Expression of DosR regulon of *Mycobacterium tuberculosis* under hypoxic condition
การแสดงออกของยีนใน DosR regulon ของเชื้อ *Mycobacterium tuberculosis* ภายใต้สภาวะออกซิเจนต่ำ

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ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), which claims approximately two million people annually, remains a serious health problem worldwide. The non-replicating or dormancy like state of Mtb is widely recognized as the key point of latent infection. The dormancy survival regulator (DosR) regulon, controls the response of Mtb to hypoxia which is basic stress condition during infection. The selected genes of DosR regulon including *ctpF*, *devR*, *fdxA*, *Rv3134c*, and *tgs1* were determined the comparative expression level under an oxygen-depleted (OD) and -replete (OR) conditions. Expression of all genes under OD condition was rapidly reduced after one week of culture and almost not-found in five week of culture. The results indicated the involvement of these genes in adaptation during early stage of the hypoxic growth condition. Previous studies proposed the essential of DosR regulon in dormancy state but the results in this study showed discrepancy especially in late state of the hypoxic condition. These results reflected the complexity of regulation of the genes in response to the stress condition during infection.

บทคัดย่อ

วัณโรคซึ่งมีสาเหตุมาจากเชื้อ *Mycobacterium tuberculosis* เชื่อว่ามีผู้ติดเชื้อนี้ถึงสองล้านคนและยังคงเป็นปัญหารุนแรงด้านสุขภาพในระดับโลก เชื้อที่อยู่ในสภาวะไม่แบ่งตัวหรือสภาวะพักเกี่ยวข้องกับการติดเชื้อแฝงของโรคนี้ กลุ่มยีน DosR ควบคุมการตอบสนองของเชื้อต่อสภาวะขาดออกซิเจนซึ่งเป็นสภาวะความเครียดพื้นฐานในการติดเชื้อ ยีนในกลุ่มนี้ที่คัดเลือกประกอบด้วย *ctpF devR fdxA Rv3134c* และ *tgs1* โดยทำการศึกษาระดับการแสดงออกในสภาวะขาดออกซิเจนในหลอดทดลอง พบว่าระดับการแสดงออกลดลงอย่างรวดเร็วในสภาวะขาดออกซิเจนหลังจากการเลี้ยงสัปดาห์แรกและเกือบตรวจไม่พบการแสดงออกในสัปดาห์ที่ 5 แสดงว่ายีนกลุ่มนี้เกี่ยวข้องกับการปรับตัว

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ในช่วงเริ่มต้นของการขาดออกซิเจน การศึกษาก่อนหน้าเสนอความจำเป็นของยีนกลุ่มนี้ในสภาวะพัก แต่ผลการทดลองครั้งนี้กลับขัดแย้ง โดยเฉพาะในช่วงท้ายของสภาวะขาดออกซิเจน ผลการทดลองเหล่านี้สะท้อนให้เห็นถึงความซับซ้อนของการควบคุมการแสดงออกของยีนในการตอบสนองต่อสภาวะเครียดในระหว่างการติดเชื้อ

Keywords: *Mycobacterium tuberculosis*, Hypoxia, Gene expression quantification
คำสำคัญ: มัยโคแบคทีเรียม ทูเบอร์คิวโลสิส ภาวะขาดออกซิเจน การวัดปริมาณการแสดงออกของยีน

Introduction

About one-third of the world's population has latent tuberculosis infection (LTBI) caused by *Mycobacterium tuberculosis* (*Mtb*). The latent infection happens after Mtb is exposed to a hostile environment; it reduces metabolism and replication and then enters a non-replicating (NRP) stage. The genes associated with this pathogenic mechanism have not been well elucidated. A hypoxic condition is a major factor frequently associated with the establishment and maintenance of LTBI. An *in vitro* oxygen-depleted model had been refined and is the most widely used to investigate NRP-Mtb [1] This study aimed to evaluate gene expression level of Mtb hypoxic related genes in this condition. The dormancy survival regulator (DosR) regulon essentially controls the response of Mtb to hypoxia which is basic stress condition during infection [2]. The selected genes of DosR regulon including *ctpF*, *devR*, *fdxA*, *Rv3134c* and *tgs1* were determined the comparative expression level under an oxygen-depleted (OD) and -replete (OR) conditions in this study. These genes were selected from the group of up-regulated genes from the high-throughput study either based on transcriptomic or proteomic analysis [1, 3]. These selected genes have never been tested the expression in the oxygen-depleted culture by qRT-PCR.

Material and methodology

Mycobacterium culture

Mycobacterium tuberculosis laboratory strain H37Rv, obtained from Mahidol University was cultured in a 20 ml of Middlebrook 7H9 broth (Difco, USA) supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) (Becton Dickinson, USA) for 7 days at 37 °C. One ml aliquots of *Mtb* H37Rv stock culture in 50% glycerol were stored at -70°C until required.

In vitro oxygen-depleted culture condition

One aliquot of stock of Mtb H37Rv was used to inoculate into 20 ml M7H9 medium supplemented with 10% OADC and grown standing at 37°C until OD_{600} = 1. To perform an *in vitro* oxygen-depleted model, the mycobacterial was cultured in four 20x150 mm-tubes of 30 ml M7H9 medium, supplemented with 10% OADC and 1.5 µg/ml of methylene blue dye which was used as an oxygen indicator to monitor oxygen-deplete conditions. Decolorization of the dye represents the oxygen level depleted as expected. The Oxygen-depleted (OD) condition group was placed in a sealed tube and standing incubated but the second set Oxygen-rich (OR) condition group, was shaken incubated at 37°C. The mycobacterial cultures were harvested at weeks 1, 3 and 5.

RNA extraction

Total RNA was extracted from the mycobacterial culture by using TriZol reagent (Invitrogen, USA). Briefly, mycobacterial pellets were recovered at different time points from 30 ml of the culture medium by centrifugation at 5000 g for 20 min. RNA was isolated from cell pellets by using a combination of vortex with zirconia bead and TriZol reagent to disrupt cells. The resulting lysate was mixed with 200 µl chloroform, shaken vigorously for 10 sec and kept at room temperature for 2 min. The mixed lysate was centrifuged at 12,000 g for 30 min at 4°C in order to separate the cell particles debris from the supernatant. The upper aqueous phase, which contains the total RNA, was carefully pipetted to a new micro-tube which contained 500 µl isopropanal. The mixture was incubated at room temperature for 10 min. The RNA was centrifuged at 12,500 g for 15 min at 4°C. The supernatant was discarded and the RNA pellet was washed once with 75% ethanol. The mixture was centrifuged at 7,500 g for five min at 4°C to remove ethanol. The pellet was air-dried and dissolved in 30 µl DEPC-treated water. The concentration of RNA was determined by spectrophotometer at 260 nm. To remove genomic DNA, the crude RNA sample was treated with DNase I prior to perform cDNA synthesis and then stored at -70°C, until required. The cDNA was pooled from a quadruplicate culture tubes.

Primers

Pairs of primers for each genes were designed by using a primer BLAST program. The primers of *ctpF*, *devR*, *fdxA*, *Rv3134c* and *tgs1* were forward 5'-CCGCTGATCTACGTTCTGCT-3', reverse 5'-TCGCATTGATCACCACCACA; forward 5'-CGCCAACTCCATTCCCTTGA-3', reverse 5'-ACGTCCTACACCTCTGACGA-3'; forward 5'-TGATGTAGAGCATTCG GGCG-3', reverse 5'-TGATCGGTAGTGAGTGCGTG-3'; forward 5'-GACTCCTGCATCAGCTTGGT-3', reverse 5'-CTGCG ACTGGTGTACGTCAT-3' and forward 5'-TGATCGGTAGTG AGTGCGTG-3', reverse 5'-AAGGCAGAAGACGTGGATCG-3', respectively.. The primers were ordered from Ward Medic (Thailand).

Evaluation of gene expression

Expression analysis of the genes was evaluated by using 25 ng RNA template in 2-step RT-PCR and performed qPCR on CFX96™ Real-Time PCR machine (Bio-Rad, USA) to determine the quantity of gene expression based on dye-based qPCR (SsoFast Evagreen, Bio-Rad, USA). The expression levels were calculated from CT value of each sample by using 2-ΔΔCT method. In this study, 16s rRNA gene was used as the internal control to normalize the RNA level in difference samples.

Results and discussion

Evaluation of gene expression by using qRT-PCR

The expression of all genes under OD condition was rapidly reduced after one week of culture and almost not-found in five week of culture. Whereas the expression of these genes in OR condition were relatively up-regulated throughout the culture (Figure 1). The results indicated the involvement of these genes in adaptation during early stage of the hypoxic growth condition.

Previous studies proposed the essential of DosR regulon in dormancy state [2]. It was found to modulate adaptive immunity and is essential for Mtb persistence [4]. The transcriptomic analysis of Mtb during adaptation to stationary phase and low-oxygen dormancy reveal highly up-regulate of these genes [5].

But the results in this study showed discrepancy especially in late state of the hypoxic condition. The growth condition might be different between laboratories and caused the different response of the genes. These results also reflected the complexity and dynamicity of regulation of the genes in response to the stress condition during infection. It might be difficult to identify common genes for being latency stage markers. In addition, the mycobacterial growth is very slow, especially in the hypoxia condition. The high amount of inoculum cells is recommended in order to gain enough amount of the extracted RNA.

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References

1. Wayne LG, Hayes LG. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence, Infection and Immunity; 1996, 64(6), P2062-9.
2. Chen T, He L, Deng W, Xie J. The Mycobacterium DosR regulon structure and diversity revealed by comparative genomic analysis, Journal of cellular biochemistry; 2013, 114(1), P1-6. Epub 2012/07/27.
3. Muttucumaru DGN, Roberts G, Hinds J, Stabler RA, Parish T. Gene expression profile of Mycobacterium tuberculosis in a non-replicating state, Tuberculosis; 2004, 84(3-4), P239-246.
4. Mehra S, Foreman TW, Didier PJ, Ahsan MH, Hudock TA, Kissee R, et al. The DosR Regulon Modulates Adaptive Immunity and Is Essential for *Mycobacterium tuberculosis* Persistence, American journal of respiratory and critical care medicine; 2015, 191(10), P1185-96. Epub 2015/03/03.
5. Voskuil MI, Visconti KC, Schoolnik GK. *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy, Tuberculosis; 2004, 84(3-4), P218-227.

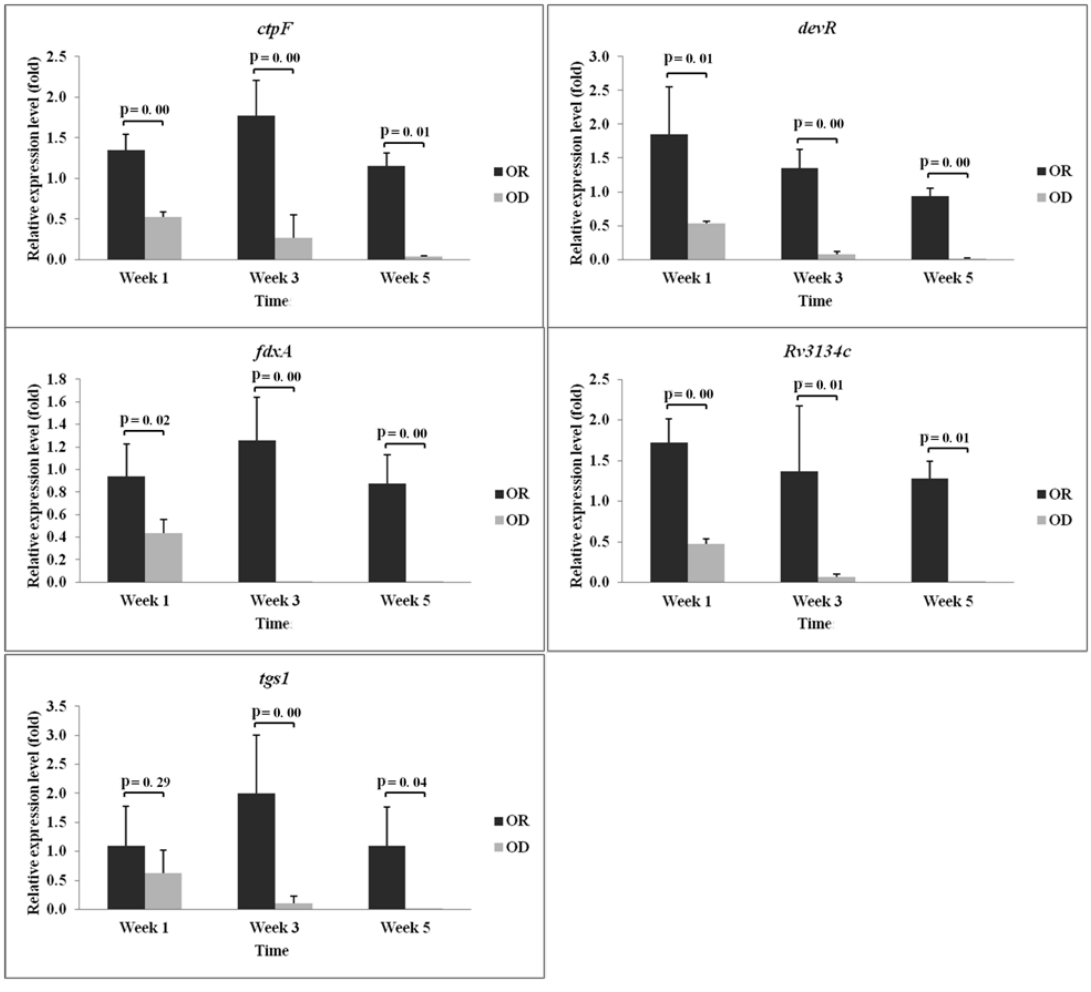

Figure 1 Relative expression levels of DosR regulon of Mtb H37Rv cultivated under oxygen-depleted (OD) and oxygen-rich (OR) conditions. The data were presented as fold change in gene expression normalized to an internal control and relative to the active growth Mtb. Data are presented mean fold-change ± SD of two independent experiments performed in triplicate. The paired Student t test was used to calculate the probability (p) that the expression of a gene had not changed. The p-value < 0.05 is considered significant