Study of the role of Mce3R on the transcription of mce genes of Mycobacterium tuberculosis

María P Santangelo¹, Federico C Blanco¹, María V Bianco¹, Laura I Klepp¹, Osvaldo Zabal², Angel A Cataldi¹ and Fabiana Bigi*¹

Address: ¹Institute of Biotechnology, CICVyA-INTA, Los Reseros y Las Cabañas, 1712 Castelar, Argentina and ²Institute of Virology, CICVyA-INTA, Castelar, Argentina

Email: María P Santangelo - psantangelo@cnia.inta.gov.ar; Federico C Blanco - fblanco@cnia.inta.gov.ar; María V Bianco - mbianco@cnia.inta.gov.ar; Laura I Klepp - lklepp@cnia.inta.gov.ar; Osvaldo Zabal - ozabal@cnia.inta.gov.ar; Angel A Cataldi - acataldi@cnia.inta.gov.ar; Fabiana Bigi* - fbigi@cnia.inta.gov.ar

* Corresponding author

Abstract

Background: mce3 is one of the four virulence-related mce operons of Mycobacterium tuberculosis. In a previous work we showed that the overexpression of Mce3R in Mycobacterium smegmatis and M. tuberculosis abolishes the expression of lacZ fused to the mce3 promoter, indicating that Mce3R represses mce3 transcription.

Results: We obtained a knockout mutant strain of M. tuberculosis H37Rv by inserting a hygromycin cassette into the mce3R gene. The mutation results in a significant increase in the expression of mce3 genes either in vitro or in a murine cell macrophages line as it was determined using promoter-lacZ fusions in M. tuberculosis. The abundance of mce1, mce2 and mce4 mRNAs was not affected by this mutation as it was demonstrated by quantitative RT-PCR. The mce3R promoter activity in the presence of Mce3R was significantly reduced compared with that in the absence of the regulator, during the in vitro culture of M. tuberculosis.

Conclusion: Mce3R repress the transcription of mce3 operon and self regulates its own expression but does not affect the transcription of mce1, mce2 and mce4 operons of M. tuberculosis.

Background

Tuberculosis (TB), a chronic illness caused by Mycobacterium tuberculosis, is still a major worldwide disease. Pathogenic mycobacteria species have demonstrated a remarkable ability to survive in diverse conditions encountered during the infection process. However, even after decades of investigation, there is still little knowledge about mycobacterial pathogenesis. Understanding the infective process at the molecular and cellular levels will lead to new strategies to control this disease and even to the development of an effective vaccine.

The analysis of the complete sequence of the M. tuberculosis H37Rv genome revealed the presence of four paralogous mce genes, all encoded in an operon structure consisting of eight genes [1]. The biological function of Mce proteins is not known, but increasing evidence has demonstrated that they are clearly related to the virulence of Mycobacterium tuberculosis complex species [2-8].

Gene regulation is considered to play a central role in host-microbe interactions, and many virulence genes are regulated in response to the host. Casali and collaborators
[9] identified a regulatory mechanism which controls mce1 expression. They have demonstrated that a homologue of the FadR subfamily of GntR transcriptional regulators, Rv0165c (designated Mce1R), is a negative regulator that intracellularly represses expression of the mce1 operon. In addition, a gene encoding a putative transcriptional factor, Rv0586, is located immediately upstream of mce2 operon and it is transcribed in the same direction as that of mce2 genes. Furthermore, it has been found that there are growth phase and tissue specific differences in the expression of mce operons in M. tuberculosis [10-12] which is in agreement with the presence of regulatory mechanisms controlling mce transcription. In a previous work, we found evidence indicating that Mce3R, a TetR family transcriptional regulator, down-regulates the mce3 operon during the in vitro growing of M. tuberculosis [13]. We have demonstrated that the overexpression of Mce3R in both M. smegmatis and M. tuberculosis abolishes the expression of a gene reporter fused to mce3 promoter. TetR family members often regulate their own synthesis [14-18]. The classic example of self regulation in members of this family protein is a repressor involved in resistance to tetracycline of Escherichia coli, which has given the name TetR to the group [19]. In a number of TetR-autoregulated systems the regulator and the structural genes are divergently transcribed and the region for protein binding overlaps the promoters placed in the intergenic region [15,18,20]. That is the case of mce3R, which is placed upstream of mce3 operon, oriented in the opposite direction and separated from it by a region of 880 bp.

In this work we validate the role of Mce3R in repressing the mce3 transcription in M. tuberculosis by analyzing gene expression in a mce3R-knockout M. tuberculosis strain. We also found that this regulation is exclusive for the mce3 operon among mce genes and that the Mce3R repressor regulates its own expression.
Results

Construction of a mce3R mutant in M. tuberculosis

As a first step to assess the mce3 operon expression in the absence of Mce3R, we obtained a knockout mutant strain of M. tuberculosis H37Rv by inserting a hygromycin cassette into the mce3R gene. The site-directed mutant strain of M. tuberculosis was obtained by two-step mutagenesis strategy by using the p2NIL shuttle plasmid [21], which carries the lacZ gene and the counter selectable marker sacB. Allelic exchange was confirmed in the selected clones (Hy8, Km5, and Sac6) by Southern blotting (Fig. 1A), since the mutant showed a hybridizing fragment of about 1.5 kb absent in the wild-type strain. This polymorphism is due to the introduction of an extra EcoRI site present in the hygromycin cassette (Fig. 1B). The mutant strain was designated Δmce3R. The mutation was complemented by transforming the plasmid pSummce3R into the mutant.

In vitro characterization of Δmce3R

To determine whether mce3R disruption introduces alterations during in vitro growth, growth curves of the Δmce3R mutant, complemented, and parental strains were compared under standard culture conditions. All assayed strains showed similar doubling time and growth characteristics throughout the culture period (Fig. 2). This result indicates that the mutation does not affect the in vitro growth of M. tuberculosis.

![Figure 2](http://www.biomedcentral.com/1471-2180/8/38)

**Figure 2**

**Effect of the mce3R mutation on in vitro growth of M. tuberculosis.** Cultures of the mutant [Δmce3R, square], the complemented [Δmce3R (pSummce3R), triangle] and the parental wild-type [H37Rv, rhombus] strains were grown to stationary phase and inoculated into fresh Dubos medium supplemented with 0.4% glucose at OD 600nm 0.005 and the OD 600nm was measured at various time points. It is shown a representative experiment from triplicate.

mce3 operon expression is repressed by Mce3R during in vitro culture and inside murine macrophages

We have previously demonstrated that the mce3 promoter allows the expression of the lacZ reporter gene in M. tuberculosis H37Rv but that this expression is completely abolished when Mce3R is overexpressed in the H37Rv strain from a multi-copy plasmid [13]. Although these findings constitute initial evidence demonstrating the role of Mce3R as a repressor of the mce3 operon transcription, the presence of an endogenous copy of the mce3R gene in M. tuberculosis did not enable us to determine the conditions in which the regulator system operates.

Here, in order to compare the expression the mce3 operon either in the absence or in the presence of Mce3R, DNA fusions of the mce3 promoter to lacZ reporter, containing or not containing mce3R were cloned within pYUB178-lacZ. The resulting plasmids, pP3-mce3R and pP3 respectively, were integrated into the chromosome of the Δmce3R strain. The β-galactosidase activity was measured at different points along cultures of M. tuberculosis grown in vitro and in a macrophages cell line. Since transcription of mce3 genes has previously shown to be increased when M. tuberculosis was grown in rich media [10-12] the expression of mce3 operon was assessed in both synthetic (7H9) and rich (Dubos) media (figure 3 and data not shown).

While hardly any β-galactosidase activity was detected either in in vitro cultures (Fig. 3) or in cell-line mice macrophages (Table 1) in the presence of Mce3R (Δmce3R::pP3-mce3R strain), the activity in the Δmce3R::pP3 strain was remarkably high in all of the conditions tested. The promoter activity increased along the in vitro cultures of Δmce3R::pP3, and peaked at 24 h of infection inside the cell. These results clearly indicate that Mce3R represses the expression of mce3 operon in M. tuberculosis in the growth conditions tested.

Assessment of the role of Mce3R in the transcription of the four mce operons

A number of reports indicate that the expression of all mce genes depends on the growth conditions [10-12]. These observations, together with the findings that regulatory proteins are involved in the expression of the mce1 and mce3 operons, suggested the idea of a broader regulatory mechanism differentially controlling the expression of mce genes. In order to test whether Mce3R is able to control the transcription of the other mce operons apart from mce3, the expression of one gene from each mce operon in the mutant strain was compared with that of the wild type. Primers were designed to amplify a 189, 168, 234, 151 and 134 bp region on mce1D (Rv0172), mce2A (Rv0589), mce3E (Rv1970), mce4A (Rv3499), and sigA
independent experiments. Results represent one of at least three
duplicate in three time points (1 early exponential phase, 2
exponential phase and 3 stationary phase). Growth curves of
Δmce3R (pP3-lacZ) (square) and Δmce3R (pP3-lacZ) (white bars) strains
in M7H9-AD-G medium. The results are presented as Miller units ± S.D. of
OD 600nm values are indicated on the right. Results represent one of at least three
independent experiments.

respectively. Amplicons of expected size were obtained with each pair of primers (data not shown).

Differences in relative gene expression between the wild
type and the mutant strains were assessed in group means
for statistical significance by a randomisation test (see
Materials and Methods). While the relative abundance of
mce3E mRNA was significantly higher (P < 0.030) in the
mutant than in the wild type strain in the conditions eval-
uated, no significant differences between both strains
were observed on the expression of mce1D (P < 0.74),
mce2A (P < 0.918) and mce4A (P < 0.511) (Fig 4).

Table 1: Effect of MceR on mce3 promoter activity in M. tuberculosis during infection of J774 Macrophage-like cell line.

| Strain             | 4 h       | 24 h      | 72 h       |
|--------------------|-----------|-----------|------------|
| Δmce3R (pP3-mce3R) | 0         | 0         | 0          |
| Δmce3R (pP3)       | 114±5     | 2571±172  | 2162±92    |

* calculated as: arbitrary β-galactosidase units/number of bacteria (CFU)/100000.
Values are presented as the mean and standard deviation (SD) of reactions performed in triplicate.

To verify that RNA samples were not contaminated with
 genomic DNA, RT-PCR reactions were performed without
the addition of reverse transcriptase. The lack of amplifi-
cation products verifies that the RT-PCR products were
amplified from RNA that had been reversely transcribed
into cDNA.

These results demonstrate that Mce3R regulates exclusively
the transcription of mce3 operon among mce genes in the conditions tested

Mce3R expression is self regulated
To investigate whether mce3R is subject to transcriptional auto-
regulation, a transcriptional fusion was constructed between the mce3R promoter and the lacZ reporter. Since
mce3R is located adjacent to mce3 operon and divergently
oriented, the mce3R promoter is situated in mce3R-yrbE3A
intergenic region. The entire intergenic region was fused to the lacZ gene within pYUB178-lacZ to create plasmid
pPR3-lacZ. The pPR3-lacZ plasmid was transformed into the wild type M. tuberculosis H37Rv and the M. tuberculosis
Δmce3R mutant, and β-galactosidase activity was measured to assess the levels of mce3R promoter activity with
and without Mce3R regulator. As shown in figure 5, in the presence of Mce3R (wild type H37Rv strain) the activity of
mce3R promoter is steadily and significantly reduced as compared with that in the absence of the Mce3R regulator
(mutant Δmce3R strain). This reduction in mce3R promoter activity was more evident during the stationary growth phase. These experiments demonstrate that the
Mce3R protein is able to transcriptionally repress expression of the mce3R promoter in M. tuberculosis during the in
vitro culture condition tested.

Discussion
Little is known about gene regulation of virulence factors
in M. tuberculosis due to its slow growth rate and the late
development of mycobacterial genetics. Prokaryotic tran-
scriptional regulators are classified in families on the basis
of sequence similarity and structural and functional crite-
ria. The TetR family, a family of transcriptional regulators
that is well represented and widely distributed among
bacteria, has a helix turn-helix (HTH) signature, the most
recurrent DNA binding motif, to bind its target DNA.
Members of the TetR family of repressors control tran-
scription of proteins involved in multidrug resistance,
enzymes implicated in different catabolic pathways, biosynthesis of antibiotics, osmotic stress, and pathogenicity of gram-negative and gram-positive bacteria. At least 40 putative TetR-family regulator genes are spread on the *M. tuberculosis* genome. Most of them are similar to the TetR/AcrR family, but just a few have been characterized. Mce3R was the first TetR-like regulator studied in *M. tuberculosis* [13]. Then, Engohang-Ndong et al. [22] found that a member of the TetR/CamR family represses the expression of *ethA* that encodes a protein that catalyses the activation of ethionamide (ETH). ETH is an important second-line anti TB drug used for the treatment of patients infected with multidrug-resistant strains.

The transcription profile of the mce3 operon in different in vitro growth conditions of *M. tuberculosis* has been addressed by RT-PCR in a number of publications and transcription of mce3 genes has been found when bacteria were cultured both on LJ and Dubos media [11,12] but not in 7H9 synthetic medium in both exponential and stationary growth phases [10]. Using similar methodology we detected mRNA of mce3 operon during in vitro culture of *M. tuberculosis* either in synthetic or in rich (data not shown) media. However, transcription from the mce3 promoter, measured as β-galactosidase activity, was completely absent in the presence of Mce3R both in in vitro conditions and inside a murine macrophages cell line. These last results, together with the finding that the elimination of Mce3R significantly increases mce3 transcription, indicate that the potential level of mce3 expression is repressed in the conditions of growth assayed.

Interestingly, the high homology among mce operons is not conserved among their regulator genes, since Mce1R, the other mce regulator described, as well as the putative regulator of the mce2 operon, belong to the GntR family [9,1]. In addition, no putative regulatory gene is placed in the vicinity of the mce4 operon; however, it was recently proposed Rv3574, a TetR-type regulator, as repressor of mce4 operon expression [23].

Here it was demonstrated that mce3R is not involved in the expression of mce1, mce2 nor mce4 operons. Therefore, it is tempting to speculate that both facts, i.e. gene redundancy and differential regulation, ensure the production of Mce proteins in different environments.

Here we demonstrated that mce3 promoter is stronger than mce3R promoter, and mce3 expression seems to be
mostly shut down during in vivo growth, but it is also likely that under unknown particular conditions of growth it would turn on.

As it happens to many other proteins of the TetR family, we found that Mce3R negatively regulates its own expression. The experiments with the mce3R promoter indicate that there is in average ten folds decreased in transcriptional activity when the Mce3R is provided. In this condition, the activity of mce3 promoter is sixty times repressed. Since the mce3R – yrbE3A intergenic region spans 880 bp, it is likely that Mce3R binds to consensus motifs located between the divergent genes in both promoter regions.

Although this study provides more insights to the role of Mce3R in the regulation of the mce operons, the information is still limited and further studies are necessary to detect any other gene regulated by this system. Elucidation of other promoters targeted by Mce3R will lead to the definition of a consensus mce3R-binding site and the possibility of define the Mce3R-regulon. Thus, one could hope to decipher the function of mce genes by the identification of the Mce3R regulon.

Conclusion
The available evidence demonstrates that while Mce3R represses powerfully the transcription of mce3 operon in vivo and inside macrophages, it does not affect the transcription of mce1, mce2 and mce4 operons during in vitro culture of M. tuberculosis. It was also demonstrated that Mce3R negatively regulates its own expression but the level of expression is lower than that observed for mce3 operon.

Methods
Bacterial strains and culture media
All cloning steps were performed in Escherichia coli DH5α. Regulation studies were performed in M. tuberculosis H37Rv. E. coli was grown in Luria-Bertani (LB) broth or on LB agar. M. tuberculosis strains were grown in Middlebrook 7H9 medium supplemented with 0.05% Tween 80, Dubos and Middlebrook 7H11, all supplemented with albumin 0.5%, dextrose 0.4%) and 0.5% glycerol (M7H9-AD-G). When necessary, 20 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹ were added to the media.

General DNA methodology
PCR amplifications from genomic DNA templates were performed as previously described [13]. Each primer contained base mismatches that introduced a restriction site suitable for directional cloning (Table 2). Chromosomal DNA samples were obtained as described vanSoolingen [24]. Purification of plasmids and DNA fragments were performed using the GeneJet Micro Plasmid Prep Kit (GE Healthcare) and DNA and Gel Band Purification Kit (GE Healthcare), respectively, according to the manufacturer’s instructions. Plasmid pYUB178-lacZ was created by insertion of the β-galactosidase gene from plasmid pMC1871 (AmershamPhar macia) into HindIII and Nhel sites of pYUB178 mycobacterial integrative vector [25]. M. tuberculosis H37Rv and M. tuberculosis Δmce3R (see below) were transformed by electroporation, as described by Parish and Stoker [26].

Construction of M. tuberculosis Δmce3R mutant strain
A genomic region containing mce3R and about 2 kb flanking 5’ and 3’ regions was obtained by PCR from M. tuberculosis H37Rv total DNA by using primers: upMutReg and lowMutReg. The amplified fragment was cloned in site NotI of p2NIL plasmid [21] and the mutant allele of mce3R was generated by inserting a cassette conferring hypromycin resistance from pUC-Hy7 (AmershamPhar macia) into a unique HindIII site internal to mce3R. The final delivery vector was generated by incorporation of the PacI cassette from pGOAL 17 into this last p2NIL recombinant vector. Mutants were constructed using a two-step strategy as described previously [21]. Chromosomal DNA was prepared from the selected clones and digested with EcoRI and then analyzed by Southern blotting by using the wild-type gene as probe. The mutant strain resulting from allelic exchange was designated M. tuberculosis Δmce3R.

DNA fragment encompassing mce3R and the intergenic region between mce3R and Rev1964 was PCR amplified with primers: mce3R-P3up and P3rev and cloned into TOPO 2.1 vector (Invitrogen). A fragment containing

|Table 2: Primer sequences used in this study.|
|---|
|Primer | Sequence* |
|P3rev | ggtccggcgcgcgcaccgctggatcga |
|mce3R-P3up | ggtccggcgcgcgcaccagctggatcga |
|upMutReg | gcggccgcgcgcaccagctgga |
|lowMutReg | gcggccgcgcgcaccagctgga |
|up3mce3RInt | gcggccgcgcgcaccagctgga |
|P3mce3RRevInt | gcggccgcgcgcaccagctgga |
|P3Int | gcggccgcgcgcaccagctgga |
|mce3Eup | gcggccgcgcgcaccagctgga |
|mce3Elow | gcggccgcgcgcaccagctgga |
|sigA1 | gcggccgcgcgcaccagctgga |
|sigA2 | gcggccgcgcgcaccagctgga |
|mce1Dup | gcggccgcgcgcaccagctgga |
|mce1Down | gcggccgcgcgcaccagctgga |
|mce2Up | gcggccgcgcgcaccagctgga |
|mce2Down | gcggccgcgcgcaccagctgga |
|mce4Up | gcggccgcgcgcaccagctgga |
|mce4Down | gcggccgcgcgcaccagctgga |
|P3anti up | gcggccgcgcgcaccagctgga |
|P3anti low | gcggccgcgcgcaccagctgga |

* Restriction enzyme site added at the end of each primer is underlined.
mce3R and its promoter was released from this last plasmid by digestion with EcoRI and BamHI and cloned into pSU M41 [27] to produce plasmid pSU mce3R. This plasmid was used to transform M. tuberculosis Δmce3R strain by electroporation. The resulting complemented strain was referred to as Δmce3R::mce3R.

RNA preparation
Culture pellets of 50 ml were resuspended in 1 ml of TRIzol (Invitrogen). Cells were disrupted using a Fastprep FP120 bead-beater (Savant) for 20 s at a speed of 6.0 m s⁻¹ with Lysing Matrix B (Q-Biogene). Then, 200 µl of chloroform (Merck) was added, and the mixture was incubated for 15 min at room temperature. Tubes were centrifuged at 10,000 × g for 15 min at 4°C, and the supernatant was extracted again with 100 µl of chloroform and 600 µl of isopropanol and 60 µl of 3 M ammonium acetate (pH 5.3) at -70°C overnight. Pellets were washed with 75% ethanol and resuspended in 50 µl of diethyl pyrocarbonate-treated water (Sigma-Aldrich). The RNA preparations were treated with DNase amplified grade (Invitrogen).

RT-Q-PCR
DNA-free RNA (1 µg) extracted from middle logarithmic-phase culture of either M. tuberculosis H37Rv or Δmce3R was mixed with 50 ng of random primers (Invitrogen) in 20 µl of final volume and reversely transcribed to total cDNA with SuperScript III reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Identical reactions lacking reverse transcriptase were also performed to confirm the absence of genomic DNA in all samples.

Q-PCR was performed in the Applied Biosystems 7000 DNA sequence detection system (Perkin-Elmer Corp.), by using Master Mix QuantiTect SYBR Green (Qiagen), 1 µl of template cDNA and the pairs of primers listed on Table 1. Each reaction was performed in duplicate. Results were presented as ratios calculated with the Relative expression software tool (REST®) application described by Pfaffl et al. [28]. Relative quantification of each target (mce) gene was performed by using sigA as reference gene and a subsequent test for significance of derived results was performed by using Pair Wise Fixed Reallocation Randomisation [29]. The value of PCR efficiency for all transcripts was 2, as calculated following the formula: E = 10^[1/slope].

Construction of β-galactosidase fusions
DNA fragments encompassing the intergenic region between mce3R and operon mce3, either containing or not containing the coding sequence of mce3R, were generated by PCR amplification with the pairs of primers upP3mce3Rint/P3mce3Rrev and upP3int/P3revint, respectively. Both DNA fragments were cloned into the HindIII or the NcoI sites of p178-lacZ, giving rise to plasmids pP3-lacZ and pP3-mce3R-lacZ, respectively. The intergenic region was also amplified by PCR using primers P3antiup/P3antiow and the DNA fragment was cloned in plasmid pYUB178-lacZ to generate pPR3-lacZ. This last plasmid is the antisense version of pP3-lacZ in which the promoter of mce3R was fused to the reporter gene. These plasmids were used to transform M. tuberculosis strains as indicated.

Measurements of β-galactosidase activity
Determination of β-galactosidase activity in M. tuberculosis recombinant strains was performed as previously described [13]. Briefly, β-galactosidase activity was measured in soluble cell extract prepared from aliquots of in vitro cultures taken at different time points. Results were expressed in Miller units [A420 × 1000/reaction time (min) xA600] [30].

Cultures of the murine macrophage-like cell line J774 were infected with recombinant M. tuberculosis H37Rv strains (free of clumps) at a m.o.i. of 5. J774-infected cells were disrupted with 1% Triton x-100, at 4, 24 and 72 h post-infection. β-galactosidase measurements were performed on the soluble cell extract by using the Chemiluminescent lacZ β-galactosidase detection kit (MGT Product M08550) and the Luminometer, Veritas 1.4 (Turner Biosystems, Inc.). β-galactosidase activity was related to the number of bacteria as determined by bacterial counting.

Authors’ contributions
MPS constructed the M. tuberculosis mutant strain and the plasmids, FCB performed the RT-QPCRs, MVB and OZ carried out cell infection experiments, AC and FB conceived of the study, and participated in its design and coordination and drafted the manuscript. All authors read and approved of the final manuscript.

Acknowledgements
The present study was supported by SECyT grant Bid 1728-OC/AR PICT 2002 -11765 and INTA grant AEIBIO3454. A.C. and F.B. are CONICET fellows. L.K. is recipient of a fellowship from CONICET.

We thank Valeria Rocha for performing plasmids constructions, Teresa Moran for technical assistance in cell cultures, and Luis Fernandez for the bibliography provided. We also thank Dr. T. Parish for providing us with p2nil and pGOAL17 plasmids.

References
1. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE III, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream M-A, Rogers J, Rutter S, Seeger K, Skelton J, Squares R,
Squares S, Sulston JE, Taylor K, Whitehead S, Barrett BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998, 393:537-544.

2. Arruda S, Bonifim G, Knights R, Huisma-Byron T, Riley LW. Cloning of an M. tuberculosis DNA fragment associated with entry and survival inside cells. Science 1993, 261:1454-1457.

3. Flesselles B, Anand NN, Remani J, Loosmore SM, Klein MH. Disruption of the mycobacterial cell entry gene of Mycobacterium bovis BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. FEMS Microbiol Lett 1999, 15:237-242.

4. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 2002, 48:77-84.

5. Gioffre A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meile V, Etchechoury I, Romano MI, Cataldi A, Hernandez RP, Bigi F. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. Microbes Infect 2005, 7:325-334.

6. Shimono N, Morici L, Casali N, Cantrell S, Sidders B, Ehrt S, Riley LW. Hypervirulent mutant of Mycobacterium tuberculosis resulting from disruption of the mce1operon. Proc Natl Acad Sci 2003, 100:15918-15923.

7. Chitale S, Ehrt S, Kawamura I, Fujimura T, Shimono N, Anand N, Lu S, Cohen-Gould E, Riley L. Intracellular Mycobacterium tuberculosis protein associated with mammalian cell entry. Cellular Microbiology 2001, 3:247-254.

8. Chitale S, Ehrt S, Kawamura I, Fujimura T, Shimono N, Anand N, Lu S, Cohen-Gould E, Riley L. Intracellular Mycobacterium tuberculosis protein associated with mammalian cell entry. Cellular Microbiology 2001, 3:247-254.

9. Uchida Y, Casali N, White AM, Moriici L, Kendall VL, Riley LW. Accelerated immunopathological response of mice infected with Mycobacterium tuberculosis disrupted in the mce1 operon. Microbiology 2001, 9:1275-83.

10. Kumar M, Bose M, Brahmacari V. Comparison of mammalian cell entry operons of mycobacteria: in silico analysis and expression profiling. FEMS Immnol Med Microbiol 2005, 43:185-195.

11. Haile Y, Bjune G, Wiker HG. Expression of the mceA, esat-6 and hspX genes in Mycobacterium tuberculosis and their responses to aerobic conditions and to restricted oxygen supply. Microbiology 2002, 148:3881-3886.

12. Santangelo MP, Alito A, Caimi K, Zabal O, Zunarraga M, Romano MI, Cataldi A, Bigi F. A negative regulator of mce3 operon in Mycobacterium tuberculosis. Microbiology 2002, 148:2997-3006.

13. Nolas JL, Martinez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R. The TetR Family of Transcriptional Repressors. Microbiology and Molecular Biology Reviews 2005, 69:326-356.

14. Chuanchuen R, Gaynor JB, Karkhoff-Schweizer R, Schweizer HP. Microbial Characterization of MexL, the Transcriptional Repressor of the mexJK Multidrug Efflux Operon in Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy 2005, 49:1844-1851.

15. Delany I, leva N, Alaimo C, Rappuoli R, Scarlato V: The iron-responsive regulator Fur is transcriptionally autoregulated and not essential in Neisseria meningitidis. J Bacteriol 2003, 185:6032-6041.

16. Sala C, Foro F, di Florio E, Canneva F, Miliano A, Riccardi G, Ghisotti D: Mycobacterium tuberculosis FurA autoregulates its own expression. J Bacteriol 2003, 185:5327-5332.

17. Guifoil PG, Hutchinson CR. The Streptomyces glaucescens TcrmR protein represses transcription of the divergently oriented tcrmR and tcaM genes by binding to an intergenic operator region. J Bacteriol 1993, 175:3659-3666.

18. McMurry L, Petrucci RE Jr, Levy SB. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. Proc Natl Acad Sci USA 1980, 77:3974-3977.

19. Orth P, Schnaptinger D, Hillen W, Saenger W, Hinrichs W: Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nat Struct Biol 2000, 7(3):215-9.

20. Parish T, Stoker N. Use of a flexible cassette method to generate a double unmarked Mycobacterium tuberculosis tlyA plABC mutant by gene replacement. Microbiology 2000, 146:1969-1975.

21. Angoh-A-Ndong J, Bailait D, Aumercier M, Bellefontaine F, Besra G, Locht C, Baulard A. EthR, a repressor of the TetR/CamR family implicated in ethionamide resistance in mycobacteria, octamerizes cooperatively on its operator. Molecular Microbiology 2004, 51:175-188.

22. Kendall SL, Witters M, Humpf CN, Moreland NJ, Gurcha S, Sidders B, Frita R, Ten Bokum A, Besra GS, Lotz JS, Stoker NG: A highly conserved transcriptional repressor controls a large region involved in lipid degradation in Mycobacterium smegmatis and Mycobacterium tuberculosis. Mol Microbiol 2007, 65:684-99.

23. van Soolingen D, Herrmans PWM, de Haas PEW, Soll DR, van Embden JDA: Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: evaluation of an insertion sequence dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol 1991, 29:2578-2586.

24. Pacsopella L, Collins FM, Martin JM Jr, Jacobs WR, Bloom BR: Identification of a genomic fragment of Mycobacterium tuberculosis responsible for in vivo growth advantage. Infect Agents Dis 1993, 2(4):282-284.

25. Parish T, Stoker NG: Electroporation of Mycobacteria. In Mycobacteria Protocols Volume 101. Method in Molecular Biology Humana Press Totowa, New Jersey; 1998:120.

26. Ainsa JA, Martin C, Cabeza M, De la Cruz F, Mendiola MV: Construction of a family of Mycobacterium/Escherichia coli shuttle vectors derived from pAL5000 and pACYC 184: their use for cloning an antibiotic-resistance from Mycobacterium fortuitum. Gene 1996, 176:23-26.

27. Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002, 30:e36.

28. Rest gene-quantification [http://www.rest-gene-quantification.info]

29. Miller JH: Experiments in Molecular Genetics Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1972.