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Functional expression of the Flp recombinase in *Mycobacterium bovis* BCG

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Abstract

Mycobacteria contain a large number of redundant genes whose functions are difficult to analyze in mutants because there are only two efficient antibiotic resistance genes available for allelic exchange experiments. Sequence-specific recombinbases such as the Flp recombinase can be used to excise resistance markers. Expression of the flp_e gene from *Saccharomyces cerevisiae* is functional for this purpose in fast-growing *Mycobacterium smegmatis* but not in slow-growing mycobacteria such as *M. bovis* BCG or *M. tuberculosis*. We synthesized the flp_m gene by adapting the codon usage to that prefered by *M. tuberculosis*. This increased the G+C content from 38% to 61%. Using the synthetic flp_m gene, the frequency of removal of *FRT-hyg-FRT* cassette from the chromosome by the Flp recombinase was increased by more than 100-fold in *M. smegmatis*. In addition, 40% of all clones of *M. bovis* BCG had lost the *hyg* resistance cassette after transient expression of the flp_m gene. Sequencing of the chromosomal DNA showed that excision of the *FRT-hyg-FRT* cassette by Flp was specific. These results show that the flp_m encoded Flp recombinase is not only an improved genetic tool for *M. smegmatis*, but can also be used in slow growing mycobacteria such as *M. tuberculosis* for constructing unmarked mutations. Other more sophisticated applications in mycobacterial genetics would also profit from the improved Flp/*FRT* system.

Keywords

sequence-specific recombination; Flp recognition target; codon usage; unmarked mutation

1. INTRODUCTION

Mycobacterium tuberculosis is a major global health problem and causes about 2 million deaths per year. To understand mycobacterial pathogenesis at the molecular level, efficient and specific genetic systems for recombination, mutagenesis and complementation are required (Pelicic et al., 1998). In particular, the ability to construct mutants by allelic exchange is imperative to characterize the function of a particular gene. Considerable progress in constructing allelic exchange mutants in mycobacteria has been achieved using conditionally replicating temperature-sensitive plasmids (Pelicic et al., 1997) or specialized transducing mycobacteriophages (Lee et al., 1991). However, the main challenge in analyzing the functions of redundant genes is that only a few resistance genes are functional in mycobacteria. Due to

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their superior efficiency, the *hyg* gene from *Streptomyces hygroscopicus* and the *aph* genes are used for almost all knock-out experiments in mycobacteria (Kana and Mizrahi, 2004).

There are two strategies to construct unmarked mutations and to simultaneously solve the problem of limited resistance markers in mycobacteria. One is based on two consecutive allelic exchange reactions. This is tedious work for both construction and analysis of the mutants. Sequence-specific recombination provides a faster and more efficient strategy. Several site-specific recombination systems are used in *E. coli*. The most frequently used system is the Flp/*FRT* system from the 2 μm plasmid of *Saccharomyces cerevisiae* (Merlin et al., 2002). In addition, the Cre/*loxP* system of the bacteriophage P1 (Hasan et al., 1994), the TnpR/*res* system of the γδ transposon (Tsuda, 1998) and the ParA/*res* system of the broad-host-range plasmid RP4 (Denome et al., 1999) are known. In our previous work, we showed that the Flp-flanked DNA was removed by the Flp recombinase in *M. smegmatis* but not in *M. bovis* BCG or *M. tuberculosis* (Stephan et al., 2004). It was discussed that the low G+C content of 38% of the *S. cerevisiae* flp_e gene may have impaired its expression in mycobacteria which have an average G+C content of >65 %. However, it was unknown why this affected expression more severely in slowly growing mycobacteria (Stephan et al., 2004). In this study, we describe the synthesis of a flp_m gene whose codon-usage was adapted for efficient translation in mycobacteria. Using this mycobacterial flp_m gene the efficiency of the Flp-mediated recombination process increased drastically both in *M. smegmatis* and in *M. bovis* BCG. The mycobacterial flp_m gene is useful both for the construction of unmarked mutants and for the analysis of essential genes in mycobacteria. Thus, it adds another genetic tool to the growing toolbox used to dissect the pathogenesis of *M. tuberculosis* on a molecular level.

2. MATERIALS AND METHODS

2.1. Chemicals, enzymes and DNA

Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck, Roche or Sigma at the highest purity available. Enzymes for DNA restriction and modification were purchased from New England Biolabs. Isolation and modification of DNA was performed as described (Ausubel et al., 1987). Oligonucleotides were obtained from Integrated DNA Technologies.

2.2. Bacterial strains and growth conditions

Escherichia coli DH5α was used for cloning experiments and was routinely grown in Luria-Bertani broth at 37°C. *M. smegmatis* strains were grown at 37°C in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween®80 or on Middlebrook 7H10 agar (Difco) supplemented with 0.2% glycerol. *M. bovis* BCG (strain Institut Pasteur) was grown in Middlebrook 7H9 broth (Difco) or on 7H10 agar plates supplemented with 0.2% glycerol and 10% OADC enrichment (BBL) at 37°C. Antibiotics were used when required at the following concentrations: hygromycin (200 μg ml^{-1} for *E. coli*; 50 μg ml^{-1} for mycobacteria) and kanamycin (50 μg ml^{-1} for *E. coli*; 30 μg ml^{-1} for mycobacteria).

2.3. Synthesis of the mycobacterial flp_m gene

To increase the expression of *S. cerevisiae* flp_e in mycobacteria, the codon usage of the flp_e gene was altered to reflect the codon usage preferred by *M. tuberculosis* H37Rv (1,368,699 codons from 4,067 CDS, http://www.kazusa.or.jp/codon/). The codons which were chosen to replace rare codons of the flp_e gene are shown in Table 1. This synthetic gene flp_m was assembled from oligonucleotides. Briefly, the oligonucleotides (two 30-mers, forty 50-mers) were synthesized on a 25 nmol scale with no purification and dissolved in water to a final concentration of 100 μM each. To assemble the oligonucleotides, PCR reactions were performed as described (Withers-Martinez et al., 1999) with minor modifications. In order to

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obtain optimal amplification for G+C rich fragments, DMSO (Sigma) was added to all PCR reactions to a final concentration of 5% (v/v). The synthetic flp_m gene was cloned into the *E. coli* pUC57 vector (Fermentas) by TA cloning and verified by DNA sequencing. This plasmid was named pUC57- flp_m. The G+C content of the mycobacterial flp gene (flp_m) and the flp_e of *S. cerevisiae* is 61% and 38%, respectively (Fig. S1).

2.4. Construction of plasmids pML116 and pML597

The flp_e expression vector pMN234 was constructed previously in our lab (Stephan et al., 2004). To obtain the flp_m expression vector, pUC57-flp_m was digested with HindIII/BamHI and the fragment was cloned into pMN234 using the same restriction sites. This plasmid was named pML597.

To integrate the *FRT*-flanked *hyg* cassette into the genomic *attB* site of mycobacteria, the plasmid pML116 (Fig. 1) was constructed by cloning the ClaI/PmeI flanked *mycgfp2+* gene of pML113 (Wolschendorf et al., 2007) into the backbone of pMN403 using the same restriction sites (Kaps et al., 2001). pML116 contains the *attP* site for the L5 integrase, a *FRT*-flanked *hyg* cassette and an expression cassette for *mycgfp2+* which encodes an enhanced green fluorescent protein (GFP) under p_{smyc} promoter. The *mycgfp2+* gene contains the same fluorescence enhancing mutations as *gfp+* (Scholz et al., 2000) and was adapted to the mycobacterial codon usage (Niederweis et al., unpublished).

2.5. Transformation and site specific recombination

To integrate the *FRT*-flanked *hyg* cassette (pML116) into the genomic *attB* site of mycobacteria, a two-plasmid system derived from mycobacteriophage L5 was used. Briefly, the replicative vector pML102 (Stephan et al., 2005) carrying the L5 integrase gene (*int*) and the counterselection marker *sacB* was transformed into *M. smegmatis* and *M. bovis* BCG. These cells were transformed with the nonreplicative vector pML116 containing the phage attachment site *attP* and the *FRT*-flanked *hyg* cassette. Since the continued expression of L5 integrase can cause the excision of the integrated vector from the genome contributing to plasmid instability (Springer et al., 2001), cells were plated on 7H10 plates containing hygromycin and 10% sucrose to select for the insertion of pML116 and to counterselect against pML102. Single colonies were plated in parallel on 7H10 plates containing hygromycin and 7H10 plates containing kanamycin to confirm the loss of pML102. The integration of the plasmid resulted in clones resistant to hygromycin and sensitive to kanamycin. Vector integration into the chromosome was visualized by GFP fluorescence and confirmed by colony PCR. The *M. smegmatis* SMR5 and *M. bovis* BCG strains with integrated pML116 were named ML17 (*attB::pML116*) and ML37 (*attB::pML116*), respectively.

To compare the efficacies of the Flp recombinases encoded by flp_e or by the synthetic flp_m genes for removal of *FRT-hyg-FRT c*assette, the plasmids pMN234 (flp_e) and pML597 (flp_m) were transformed into competent cells of *M. smegmatis* ML17 and *M. bovis* BCG ML37 and plated on 7H10 plates containing kanamycin. One colony was picked from each plate and cultured in 7H9-kan medium for two days (*M. smegmatis*) or two weeks (*M. bovis* BCG) to saturation. Then serial dilutions in 7H9 medium ranging from 10^{-4}–10^{-8} were plated on 7H10-kan plates. Two hundred colonies were picked from each plate and streaked in parallel on 7H10 and 7H10-hyg plates. The colonies which grew on 7H10 but not on 7H10-hyg plates were counted.

2.6. Analysis of chromosomal DNA by colony PCR

Colony PCR was used to analyze plasmid integration into the chromosome and removal of *FRT-hyg-FRT* cassette using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) according to the manufacturer's protocol with the primers MGR19 (5′-

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CGACCAGGATCGGGACGACGCCGGTGAACAGCTCCTCGCC-3′) and downattP (5′-TGATCTGCGACGAACCACGACCTTGGTG-3′) for *M. smegmatis,* and primers MGR19 and attBbcg01 (5′-CAGGTTGACGACAAGATCCCCGTCGA-3′) for *M. bovis* BCG, respectively. The PCR products were gel-purified using GFX™ PCR DNA and gel band purification kit (GE Healthcare) and sequenced to verify the removal of the *hyg* gene using the specific primers downattP and attBbcg01.

3. RESULTS AND DISCUSSION

3.1. Expression system for a synthetic flp gene with a mycobacterial codon-usage

The goal of our work was to establish a system which can be used for consecutive genomic deletions in mycobacteria. Since resistance markers suitable for selection of allelic mutants are limited to hygromycin and kanamycin in these organisms, it is necessary to remove the marker from the chromosome for subsequent modifications. We chose the Flp/*FRT* system as it represents one of the best characterized recombination systems (Schweizer, 2003). In its resolvase reaction, the Flp recombinase binds to two recognition sites and excises the sequence between them, thereby leaving one *FRT* site in the chromosome without polar effect (Merlin et al., 2002). However, the native flp_e gene of *S. cerevisiae* does not appear to be expressed in slowly growing mycobacteria (Stephan et al., 2004). The factors which could play a role for heterologous gene expression include codon bias (Kane, 1995), transcriptional promoters (Morris and Miller, 1992), the nucleotide sequences surrounding the N-terminus (Deana et al., 1998), mRNA stability (Stenstrom and Isaksson, 2002) and product toxicity (Cherepanov and Wackernagel, 1995). Analysis of the codon usage revealed that the flp_e gene contains 57 codons (out of 424) used in mycobacteria with a frequency of less than 5 % (Table 1) and are therefore considered to be rare codons (Kane, 1995). Since rare codon gene expression can lead to misincorporation of amino acids, termination of translation, or frameshift errors (Calderone et al., 1996), we redesigned and synthesized the flp_e gene using codons preferred by *M. tuberculosis*. To avoid an inbalanced tRNA pool due to the usage of only one codon for a particular amino acid (Kurland and Gallant, 1996), we used two codons preferred by mycobacteria for the amino acids Ala, Arg, Leu and Thr (Table S1). In addition, we also removed the singular restriction sites within the flp_m gene, which increases the compatibility of the gene with various expression vectors. The flp_e gene of *S. cerevisiae* is expressed from the constitutive promoter p_{imyc} in the plasmid pMN234 (Stephan et al., 2004). We exchanged the flp_e gene with the flp_m gene to obtain pML597. Both plasmids contain the *aph* gene and are thereby selectable in hygromycin resistant strains. They also contain the *rpsL* gene as a counter-selectable marker for easy removal in *rpsL* mutants of mycobacteria which are streptomycin-resistant (Sander et al., 1995).

3.2. Integration of the marker plasmid into the chromosome of M. smegmatis and Flp-mediated removal of the hyg cassette

We constructed the integration vector pML116 as a carrier for the *FRT*-*hyg*-*FRT* cassette with two minimal *FRT* sites in direct orientation and a *gfp* reporter gene (Fig. 1). The integration was mediated by the L5 recombinase on the plasmid pML102 which was removed after integration by counterselection using 10% sucrose. The correct integration of the plasmid pML116 into the *attB* site was verified for 20 clones both by colony PCR and GFP fluorescence (Fig. 2A, B and C). One of the resulting strains was named *M. smegmatis* ML17 (*attB::pML116*). To compare the excision efficiencies mediated by the Flp recombinase encoded by the flp_e and flp_m genes, *M. smegmatis* ML17 containing the *FRT*-flanked *hyg* gene in the *attB* site was transformed with the plasmids pMN234 and pML597, respectively. The transformants were selected on plates containing kanamycin. One clone was chosen and grown for two days to saturation in liquid medium and then plated on plates containing kanamycin. To test whether *FRT*-*hyg*-*FRT* was excised, 200 clones were streaked in parallel on plates with

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and without hygromycin. Out of the 200 clones containing the flp_e gene of *S. cerevisiae* only one clone did not grow on hygromycin plates indicating the loss of the *hyg* gene with a frequency of 0.5% (Table 1). These low excision frequencies upon expression of flp_e were confirmed in multiple experiments using the *FRT-hyg-FRT* cassette in different chromosomal locations. The reason for the 10-fold higher excision frequencies as observed in previous experiments is unknown (Stephan et al., 2004). One explanation may be that the expansion of an early clone which has lost the *hyg* cassette would result in more *hyg* negative cells and in an apparently higher recombination frequency. If such an event contributed to the number *hyg* negative clones, the recombination frequency should be different for different excision experiments. Therefore, two clones were selected after transformation with the flp_m expression plasmid pML597 and grown for two days to saturation in liquid medium before plating the culture on plates containing kanamycin. In experiments with both clones, the *hyg* gene was lost with a similar frequency of more than 60% (Table 1). The Flp-mediated removal of the *hyg* cassette was verified by colony PCR for eight of these clones (Fig. 2C). Clone #1 was named *M. smegmatis* ML27 (*attB*::pML116 *hyg*). Sequencing of the chromosomal DNA of the ML27 strain confirmed that the *hyg* gene of ML17 was specifically excised and replaced by one *FRT* site (Fig. 2D). These results demonstrate that the codon-usage adapted flp_m gene confers a much higher recombination efficiency in *M. smegmatis* than the flp_e gene of *S. cerevisiae*.

3.3. Integration of the marker plasmid into the chromosome of M. bovis BCG and Flp-mediated removal of the hyg cassette

The integration vector pML116 was also used as a carrier for the *FRT-hyg-FRT* cassette and the *gfp* reporter gene in *M. bovis* BCG (Fig. 1). The integration was mediated by the L5 recombinase on the plasmid pML102 which was removed after integration by counterselection using 2% sucrose. The correct integration of the plasmid pML116 into the *attB* site was verified for 20 clones both by colony PCR and GFP fluorescence (Fig. 3A, B and C). One of the resulting strains was named *M. bovis* BCG ML37 (*attB::pML116*). To compare the efficacies of the Flp recombinases encoded by the flp_e gene from *S. cerevisiae* and the flp_m gene, *M. bovis* BCG ML37 containing the *FRT*-flanked *hyg* gene in the *attB* site was transformed with the plasmids pMN234 and pML597, respectively. The transformants were selected on plates containing kanamycin. One clone was chosen and grown for two weeks to saturation in liquid medium and then plated on plates containing kanamycin. To test whether *FRT-hyg-FRT* was excised, 200 clones were streaked in parallel on plates with and without hygromycin. Out of the 200 clones containing the flp_e gene of *S. cerevisiae*, all of them grew on hygromycin plates indicating the loss of the *hyg* gene with a frequency of 0% (Table 1). Two clones were selected after transformation with the flp_m expression plasmid pML597 and grown for two weeks to saturation in liquid medium before plating the culture on plates containing kanamycin. In both experiments, the *hyg* gene was lost with a frequency of at least 40% (Table 1). The Flp-mediated removal of the *hyg* cassette was verified by colony PCR for eight of these clones (Fig. 3C). Clone #1 was named *M. bovis* BCG ML47 (*attB*::pML116Δ*hyg*). Sequencing of the chromosomal DNA of the ML47 strain confirmed that the *hyg* gene of ML37 was specifically excised and replaced by one FRT site (Fig. 3D). These results demonstrated that the codon-usage adapted flp_m gene confers a much higher recombination efficiency in *M. bovis* BCG than the flp_e gene of *S. cerevisiae*. These results further demonstrate that expression of the Flp recombinase is not toxic in mycobacteria as it was found for other bacteria including *E. coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Cherepanov and Wackernagel, 1995).

3.4. Role of codon usage for the expression of heterologous genes in mycobacteria

The codon distribution, genome G+C content, and the changes in codon usage are at least partly explained by a mutation-selection equilibrium between the different synonymous codons in

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each organism (Gustafsson et al., 2004). Although codon bias is not the only factor involved in protein expression, it has become increasingly clear that codon biases can have profound impacts on the expression of heterologous proteins (Kane, 1995). Indeed, the result that the codon-usage adapted flp_m gene confers a much higher recombination efficiency both in *M. smegmatis* and *M. bovis* BCG than the flp_e gene of *S. cerevisiae*, strongly indicates that mRNA transcribed from the flp_e gene of *S. cerevisiae* was not efficiently translated in both organisms. This is consistent with previous observations that codon usage adaptation improved the expression of other heterologous genes with low G+C content in mycobacteria, such as Sm14 antigen of *Schistosoma mansoni* (Varaldo et al., 2006) and human immunodeficiency virus type 1 Gag (Kanekiyo et al., 2005). Taken together, these studies indicate that codon usage adaptation may be a generally useful approach to increase expression of heterologous genes in mycobacteria. It may also alleviate putative growth-inhibiting effects of depleting the tRNA pool of mycobacteria from rare tRNAs which may be needed for the synthesis of essential housekeeping genes or are involved in post-transcriptional regulatory processes.

3.5. Comparison of the sequence-specific recombination systems available for mycobacteria

In addition to the Flp/*FRT* system, the use of two other sequence-specific recombination systems in mycobacteria has been described: the Cre/*loxP* system of the bacteriophage P1 (Hasan et al., 1994) and the TnpR/*res* system of the γδ transposon (Tsuda, 1998). The minimal target sites of the Flp recombinase (423 amino acids) and the Cre recombinase (343 amino acids) have identical lengths of 34 bp ((Sternberg and Hamilton, 1981; McLeod et al., 1986), Table 2). Expression of these recombinases in a host which harbors two target sites in direct orientation results in excision of the flanked DNA fragment and leaves a single target site in the chromosome. We have shown that the excision frequencies for the Flp recombinase encoded by the flp_m gene are similar to those for the Cre recombinase (unpublished data, Table 2). A notable difference between the target sites was revealed by sequence analysis: the *FRT* site has stop codons in reading frames 1 and 3, while the *loxP* site has stop codons in reading frames 2 and 3 with respect to the reading frame of the gene in which the site was inserted. This difference might become important if DNA within an operon should be deleted. In-frame deletions without interfering with translation was shown to be essential for efficient expression of downstream genes in operons (Liberati et al., 2006). The transposon γδ resolvase TnpR is also functional in mycobacteria (Malaga et al., 2003). However, the *res* site is 122 bp long (Heffron et al., 1979) and contains seven stop codons which are present in all three reading frames (Table 2). This may represent a disadvantage for the use of the γδ resolvase for small in-frame deletions or similar applications. The drastic increase of the excision frequency by the Flp recombinase expressed from the codon-usage adapted flp_m gene indicates that the expression level is the major determinant of the activities of these enzymes in host cells.

4. CONCLUSIONS

Future, more sophisticated expression and gene deletion constructs for mycobacteria require more tools. The flp_m based Flp/*FRT* system provides an efficient site-specific recombination system which can be used for multiple applications including constructing unmarked mutants in fast- and slow-growing mycobacteria. Although the same *FRT* cassette can be repeatedly used for construction of consecutive mutations in the same chromosome, the presence of multiple chromosomal *FRT* sites can possibly lead to undesirable secondary effects, such as inversions or deletions between these sites. In *P. aeruginosa*, the combination of two mutations caused the Flp-mediated inversion of a 1.59-Mb chromosomal region at fairly high frequencies (Barekzi et al., 2000). Therefore, the combinatorial use of Cre*/loxP,* TnpR/res and Flp*/FRT* provides a new perspective for constructing consecutive deletions and other genetic modifications in mycobacteria.

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Supplementary Material

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Abbreviations

bp
: base pair(s)

kbp
: 1000 bp

Flp
: *S. cerevisiae* recombinase

FRT
: Flp recognition target

CDS
: coding sequence

hyg
: hygromycin phosphotransferase encoding gene

ori
: origin of DNA replication

PCR
: polymerase chain reaction

wt
: wild-type

kan
: kanamycin

hyg
: hygromycin

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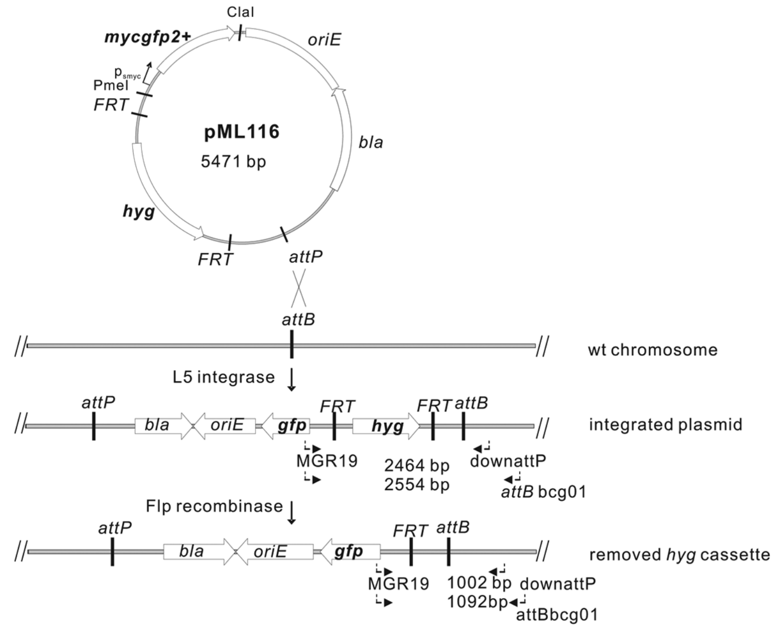

Fig. 1. Representation of the Flp-mediated excision of the *hyg* gene from a plasmid integrated into mycobacterial chromosomes

The plasmid pML116 carries a hygromycin resistance gene (*hyg*) flanked by two *FRT* sites in direct orientation, a *gfp* reporter gene (*mycgfp2+*), the *attP* site of mycobacteriophage L5 used for site-specific integration into mycobacterial chromosomes, an ampicillin resistance gene (*bla*) and an origin of replication for *E. coli* (*oriE*). The localization of the oligonucleotides used for PCR and the size of amplified fragments in *M. smegmatis* and *M. bovis* BCG are indicated.

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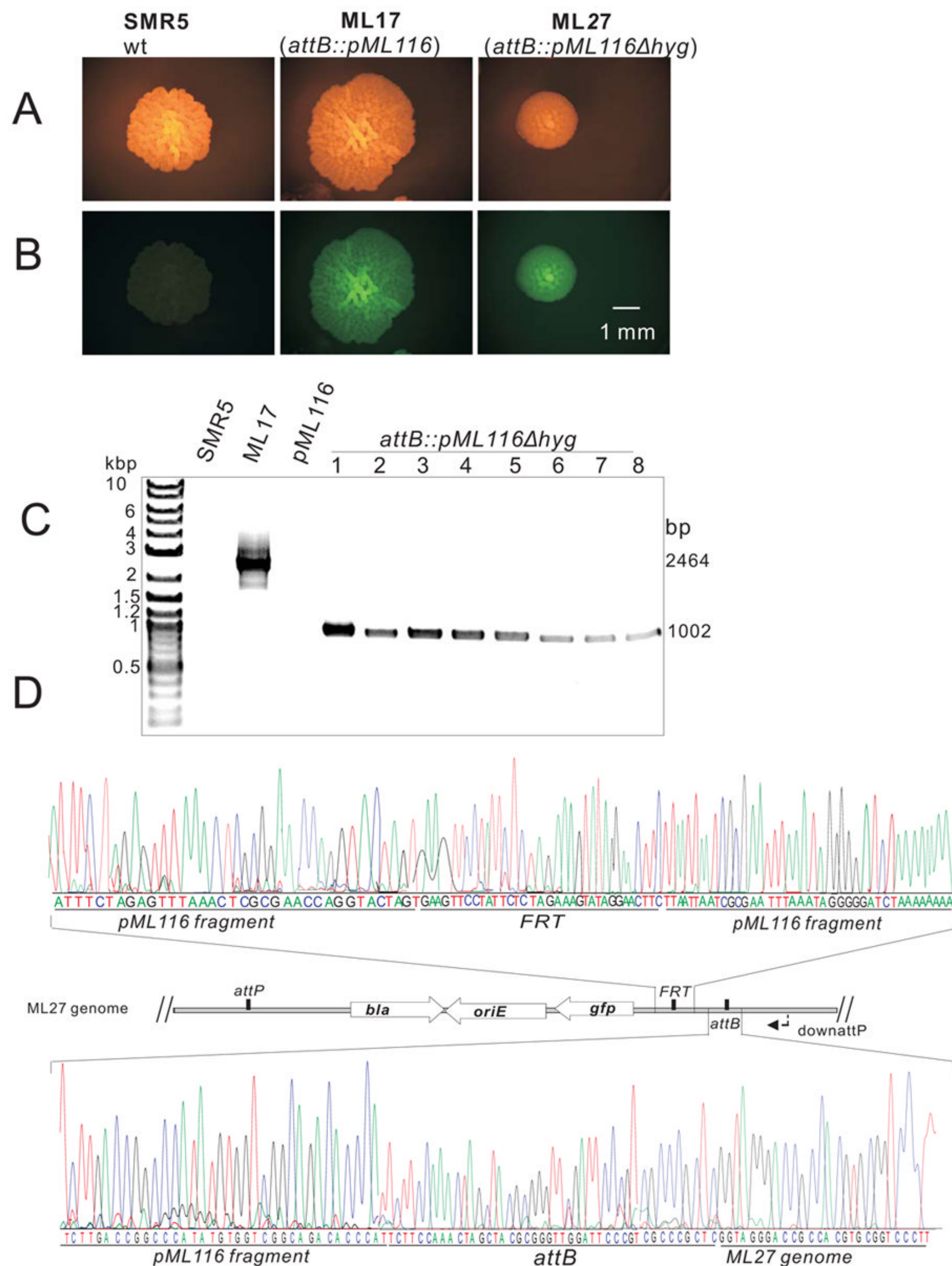

Fig. 2. Integration and removal of a *FRT-hyg-FRT* cassette from *M. smegmatis*

A and B: Microscopy of single colonies of *M. smegmatis* SMR5 (wt), ML17 (*attB::pML116*) and ML27 (*attB::pML116*Δhyg). Pictures of the same colonies were taken with an Olympus SZX12 (Model U-ULH) stereomicroscope equipped with a Zeiss AxioCam MRc camera and an Olympus fluorescence illuminator (100W mercury lamp) using a 20-fold magnification. A fluorescence filter cube (excitation: 470 nm, emission: 500 nm) was used to reveal fluorescence of the colonies. Note that the sizes of the colonies are different because they were incubated for different days. The scale bar is shown.

C: Agarose gel (1%) analysis of the PCR products amplified from chromosomal DNA. The primer pair MGR19 and downattP yielded no fragments for SMR5 and pML116 and fragments of 2,464 bp and 1,002 bp for ML17 and 8 different clones picked after *flp*$_m$ expression.
D: Partial sequencing results of the amplified 1,002 bp fragment from clone #1 which was named *M. smegmatis* ML27 (lane 1 of C) using primer downattP. The locations of the *FRT* and *attB* sites and parts of the pML116 fragment in the genome of ML27 are shown in the map.

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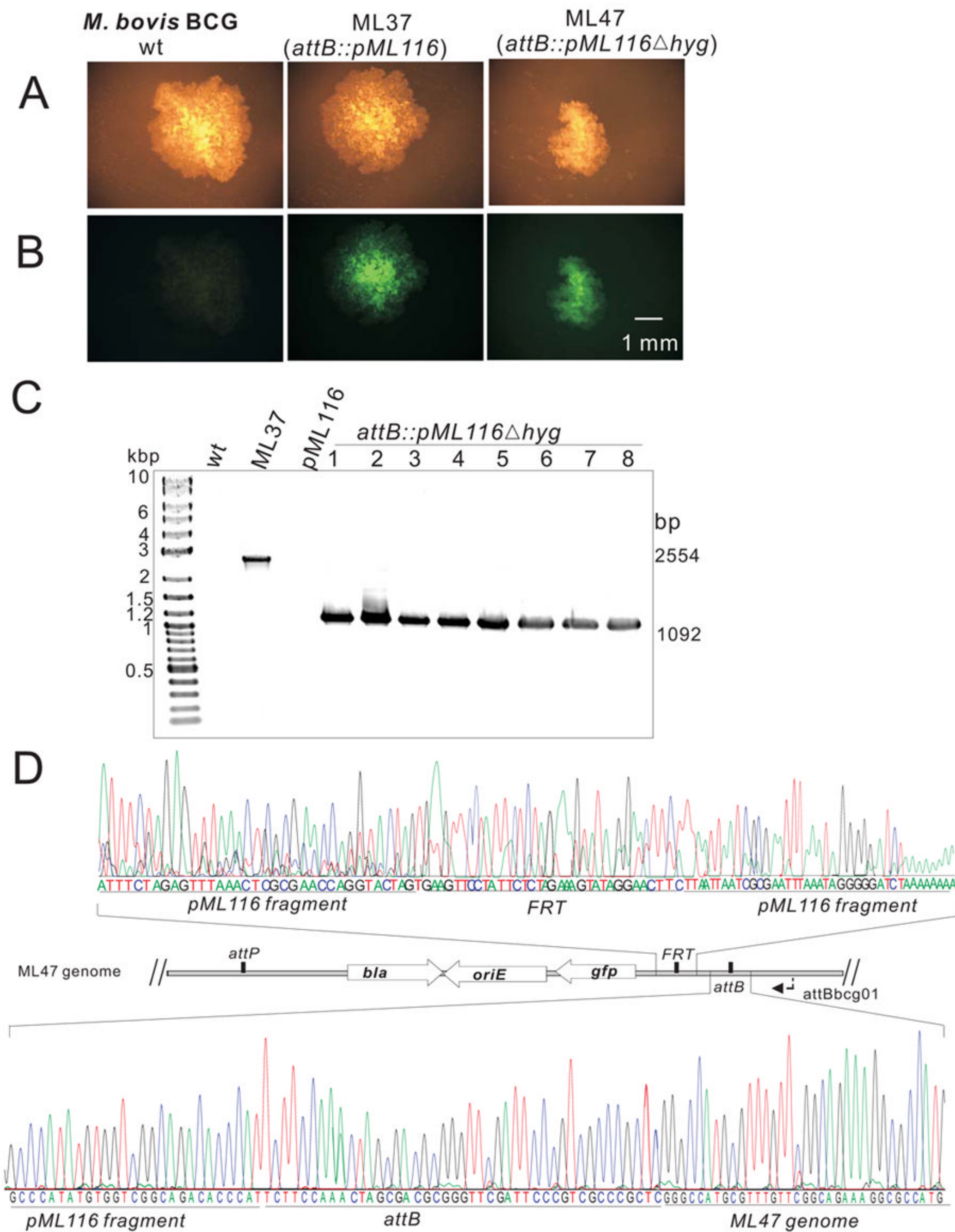

Fig. 3. Integration and removal of *FRT-hyg-FRT* cassette from *M. bovis* BCG

A and B: Microscopy of single colonies of *M. bovis* BCG (wt), ML37 (*attB::pML116*) and ML47 (*attB::pML116Δhyg*). Pictures of the same colonies were taken with an Olympus SZX12 (Model U-ULH) stereomicroscope equipped with a Zeiss AxioCam MRc camera and an Olympus fluorescence illuminator (100W mercury lamp) using a 20-fold magnification. A fluorescence filter cube (excitation: 470 nm, emission: 500 nm) was used to reveal fluorescence of the colonies. Note that the sizes of the colonies are different because they were incubated for different days. The scale bar is shown.

C: Agarose gel (1%) analysis of the PCR products amplified from chromosomal DNA. The primer pair MGR19 and attBbcg01 yielded no fragments for *M. bovis* BCG wt and pML116

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and fragments of 2,554 bp and 1,092 bp for ML37 and 8 different clones picked after flp_m expression.

D: Partial sequencing results of the amplified 1,092 bp fragment from clone #1 which was named *M. bovis* BCG ML47 (lane 1 of C) using primer attBbcg01. The locations of the *FRT* and *attB* sites and parts of the pML116 fragment in the genome of ML47 are shown in the map.

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Table 1

Frequency of excision of the *FRT-hyg-FRT* cassette by Flp recombinase encoded by flp_e and flp_m.

Gene	Strain	Number of clones	Excision frequency	References
flp$_e$	*M. smegmatis*	40	5%	(Stephan et al., 2004)
	M. smegmatis	40	10%	(Stephan et al., 2004)
	M. smegmatis	200	0.5%	This study
	M. bovis BCG	ND	0%	(Stephan et al., 2004)
	M. bovis BCG	200	0%	This study
flp$_m$	*M. smegmatis*	200	64%	This study
	M. smegmatis	120	63%	This study
	M. bovis BCG	200	40%	This study
	M. bovis BCG	32	59%	This study

ND: not determined

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Table 2

Comparison of *FRT*, *loxP* and *res* recombination sites

Recombinase (amino acids)	Target sites	Sequence (5′-3′)	Recombination frequency (%)		Length (bp)
			Msmeg	BCG/*Mtb*	
FLP (423)	FRT	*GAAGTTCCTA*TACTTTC**TAG**AGAA***TAG****GAACTTC*	>60[a]	>40[a]	34[b]
Cre (343)	loxP	*A**TAA**CTTCGTA***TAG**CATACAT*TATACGAAGTTAT*	90[c]	40[c]	34[d]
γδ TnpR (186)	res	AACCGTCCGAAATATTA**TAA**ATTATCGCACACA**TAA**AAACAG TGCTGT**TAA**TGTGTCTAT**TAA**ATCGATTTTTGTTA**TAA**CAGACA CTGCTTGTCCGATATT**TGA**TT**TAG**GATACATTTTT	100[e]	3–5[f]	122[g]

The core regions of the *FRT* and *loxP* sites are underlined. The palindromic sequences of *FRT* and *loxP* are indicated with italic letters. There are three resolvase binding sites at the internal of *res*, which are indicated with box. The stop codons of translation in 5′-3′ orientation and 3′-5′ orientation are indicated with bold and shadow letters, respectively, for each recombination site.

[a] this study

[b] (McLeod et al., 1986)

[c] Song et al. (unpublished data using the plasmid pCreSacB1 and a *hyg* expression cassette flanked by *loxP* sites)

[d] (Sternberg and Hamilton, 1981)

[e] (Malaga et al., 2003)

[f] (Bardarov et al., 2002)

[g] (Kitts et al., 1983)