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 dosR and have shown dosR expression is restored to wild-type level.

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

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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 dosR constitutively in Tame 16 (dosR mutant) and have shown restoration of dosR 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 BglIII-BamHI and the 275 bp fragment, containing the Ag85 promoter, was then isolated and cloned into the BamHI 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 are 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 BamHI restriction sites for both N and C termini, followed by digestion with BamHI (if a BamHI site exits within the gene, BglIII can be used), and final cloning into the BamHI site of pFM209. In the second step, the 3 kb HindIII fragment of pUC-Gm-int (Mahenthiralingam et al., 1998), containing the attP site, Gm cassette and L5 integrase gene should be cloned into the HindIII site of pFM209. In order to produce the integrated version. If a HindIII site exists within the gene of interest, a PvuII cassette of pUC-Gm-int can be cloned into the PvuII site of pFM209. Cloning the HindIII cassette of pUC-Gm-int, prior to that of the gene of interest, is inadvisable since this cassette has a BamHI 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 GTGTTAAAAGGTC) and dosR-Bam2 (CGAGGGATC TCAATGGTCCCATCA). 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 BamHI and cloned into the BamHI site of pFM209. The resulting clones were subjected to restriction digestion using several enzymes, and were run on a gel to select for the correct orientation of the gene, thereby producing pFM210. The HindIII cassette of pUC-Gm-int, carrying the int and gm genes, was cloned into the HindIII site of pFM210 to produce pFM211. The final plasmid was sequenced to confirm that no mutations had been introduced, during either dosR amplification, or pFM211 construction. The plasmids used in this study are summarized in Table 1.

RTq-PCR experiments were carried out to determine the level of dosR mRNA expression in exponential cultures of H37Rv and a dosR mutant with constitutive expression of dosR under the Ag85 promoter. Expression levels were normalized to those of sigA mRNA and calculated based on the RNA used for reverse transcription. RNA was prepared from an exponential (7-day) rolling culture of M. tuberculosi 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 1 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 (dosR), or 62 °C (sigA) 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 dosR is constitutively expressed in the mid-exponential phase in Fame101 (AdosR::pFM211), the level of dosR mRNA is 0.54 (95%
Table 1 - Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F–Δ80lacZAM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (K–, mK+) phoA supE44 Δ– thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>wild-type laboratory strain</td>
<td>ATCC 25618</td>
</tr>
<tr>
<td>Tame 16</td>
<td>ΔdosR</td>
<td>Parish et al., 2003</td>
</tr>
<tr>
<td>Fame101</td>
<td>ΔdosR::pFAME101</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-Gm-int</td>
<td>pUC-based plasmid with HindIII cassette carrying gm and L5 int</td>
<td>Mahenthiralingam et al., 1998</td>
</tr>
<tr>
<td>p2NIL</td>
<td>gene manipulation vector, kan^R</td>
<td>Parish and Stoker, 2000</td>
</tr>
<tr>
<td>pEM37</td>
<td>P_Ags-lacZ in Mycobacterium- E. coli shuttle vector</td>
<td>Edith Machowski, Johannesburg, South Africa</td>
</tr>
<tr>
<td>pFM209</td>
<td>Ag85 promoter in p2Nil</td>
<td>This study</td>
</tr>
<tr>
<td>pFM210</td>
<td>dosR in pFM209</td>
<td>This study</td>
</tr>
<tr>
<td>pFM211</td>
<td>pFM210:: int gm</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

confidence interval 0.38-0.75) to that of sigA. A t-test was run and no significant difference was observed between the constitutively expressed dosR in the dosR mutant and in the wild-type. In H37Rv the level of dosR mRNA is 0.55 (95% confidence interval 0.47-0.65) to that of sigA (Figure 2). As expected, no dosR 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 hsp60 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).

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References


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