Tandem repeat coupled with endonuclease cleavage (TREC): a seamless modification tool for genome engineering in yeast

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ABSTRACT

The complete synthetic Mycoplasma genitalium genome (~583 kb) has been assembled and cloned as a circular plasmid in the yeast Saccharomyces cerevisiae. Attempts to engineer the cloned genome by standard genetic methods involving the URA3/5-fluoroorotic acid (5-FOA) counter-selection have shown a high background of 5-FOA resistant clones derived from spontaneous deletions of the bacterial genome maintained in yeast. Here, we report a method that can seamlessly modify the bacterial genome in yeast with high efficiency. This method requires two sequential homologous recombination events. First, the target region is replaced with a mutagenesis cassette that consists of a knock-out CORE (an18-bp I-SceI recognition site, the SCEI gene under the control of the GAL1 promoter, and the URA3 marker) and a DNA fragment homologous to the sequence upstream of the target site. The replacement generates tandem repeat sequences flanking the CORE. Second, galactose induces the expression of I-SceI, which generates a double-strand break (DSB) at the recognition site. This DSB promotes intra-molecular homologous recombination between the repeat sequences, and leads to an excision of the CORE. As a result, a seamless modification is generated. This method can be adapted for a variety of genomic modifications and may provide an important tool to modify and design natural or synthetic genomes propagated in yeast.

INTRODUCTION

The yeast Saccharomyces cerevisiae has been developed as a host capable of cloning large DNA fragments, as both linear and circular yeast artificial chromosomes (YACs) (1,2). Once cloned in yeast, YACs can be manipulated using standard yeast genetic tools. Transfer of this modified DNA back to host cells allows the functional study of genes and their regulation (1–3). Recent progress on the cloning of whole bacterial genomes in yeast, and subsequent transplantation of such genomes back into their original cellular environments (4), has extended this application from the gene to the genome level.

One common technique for DNA modification in yeast is gene replacement with a counter-selectable marker that can be subsequently removed. This usually involves two homologous recombination events. First, a counter-selectable marker is recombined into a target site. Second, a DNA fragment containing the desired alteration is recombined in place of the marker. The most frequently used marker in this procedure is the URA3 gene, which restores uracil prototrophy. Counter-selection for the replacement of the URA3 maker is performed by treatment with 5-fluoroorotic acid (5-FOA) (5). This method is of particular importance for two reasons. First, it restores uracil auxotrophy, which can then be used again for a further round of modification. Second, it creates a seamless modification. The basic URA3 replacement method has been improved in a number of ways. One improvement, the tandem repeat pop-out method, is widely used for gene deletion and subsequent removal of a counter-selectable marker to produce a seamless genome modification (6–10). Another approach is to utilize the formation of a double-strand break (DSB), generated by the rare-cutting endonuclease enzyme I-SceI, near the targeted locus to stimulate the efficiency of homologous recombination repair (11). The methods described above can be adapted for deletions, point mutations or gene replacements.

We have previously demonstrated the assembly and cloning of the synthetic Mycoplasma genitalium genome as a circular YAC in yeast (12,13). This potentially allows us to use yeast as a platform to directly engineer or redesign synthetic bacterial genomes in vivo. At first, we...
attempted to engineer the synthetic *M. genitalium* genome in yeast by the traditional two-step method of replacement and a tandem repeat pop-out method to produce a point mutation and a deletion, respectively. Unfortunately, we were not able to isolate clones with the desired modifications in the *M. genitalium* genome due to a high background of non-specific loss of the *URA3* marker during the course of manipulations. Therefore, we designed a method to place both tandem repeat sequences and a DSB near the target site to enhance the efficiency of target-specific recombination. We have termed this the TREC (tandem repeats coupled with endonuclease cleavage) method, and find that it can be used to seamlessly engineer bacterial genomes that are cloned in yeast.

**MATERIALS AND METHODS**

**Yeast strain and media**

*Saccharomyces cerevisiae* yeast strain VL6-48N (*MATα his3Δ200 trp1Δ1 ura3Δ1 lys2 ade2-101 met14*) housing a 0.6 Mb *Mycoplasma genitalium* whole genome YAC was constructed previously (12). Yeast cells were grown in standard rich medium containing glucose (YEPD) or galactose (YEPA); or in synthetic minimal medium containing dextrose (SD) or galactose (SG) (14). SD medium supplemented with 5-fluoroorotic acid (5-FOA) was used to select for loss of the *URA3* marker (5).

**Preparation of mutagenesis cassettes**

Primers used for construction of all mutagenesis cassettes are listed in Supplementary Table S1. They were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primers longer than 60 bp were purified by polyacrylamide gel electrophoresis. All polymerase chain reactions (PCRs) were performed with Takara Ex *Taq* DNA polymerase (Takara Bio Inc.) using the conditions recommended by the manufacturer. The *URA3* marker (1066 bp) was amplified from the plasmid pRS306 (15); the 1184-bp fragment containing the *GAL1* promoter (450 bp) was amplified from the plasmid pYES2 (Invitrogen, Carlsbad, CA, USA); the 1184-bp fragment containing the *GAL1* promoter and the *SCEI* gene was amplified from the plasmid pGSKU (11); and the *Cre* recombinase gene (1032 bp) was amplified from the plasmid pBS185 (16). Assembly of linear DNA fragments were performed by a PCR-fusion technique (17). In each case of PCR-based fusion, complementary ends overlapped by 40 bp (Supplementary Table S1). To generate each final mutagenesis cassette, a fusion product was PCR-reamplified with chimeric primers, each containing 50 bp of homology to the target site (Supplementary Table S1). The final constructs are illustrated in Figures 1A, 2A, 15 and 28.

**Transformation and PCR analysis**

Lithium acetate integrative transformation was performed according to a published method (18). Two to three micrograms of integrative construct DNA and 25 μg of carrier DNA (salmon testis DNA, Sigma) were used in routine experiments. Isolation of total DNA from yeast for PCR analysis was performed according to a published protocol (8). Correct integration of each mutagenesis cassette was verified by PCR using diagnostic primers located upstream and downstream of the target site (described in figure legends). Multiplex PCR (MPCR) was used to confirm completeness of *M. genitalium* genomes as described previously (13). The primer set (set 3) used for MPCR was designed to produce 10 amplicons (ranging from 125 to 1025 bp in 0.1-kb increments) distributed around the *M. genitalium* genome approximately every 60 kb (13).

**RESULTS**

**Nonspecific deletions in a bacterial genome cloned in yeast**

We attempted to correct a point mutation in the MG259 locus of a synthetic *M. genitalium* genome maintained in yeast by the traditional method involving two homologous recombination procedures (Figure 1A). After the first homologous recombination, the exact replacement of a target region with the *URA3* marker was confirmed by PCR (data not shown). After the second round of homologous recombination, however, we were not able to identify the correct replacement of the *URA3* marker with the 328-bp DNA segment by PCR screening from 97 5-FOA resistant colonies (Figure 1B and Table 1). These results suggest that the loss of the *URA3* marker might be due to unexpected deletions. The *M. genitalium* genome propagated as a circular YAC in yeast does not have functional complementation with its host, except histidine prototrophy. Any deletions or rearrangements in the bacterial genome are likely neutral for the yeast viability. Multiplex PCR was used to evaluate the integrity of the *M. genitalium* genome in yeast. The primer set was designed to produce 10 amplicons (ranging from 125 to 1025 bp in 0.1-kb increments) distributed around the *M. genitalium* genome approximately every 60 kb. Total DNA prepared from twenty-two 5-FOA-resistant colonies did not produce all 10 amplicons (Figure 1C). Two amplicons, 0.525 kb and 0.625 kb (separated from each other by ~60 kb), were absent in all clones. The MG259 locus lies between these two amplicons. This result demonstrates that some spontaneous deletions or rearrangements occur in the *M. genitalium* genome propagated in yeast. The loss of the *URA3* marker could result from homologous recombination among repetitive sequences in the *M. genitalium* genome. As a result, the cells with spontaneous deletions of the *URA3* gene could survive on 5-FOA medium. In this case, the probability of nonspecific loss of the *URA3* gene was higher than that of the *URA3* gene replacement by the incoming DNA fragment (Figure 1D). In agreement with this hypothesis, the frequency of nonspecific loss of the *URA3* gene placed in the MG259 locus was ~0.3% (data not shown).

We also tried to make a seamless deletion of a 450-bp region within the MG259 locus by the tandem repeat pop-out approach (Supplementary Figure S1). Again, we were not able to find the correct modification by PCR screening (Table 1). Multiplex PCR showed that eight out of nine 5-FOA resistant clones contained incomplete
Therefore, we concluded that these methods were not efficient enough to engineer the *M. genitalium* genome in yeast and that the 5-FOA-resistant cells were likely derived from cells that nonspecifically lost the *URA3* marker during the course of manipulations.

**Combination of TREC**

DSBs have been introduced near the targeted-locus to stimulate the efficiency of homologous recombination repair in yeast and higher eukaryotic cells (11,19–22). Therefore, we believed that the frequency of recombination between two tandem repeats should be enhanced by a DSB near the target site. A new mutagenesis construct was designed. It contains a CORE cassette (consisting of the 18-bp I-SceI recognition site, the *GAL1* promoter, a gene encoding the I-SceI endonuclease and the *URA3* gene) and 378 bp of DNA homologous to the region upstream of the target site. Two terminal sequences homologous to the target site were added into the construct by PCR (Supplementary Table S1). Replacement of the 450-bp target region with this construct would...
produce two repeat sequences flanking the CORE, and homologous recombination between the repeat sequences would result in a seamless deletion (Figure 2A).

Following transformation of the mutagenesis construct into yeast, the expression of I-SceI endonuclease was induced by galactose. After a 2-day incubation, cells were replica-plated onto SD-His+ 5-FOA plates. Cells with galactose induction produced significantly more colonies on SD-His+ 5-FOA medium than un-induced cells (Figure 2B). 5-FOA-resistant cells derived from both induced and un-induced cells were re-streaked, and single colonies were then selected and analyzed. Transformants with the correct deletion were identified by PCR screening. DNA with precise removal of the CORE cassette would result in the generation of a proper PCR product. (Figure 2C left panel) suggests that all 24 galactose-induced clones contain the correct modification in the M. genitalium genome, while only two PCR-positive clones isolated from un-induced cells do (Figure 2C, right panel). Furthermore, the integrity of the M. genitalium genomes was evaluated by multiplex PCR. DNA from 10 galactose-induced clones produced the complete set of 10 amplicons (left panel, Figure 2D). DNA from un-induced clones did not generate the complete set of 10 amplicons (Figure 2D, right panel). Hence, results from both PCR analyses demonstrate that the TREC method can perform a seamless deletion on a bacterial genome cloned in yeast with high efficiency (Table 1).

The Cre-loxP system has been demonstrated to produce marker excision in yeast with high efficiency (23). Therefore, we compared the efficiency of the TREC method with that of the Cre-loxP system for gene deletion in a M. genitalium genome cloned in yeast. The Cre-loxP mutagenesis construct consists of the Cre gene under the control of the GAL1 promoter, the URA3 gene, and two mutant loxP sites flanked by the two terminal sequences homologous to the target site (Supplementary Figure S2). The mutant loxP sites prevent a reverse recombination event (24). The same region deleted by TREC was targeted by this construct. Following transforming, Ura^- clones were grown on galactose medium to induce the expression of Cre recombinase. In turn, this excises most of mutagenesis cassette, including the URA3 gene, but leaves a 34-bp mutant loxP element in the target site. PCR analysis showed that 93% (28/30) of the 5-FOA resistant clones contained the desired deletion. Multiplex PCR indicated that 100% (4/4) of the correct deletion clones contained the complete M. genitalium genome (Table 1). In conclusion, we find that the efficiency of the TREC method is comparable with that of the Cre-loxP system in engineering an M. genitalium genome cloned in yeast.

### DISCUSSION

Several existing methods that adapt the URA3/5-FOA counter-selection have been successfully demonstrated for modification of yeast chromosomes (1–3). However, the two methods that we have tried are not efficient enough for engineering an M. genitalium genome episomally maintained in yeast. The M. genitalium genome seems to be relatively stable in yeast even though the genome contains up to 4% of repetitive sequences (25), but spontaneous deletions or rearrangements still occurs at a low frequency ~2% (data not shown). This would potentially generate undesired URA3-negative clones during the course of manipulations and therefore complicate 5-FOA selection for site-specific mutagenesis.

We have demonstrated that the TREC method is a very efficient tool to produce seamless modifications in the M. genitalium genome in yeast. It is a simple method that only needs a single transformation and is adaptable to other kinds of modifications (insertions, gene replacements or point mutations). The high frequency of homologous recombination of the TREC method is mainly attributable to the fact that every cell, in principle, is engaging in repair during the induction of the DSB and that the repair substrates (repeat sequences and DSB) are in close proximity. The performance of TREC is comparable with the Cre-loxP system. However, since TREC does not leave a scar, the TREC method offers a significant advantage over the Cre-loxP system in genomic engineering. Recently, a new method, called MIRAGE, was reported for generating a seamless modification in the S. cerevisiae yeast genome with high efficiency (26). This method is based on the introduction of an inverted repeat near the target site, flanked by two short tandem repeats. The unstable inverted repeat greatly promotes an excision between the two tandem repeats. However, the inverted repeat sequences may lead to imprecise deletions due to replication slippage (27,28). Furthermore, the MIRAGE method requires a complicated knockout construct to be generated, which may take more than a day to prepare. In contrast, the TREC construct can be generated and transformed into yeast in the same day.

A similar strategy has been developed for making seamless modifications in Escherichia coli (29,30). It also involves the introduction of both I-SceI induced DSB and tandem repeat sequences near the target site and requires the assistance of lambda red for homologous recombination. This method makes E. coli a powerful host for molecular engineering. However, unlike the mega-base pair cloning capacity of yeast, cloning foreign DNA

### Table 1. Efficiency of several DNA modification methods for engineering M. genitalium genomes in yeast

| Method                      | Fraction of clones with the correct modification | Fraction of clones with a complete genome |
|-----------------------------|--------------------------------------------------|------------------------------------------|
| Traditional sequence        | 0/97                                             | 0/22                                     |
| replacement                 |                                                  |                                          |
| Tandem repeat pop-out       | 0/38                                             | 1/9                                      |
| Tandem repeat endonuclease  | 28/28                                            | 10/10                                    |
| cleavage (TREC)             |                                                  |                                          |
| Cre-loxP recombinase        | 28/30                                            | 4/4                                      |

*Estimated by diagnostic PCR.

*Estimated by multiplex PCR assay.
Figure 2. Seamless deletion using the TREC method. (A) The outline of the TREC method. Through homologous recombination, a 450-bp region located at the MG259 locus is replaced with a mutagenesis cassette that consists of a knock-out CORE (an 18-bp I-SceI recognition site, the SCEI gene under the control of a GAL1 promoter, and the URA3 marker) and a DNA fragment (shown in white arrow) identical to a region upstream of the target site. The replacement generates tandem repeat sequences flanking the CORE. Galactose induces the expression of I-SceI, which generates a double-strand break (DSB) at the I-SceI site near the target locus. The DSB promotes an intra-molecular homologous recombination (dash line) between the repeat sequences, leading to an excision of the CORE. (B) Replica-plating steps used for selection of M. genitalium genome modification. URA3 positive transformants were grown on SD-HIS-URA medium, followed by replica plating to either galactose or glucose plates. After a 2-day incubation, cells were replica-plated onto SD-HIS containing 5-FOA. 5-FOA-resistant cells were re-streaked out to produce single colonies for PCR analyses. (C) PCR analysis using the diagnosis primers, Seq-F and M2-det1(aagtaactagcaatttgttg), for excision of the mutagenesis cassette. DNA was prepared from 24 colonies replica-plated from either galactose or glucose plate, respectively. DNA with a precise deletion would give rise to a 0.55-kb PCR product, compared to a 1-kb PCR product from un-modified DNA. (D) Analysis of the integrity of the M. genitalium genome. Ten DNA samples from galactose-induced and un-induced 5-FOA resistant clones in (C) were further analyzed by multiplex PCR using the same primer sets described in Figure 1C. M, 100-bp DNA ladder. C, DNA purified from Ura+ transformants before galactose induction and 5-FOA selection.
>300 kb in *E. coli* is not very common, which limits its application.

Delivering an engineered YAC back to its original cell can determine the function and regulation of genes and gene clusters (1–3). Seamless modification is a favorable means of engineering a YAC, since additional sequences remaining in engineered site could potentially cause unexpected consequences. In addition, chromosomes of many higher eukaryotic cells contain a high fraction of repetitive sequences. The method described here should be beneficial for modifying their gene(s) cloned in yeast. Furthermore, we have also applied TREC method to easily generate a variety of genomic modifications, including gene insertion, deletion and mutation correction, in both the synthetic *M. genitalium* genome and a *Mycoplasma mycoides* (*M. mycoides*) genome (~1 Mb) cloned in yeast (unpublished data). One of these engineered clones, YCpMMyc1.1*A-typeIII*res, is a seamless deletion of a Type III restriction enzyme gene. The genome purified from this clone has been transplanted into a *Mycoplasma capricolum* to produce an *M. mycoides* cell with the desired genome modification (4). Together, since the yeast *S. cerevisiae* has been successfully demonstrated as a host for the assembly of the synthetic *M. genitalium* genome, TREC becomes an important tool in yeast to engineer synthetic genomes, which could be used to produce synthetic cells.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**

1. Kouprina, N. and Larionov, V. (2003) Exploiting the yeast Saccharomyces cerevisiae for the study of the organization and evolution of complex genomes. *FEMS Microbiol. Rev.*, 27, 629–649.
2. Shashikant, C.S., Carr, J.L., Bhargava, J., Bentley, K.L. and Rudolf, F.H. (1998) Recombinogenic targeting: a new approach to genomic analysis—a review. *Gene*, 223, 9–20.
3. Peterson, K.R. (2003) Transgenic mice carrying yeast artificial chromosomes. *Expert Rev. Mol. Med.*, 5, 1–25.
4. Lartigue, C., Vashee, S., Algire, M.A., Chuang, R.Y., Benders, G.A., Ma, L., Noskov, V.N., Denisova, E.A., Gibson, D.G. and Assad-Garcia, N. et al. (2009) Creating bacterial strains from genomes that have been cloned and engineered in yeast. *Science*, 325, 1693–1696.
5. Bocke, J.D., LaCroute, F. and Fink, G.R. (1984) A positive selection for mutants lacking orotidine-5’-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.*, 197, 345–346.
6. Scherer, S. and Davis, R.W. (1979) Replacement of chromosome segments with altered DNA sequences constructed in vitro. *Proc. Natl Acad. Sci. USA*, 76, 4951–4955.
7. Alani, E., Cao, L. and Kleckner, N. (1987) A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics*, 116, 541–545.
8. Noskov, V., Kouprina, N., Leem, S.H., Koriabine, M., Barrett, J.C. and Larionov, V. (2002) A genetic system for direct selection of gene-positive clones during recombinational cloning in yeast. *Nucleic Acids Res.*, 30, E8.
9. Akada, R., Kitagawa, T., Kaneo, S., Toyona, D., Ito, S., Kakkara, Y., Hoshida, H., Morimura, S., Kondo, A. and Kida, K. (2006) PCR-mediated seamless gene deletion and marker recycling in Saccharomyces cerevisiae. *Yeast*, 23, 399–405.
10. Hirashima, K., Iwaki, T., Takegawa, K., Giga-Hama, Y. and Toida, H. (2006) A simple and effective chromosome modification method for large-scale deletion of genome sequences and identification of essential genes in fission yeast. *Nucleic Acids Res.*, 34, e11.
11. Storici, F., Durham, C.L., Gordenin, D.A. and Resnick, M.A. (2003) Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. *Proc. Natl Acad. Sci. USA*, 100, 14994–14999.
12. Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A. et al. (2006) Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome. *Science*, 319, 1215–1220.
13. Gibson, D.G., Benders, G.A., Axelrod, K.C., Zaveri, J., Algire, M.A., Moodie, M., Montague, M.G., Venter, J.C., Smith, H.O. and Hutchison, C.A. III (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic Mycoplasma genitalium genome. *Proc. Natl Acad. Sci. USA*, 105, 20404–20409.
14. Amberg, D.C., Burke, D.J. and Strathern, J.N. (2005) Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 230.
15. Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, 122, 19–27.
16. Sauer, B. and Henderson, N. (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biologist*, 2, 441–449.
17. Shevchuk, N.A., Bryksin, A.V., Nusinovich, Y.A., Cabello, F.C., Sutherland, M. and Ladisch, S. (2004) Construction of long DNA recombinase. *New Biologist*, 2004–2009.
18. Gietz, D., St Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, 20, 1425.
19. Rouet, P., Smith, F. and Jasmin, M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl Acad. Sci. USA*, 91, 6664–6668.
20. Plessis, A., Perrin, A., Haber, J.E. and Dupin, B. (1992) Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. *Genetics*, 130, 451–460.
21. Gong, W.J. and Golic, K.G. (2003) Ends-out, or replacement, gene targeting in Drosophila. *Proc. Natl Acad. Sci. USA*, 100, 2556–2561.
22. Haber, J.E. (2002) Uses and abuses of HO endonuclease. *Methods Enzymol.*, 350, 141–164.
23. Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D., and Hegemann, J. H. (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.*, 30, e23.

24. Araki, K., Araki, M. and Yamamura, K. (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.*, 25, 868–872.

25. Peterson, S. N., Bailey, C. C., Jensen, J. S., Borre, M. B., King, E. S., Bott, K. F. and Hutchison, C. A. III (1995) Characterization of repetitive DNA in the Mycoplasma genitalium genome: possible role in the generation of antigenic variation. *Proc. Natl Acad. Sci. USA*, 92, 11829–11833.

26. Nair, N. U. and Zhao, H. (2009) Mutagenic inverted repeat assisted genome engineering (MIRAGE). *Nucleic Acids Res.*, 37, e9.

27. Lobachev, K. S., Shor, B. M., Tran, H. T., Taylor, W., Keen, J. D., Resnick, M. A. and Gordenin, D. A. (1998) Factors affecting inverted repeat stimulation of recombination and deletion in Saccharomyces cerevisiae. *Genetics*, 148, 1507–1524.

28. Gordenin, D. A., Malkova, A. L., Peterzen, A., Kulikov, V. N., Pavlov, Y. I., Perkins, E. and Resnick, M. A. (1992) Transposon Tn5 excision in yeast: influence of DNA polymerases alpha, delta, and epsilon and repair genes. *Proc. Natl Acad. Sci. USA*, 89, 3785–3789.

29. Yu, B. J., Kang, K. H., Lee, J. H., Sung, B. H., Kim, M. S. and Kim, S. C. (2008) Rapid and efficient construction of markerless deletions in the Escherichia coli genome. *Nucleic Acids Res.*, 36, e84.

30. Tischer, B. K., von Einem, J., Kaufer, B. and Osterrieder, N. (2006) Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. *Biotechniques*, 40, 191–197.