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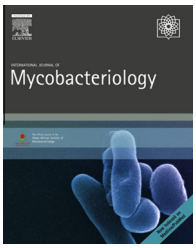

Full Length Article

Expression profile of *mce4* operon of *Mycobacterium tuberculosis* following environmental stress

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ABSTRACT

Background: The *mce4* operon is one of the four mce operons with eight genes (*yrbE4A*, *yrbE4B*, *mce4A*, *mce4B*, *mce4C*, *mce4D*, *mce4E* and *mce4F*) of *Mycobacterium tuberculosis*. It expresses in the later phase of infection and imports cholesterol for long term survival of the bacilli. To cause latent infection, *M. tuberculosis* undergoes metabolic reprogramming of its genes to survive in the hostile environment like low availability of oxygen and nutrition depletion inside the host.

Objective: To analyze real time expression profile of *mce4* operon under various stress conditions.

Methods: *M. tuberculosis* H37Rv was exposed to surface stress (0.1% SDS for 30 min and 90 min in late log and stationary phase of culture), hypoxia (5, 10, 15 and 20 days) and grown in the presence of either glycerol or cholesterol as sole source of carbon. The expression profile of genes of *mce4* operon was analyzed by real time PCR.

Results: Surface stress induced expression of *mce4C* and *yrbE4B* in late log phase on 30 min and 90 min exposure respectively. The SDS exposure for 30 min induced *mce4C*, *mce4D* and *mce4F* in stationary phase. All eight genes were induced significantly on 10th and 15th days of hypoxia and in the presence of cholesterol.

Conclusion: Hypoxia and cholesterol are potent factors for the expression of *mce4* operon of *M. tuberculosis*.

Introduction

Mycobacterium tuberculosis, encounters robust resistance inside the host cell. Various environmental stress conditions presented to *M. tuberculosis* within the host cell include oxidizing agents, namely reactive oxygen intermediates and reactive nitrogen intermediates, which are produced by the infected and activated macrophages. The bacterium may also

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be exposed to a low pH inside the host cell due to phagosome acidification [1]. In addition, host cells damage surface structures of the bacilli by releasing surfactants. Alveolar surfactant is a mild detergent with antibacterial activity and could damage the structure of the bacterium's fatty acid-rich cell envelope. Also, toxic peptides and proteins such as granulysin, thought to act at the level of the bacterial surface, are released by activated macrophages and natural killer cells [2]. Low availability of oxygen, especially inside granulomas and the phagosome, is the best known environmental condition for the induction of persistence, a phenomenon of great importance in *M. tuberculosis* pathogenesis [3]. However, this fact needs to be explored in detail at the molecular level. Reduced availability of micronutrients inside the granuloma is another major constraint encountered by the bacilli. During such stress, the bacterium adapts itself to utilize the compounds scavenged from the host. *M. tuberculosis* has the unusual ability to use the host cholesterol as a source of carbon and energy, and this unusual capacity seems to be responsible for persistence with in the animal tissues [4].

In our previous study, we reported surface stress, hypoxia, and the availability of cholesterol (as the sole source of carbon) as inducible factors for the promoter region of the *mce4* operon in *Mycobacterium smegmatis* [5]. In the present study expression profiles of the genes of the *mce4* operon were analyzed in real time to understand the role of *mce4* operon genes in the biology of *M. tuberculosis* under various physiological stresses.

Materials and methods

Bacterial strain and culture conditions

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.2% (volume/volume) glycerol and 10% (volume/volume) oleic acid, albumin bovine, fraction V, dextrose, and catalase (Difco Laboratories). The optical density of the cultures was measured with Infinitepro F200 (Tecan, Männedorf, Zürich, Switzerland) spectrophotometer.

Surface stress to M. tuberculosis

M. tuberculosis H37Rv was grown up to late log phase (Day 12) and stationary phase (Day 20) of culture and exposed to 0.1% sodium dodecyl sulfate (SDS) for 30 min and 90 min. Cells were pelleted down and rinsed with phosphate-buffered saline twice before RNA isolation. Unexposed *M. tuberculosis* H37Rv was taken as a control for the study.

Hypoxic stress to M. tuberculosis

M. tuberculosis H37Rv was grown up to $OD_{600} = 0.4$ in Dubos tween albumin medium. Three milliliters of culture was injected into 5-mL uncoated vacutainer tubes and incubated in a static position at 37 °C. Control cultures contained methylene blue (1.5 μg/mL) to monitor the depletion of oxygen. Cells were harvested after 5 days, 10 days, 15 days, and 20 days of hypoxia and processed for RNA isolation.

Nutritional stress and cholesterol supplementation

M. tuberculosis H37Rv was grown in minimal medium (asparagine 0.5 g/L, KH_2PO_4 1.0 g/L, Na_2HPO_4 2.5 g/L, ferric ammonium citrate 50 mg/L, $MgSO_4.7H_2O$ 0.5 g/L, $CaCl_2$ 0.5 g/L, and $ZnSO_4$ 0.1 mg/L) supplemented with either 0.1% glycerol or 0.01% water soluble cholesterol (Sigma Aldrich, USA) up to late log phase and stationary phase of culture and processed for RNA isolation.

Isolation of RNA from M. tuberculosis H37Rv and complementary DNA synthesis

M. tuberculosis H37Rv (3×10^8 cells) from cultures grown under different stress conditions and normal conditions were pelleted down. The pellet was suspended in 1-mL RNA protection buffer (Qiagen GmbH, Hilden, Germany), incubated for 10 min at room temperature, and processed for RNA isolation using RNeasy Minikit (Qiagen GmbH) according to the manufacturer's instructions. DNA contamination from RNA was removed by DNase I (Thermo Fischer Scientific Inc., Waltham, MA, USA). Absence of DNA in the RNA sample was confirmed by polymerase chain reaction (PCR) reaction, having purified RNA as a template for amplification of *sigA* (Table 1). The RNA was quantified through spectrophotometry (A_{260}/A_{280}). Complementary DNA was synthesized by using 1 μg of RNA with random hexamers of First Strand Complementary DNA Synthesis Kit (Thermo Fischer Scientific Inc.).

Expression analysis by quantitative reverse transcription-PCR

Real-time PCR was performed to quantify the expression of all eight genes of *mce4* operon, namely *yrbE4A*, *yrbE4B*, *mce4A*, *mce4B*, *mce4C*, *mce4D*, *mce4E*, and *mce4F* using QuantiTect SYBR Green Master Mix Kit (Roche Applied Science, Indianapolis, IN, USA) in a Light Cycler 480 II Real-time PCR system (Roche Applied Science) using the primers as listed in Table 1. All primers were designed to anneal at 60 °C, using Gene Runner version 3.01 software (Hastings Software, Inc., Hastings, NY). The reaction conditions included preincubation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and melting curve analysis at 95 °C for 5 s, 70 °C for 1 min, continued to cooling down at 40 °C for 10 s. The housekeeping gene *sigA* was used as an internal control to normalize messenger RNA levels [6]. Each quantitative reverse transcription-PCR (qRT-PCR) experiment was performed with duplicate samples that were each assayed in triplicate. The data was analyzed using the inbuilt quantification software in the lightcycler. A relative expression "one" indicated identical expression level of genes in normal and stress conditions.

Statistical analysis

In qRT-PCR, >2-fold change in expression profile of genes was considered significant. Results of qRT-PCR were compared using Graph Pad Prism software (version 5.0 for windows) (GraphPad Software, Inc., USA) by one-way analysis of vari-

Table 1 – List of primers used in the study.

Primer	Sequence	Size of amplicon (bp)
yrbE4AFP	GGCGTTCACCCTCAACATTCTG	227
yrbE4ARP	GGACCACGAGCCGTTGAATTG	
yrbE4BFP	ATGCGGGTCTCTGAGGAGATCG	218
yrbE4BRP	GACTGCCCATTGACGAACACC	
mce4AFP	TAAGGCTGGGCTGTTCACCTC	201
mce4ARP	AACGGCTGGTATGGGATCAAAG	
mce4BFP	TCAAGCTCAACCCAGACCACAG	216
mce4BRP	GCAACGCATCGAGATCCAGTG	
mce4CFP	CAACGCCAACGACCTGAATAGG	187
mce4CRP	CACCGATGTCACCGACTTGG	
mce4DFP	TGGAATGGGACGAGGTGAAAG	216
mce4DRP	TCTTGACGGTGCCGAAGATG	
mce4EFP	ACCTCGCTGCTGGGTTCGTTAC	204
mce4ERP	CTGGTGGGTCTCGTCAATGATC	
mce4FFP	CGTTCCTGCAAGCCCAGATC	180
mce4FRP	CGGGAAACGAAGGACGAATC	
sigAFP	GTGGCAGCGACCAAAGCAAG	200
sigARP	GTGTCCTGGGGTGCCGAG	

ance. All comparisons were performed by Bonferroni's multiple comparison test. All experiments were performed in duplicate and standard deviation was presented by error bars. Tests where (*) $p < .05$ were considered significant.

Results

Expression of mce4 operon in the presence of mild surface stress

The expression profile of eight genes of the *mce4* operon by real-time PCR analysis (Fig. 1 and Table S1) showed that in the late log phase of culture, on 30 min of SDS exposure, only the *mce4C* gene was expressed by 2-fold. The remaining seven genes demonstrated an expression level similar to the control. On further exposure to SDS up to 90 min, there was a significant induction of gene *yrbE4B* (3.06-fold). However, although *mce4B*, *mce4D*, *mce4E*, and *mce4F* were also induced, the increase was not significant. In the stationary phase of growth, after 30 min of exposure to SDS, *mce4C*, *mce4D*, and *mce4F* demonstrated 2.35-fold, 3.80-fold, and 5.95-fold higher expressions, respectively, in comparison to unexposed culture. Other genes of the operon were also induced but not significantly. On increasing the SDS exposure up to 90 min in the stationary phase, expression of each gene of the operon reduced significantly.

Oxygen depletion induces expression of genes of mce4 operon

On the 5th day of hypoxic stress, six genes of the *mce4* operon (*yrbE4A*, *yrbE4B*, *mce4A*, *mce4B*, *mce4C*, and *mce4E*) were expressed (Fig. 2), whereas there was no expression of *mce4D* and expression of *mce4F* was negligible (0.06-fold). On the 10th and 15th day of hypoxic stress, >2-fold and >3-fold induction was observed, respectively, for all genes of the operon. Interestingly, expression of all genes of the operon was significantly higher on the 15th day in comparison to the 10th day of hypoxia. On the 20th day of hypoxic stress, 6 genes of the operon showed reduced expression in comparison to the 15th day, while *mce4D* (21 fold) and *mce4F* (2.66 fold) showed higher expression compared with oxygenated

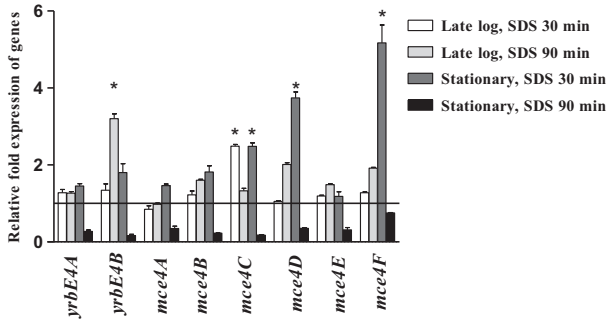

Fig. 1 – Relative fold expression of all genes of the *mce4* operon in the presence of 0.01% sodium dodecyl sulfate (SDS) in comparison to unexposed culture of *Mycobacterium tuberculosis*.

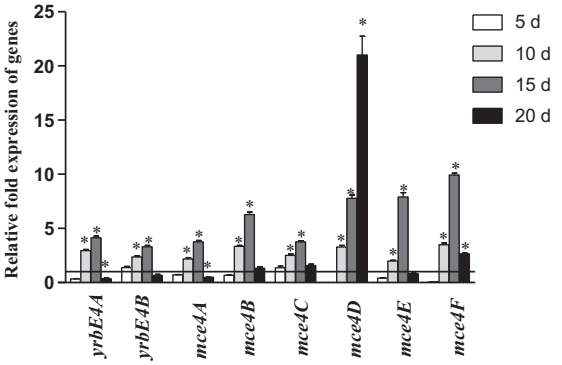

Fig. 2 – Relative fold expression of genes of the *mce4* operon in low availability of oxygen versus oxygenated culture of *Mycobacterium tuberculosis*. Note. d = days.

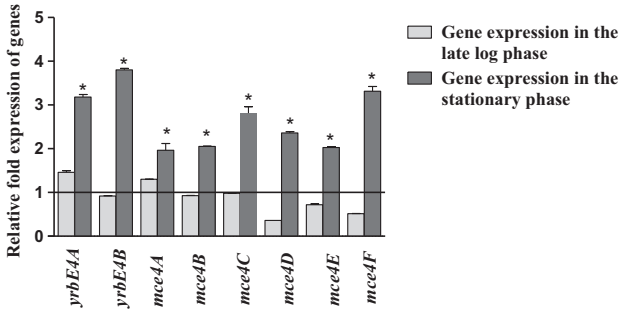

Fig. 3 – Relative fold expression of genes of the *mce4* operon in the presence of cholesterol in comparison to glycerol as the sole source of carbon in the late log phase as well as in the stationary phase culture of *Mycobacterium tuberculosis*.

culture (Fig. 2 and Table S2). With increasing days of incubation, fading of the blue color was observed in culture tubes supplemented with methylene blue, verifying increasing hypoxic stress to the bacteria.

Expression profile of genes of the mce4 operon of M. tuberculosis H37Rv in the presence of cholesterol or glycerol as the sole carbon source

M. tuberculosis H37Rv was grown in minimal medium supplemented with either glycerol or cholesterol to provide a carbon source. In the late log phase, no significant difference in the expression of the genes of the *mce4* operon was observed in the presence of cholesterol as the sole source of carbon when compared with expression in the presence of glycerol. As opposed to this expression of all genes of the operon was enhanced 2–3-fold in the presence of cholesterol compared with glycerol as the sole source of carbon in the stationary phase of culture (Fig. 3 and Table S3).

Discussion

Understanding the gene transcription profile of *M. tuberculosis* in the intracellular environment can provide an insight to deal with tuberculosis infection. In this report we described the transcription profile of the *mce4* operon genes under different stress conditions faced by the bacterium postentry. *M. tuberculosis* possesses four homologous *mce* operons, out of which *mce4* operon is reportedly a cholesterol importer in the nutrition-deficient stage of infection [7] and Mce4A plays an important role in the entry of the bacilli into the host cell in the later phase of infection [8] ostensibly to maintain the infected status of the host.

To understand the factors influencing the induction of *mce4* operon genes that facilitate long-term survival, *M. tuberculosis* H37Rv was exposed to mild surface stress and hypoxia. Surface stress is thought to be the initial stress encountered by the bacilli in the lung alveoli coated with pulmonary surfactant [2]. Additionally, *M. tuberculosis* H37Rv was exposed to nutritional stress along with supplementation with cholesterol or glycerol as the sole source of carbon.

We observed that exposure to SDS in the late log phase for 30 min and 90 min led to a significant induction of *mce4C* and *yrbE4B*, respectively. In the stationary phase of culture, in addition to *mce4C*, *mce4D* and *mce4F* were induced after 30 min of SDS exposure. However, exposure of SDS for 90 min was inhibitory to the expression of all genes of the operon. To date the function of each gene of the *mce4* operon has not been worked out. Further investigation will only be able to decipher the exact reason for such an observation.

Under hypoxic stress, on the 5th day, there was no expression of *mce4D*, while *mce4F* was significantly reduced; however, there was no significant change in the expression of the other genes of the *mce4* operon compared with the oxygenated culture. By contrast, on the 10th day and 15th day of hypoxic stress, all the genes of the *mce4* operon were overexpressed in comparison to the oxygenated culture. On the 20th day of hypoxia the expression level of all genes of the *mce4* operon lowered significantly except *mce4D* and *mce4F*, which were overexpressed. Thus, under surface stress as well as hypoxia, *mce4D* and *mce4F* were expressed differentially in comparison to the other genes of the operon. An observation from the above data suggests the possibility of the presence of some additional regulatory elements for *mce4D* and *mce4F* gene of the *mce4* operon of *M. tuberculosis* H37Rv, although these two genes are not functionally well characterized as yet. *In silico* analysis predicts involvement of these genes in host cell invasion and lipid catabolism (http://genolist.pasteur.fr/Tuberculist/).

Within the granuloma *M. tuberculosis* and host cells come close to each other and interact, leading to cell proliferation and aggregation. The cellular aggregates thus formed restrict *M. tuberculosis* from spreading and this leads to a period of latency simulating the stationary phase of growth [9]. Cholesterol may be one of the abundant carbon sources available in such tuberculous lesions. Presence of cholesterol in the plasma membrane of host cells is important for efficient entry of the mycobacteria within the host cells [10]. In our experiments we observed that the expression of the genes of the *mce4* operon was significantly higher when cholesterol was used to replace glycerol as the sole source of carbon in the stationary phase of culture.

The accumulated observations from the present study on the gene-wise dissection of the *mce4* operon indicate that the low oxygen concentration in the intracellular environment of the activated macrophages post-*M. tuberculosis* invasion and the abundant presence of host cholesterol are potent inducible factors for the expression of the *mce4* operon. We reported earlier that the *mce4* operon is expressed in the later phase of infection [11] when the bacteria possibly enter into a dormant state. The gene-wise expression profile presented here would add to the understanding of the life of *M. tuberculosis* following host cell invasion that may be useful to further strategize the approach to combat dormant *M. tuberculosis* within the host tissue.

Conflicts of interest

The authors have nothing to disclose.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmyco.2016.08.004.

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