	5′-TTATTTTTTAGCAGCAGCTT-3′
	ExIn’	5′-AATAATGGCCATTATTGAGGG-3′

Table 2 Gene targeting in dIR1 (diploid)

Target gene	Co-transformed oligomers	Concentration of co-transformed oligomers (μM)	Number of transformants (among 2×10^7 cells)	Number of homothalic homologous recombinants (−/−) (%transformants)
pkaC	None	0	130 ± 8	0 ± 0
	In and In’	100	223 ± 7	28 ± 3
	Out and Out′	100	143 ± 6	0 ± 0
gbfA	None	0	3 ± 1	0 ± 0
	In and In’	100	10 ± 4	2 ± 1
	Out and Out′	100	3 ± 1	0 ± 0

The results represent the mean ± standard deviation of 3 independent experiments.