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Efficient construction of unmarked recombinant mycobacteria using an improved system

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ABSTRACT

The genetic study of mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*, is hampered heavily by their slow growth. We have developed efficient, versatile, and improved genetic tools for constructing unmarked recombinant mycobacteria more rapidly including generating multiple mutants using the same antibiotic marker in both fast- and slow-growing mycobacteria.

1. Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB), is one of the most serious single infectious diseases of mortality worldwide. The 8 million incident cases of TB, 1.45 million deaths from TB patients in 2011 alone (Nahid and Menzies, 2012), and the appearance of multidrug-resistant (MDR), extensively drug-resistant (XDR) and even totally drug-resistant (TDR) TB (Loewenberg, 2012) provide a striking reminder of the magnitude of destruction caused by TB. However, the laboratory research of MTB is highly impeded by the slow growth rate of the MTB strains and lack of convenient and efficient genetic tools for recombination and mutagenesis (van Kessel et al., 2008).

The development and application of genetic manipulation in mycobacteria have accelerated the study of mechanisms of TB pathogenesis and have been used extensively in the generation of potential recombinant live vaccines and in understanding the mechanisms of drug action and resistance. Appropriate antibiotic resistance genes are required for direct selection of the subset of bacteria that have taken up the DNA materials used for constructing recombinant mycobacteria, such as plasmids, cosmids and phasmids (Jacobs et al., 1987). However, the use of antibiotic resistance markers has some drawbacks. Firstly, there are only a few antibiotic resistance genes, especially for mycobacteria. In fact, only kanamycin (KAN) and hygromycin B (HYG) resistance marker genes are used in genetic experiments with strains of the MTB complex (Malaga et al., 2003). Some MTB clinical isolates are already resistant to KAN, which makes KAN resistance gene not suitable for them. Secondly, if a recombinant mycobacterial strain already has a resistance cassette, this excludes the marker for further use in it. This can greatly hamper the study of the functions of redundant genes in mycobacteria in which genomic studies have revealed extensive gene duplications with highly conserved sequences (Garnier et al., 1998) since disruption of one gene may be compensated by its homologous gene (Puech et al., 2002; Agarwal et al., 2009). Therefore, multiple mutation and complementation experiments would be required for uncovering the biological roles of a gene or a series of genes having the same or compensatory functions. Thirdly, the insertion of a resistance gene into an operon could affect the expression of the

Abbreviations: CST1, *dif*-ΩHYG-*dif* CASSETTE V1; CST2, *dif*-ΩHYG-*dif* CASSETTE V2; HYG, hygromycin B; *Hyg*, HYG-resistant gene; KAN, kanamycin; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis; *sacB*, a *Bacillus subtilis* gene encoding the enzyme levansucrase.

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corresponding downstream genes and the polar effect can complicate the characterization of each gene's roles. Fourthly, our previous study found that HYG resistance gene (*Hyg*) may disrupt the virulence of *Mycobacterium ulcerans*. Recombinant *M. ulcerans* strains integrated with a plasmid containing *Hyg* could not cause mouse footpad swelling. However, *M. ulcerans* strains with KAN resistance gene caused the swelling (unpublished data). Therefore, HYG resistance gene might affect its virulence. Lastly, in some special situations, for example, the construction of reporter mycobacterial strains for screening and evaluating drug activity, the resistance marker is not desired because of the potential cross resistance or the inability to test the activity of combined drugs.

The unmarked mutations can be obtained by a two-step strategy in which the first step is to only select one single cross-over recombinant event while the second step is to select the cells in which the second cross-over has occurred. This involves the use of multiple markers for selection and counter-selection of the different allelic exchange events (Parish and Stoker, 2000). In this case, only some of the selected cells would be mutated with the others recovered as wild type. Thus, this method is problematic and more applicable where the mutant has a distinct phenotype, such as auxotrophy. A common way to circumvent this problem is by the production of unmarked mutations which can be realized by developing an antibiotic resistance cassette flanked by two short DNA sequences in direct orientation which can be recognized and catalyzed by a recombinase or resolvase. The recombination of these two short sequences allows the removal of the resistance marker after it has been used for the positive selection and its use in another round of genetic manipulation. These site-specific recombination systems are usually derived from bacteriophages or transposons. Three such systems have been successfully used with mycobacteria and were summarized before (Cascioferro et al., 2010). However, all the systems require the expression of an exogenous resolvase or recombinase from a plasmid to allow the excision of the resistance marker which makes the procedure complicated and very time-consuming.

Recently, a new sequence-specific recombinase system based on the endogenous Xer recombinases (Xer-cise) was successfully adapted to mycobacteria (Cascioferro et al., 2010). In this system, the antibiotic resistance cassette was flanked by *dif* sites which can be recognized and resolved by the endogenous recombinases XerC and XerD. The system does not need the introduction and subsequent elimination of the extrachromosomal plasmids containing exogenous genes for removing the resistance cassette, which makes it extremely simple and practical (Cascioferro et al., 2010). However, the *Hyg* cassette they used was not optimized for the length, the promoter of *Hyg* and the restriction sites in and surrounding it. In addition, the reuse of the same cassette in a single strain was not demonstrated before.

In this study, we constructed an artificial, versatile, *dif*-containing resistance cassette based on the shortened, optimized *Hyg* flanked with artificial promoter and multi-cloning sites. It could be an efficient, versatile tool for constructing unmarked mycobacteria especially combined with the recombineering technique. Recombineering is an important progress in mycobacterial genetic manipulation by inducible expression of recombination proteins derived from mycobacteriophage Che9c for facilitating allelic exchange through increasing the recombination rate (van Kessel and Hatfull, 2007).

This novel cassette and the improved recombineering system will be helpful in stimulating mycobacterial genetic research.

2. Material and methods

2.1. Bacterial strains and culture conditions

Escherichia coli strain DH5α or ET12567 was grown at 37 °C in Luria–Bertani (LB) broth or on LB agar. *Mycobacterium smegmatis* mc^2 155 (MS) and MTB H37Ra were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% oleic acid albumin dextrose catalase (OADC, Becton Dickinson), carbenicillin 50 μg/ml, cycloheximide 10 μg/ml and 0.05% tween 80 as indicated, or on LB agar or on solid Middlebrook 7H11 medium (Difco) supplemented with OADC (or ADC for MS) or containing 10% sucrose if necessary. Ampicillin (Sigma) 100 μg/ml for *E. coli*, KAN (Invitrogen) 40 μg/ml for all species or HYG (Roche Diagnostics) 200 μg/ml for *E. coli*, 150 μg/ml for MS and 50 μg/ml for MTB were added to the agar plates when required. About 20 μg/ml KAN and 100 μg/ml HYG for MS and 10 μg/ml for MTB were added to liquid broth when required. 7H9 induction medium was 7H9 broth supplied with 0.2% succinate and KAN at 20 μg/ml prepared as described before (van Kessel and Hatfull, 2008).

2.2. Plasmid construction

2.2.1. The dif-ΩHYG-dif cassette V1 (CST1) and pTY95

Two copies of the *dif* site were synthesized as DNA oligonucleotides (Table 1) and were inserted into plasmid pUC19 at *Hin*dIII–*Kpn*I sites by 3-fragment-ligation (Zhang et al., 2006a, 2006b) resulting in pTYd. A primer Hse (Table S1) was designed for sequencing the upstream of *Hyg* gene in pNBV1 (Howard et al., 1995). The *Hyg* gene was amplified successfully by PCR from pNBV1 (Howard et al., 1995) with primers Hygf2 and Hygr727 (Table S1), cut with *Xba*I and inserted into pTYd at *Xba*I site to give pTYdH. Its identity was verified by restriction digestion and sequencing. The promoters of the wild type *Hyg* cassette and the artificial promoter in the CST1 (Fig. 1A) were analyzed by the online Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). The plasmid pTYdHm was obtained by removing *Kpn*I and *Eco*RI sites in the *Hyg* in pTYdH using site-directed mutagenesis (Generay, China).

To test if the *Hyg* gene could cause resistance in mycobacteria and the excision efficiency of the CST1, we replaced the KAN resistance gene of the integrative plasmid, pMH94 (Lee et al., 1991), with CST1 at the *Hin*dIII sites to give pTY95 (Fig. 1B).

2.2.2. pJV53Ts and pTY46H

We digested the pJV53 plasmid with *Xba*I–*Spe*I and inserted the larger fragment containing the inducible promoter and *gp60/61* genes into thermosensitive pPR23 (Pelicic et al., 1997) which was cut with the same enzymes and dephosphorylated to avoid the self-ligation as *Xba*I and *Spe*I are isoaudamers. The recombinant plasmid having the

Table 1
DNA oligonucleotides used in this study except those related to *Hyg* gene amplification.

DNA oligo	Nucleotide sequence (5′–3′)
Dlup	(p)AGCTTCTCGAGTAAGCCGATAAGCGACATTATGTCAAGTCCCGGGT
DlLow	(p)CTAGACCCGGGACTTGACATAATGTCGCTTATCGGCTTACTCGAGA
DrUP	(p)CTAGATCGATTAAGCCGATAAGCGACATTATGTCAAGTCTCGAGAA GCTTGGTAC
DrLow	(p)CAAGCTTCTCGAGACTTGACATAATGTCGCTTATCGGCTTAATCGAT
Hyg72F	AGAGCACCAACCCCGTACTG
Hyg72R	GTGAAGTCGACGATCCCGGT
Int72F	TTCATGTGCGCTCGGATCAT
Int72R	TCACGCTGGAGGAGTACACC
Ms614f	TAAGAATTCGCTAGCCGACCTTCGTGATGACTGGC
Ms614r	GATCTGCAGAGGATCCTGGTACCAGGTGGCGAAACGGCTGA
Ms616f	CCACTGCAGCTCGAGTGCGGGTCGGGGAACAAC
Ms616r	ACCAAGCTTACTAGTTGCAGCCCATCGGACAACA
HV2f	GCAGGATCCGATATCGAATTCCATATGCCCAAGCTTCTCGAGACT
HV2r	GCTGGTACCGCTAGCGCTGCAGCCATGGCAAGCTTCTCGAGTAAG
GYFf	GGTCCATGGTGAGCAAGGGCGAGG
GYFr	GCGGGTACCTTACTTGTACAGCTCGTCCATGC
a	GTTAGCTTAGGCATACATAAGG
b	ATCTCGGAGGTCTTGACCAT
c	CCTGGATTCGCGTGCCTACC
d	TACGGGGTTGGTGCTCTCGC
Hygdf	GCCGCCTATGTAGAGCTGGTCGTT
Hygdr	CTTGCCGGTGGTGCAGATGA