Markerless gene replacement in Escherichia coli stimulated by a double-strand break in the chromosome

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ABSTRACT

A simple and efficient gene replacement method, based on the recombination and repair activities of the cell, was developed. The method permits the targeted construction of markerless deletions, insertions and point mutations in the Escherichia coli chromosome. A suicide plasmid, carrying the mutant allele and the recognition site of meganuclease I-SceI, is inserted into the genome by homologous recombination between the mutant and the wild-type (wt) alleles. Resolution of this cointegrate by intramolecular recombination of the allele pair results in either a mutant or a wt chromosome which can be distinguished by allele-specific PCR screening. The resolution process is stimulated by introducing a unique double-strand break (DSB) into the chromosome at the I-SceI site. Cleavage by the nuclease not only enhances the frequency of resolution by two to three orders of magnitude, but also selects for the resolved products. The DSB-stimulated gene replacement method can be used in recombination-proficient E. coli cells, does not require specific growth conditions, and is potentially applicable in other microorganisms.

INTRODUCTION

Microbial genome sequencing projects uncover a large number of potentially new genes. Functional analysis of these genes requires simple and efficient gene manipulation methods, which allow targeted modifications of particular sequences in their chromosomal location. Mutant alleles can be constructed in vitro by standard recombinant DNA techniques. To test the effect of a mutation, the mutant allele is introduced into the cell where it can replace the wild-type (wt) gene by homologous recombination. The effect of the mutation can then be studied by expressing the gene at its native location, under normal conditions. Two basic approaches have been used to control the gene replacement process and to select for cells harboring the desired mutant gene.

The mutant allele can be delivered into the cell on a suicide plasmid (1–3). Insertion of the circular molecule into the chromosome requires a single crossover between the mutant and the wt allele. This cointegrate can be resolved by spontaneous recombination of the allele pair resulting in cells with either a wt or a mutant allele in the chromosome (1–3). Since the resolution event is rare, effective counterselection is needed to eliminate cells which retain the cointegrate structure and carry a counterselectable gene located on the inserted plasmid (4–6). The most widely used scheme is the sacB/sucrose counterselection system (5), but the use of the method is limited by its strain-, medium- and temperature-dependence (2,3).

An alternative way is to deliver the mutant allele into the cell on a linear DNA-fragment (7–9). Replacement of the wt gene by the mutant allele requires a double crossover. Variations of the method rely on specifically altered host cells, require extensive DNA engineering using long PCR primers, and introduce a marker along with the mutant gene into the genome. This marker can have polar effects or can prevent multiple manipulations of the genome. The marker can be removed only in a second round of allele replacement (9). This step, again, requires the use of a counterselection system with its intrinsic limitations.

Here we present a simple and more general gene replacement method which can be used in recombination-proficient (wt) Escherichia coli, and produces markerless replacements at high efficiency. The method involves the integration of the mutant gene, carried on a circular plasmid, at a homologous locus into the chromosome resulting in a direct duplication. Resolution of this cointegrate via intramolecular recombination is controlled by introducing a unique double-stranded break (DSB) into the chromosome by the meganuclease I-SceI. The enzyme recognizes an 18-bp sequence and generates a DSB with a 4-base 3’ hydroxyl overhang (10).

MATERIALS AND METHODS

Plasmids

The suicide plasmids pST76-A/C/K and pSG76-A/C/K were described (11) and their nucleotide sequences are available under accession numbers Y09895–Y09897 and Y09892–Y09894.

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respectively. pST76-based plasmids carry a temperature-sensitive replicon and cannot replicate at 37–42°C (11,12). pSG76-based plasmids require the product of the pir gene for replication (11,13). pST76-KSac, containing the sacBII gene (14) of Bacillus amyloliquefaciens on a 1.7-kb EcoRI fragment, was constructed by M. Messerle (University of Muenchen).

The complete nucleotide sequences of the I-SceI-expressing plasmids pUC19RP12, pST76-AsceP and pST98-AS are available under accession numbers AF170481, AF170482 and AF170483, respectively. These plasmids, depicted schematically in Figure 2, were constructed in a multistep process. Briefly, the Clal–EcoRI fragment of pFT-A (11), carrying tetR of Tn10 transposon (15,16) was cloned between the Clal and EcoRI sites of pSG76-A, resulting in pSG76-Atet. The BamHI–PstI fragment carrying the universal core sequence of the I-SceI gene (17) was isolated from pScm525 (a gift of B. Dujon) and cloned between the BamHI and PstI sites of pSG76-Atet. Next, nucleotide sequence between PstI (18) and the initiator codon of the nuclease gene was shortened by PCR. A linear fragment was amplified on the pSG76-Atet template using primers RP1 and RP2, then blunted by T4 DNA polymerase, and ligated to form a circular plasmid, resulting in pSG76-ARP12. At this step a rearrangement occurred which altered codons 2–5 of the I-SceI gene. As a consequence, the N-terminal amino acid sequence of the nuclease changed from MKNIK to MH–Q. All further constructs carry this change of the sequence, nevertheless, the modified nuclease remained active. pUC19RP12 was constructed by cloning the Xbal–PstI fragment of pSG76-ARP12 carrying PstI and the I-SceI gene between the Xbal and PstI sites of pUC19. The same fragment was cloned between the Xbal and PstI sites of pST76-A to create pST76-AsceP. pST98-AS was constructed by ligating the large NotI–PstI fragment of pSG76-ARP12 and the NotI–BstI fragment carrying the replication origin of pST76-A. The incompatible PstI and BstI fragment ends were blunted by DNA polymerase I large fragment before ligation.

Plasmids used for obtaining chromosomal insertions were based on the suicide plasmid series pSG76 and pST76 (11). A 1.5-kb chromosomal fragment containing the att site was amplified by PCR (primers GT1/GT2), cleaved by BamHI and cloned into the BamHI site of pST76-K. Using primers GT3/GT4, a 17-bp deletion was constructed in the core sequence of the att site, resulting in plasmid pST76-KΔGT34. To construct pSG76-CMB45, a 542-bp BglII–Clal fragment of the PCR product obtained by primers MJ/M7 was cloned between the BamHI and Clal site of pSG76-C, followed by ligation of a 614-bp Clal fragment of another PCR product, obtained with primers M4/M5, into the Clal site. This latter Clal fragment was cloned into the Clal site of pST76-K to obtain pST76-KM45.

PCR primers

Nucleotides representing chromosomal sequences are in capitals. MG1655 genomic coordinates (19) of the 3′ nucleotides are given next to the primers where applicable.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Coordinates</th>
</tr>
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<tbody>
<tr>
<td>GT1:</td>
<td>5′-cggggactccggatggtttacgtcat</td>
<td>805853</td>
</tr>
<tr>
<td>GT2:</td>
<td>5′-gaaaccactcatacggtg</td>
<td>807360</td>
</tr>
<tr>
<td>GT3:</td>
<td>5′-cgcccctcaacaaggtcat</td>
<td>806534</td>
</tr>
<tr>
<td>GT4:</td>
<td>5′-ggccggagacccggagagtta</td>
<td>808887</td>
</tr>
<tr>
<td>M1:</td>
<td>5′-ccgggtcactccgaggcttgaattataat</td>
<td>261260</td>
</tr>
<tr>
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<td>324656</td>
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<tr>
<td>M5:</td>
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<td>325223</td>
</tr>
<tr>
<td>M6:</td>
<td>5′-ccgacccggacccagcat</td>
<td>325251</td>
</tr>
</tbody>
</table>

**Strains and media**

Plasmids were generally prepared from *E. coli* DH5α (20). pSG76-based plasmids were maintained in DH5α pir(TcK). This strain carries the pir gene of plasmid R6K (13), inserted into the chromosome, and was obtained from M. Koob (University of Minnesota). All gene-replacement experiments were done in MG1655 (19).

Standard laboratory media (LB, SOC) and agar plates were used. Antibiotics were used at the following concentrations: ampicillin (Ap) 50 μg/ml, chloramphenicol (Cm) 25 μg/ml, kanamycin (Km) 25 μg/ml, rifampicin (Rf) 100 μg/ml.

Heat-treated chlor-tetracycline (cTc) was used to inactivate the Tet repressor. The inducer cTc was suspended in LB at a concentration of 400 μg/ml and autoclaved in a pressure cooker for 20 min, then stored in the dark. Induction of P\text{car} was achieved by adding 1/20 volume of the cTc stock to the medium.

**Electroporation**

Electroporation-competent cells were prepared as described in the Invitrogen Electroporator II Manual (http://www.invitrogen.com). Briefly, a 500-ml *E. coli* culture was grown to an OD\text{550} of 0.5–0.6, then cells were harvested by centrifugation and washed twice in ice-cold water and once in ice-cold 10% glycerol by repeated centrifugation and suspension. At the final step the cell pellet was suspended in 0.4 ml 10% glycerol, aliquoted in 40 μl portions into microfuge tubes and stored at −80°C.

The cells were typically electroporated with nanogram quantities of plasmid DNA at 1.8 kV and a resistance of 150 Ω in a 0.1-cm electroporation cuvette using the Electroporator II device (Invitrogen). Cells were then diluted with 1 ml SOC medium, incubated in a shaker at an appropriate temperature for 1 h, and plated on selective medium.

**Insertion of suicide plasmids into the chromosome**

pST76-based suicide plasmids carrying a mutant allele were delivered into the target cell by electroporation. Cells were spread on LB plates supplemented with an appropriate antibiotic (LBAb) and incubated at 30°C overnight. Several colonies were then picked and restreaked on a LBAb plate. This plate was incubated at 42°C for 7–9 h, then transferred to 37°C for an additional 12–24 h. Typically many large colonies are formed over the background of small colonies. These large colonies carry the suicide plasmid inserted into the chromosome; however, at this point usually the colonies contain cells harboring unintegrated plasmids as well. To obtain cells that are cured of the free plasmid, a few large colonies are picked individually, restreaked on LBAb plates and grown at 37°C overnight. Colonies should be uniform in size this time, and the site of insertion can be verified by PCR using an appropriate primer pair.

Insertion of pSG76-based suicide plasmids can be achieved in a similar way, except that the target cell should carry a helper plasmid supplying Π protein (11), and longer incubation time is needed at 42°C to stop replication of the helper plasmid and to dilute the Π protein in the growing cells.

**References**

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M7: 5′-cggggactccggatggtttacgtcat | 263060 |
RP1: 5′-catttcagatgtttcaggaagtgtcgg |
RP2: 5′-catttcagatgtttcaggaagtgtcgg |
S: 5′-ccgctgtcgggtttactacagtgcgc
RESULTS

The DSB-stimulated gene replacement method

Steps of the procedure are depicted in Figure 1. The mutant allele is cloned in a suicide plasmid, which carries a temperature-sensitive replicon (pST-76 plasmid series), an antibiotic resistance gene and a recognition site for I-SceI. This plasmid is electroporated into the target cell where cointegrates of the chromosome and the suicide plasmid can form via homologous recombination. Cointegrates are selected at 42°C and the plasmid can form via homologous recombination.

The DSB-stimulated gene replacement method

Figure 1. General scheme of the DSB-stimulated gene replacement procedure. Cointegrates of the chromosome and the suicide plasmid can form via homologous recombination between the mutant and the wt alleles of the target gene. The plasmid carries a temperature-sensitive (ts) replicon, an antibiotic (Ab) resistance gene and the recognition site for I-SceI. Cointegrates are selected by their Ab resistance at the non-permissive temperature for plasmid replication. Next, cleavage of the chromosome by I-SceI is induced. The DSB stimulates recombination between the duplications resulting either in a reversion to the wt chromosome or in a markerless gene replacement event.

Expression of I-SceI in E. coli cells

Three plasmids, expressing I-SceI, were constructed (Fig. 2). In all cases, transcription of the I-SceI gene is driven by P_{tetA} of Tn10. Plasmids pUC19RP12 and pST76-AsceP express the nuclease constitutively. In pST98-AS suicide plasmid, where the product of tetR causes tight repression of P_{tetA}, expression is induced by adding heat-treated cTc to the medium. Since the same cassette expresses the nuclease in all three constructs, differences in the effects of I-SceI cleavage on the cell can be attributed to differences in gene dosage due to the copy number of the plasmids.

Although the E. coli genome does not contain a I-SceI site, potential effect of cleavage at secondary sites in the chromosome was tested by electroporating pUC19RP12 into MG1655. No differences were observed in the phenotype of the colonies or in the growth rate in LBAp at various temperatures (30, 37, 42°C), as compared to cells harboring pUC19 control plasmid.

Stimulation of intramolecular recombination between direct repeats by a DSB

To test the efficiency of the allele replacement, we attempted to delete the λ att site core sequence (21) of the E. coli MG1655 genome. A 1.5-kb segment of the chromosome, centered by the λ att site, was amplified by PCR. This fragment was modified by deleting 17 bp of the core sequence by recombinant PCR, and cloned in a suicide plasmid resulting in pST76-KaGT34. The plasmid was electroporated into MG1655 and cointegrants, designated MGGTatt, were selected at 42°C. The site of integration in the chromosome was verified by PCR using primers S/GT2. MGGTatt electroporation-resistant cells, grown in LBKm, were prepared, and electroporated by either pUC19RP12 or pST76-AsceP. I-SceI, expressed by the plasmids, cleaves the chromosome in the plasmid sequence and cells can survive only by repairing the DSB or by deleting the I-SceI site by intramolecular recombination between the direct repeats. The number of surviving transformants was compared to the potential total number of transformants, which was calculated from a set of control electroporations (MG1655/pUC19, MG1655/pUC19RP12, MGGTatt/pUC19). Resolution of the cointegrate was checked by replica plating several hundred colonies on LBAp and LBApKm plates, as well as by PCR using primers S/GT2. Results were obtained from three independent experiments and are summarized in Figure 2. When electroporating MGGTatt cells with pUC19RP12 (high meganuclease gene dosage), the fraction of the surviving transformants ranged from 8 to 10%, and all cells showed deletion of the segment between the repeats. All potential transformants (100%) survived when MGGTatt cells were electroporated with pST76-AsceP (low meganuclease gene dosage). In this case, primary colonies resulting from the transformation on LBAp plates proved to be mixtures of cells either retaining or deleting the insertion. On LBApKm plates, colonies survived but showed a reduced growth rate. Secondary colonies, obtained by restreaking the primary colonies on LBAp plates, showed in most cases (∼90%) deletion of the insert.

In a third variation of the experiment, the insertion was created using the suicide plasmid vector pST98-AS. I-SceI crossover. Using an allele-specific primer, cells carrying the mutant allele can be identified by PCR screening.

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expression was then induced and cells were grown in LBcTc at 37°C with repeated re-inoculation for several generations. At every tenth generation an inoculum was withdrawn, diluted, and spread on LB. Several hundred colonies were then replica plated on LB and LBKm plates. A linear increase in the number of colonies that lost the insert (KmS) was observed. After 60 generations, all cells proved to be KmS. The percentage of KmS cells in a control experiment, using LB medium without inducer, was <1% after 60 generations of growth.

Next, the frequency of spontaneous recombination (without DSB) between the repeat sequences was determined. MGGTatt cells, grown in LBKm, were electroporated with pUC19 and spread on LBAp plates. From the resulting transformants 1200 colonies were replica plated on LBAp and LBApKm plates. Two colonies proved to be KmS, thus, frequency of spontaneous intramolecular recombination was $1.7 \times 10^{-3}$/generation.

In conclusion, introducing a DSB into the chromosome between 1.5-kb direct repeats, separated by 3.4 kb, increased the frequency of intramolecular recombination by two to three orders of magnitude. This effect depended on the meganuclease concentration in the cell and on the length of time during which the meganuclease could exert its effect.

Symmetry of the resolution of the cointegrate

The experimental setting depicted in Figures 2 and 3 was used to assess the symmetry of the resolution of the cointegrate. The 17-bp deletion divides the repeat sequence into two segments of nearly identical size (734 and 746 bp), thus the frequencies of recombination events resulting in wt (A) or mutant (B) chromosome are expected to be equal. However, preliminary experiments showed that the RecBCD complex is involved in the DSB-stimulated recombination, therefore the presence of a RecBCD-activating chi site (22) might influence the distribution of actual crossover sites.

There is a single properly oriented (22) chi site in the chromosomal region shown in Figure 3. Using primers GT1/GT2, PCR-amplified fragments were prepared from colonies which lost the insert by intramolecular recombination (MGGTatt/pUC19RP12 cells). The fragments were digested by PstI and MluI resulting in three fragments. A 17-bp size reduction of the largest fragment indicates if the deletion mutant allele was retained in the chromosome. Of 66 colonies studied, 31 revealed wt pattern and 35 proved to be mutant. We concluded that distribution of crossover sites in DSB-stimulated intramolecular recombination between direct repeats was not significantly influenced by a chi site.
Construction of a 62 kb chromosomal deletion

From the technical point of view, introduction of small deletions, insertions or point-mutations into the chromosome are equivalent processes. However, construction of a large deletion (e.g. deletion of a pathogenicity island) poses a specific problem. Since the frequency of intramolecular recombination between direct repeats depends on the physical distance on the chromosome (23), resolution of the cointegrate would preferentially lead to reversion to wt (Fig. 4). In a preliminary experiment, using sacB/sucrose counterselection, short-distance deletion leading to wt was heavily favored over a long-distance deletion (46 kb), which could not be detected by PCR in a pool of several hundred colonies (data not shown).

To test the DSB-stimulated deletion method, pSG76-CMB45 was inserted into the genome of MG1655 (Fig. 4), resulting in cells designated MGCH. The plasmid carried PCR-amplified copies of two, relatively short segments (546 and 614 bp) flanking a 62-kb chromosomal region that coded for putative prophages and was assumed dispensable. Plasmid pUC19RP12 was electroporated into MGCH and surviving transformants were tested by PCR, using primers M1/M6 and M1/M7. Of 100 colonies investigated, 87 reverted to wt and 13 contained the large deletion.

As a more effective but more laborious alternative, a second insertion, using pST76-KM45, was introduced into the genome of MGCH, resulting in strain MGCK (Fig. 4). Plasmid pUC19RP12 was electroporated into MGCK. Based on three independent experiments, a small number (0.5–1%) of the potential transformants survived, but an average of 33% of the cells carried the desired deletion. In a similar experiment, using pST76-AsceP, nearly 100% of the transformants survived, and the fraction of the deletion mutants varied between 6 and 18% in three experiments.

Figures 3 and 4. Deletions of 17 bp of the att site core sequence and the symmetry of the resolution of the cointegrate. The 5' and the 3' nucleotides of the homologous segments, as well as the 3' nucleotide of the chi site are marked by the corresponding MG1655 genomic coordinates. The yellow box represents the 17-bp deletion. Colonies which resolved the cointegrate were analyzed by a MluI and PstI double digest of the fragment obtained by PCR using GT1 and GT2 primers. Of 66 colonies analyzed, 31 reverted to wt (A) and 35 retained the desired deletion (B).

Figure 4. Construction of a 62-kb deletion of the MG1655 chromosome. (I) Deletion of the 62-kb segment using a single insertion. The genomic coordinates of the 5' nucleotides of the homologous segments are shown in italics. Arrowheads marked M1, M7, M4, M5 and M6 are PCR primers. Type A recombination event, found in 87 of 100 colonies, deletes the suicide plasmid; type B deletion, found in 13 of 100 colonies, removes the large, 62-kb chromosomal region. (II) Deletion of the 62-kb segment with the help of two insertions. DSB-stimulated resolution of the cointegrates results in the loss of the chromosomal segment in 33% of the cells (average of three experiments). In all cases, I-SceI was expressed from pUC19RP12.
Rate of mutation in cells subjected to DSB-stimulated gene-replacement

Preliminary results indicated that a functional LexA repressor, and thus, induction of the SOS system is absolutely necessary for eliminating the DSB by intramolecular recombination (data not shown). Induction of the SOS response is normally coupled with error-prone DNA synthesis (24). Since an increase in the number of unwanted chromosomal mutations would seriously limit the use of the method, we measured the rate of spontaneous mutations by counting rifampin resistant (RpoB) mutants (25) in the cell population. MGCH cells were transformed by either pUC19 or pUC19RP12 and plated on LBAp plates. Colonies from these plates were suspended in LB and dilutions were spread both on LBAp and LBApRf plates. Spontaneous rifampicin resistant mutants arose at rates of $1.5 \times 10^{-5}$ and $1.7 \times 10^{-5}$ in MGCH/pUC19 and MGCH/pUC19RP12 cells, respectively. In conclusion, repair of a DSB by intramolecular recombination did not significantly increase the rate of mutations in the cell.

DISCUSSION

The DSB-stimulated gene replacement method offers a simple and efficient way to manipulate chromosomal sequences. Small, markerless deletions, insertion, point-mutations, as well as large deletions can be created virtually at will. Two sets of suicide plasmids, equipped with an I-SceI recognition site, are available for inserting mutant alleles into the chromosome (11). Insertion creates a duplication of the target sequence. Cells which resolve this cointegrate can be selected by various counterselection methods. The novelty of the method presented here is that I-SceI cleavage serves not only as a selection tool, but also as a stimulator of the resolution process, increasing the efficiency of resolution by two to three orders of magnitude. Additional advantages are that the method works in recombinase-proficient (wt) E.coli and no special growth conditions are required. This method has been used in our laboratory in various E.coli strains, including pathogens, to introduce insertions, deletions and a point mutation into the genome at six different loci (data not shown). The efficiency of the procedure was similar in all experiments, although a detailed analysis was carried out only in the cases presented in this paper. We note that the sacB/sucrose counterselection method did not work in the pathogen O157:H7, in contrast to the DSB method. Since DSBs are known to stimulate homologous recombination in a wide range of systems, it can be assumed that modified versions of this system can be applied to various microorganisms.

The procedure does not directly result in cells with the mutant allele and a proper allele-specific PCR screen was conveniently used to identify the desired product. Wt and mutant progenies arise in equal numbers, provided the mutation is small and is in the middle of the repeat sequence. We showed that the presence of a chi site, a hotspot for the RecBCD recombination pathway, does not influence this symmetry of resolution.

Construction of large, chromosomal deletions is difficult because reversal of the cointegrate to wt is heavily favored due to the physical distance differences between the repeat sequences. Using the DSB-stimulated replacement method, we were able to delete in MG1655 a 62-kb chromosomal segment carrying putative prophages (19). Since reversal of the cointegrate to wt was only about eight times more frequent than the resolution resulting in the large deletion, screening of a relatively small number of colonies was needed to obtain the desired product.

Introduction of a DSB into the chromosome induces the SOS response and this might lead to error-prone DNA synthesis (24). As a consequence, unwanted mutations might accumulate in the genome. However, SOS-induced mutagenesis is usually studied in cells that are irradiated by UV or ionizing radiation and suffer a number of DNA lesions. The principal cause of SOS-induced mutations are these lesions, where the error-prone DNA polymerase incorporates wrong nucleotides (24). When applying the DSB-stimulated gene replacement method, a single DNA lesion is inflicted on the chromosome, thus there are no other hotspots for mutation. Indeed, no increase of the mutation rate was observed in cells subjected to DSB-stimulated gene replacement.

Three alternative I-SceI sources are presented in this paper. In the first two cases, following the integration step, I-SceI-producing plasmids must be delivered into the cell by electroporation. When the high copy-number plasmid pUC19RP12 is used, 8–10% of the potential transformants survive, and all of them resolve the cointegrate. Using the low copy-number plasmid pST76-ASceP, a higher number of transformants survive, but colonies must be restreaked once to obtain resolution in >90% of the cells. Since pST76-ASceP carries a temperature-sensitive replicon, cells can be easily cured of the plasmid after the gene replacement was carried out. No helper plasmid delivery step is needed when the third alternative, the pST98-AS suicide plasmid is used. It carries the I-SceI gene repressed by the tet repressor and, after insertion, I-SceI production is induced by adding cCt to the medium. Accumulation of I-SceI in the cell and the irreversible nature of the intramolecular recombination process lead to an increasing fraction of the cells resolving the cointegrate. A slight disadvantage is that growth for about 60 generations is needed to achieve a resolution close to 100%. We plan to replace P_{tet} with a stronger, but similarly repressible promoter (26) to increase I-SceI production and thus shorten the growth period.

The three plasmids, used as meganuclease sources, give rise to different I-SceI gene dosages in the cell. This leads to differences in the number of surviving cells and in the rate at which these cells resolved the cointegrate. These effects can be explained by assuming that higher I-SceI level leads to simultaneous cleavage of all chromosome copies in the growing cell, while lower concentration of the nuclease results in intact and cleaved copies of the chromosome in the same cell. In the latter case, two competing processes take place. On one hand, recombinational DSB repair restores the I-SceI site by using sequence information from the intact copy of the chromosome (27). On the other hand, intramolecular recombination deletes the inserted sequences. Since this intramolecular recombination process is irreversible, it gradually becomes dominant and the fraction of cells resolving the cointegrate is increasing generation by generation. A detailed analysis of these processes, including the involvement of various recombinase and repair genes (recA, recB, recF, recN, lexA, ligase) will be published elsewhere.
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REFERENCES