An improved Xer-cise technology for the generation of multiple unmarked mutants in *Mycobacteria*

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ABSTRACT

Xer-cise is a technique using antibiotic resistance cassettes flanked by *dif* sites allowing spontaneous and accurate excision from bacterial chromosomes with a high frequency through the action of the cellular recombinase XerCD. Here, we report a significant improvement of Xer-cise in *Mycobacteria*. Zeocin resistance cassettes flanked by variants of the natural *Mycobacterium tuberculosis* *dif* site were constructed and shown to be effective tools to construct multiple unmarked mutations in *M. tuberculosis* and in the model species *Mycobacterium smegmatis*. The *dif* site variants harbor mutations in the central region and can therefore not recombine with the wild-type or other variants, resulting in mutants of increased genetic stability. The herein described method should be generalizable to virtually any transformable bacterial species.

METHOD SUMMARY

dif-Zeo^R-*dif* cassettes are used to replace non-essential genes in mycobacterial genome through recombineering. Spontaneous excision of the cassette is carried out under the action of the recombinase XerCD, resulting in unmarked deletions. Subsequent rounds of mutagenesis using cassettes flanked by a range of *dif* site variants allow construction of multiple mutants in which the different *dif* sites cannot recombine with each other, yielding stable genetic constructs.

KEYWORDS

dif site • excisable cassette • mutagenesis • *Mycobacterium* • recombineering • unmarked deletions • Xer-cise

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Genetic studies of bacterial evolution and physiology require efficient and reliable tools for the construction of specific gene deletion mutants. A range of tools are available to manipulate *Mycobacteria*, such as *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis. In particular, the development of recombineering has been a considerable improvement over previous methods [1]. This technique allows replacement of a specific gene by an antibiotic resistance cassette through homologous recombination between linear DNA fragments, so-called allelic exchange substrates (AES) and chromosomal DNA catalyzed by RecET-like mycobacteriophage proteins gp60 and gp61 (Figure 1D). However, only a limited number of selectable marker genes are available for mycobacteria [2] and, for safety reasons, strains used for medical studies must not carry resistance to multiple antibiotics.

One way to overcome these problems is to use excisable cassettes that can be removed by recombination between two specific sites repeated at both sides of the cassette. The so-called Xer-cise technique is a convenient way to achieve auto-excision, because the recombination between two deletion-induced filamentation (*dif*) sites is catalyzed by the XerCD recombinase present and active in most bacterial species, as it is required for chromosome maintenance [3]. Xer-cise has been adapted to *Mycobacteria* and shown to be efficient both in *M. tuberculosis* and in the fast-growing model species *Mycobacterium smegmatis* [2,4,5]. However, these studies used the wild-type *dif* sequence of *Mycobacteria* [4,6], leading to two possible drawbacks. First, XerCD-mediated recombination may occur between the *dif* sites on the AES and the endogenous chromosomal *dif* site, which

may generate false positives during the selection of antibiotic resistant recombinant clones. Second, after completion of the recombination events, the presence of several copies of the *dif* site on the bacterial chromosome may be deleterious because recombination between these sites may generate deletions or inversions on the chromosome, depending on the relative orientation of the *dif* sites [7].

Here, we report an improvement of the Xer-cise technique in *Mycobacteria*. We designed and validated a set of variants of the natural *M. tuberculosis* *dif* site (Table 1) that can recombine with another copy of the same sequence, prompting excision of the antibiotic resistance cassette, but should recombine neither with the wild-type nor with the other variant *dif* sites, yielding stable, unmarked multiple mutants.

DIF SITE VARIANTS

dif sites have been identified in *M. tuberculosis* and *M. smegmatis* [4,6]. The minimal site is 28-bp long, with a XerC-binding left arm, a XerD-binding right arm and a 6-bp central region (Table 1). The mechanism of XerCD-mediated recombination has been well described in *Escherichia coli* [8,9]. It involves the formation of a synaptic complex gathering two *dif* sites in a head-to-tail conformation inside a XerCD hetero-tetramer. The two XerD protomers create single-strand cleavage on each *dif* site, at the border of the central region, then promote strand transfer at the equivalent positions of the other site, leading to a Holliday junction-containing intermediate. By analogy with other site-specific recombinases of the Y-recombinase family [8], efficient strand transfer requires that the first two to three nucleotides of the central region can pair. Indeed, recombination between sites with heterologous central region is inefficient [10,11]. After isomerization of the

Figure 1. Construction of unmarked deletions in *Mycobacteria* by recombineering using zeocin-resistance cassettes flanked by *dif* site variants. (A) PCR amplification of *Zeo^R* cassettes with oligonucleotides introducing *dif* site variants on each side (red arrows). (B) PCR amplification of DNA fragments flanking the gene to be deleted. Blue boxes show sequence extensions identical to the left or right ends of the *Zeo^R* cassette. (C) Three-fragment PCR amplification yielding the AES. (D) Recombineering in *Mycobacteria* carrying plasmid pJV53H yields *Zeo^R* recombinant clones. (E) Spontaneous XerCD-dependent recombination between the tandem *dif* site variants generates unmarked deletion with a 78-bp scar replacing the deleted sequences. AES: Allelic exchange substrate.

intermediate, XerC protomers cleave both *dif* partners on the other side of the central region and transfer the second pair of strands by the same mechanism, allowing resolution of the intermediate and completion of the reaction. Both steps of reciprocal strand transfer require complementary sequences in the two recombination partners. Therefore, concerted modifications of the first two nucleotides on both sides of the central region result in *dif* site variants that are still able to recombine with an identical site, because complementarity is conserved, but not with the wild-type sequence with which base pairing at the border of the central region is no longer possible. Four variants, named *dif4* to *dif7*, were designed following this rationale (Table 1). These variants should not be able to recombine with each other, allowing construction of stable strains carrying multiple variants in their genome. Using specific oligonucleotides (Table 2) and plasmid pGET-*Zeo^R* as a template, we

amplified by PCR a set of Zeocin-resistance cassettes [12] flanked by the four variants of the *dif* site (Figure 1A).

STRATEGY TO CONSTRUCT UNMARKED DELETED MUTANTS

To delete a gene of interest, ~500-bp-long regions of homology corresponding to the flanking upstream and downstream sequences were amplified by PCR using oligonucleotides that introduce sequences overlapping the right or left end of the *dif-Zeo^R-dif* cassettes, outside the *dif* sites (Figure 1B, Table 2, Supplementary Tables 1 & 2). Next, these fragments were mixed with one of the *dif-Zeo^R-dif* cassettes for a three-fragment PCR, allowing amplification of an ~1700-bp AES (Figure 1B & C). The purified AES were used to transform *M. tuberculosis* (strain H37Rv, ATCC27294) or *M. smegmatis* (strain mc² 155, ATCC700084) carrying the plasmid pJV53H, a derivative of pJV53 [1], which expresses a mycobacteriophage

recombinase and confers resistance to hygromycin (Figure 1D & E). Transformation protocol was performed as described [1], performed with 100 ng of AES DNA, as recommended. Transformants were plated on Middlebrook 7H11 agar (Difco, Detroit, MI) supplemented with 10% oleic acid–albumin–dextrose–catalase (Difco) (complete 7H11 medium) and zeocin (25 µg ml⁻¹; Thermo Fisher Scientific, MA, USA). Zeocin-resistant (*Zeo^R*) clones were restreaked on the same medium, and single *Zeo^R* colonies were inoculated in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin–dextrose–catalase (Difco) and 0.05% Tween 80 (complete 7H9 medium) without antibiotic for growth, DNA extraction and verification by PCR. *Zeo^R* clones exhibited replacement of the band corresponding to the wild-type sequence by bands of the expected size for the *dif-Zeo^R-dif* cassette insertion. Several of these clones were streaked on complete 7H11 agar without antibiotic and grown at 37°C. Fifty single colonies were then phenotypically tested in grids on complete 7H11 agar, with or without zeocin, to screen for spontaneous excision of the cassette. At this stage, the number of *Zeo^S* colonies appeared variable from clone to clone with the same *dif* variant, presumably depending on how early excision events had occurred during the preculture of the candidates. However, we systematically observed *Zeo^S* among the 50 clones tested, regardless of the *dif* variant used, demonstrating that all of the variants are functional and can recombine with efficiencies falling into the same range. *Zeo^S* clones were inoculated ►

Table 1. Sequence of wild-type and variant *dif* sites[†].

<i>dif</i> site	Sequence
<i>M. smegmatis dif</i> [†]	AGTACCGATAA GCTACA TTATGTCAACT
<i>M. tuberculosis dif</i> [†]	TAAGCCGATAA GCGACA TTATGTCAAGT
<i>M. tuberculosis dif4</i>	TAAGCCGATAA ATGATG TTATGTCAAGT
<i>M. tuberculosis dif5</i>	TAAGCCGATAA TAGAGT TTATGTCAAGT
<i>M. tuberculosis dif6</i>	TAAGCCGATAA CGGAAC TTATGTCAAGT
<i>M. tuberculosis dif7</i>	TAAGCCGATAA CTGATC TTATGTCAAGT

[†]Left and right arm of the 28-bp *dif* sites are shown in black. The 6-bp central sequence is shown in red. Nucleotides modified in each variant are in bold letters.

Table 2. Oligonucleotides used to amplify Zeocin resistance cassette flanked by variant *dif* sites†.

Primers	Sequence (5' to 3')
Zeo-Dif4-Fw	CTCCACGTGGATCGACTGCCAGGCACTTGACATAA CATCAT TTATCGGCTTAATCAAATAAAACGAAAGGCTC
Zeo-Dif4-Rv	GAGCACGTGGGTCTGACGCTCAGTGGTAAGCCGATAA ATGATG TTATGTCAAGTAACGAAAACTCACGTTAAGGG
Zeo-Dif5-Fw	CTCCACGTGGATCGACTGCCAGGCACTTGACATAA ACTCTA TTATCGGCTTAATCAAATAAAACGAAAGGCTC
Zeo-Dif5-Rv	GAGCACGTGGGTCTGACGCTCAGTGGTAAGCCGATAA TAGAGT TTATGTCAAGTAACGAAAACTCACGTTAAGGG
Zeo-Dif6-Fw	CTCCACGTGGATCGACTGCCAGGCACTTGACATAA GTTCCG TTATCGGCTTAATCAAATAAAACGAAAGGCTC
Zeo-Dif6-Rv	GAGCACGTGGGTCTGACGCTCAGTGGTAAGCCGATAA CGGAAC TTATGTCAAGTAACGAAAACTCACGTTAAGGG
Zeo-Dif7-Fw	CTCCACGTGGATCGACTGCCAGGCACTTGACATAA GATCAG TTATCGGCTTAATCAAATAAAACGAAAGGCTC
Zeo-Dif7-Rv	GAGCACGTGGGTCTGACGCTCAGTGGTAAGCCGATAA CTGATC TTATGTCAAGTAACGAAAACTCACGTTAAGGG

†Sequence corresponding to the *dif* site are shown in bold letters. Red letters show the nucleotides modified in each variant of *dif*.

in complete 7H9 medium for growth, DNA extraction and PCR verification. For construction of multiple mutants, the verified Zeo^S Hyg^R clones were submitted to a subsequent round of mutagenesis, using a *dif*-Zeo^R-*dif* cassette with a different *dif* site variant. Finally, clones verified to carry the expected mutation(s) were streaked on complete 7H11 agar without antibiotic, and single colonies were pheno-

typically tested in grids on complete 7H11 agar, with or without hygromycin, to screen for spontaneous loss of the pJV53H plasmid (Hyg^S). The efficiency of this step could be improved by the use of a counter-selection marker or a visual phenotype screening associated to plasmid pJV53H, as described [5]. A final DNA extraction and PCR verification of each mutant was performed on the Zeo^S Hyg^S clones

(Figures 2 & 3).

CONSTRUCTION OF MULTIPLE UNMARKED DELETIONS IN *M. SMEGMATIS*

To validate the functionality of this system, we constructed deletion mutants in *M. smegmatis* mc² 155. Successive rounds of recombineering, using AES constructed with appropriate primers (Supple- ▶

Figure 2. Verification of the quadruple mutant of *M. smegmatis* mc² 155 ΔMSMEG_3155::*dif*6 ΔMSMEG_1517::*dif*4 ΔMSMEG_4576::*dif*5 ΔMSMEG_6059-6058::*dif*7. (A) Schematics of the primers (arrowheads) and PCR products used to verify the four deletions. Brown arrowheads indicate primers internal to the sequences to be deleted. Red numbers in front of each pair of primers refer to the lanes in (B). (B) Agarose gel analysis of the amplified fragments. PCR was performed using DNA from wild-type mc² 155 (odd numbers) or the triple mutant strain (even numbers). M: 1 kb DNA ladder (Thermo Scientific, MD, USA), bands of higher intensity correspond to 1, 3 and 6 kbp.

Figure 3. Construction and verification of H37Rv Δ Rv0968::*dif5* Δ Rv1993c::*dif6* Δ Rv3269::*dif4*, a triple in-frame deletion mutant of *M. tuberculosis*. (A) Translation of the 78-bp *dif4* scar left after cassette excision. Nucleotides in bold indicate the *dif* site central region. Nucleotides in red correspond to the *dif4* modifications. Stop codons in ORF 0 and +2 are underlined. (B) In-frame deletion resulting from fusion of the 5' and 3' ends of the gene of interest (blue nucleotides) to the +1 ORF of the *dif*-scar (black nucleotides and bracket). (C) Genetic organization of the operons encoding *ctpC*, *ctpG* and *ctpV*. (D) Schematics of the primers (arrowheads) and PCR products used to verify the three deletions. Brown arrowheads indicate primers internal to the sequences to be deleted. Red numbers in front of each pair of primers refer to the lanes in 3E. (E) Agarose gel analysis of the amplified fragments. PCR were performed using DNA from wild-type H37Rv (odd numbers) or the triple mutant strain (even numbers). M: 1 kb DNA ladder (Thermo Scientific), bands of higher intensity correspond to 1, 3 and 6 kbp.

mentary Table 1), and a different *dif* variant for each gene to be deleted allowed construction of multiple deletions. For example, we constructed a quadruple mutant, mc² 155 Δ MSMEG_1517::*dif4* Δ MSMEG_4576::*dif5* Δ MSMEG_3155::*dif6* Δ MSMEG_6059-6058::*dif7*. DNA extracted from this mutant was amplified in PCR reactions with pairs of external primers or with an external primer and a second one internal to the deleted sequence (Figure 2A & Supplementary Table 1). Analysis of the PCR products confirmed the genetic structure of the mutant (Figure 2B).

CONSTRUCTION OF MULTIPLE UNMARKED IN-FRAME DELETIONS IN *M. TUBERCULOSIS*

Deletion of genes embedded within operons often results in polar effects on the downstream genes, and an interesting advantage of excisable cassettes is the

possibility to construct in-frame deletions, which circumvent such effect [4,13]. After excision, the *dif*-Zeo^R-*dif* cassettes used here leave a 78-bp scar with no stop codon in the +1 translation frame (Figure 1E & 3A). Therefore, provided that internal deletions of the target gene were designed in-frame with this +1 frame, the final construction fuses the translation start to the 26 aa translated from this frame and the last codons of the deleted gene (Figure 3B), which should not affect expression of the downstream genes. For example, the genes *ctpC*, *ctpG* and *ctpV*, encoding P-type ATPases involved in efflux of heavy metal ions in *M. tuberculosis* [14–16], are organized in operons containing an upstream gene, encoding a small protein of unknown function, Rv3269, Rv1993c or Rv0968, respectively (Figure 3C). A triple mutant of *M. tuberculosis* deleted of Rv0968, Rv1993c and Rv3269, namely H37Rv Δ Rv0968::*dif5*

Δ Rv1993c::*dif6* Δ Rv3269::*dif4*, was constructed in three rounds of recombineering with appropriate AES (Supplementary Table 2). The PCR verification of this triple mutant is shown in Figure 3E. Sequencing of the PCR fragments encompassing the variant *dif* sites confirmed the in-frame nature of the three deletions and complementation experiments (data not shown) confirmed that the CtpC efflux pump is still expressed in the Δ Rv3269::*dif4* mutant.

In conclusion, we believe that the improvement of the Xer-cise technique described here can be useful to construct mutants in all mycobacterial species prone to recombineering. Our approach is applicable to any antibiotic resistance cassette by adapting the primers used to amplify the cassette (Table 2). It can also be extended to the many bacterial species in which XerCD, or the functionally equivalent XerS or XerH systems are present, ►

and to archaeal species in which a Xer-like system operates [17,18].

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0119

AUTHOR CONTRIBUTIONS

Y-M Boudehen, M Wallat and C Gutierrez performed experiments. O Neyrolles acquired funding. C Gutierrez and Y-M Boudehen wrote the manuscript; O Neyrolles and P Rousseau edited the manuscript. All the authors contributed to conception and design, analysis and interpretation of data.

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