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Short Communication

A two-step strategy for the complementation of *M. tuberculosis* mutants

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Abstract

The sequence of *Mycobacterium tuberculosis*, completed in 1998, facilitated both the development of genomic tools, and the creation of a number of mycobacterial mutants. These mutants have a wide range of phenotypes, from attenuated to hypervirulent strains. These phenotypes must be confirmed, to rule out possible secondary mutations that may arise during the generation of mutant strains. This may occur during the amplification of target genes or during the generation of the mutation, thus constructing a complementation strain, which expresses the wild-type copy of the gene in the mutant strain, becomes necessary. In this study we have introduced a two-step strategy to construct complementation strains using the Ag85 promoter. We have constitutively expressed *dos*R and have shown *dos*R expression is restored to wild-type level.

Key words: tuberculosis, mutagenesis, constitutive expression, complementation.

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The completion of the entire sequence of *Mycobacterium tuberculosis* (Cole *et al.*, 1998) launched a new era in tuberculosis research. In order to study the function of *M. tuberculosis* genes, several mutants have been produced by homologous recombination and studied in animal models (Parish and Stoker 2000; Movahedzadeh *et al.*, 2004, 2008, 2010). There is a wide range of phenotypes, from highly attenuated mutants (Smith *et al.*, 2001; Movahedzadeh *et al.*, 2004) to hypervirulent strains (McAdam *et al.*, 2002; Parish *et al.*, 2003). These phenotypes require confirmation by the generation of complementation strains, whereby the wild-type copy of the gene is re-introduced into the mutant strain. By complementation of the mutant strain, one can ensure that the observed mutant phenotype, *e.g.* increased virulence of *M. tuberculosis* with the loss of *dos*R, is actually due to the loss of *dos*R and not to secondary mutations that may have arisen during the creation of the mutant strain.

Several cloning systems for mycobacteria have been developed, based on *M. fortuitum* plasmid pAL5000 (Labidi *et al.*, 1985); such plasmids are generally called shuttle plasmids. Although these have a low copy number in mycobacteria, where replication is driven by the mycobacterial Ori, they have high copy number in *E. coli*. For successful complementation, the wild-type gene must be stably expressed in the mutant strain. This may be accomplished by using a replicating plasmid that contains the mycobacterial Ori, or by an integrating plasmid into the genome of mycobacteria. A competition experiment with *M. bovis* BCG using pMV261 and pMV361 vectors (Stover *et al.*, 1991), showed that only pMV361 was stably maintained. These two plasmids both have an *E. coli* origin of replication, a kanamycin (Km) resistance gene and the *hsp*60 promoter. The difference is that pMV261 is a replicative vector containing the pAL5000 origin of replication and pMV361 is an integrative vector containing an attachment site and the origin of replication genes from mycobacteriophage L5. The use of integrative plasmids for complementation proved to be more advantageous (Stover *et al.*, 1991; Kumar *et al.*, 1998), and they have been successfully developed for mycobacteria, using *attP* and *int* gene of the temperate mycobacteriophage L5, such as pMV306 (MedImmune, MD) and pUC-Int (Mahenthiralingam *et al.*, 1998). One reported disadvantage of the L5 integration vector is that the integration locus would be unfavourable for transcription of integrated genes, when using native promoters (Murry *et al.*, 2005). This is even more marked, when the gene being expressed under its native promoter is not the first gene in the operon. The *Ag*85 promoter has been used in our laboratory and many others (Abdallah *et al.*, 2006; Hu *et al.*, 2006). The 85A antigen is part of a secreted antigenic complex, and represents one of the major secreted proteins from slowly growing mycobacteria (Wi-

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ker and Harboe, 1992). The promoter region controlling the expression of this antigen has already been analyzed (Kremer *et al.*, 1995). In this study, a two-step strategy for constructing complementation strains using the Ag85 promoter was introduced. We have used this vector to express *dos*R constitutively in Tame 16 (*dos*R mutant) and have shown restoration of *dos*R expression to wild-type levels. Deletion of *dosR* (Rv3133c), a transcription factor that mediates the hypoxic response of *M. tuberculosis* (Park *et al.*, 2003), resulted in increased virulence in *M. tuberculosis* (Parish *et al.*, 2003). *dosR* is part of an operon encoding three genes: Rv3134c (*dosT*), Rv3133c (*dosR*), and Rv3132c (*dosS*).

pEM37 (donated by E. Machowski) was digested with *Bg*lII-*Bam*HI and the 275 bp fragment, containing the Ag85 promoter, was then isolated and cloned into the *Bam*HI site of p2Nil (Parish and Stoker, 2000). This plasmid containing the Ag85 promoter consists of a multiple cloning site (MCS) and a kanamycin resistance gene with an origin of replication from *E. coli*.

To permit in-frame cloning into pFM209 under the Ag85 promoter, the following steps is advised: In the first step, the coding sequence of the gene, plus one extra base pair of the upstream sequence should be amplified (high fidelity PCR kit, Boehringer Mannheim), using primers to introduce *Bam*HI restriction sites for both N and C termini, followed by digestion with *Bam*HI (if a *Bam*HI site exits within the gene, *Bg*lII can be used), and final cloning into the *Bam*HI site of pFM209. In the second step, the 3 kb *Hind*III fragment of pUC-Gm-int (Mahenthiralingam *et al.*, 1998), containing the *attP* site, Gm cassette and L5 integrase gene should be cloned into the *Hind*III site of pFM209, in order to produce the integrated version. If a *Hind*III site exists within the gene of interest, a *Pvu*II cassette of pUC-Gm-int can be cloned into the *Pm*lI site of pFM209. Cloning the *Hind*III cassette of pUC-Gm-int, prior to that of the gene of interest, is inadvisable since this cassette has a *Bam*HI restriction site. However, having the gentamycin marker in this cassette facilitates screening of the correct clone in the second stage. This is summarized in Figure 1.

In this study, *dosR* was constitutively expressed by using this vector. *dosR* was amplified with the Expand High Fidelity PCR system using *M. tuberculosis* H37Rv genomic DNA as template and DMSO at 5%. The primers used (each at 300 nM) were dosR-Bam1 (CGCGGATCC GGTGGTAAAGGTC) and dosR-Bam2 (CGAGGATCC TCATGGTCCATCA). The temperature cycle used was as follows: an initial 3 min at 94 °C to denature the DNA; then 10 cycles of 45 s at 94 °C, 1 min at 63 °C and 1 min at 72 °C; then 25 cycles of 45 s at 94 °C, 1 min at 63 °C and 1 min plus 20 s per cycle at 72 °C; and finally an extension step of 72 °C for 7 min. The resulting PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pFM209. The resulting clones were subjected to restriction digestion

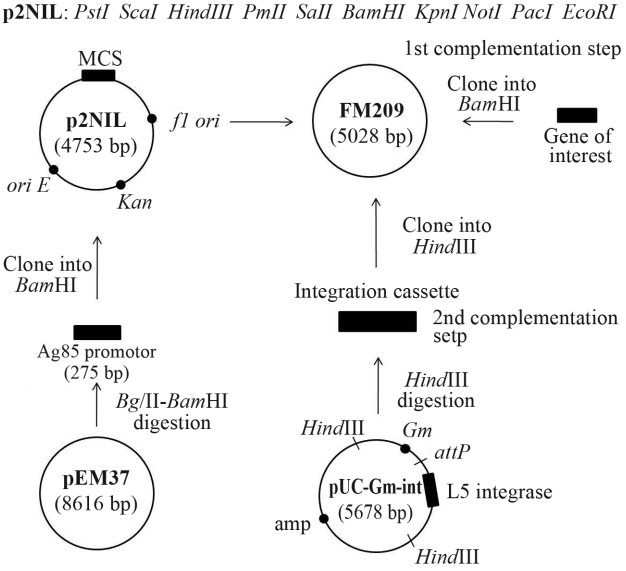

Figure 1 - Construction of pFM209 and the two-step strategy for complementation.

using several enzymes, and were run on a gel to select for the correct orientation of the gene, thereby producing pFM210. The *Hind*III cassette of pUC-Gm-int, carrying the *int* and *gm* genes, was cloned into the *Hind*III site of pFM210 to produce pFM211. The final plasmid was sequenced to confirm that no mutations had been introduced, during either *dos*R amplification, or pFM211construction. The plasmids used in this study are summarized in Table 1.

RTq-PCR experiments were carried out to determine the level of *dos*R mRNA expression in exponential cultures of H37Rv and *a dos*R mutant with constitutive expression of *dos*R under the Ag85 promoter. Expression levels were normalized to those of *sig*A mRNA and calculated based on the RNA used for reverse transcription. RNA was prepared from an exponential (7-day) rolling culture of *M. tuberculosis* H37Rv (Betts *et al.*, 2002) and cDNA synthesis was carried out using Superscript II (Invitrogen), according to the manufacturer's protocol. Reverse-transcription quantitative PCR (RTq-PCR) reactions were set up using the DyNAmo SYBR Green qPCR kit (MJ Research), and performed using the DNA Engine Opticon 2 System (Genetic Research Instrumentation). Reactions containing 1X DNA Master SYBR Green I mix, 1 μL cDNA product and 0.3 mM of each primer in 20 μL, were set up on ice. Samples were heated to 95 °C for 10 min before cycling for 35 cycles of 95 °C for 30 s, 60 °C (*dos*R), or 62 °C (*sig*A) for 20 s, and 72 °C for 20 s. Fluorescence was captured at the end of each cycle, after heating to 80 °C to ensure the denaturation of primer dimers. The experiment was repeated twice using cDNA from each of the two independent RNA preparations. We showed that when *dos*R is constitutively expressed in the mid-exponential phase in Fame101 (ΔdosR::pFM211), the level of *dos*R mRNA is 0.54 (95%

Table 1 - Strains and plasmids used in this study.

Strains/plasmids	Characteristics	Source
E. coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
M. tuberculosis H37Rv	wild-type laboratory strain	ATCC 25618
Tame 16	Δ*dos*R	Parish *et al.*, 2003
Fame101	Δ*dos*R::pFM211	This study
pUC-Gm-int	pUC-based plasmid with *Hind*III cassette carrying gm and L5 *int*	Mahenthiralingam *et al.*, 1998
p2NIL	gene manipulation vector, *kan*^{R}	Parish and Stoker, 2000
pEM37	P $_{Ag85}$ -*lacZ* in *Mycobacterium- E. coli* shuttle vector	Edith Machowski, Johannesburg, South Africa
pFM209	Ag85 promoter in p2Nil	This study
pFM210	*dos*R in pFM209	This study
pFM211	pFM210:: *int gm*	This study

Gm – Gentamycin resistant; kan^R – Kanamycin resistant.

confidence interval 0.38-0.75) to that of *sig*A. A t-test was run and no significant difference was observed between the constitutively expressed *dos*R in the *dos*R mutant and in the wild-type. In H37Rv the level of *dos*R mRNA is 0.55 (95% confidence interval 0.47-0.65) to that of *sig*A (Figure 2). As expected, no *dos*R expression was observed under the above conditions (not detectable, below 50,000 copy). We have also used this method to complement one of the unknown *M. tuberculosis* genes, located in the middle of an operon. Complementation of this mutant restored the attenuation phenotype observed in a mouse model (unpublished data).

The method described here is useful for the complementation of mycobacterial mutants, especially for genes located in the middle of an operon, where it would be difficult to use its own promoter. Furthermore, by using the integrative approach, the instability previously reported in certain shuttle vectors (Stover *et al.*, 1991; Kumar *et al.*, 1998), would be avoided. Although the *hsp*60 promoter has been used successfully for gene expression in mycobacteria, some problems have been reported (Nicola Casali, PhD thesis, 1998) when expressing *lacZ* under this promoter, such as colonies rapidly losing color. When the plasmids were extracted, some rearrangements or deletions were found. Possibly, there are two potentially related but separate problems: the expression being too high (causing toxicity problems to the cells), and the tendency of the promoter DNA to rearrange. We favour the use of Ag85 promoter since it has been shown by others to be more stable than the Hsp60 promoter (Haeseleer, 1994; Al-Zarouni and Dale, 2002).

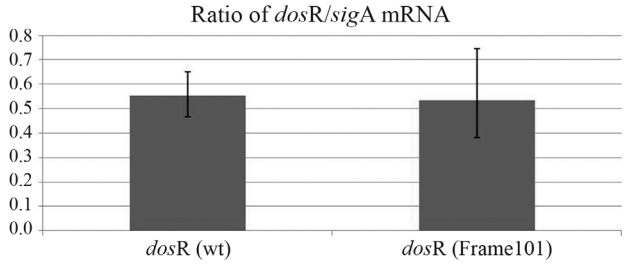

Figure 2 - Expression level of *dos*R using RTq-PCR. RNA was extracted from both 7-day rolling cultures of *M. tuberculosis* H37Rv and Fame101, then reverse transcribed to cDNA. RTq-PCR was then carried out using the DyNAmo SYBR Green qPCR kit and DNA Engine Opticon 2 System. Expression levels were normalized to those of *sig*A mRNA. We found that the ratio of *dos*R/*sig*A mRNA of the constitutively expressed *dos*R in Fame 101 (0.54), is not significantly different from that of the wild-type (0.55). Levels of *dosR* were undetected in the mutant.

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References

Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, Eisenberg D, Musters RJ, Vandenbroucke-Grauls CM, Appelmelk BJ, Luirink J, *et al*. (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 62:667-679.

Al-Zarouni M and Dale JW (2002) Expression of foreign genes in *Mycobacterium bovis* BCG strains using different promoters reveals instability of the hsp60 promoter for expression of foreign genes in *Mycobacterium bovi*s BCG strains. Tuberculosis (Edinb) 82:283-291.

Betts JC, Lukey PT, Robb LC, McAdam RA and Duncan K (2002) Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol 43:717-731.

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry 3rd CE, *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537-544.

Haeseleer F (1994) Structural instability of recombinant plasmids in mycobacteria. Res Microbiol 145:683-687.

Hu Y, Movahedzadeh F, Stoker NG and Coates AR (2006) Deletion of the *Mycobacterium tuberculosis* alpha-crystallin-like hspX gene causes increased bacterial growth *in vivo*. Infect Immun 74:861-868.

Kremer L, Baulard A, Estaquier J, Content J, Capron A and Locht C (1995) Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. J Bacteriol 177:642-653.

Kumar D, Srivastava BS and Srivastava R (1998) Genetic rearrangements leading to disruption of heterologous gene expression in mycobacteria: An observation with E*scherichia coli* beta-galactosidase in *Mycobacterium smegmatis* and its implication in vaccine development. Vaccine 16:1212-1215.

Labidi B, Gregoire M, Hernandez-Verdun D and Bouteille M (1985) Procedure for isolating micronuclei from rat kangaroo cultured cells containing individualized chromosomes. Eur J Cell Biol 38:165-170.

Mahenthiralingam E, Marklund BI, Brooks LA, Smith DA, Bancroft GJ and Stokes RW (1998) Site-directed mutagenesis of the 19-kilodalton lipoprotein antigen reveals no essential role for the protein in the growth and virulence of *Mycobacterium intracellulare*. Infect Immun 66:3626-3634.

McAdam RA, Quan S, Smith DA, Bardarov S, Betts JC, Cook FC, Hooker EU, Lewis AP, Woollard P, Everett MJ, *et al.* (2002) Characterization of a *Mycobacterium tuberculosis* H37Rv transposon library reveals insertions in 351 ORFs and mutants with altered virulence. Microbiology 148:2975-2986.

Movahedzadeh F, Wheeler PR, Dinadayala P, Av-Gay Y, Parish T, Daffe M and Stoker NG (2010) Inositol monophosphate phosphatase genes of *Mycobacterium tuberculosis*. BMC Microbiology 10:50.

Movahedzadeh F, Smith DA, Norman RA, Dinadayala P, Murray-Rust J, Russell DG, Kendall SL, Rison SC, McAlister MS, Bancroft GJ, *et al.* (2004) The *Mycobacterium tuberculosis ino1* gene is essential for growth and virulence. Mol Microbiol 51:1003-1014.

Movahedzadeh F, Williams A, Clark S, Hatch G, Smith D, ten Bokum A, Parish T, Bacon J and Stoker N (2008) Construction of a severely attenuated mutant of *Mycobacterium tuberculosis* for reducing risk to laboratory workers. Tuberculosis (Edinb) 88:375-381.

Murry J, Sassetti CM, Moreira J, Lane J and Rubin EJ (2005) A new site-specific integration system for mycobacteria. Tuberculosis (Edinb) 85:317-323.

Parish T and Stoker NG (2000) Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* tlyA plcABC mutant by gene replacement. Microbiology 146:1969-1975.

Parish T, Smith DA, Kendall S, Casali N, Bancroft GJ and Stoker NG (2003) Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. Infect Immun 71:1134-1140.

Park HD, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik GK and Sherman DR (2003) Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. Mol Microbiol 48:833-843.

Smith DA, Parish T, Stoker NG and Bancroft GJ (2001) Characterization of auxotrophic mutants of *Mycobacterium tuberculosis* and their potential as vaccine candidates. Infect Immun 69:1142-1150.

Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF, *et al.* (1991) New use of BCG for recombinant vaccines. Nature 351:456-460.

Wiker HG and Harboe M (1992) The antigen 85 complex: A major secretion product of *Mycobacterium tuberculosis*. Microbiol Rev 56:648-661.

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