Available online at www.sciencedirect.com
ScienceDirect
Gene 399 (2007) 112–119
GENE
www.elsevier.com/locate/gene

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

Houhui Song, Michael Niederweis *

*Department of Microbiology, University of Alabama at Birmingham, 609 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294, USA*

Received 30 March 2007; received in revised form 3 May 2007; accepted 4 May 2007
Available online 21 May 2007
Received by R. Britton

**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 preferred 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 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

*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.

* Corresponding author. Tel.: +1 205 996 2711; fax: +1 205 934 9256.
*E-mail address:* mnieder@uab.edu (M. Niederweis).

0378-1119/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2007.05.005

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 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 4067 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 S1. 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 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).

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.

### 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 continued expression of L5 integrase can cause excision of the integrated vector from the genome contributing to plasmid instability (Springer et al., 2001), cells