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Functional analysis of *mce4A* gene of *Mycobacterium tuberculosis* H37Rv using antisense approach

Amita Chandolia[a,1], Nisha Rathor[a], Monika Sharma[b], Neeraj Kumar Saini[a,2], Rajesh Sinha[a], Pawan Malhotra[c], Vani Brahmachari[d], Mridula Bose[a,*]

[a] Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India
[b] Department of Zoology, Miranda House, University of Delhi, Delhi 110007, India
[c] International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India
[d] Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India

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ABSTRACT

Antisense strategy is an attractive substitute for knockout mutations created for gene silencing. mce genes have been shown to be involved in mycobacterial uptake and intracellular survival. Here we report reduced expression of *mce4A* and *mce1A* genes of *Mycobacterium tuberculosis* using antisense technology. For this, 1.1 kb region of mce4A and mce1A was cloned in reverse orientation in pSD5 shuttle vector, resulting into antisense constructs pSD5-4AS and pSD5-1AS, respectively. In *M. tuberculosis* H37Rv approximately 60% reduction in Mce4A and 66% reduction in expression of Mce1A protein were observed. We also observed significantly reduced intracellular survival ability of both antisense strains in comparison to *M. tuberculosis* containing pSD5 alone. RT-PCR analysis showed antisense did not alter the transcription of upstream and downstream of mceA genes of the respective operon. The colony morphology, *in vitro* growth characteristics and drug susceptibility profile of the antisense construct remained unchanged. These results demonstrate that antisense can be a promising approach to assign function of a gene in a multiunit operon and could be suitably applied as a strategy.

1. Introduction

The study of factors responsible for mycobacterial survival and persistence inside its host are of great interest as the success of *Mycobacterium tuberculosis* lies in its inherent ability to survive intracellularly, inside macrophages and to persist in latent form in its host for decades.

Techniques of creating mutant are valuable tools to explore the function of genes but it has remained difficult to achieve for mycobacterium, probably due to low rate of homologous recombination (Muttucumaru and Parish 2004). With knockout mutant, function of non essential gene may be analyzed but this approach is not suitable for essential genes. Gene inactivation by creating mutant may not be suitable to characterize function of individual gene if the gene of interest is present in an operon, due to possibility of polar effect of the interrupted DNA sequence. The knockout strains of *mce1A* or *yrbE4A* genes have been shown to disrupt downstream genes of *mce1* operon (Shimono et al. 2003). Here we applied the antisense method to block the *mce4A* gene of *mce4* operon of *M. tuberculosis*. The antisense strategy has traveled a long path and has been proven useful within a short period of time since 1984 (Mizuno et al. 1984; Tatout et al. 1998; Rasmussen et al. 2007). Being extensively used in case of eukaryotes, it has gained acceptance as first anticancer drug that has been patented (de Smet et al. 1999). In case of prokaryotes, it also showed promise to study and characterize function of genes playing essential roles for their host. As knockout becomes lethal, antisense reduces expression from 10% to 65% of its target gene leading to the study function of essential genes in mycobacteria (Harth et al. 2000, 2002; Wilson et al. 1998; Greendyke et al. 2002; Deol et al. 2005). Being less tedious and less time consuming are other advantages of this strategy.

Therefore, this study aimed to investigate whether antisense strategy provide useful information about function of a gene when gene is present in an operon and if targeting of this protein in mycobacterium will affect its virulence. We selected *mce4A (Rv 3499)* for this purpose. *mce4A* gene is the third gene of *mce4* operon of *M. tuberculosis*. This gene was found to be associated with entry

* Corresponding author. Tel.: +91 73 51061110; fax: +91 11 27666549.
E-mail addresses: amita.micro@gmail.com (A. Chandolia), microknp@gmail.com (N. Rathor), monika_sharma18@rediffmail.com (M. Sharma), neeraj.saini@einstein.yu.edu (N.K. Saini), rajeshsinhadu@gmail.com (R. Sinha), pawanmal@gmail.com (P. Malhotra), vani.brahmachari@gmail.com (V. Brahmachari), mridulabose@hotmail.com (M. Bose).
[1] Present address: Department of Biomedical Sciences, Acharya Narendra Dev College, University of Delhi, Govindpuri Kalkaji, New Delhi 110019, India.
[2] Present address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA.

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of the bacilli inside host cell and to assist the pathogen in intracellular survival (Saini et al. 2008). It has also been shown to be required during chronic phase of murine infection (Pandey and Sassetti 2008). *mce1A* was taken as control. Thus, *mce4A* and *mce1A* genes were targeted with a plasmid based shuttle vector harboring a 1.1 kb region of *mce4A* (pSD5-4AS) and *mce1A* (pSD5-1AS) gene in antisense orientation, to block the expression of *mce4A* and *mce1A* in *M. tuberculosis*. Effect of reduced expression of Mce4A and Mce1A in *M. tuberculosis* antisense strains on intracellular survival, was studied using THP-1 cell line as a model. Effect of antisense RNA on (a) stability of target mRNA (b) expression of other *mce* operons and (c) on expression of nine other virulence associated genes which have been shown to be expressed during intracellular survival of *M. tuberculosis* inside macrophages (Graham and Clark-Curtiss 1999), were also investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids, cell lines and growth conditions

Liquid cultures of *M. tuberculosis* strains were grown in Middlebrook 7H9 medium (Becton Dickinson) supplemented with 0.2% glycerol (Sigma), 0.05% Tween 80 (Sigma), and 10% oleic acid albumin-dextrose-catalase (OADC) enrichment media (Becton Dickinson). For log phase culture bacteria were grown to OD_{600} = 0.4–0.5 and for stationary phase OD_{600} = 0.8 was used. For the determination of the number of colony forming units (CFU) and examination of growth on solid media, Middlebrook 7H11 agar medium (Becton Dickinson) supplemented with 0.5% glycerol and 10% OADC was used. Plasmid pSD5 *Mycobacterium* shuttle vector (has origin of replication for mycobacteria and *Escherichia coli*) containing the kanamycin resistance gene as a selectable marker and the promoter from the *hsp60* gene for expression of integrated genes (Deol et al. 2005) was used for antisense cloning.

E. coli DH5α and BL-21 (DE3) cells were grown in Luria-Bertani (LB) broth or on LB agar. *M. tuberculosis* and *E. coli* strains were grown in presence of kanamycin wherever appropriate. THP-1 monocyte cell line was cultured and maintained in RPMI 1640 (Sigma) supplemented with 10% FCS (fetal calf serum, Gibco) and 2 mM L-glutamine (Sigma) and grown at 37 °C in presence of 5% CO_2. PMA (phorbolmyristate acetate, Sigma) at a concentration 20 ng/ml, was used for the adhesion of THP-1 cells.

2.2. DNA and RNA manipulations

Molecular biology techniques were carried out according to standard protocols (Maniatis et al. 1982) or according to the recommendations of the manufacturers of kits and enzymes. PCR was performed using Taq polymerase (Bangalore Genei) under standard conditions in a total volume of 50 μl. dNTPs were used at a concentration of 0.2 mM each (MBI Fermentas); 10 pM of each primer was used (Microsynth, Germany). The protocol used for amplification was as follows; Initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1 min, reannealing for 1 min at 56–60 °C depending on the primer sequence, and elongation at 72 °C for 1 min, with a final extension at 72 °C for 10 min. A total of 10 ng of genomic DNA of *M. tuberculosis* was used as template. Restriction enzymes were obtained from MBI Fermentas and Biolabs. We used the QIAquick Gel Extraction kit (Qiagen) to elute DNA fragments from agarose gels. Purification of DNA samples to remove enzymes, nucleotides or salts was achieved by using the QIAquick PCR Purification kit (Qiagen). Ligation reactions were carried out with the T4-DNA-Ligase from Quick ligation kit from Biolabs. *E. coli* DH5α and BL-21 (DE3) were transformed as described previously (Maniatis et al. 1982). *M. tuberculosis* H37Rv was transformed by electroporation (Parish and Stoker 1998). Plasmids were isolated from *E. coli* with the QIAGEN® Plasmid Mini kit (Qiagen) or by alkaline lysis method (Maniatis et al. 1982). Sequencing reactions were carried out by using the Prism Big Dye™ FS Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems.

2.3. Construction of a M. tuberculosis H37Rv derivative containing the mce4A-antisense plasmid pSD5-4AS and mce1A-antisense plasmid pSD5-1AS

1100 bp fragment of H37Rv-DNA was amplified, covering the most of the coding sequence from the *mce4A* and *mce1A* in separate PCR reactions. The PCR primers used (4ASFP, 4ASRP; 1ASFP, 1ASRP; Table 1) were designed with restriction sites for the restriction enzymes *MluI* (4ASFP and 1ASFP) or *NdeI* (4ASRP and 1ASRP) to allow insertion of the amplified DNA into the *MluI*/*NdeI* digested vector pSD5. This procedure resulted in an insertion of 1.1 kb region of the *mce4A* and *mce1A* in antisense orientation with respect to the *hsp*60 promoter in pSD5 vector. Sequencing of the insert of the recombinant plasmid pSD5-4AS confirmed the antisense orientation and the absence of mismatches. The recombinant plasmid (pSD5-4AS and pSD51AS) as well as the empty vector pSD5 were introduced into *M. tuberculosis* H37Rv by electroporation. Experimental proof of transformation of the plasmids in H37Rv was achieved on the basis of kanamycin resistance.

2.4. Double transformation experiment to test mce4A antisense constructs efficacy in E. coli

The *mce4A* gene from *M. tuberculosis* H37Rv was PCR amplified and cloned into pGEX-5 plasmid (Novagen), yielding pGEX-4A containing a GST fusion protein. After confirmation with restriction digestion and DNA sequencing, pGEX-*mce4A* was transformed into *E. coli* BL21 CodonPlus(DE3)-RIL cells (Stratagene). The resulting strain was again made $CaCl_2$ competent and transformed with either pSD5 or pSD5-4AS and selected on Ampicillin (100 μg/ml), and Kanamycin (25 μg/ml). The resulting colonies were grown

Table 1
List of primers used in the study. Four mce operons junctions primers (mce1, mce2, mce3, mce4) and upstream and downstream regions of antisense targeted operons mce4 (mce4.2 and mce4.12) and mce1 (mce1.2 and mce1.7) are used to analyze expression of respective operon.

Primer name	Forward primer binding site	Sequence (5′–3′)	Reverse primer binding site	Sequence (5′–3′)	Product size (bp)	References
4ASFP	*mce4A*	TCTCGACGCACGCGTGTCAG	*mce4A*	CGCCGCATATGGTGTTTC	1100	This study
1ASFP	*mce1A*	AAGCTGAACAACGCGTGAGTG	*mce1A*	GGTAGGGCTCCCGCATATGATTTG	1100	This study
mce1	*yrbE1B*	ATTATGTCGTTCCTGTCCCC	*mce1A*	GGTGAGCGTCTGGAACAAC	789	Kumar et al. (2003, 2005)
mce2	*yrbE2B*	CGACATGGCTTTCACCTCTG	*mce2A*	CCGACCCCACATCAATCAC	750	Kumar et al. (2003, 2005)
mce3	*yrbE3A*	CAACACCCGCGAGATTCAG	*mce3A*	GTTCTTCGAATGCAGTACC	946	Kumar et al. (2003, 2005)
mce4	*yrbE4B*	CACCTTCCTCATCCCCTC	*mce4A*	GATGAGCGATTGGAACAAC	702	Kumar et al. (2003, 2005)
mce4.2	*yrbE4A*	GACGTTGCTGGTGTCCATTC	*yrbE4A*	GATCTCCGCCAGTATCAATTCG	436	Pasricha et al. (2011)
mce4.12	*lprN*	GGTCAACAAGGGTAACGTC	*lprN*	AAGTTGGGAAATGGGAAC	452	Pasricha et al. (2011)
mce1.2	*yrbE1A*	CTGCAGTGCTGGTTCATCATGG	*yrbE1A*	ACCGATGGTGGTCAAGATCACG	632	Pasricha et al. (2011)
mce1.7	*mce1B*	TACCTGGACGCTATTCAGC	*mce1C*	TCGGAGAACTTAGCCACC	780	Pasricha et al. (2011)

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