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Research Paper

# Genome-wide transcription analyses in *Mycobacterium tuberculosis* treated with lupulone

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## Abstract

*Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis, still causes higher mortality than any other bacterial pathogen until now. With the emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR-TB) strains, it becomes more important to search for alternative targets to develop new antimycobacterial drugs. Lupulone is a compound extracted from Hops (Hurnulus lupulus), which exhibits a good antimicrobial activity against *M. tuberculosis* with minimal inhibitory concentration (MIC) value of 10 μg/mL, but the response mechanisms of lupulone against *M. tuberculosis* are still poorly understood. In this study, we used a commercial oligonucleotide microarray to determine the overall transcriptional response of *M. tuberculosis* H37Rv triggered by exposure to MIC of lupulone. A total of 540 genes were found to be differentially regulated by lupulone. Of these, 254 genes were upregulated, and 286 genes were downregulated. A number of important genes were significantly regulated which are involved in various pathways, such as surface-exposed lipids, cytochrome P450 enzymes, PE/PPE multigene families, ABC transporters, and protein synthesis. Real-time quantitative RT-PCR was performed for choosed genes to verified the microarray results. To our knowledge, this genome-wide transcriptomics approach has produced the first insights into the response of *M. tuberculosis* to a lupulone challenge.

**Key words:** antimycobacterial avticity, lupulone, DNA microarray.

## Introduction

Among infectious diseases, tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is the major leading reason of death in the world, killing nearly 3,000,000 people annually (Ates *et al.*, 2008). Accompany with the human immunodeficiency virus (HIV), together with the emergence of multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) strains, has represented mycobacteria as a primary public health threat. Thus, the new drugs against TB owning new target are urgently needed (Youm and Saier, 2012).

Plants and other natural materials may prove to be valuable sources of useful new antimycobacterial drugs (Cantrell *et al.*, 2001). Lupulon (structure shown in Figure 1), a compound extracted from Hops (*Hurnulus lupulus*), was described as the antibiotic constituents (Lewis *et al.*,

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1949). It was identified to be bacteriostatic against multiple human (Chin *et al.*, 1949; Sacks and Humphreys, 1951) and veterinary bacterial pathogens (Siragusa *et al.*, 2008). The use of lupulone for TB treatment was documented in the scientific literature as early as in 1951 (Erdmann and Phytoncides, 1951). Interestingly, previous reports showed that lupulone inhibit nitric oxide production in RAW cells (Zhao *et al.*, 2003) and have potent radical scavenging activity and lipid peroxidation inhibitory activity (Tagashira *et al.*, 1995). It was suggested that lupulone acts by causing membrane leakage (Teuber and Schmalreck, 1973). However, the further research on action mechanism of lupulone is very poor, this prohibit the further availability of lupulone to be the mainstream antibiotics that treat TB.

In the past several years, DNA microarray technology has been used to discover gene functions, to understand biochemical pathways and to discver drug targets (Yu *et al.*, 2007, 2008, 2010; Liang *et al.*, 2011). This study is designed to analyze the genome-wide transcriptional changes in response to lupulone against *M. tuberculosis* with commercial agilent microarrays, and a subset of the microarray results were verified by real-time RT-PCR.

## Materials and Methods

### Bacterial strain and materials

The *M. tuberculosis* strain H37Rv (American Type Culture Collection 27294) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. Middlebrook 7H9 broth and OADC (oleic acid, albumin, dextrose and catalase) was purchased from BD Biosciences, Inc., Sparks, MD. Alamar Blue was obtained from Trek Diagnostic Systems (Westlake, OH, US). TRIzol was purchased from Invitrogen. Tween 80 was purchased from Sigma-Aldrich. Lupulone was obtained from Sigma-Aldrich. Stock solutions of lupulone were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

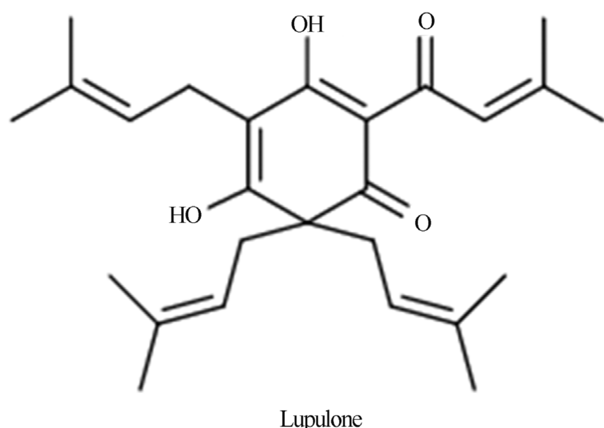


**Figure 1** - Chemical formula of lupulone.

### The minimal inhibitory concentration (MIC) determinations

The activity of lupulone against the aforementioned *M. tuberculosis* strain was tested using a microplate Alamar Blue assay (MABA) according to the method of Franzblau *et al.* (1998), as modified by Jiménez-Arellanes *et al.* (2003). Briefly, sterile distilled water (200 μL) was poured into the outer perimeter wells of the microplate. All other wells received 100 μL of supplemented Middlebrook 7H9 broth. Then, working extract solutions (100 μL) were poured into the first well of each row and two-fold dilution series were made from these solutions through the microplate column. The test inoculum (100 μL) was added to all testing wells, as well as to the drug-free control wells. The final concentration of DMSO in the wells was approximately 1% v/v. At the same time, controls diluted 10:100 and 1:100 were prepared from the bacterial suspension, representing the growth of 10% and 1% of the bacterial population tested, respectively. The final concentrations of lupulone tested ranged from 160 to 0.3125 μg/mL. Each concentration was assayed in duplicate. Each microplate was incubated for five days at 37 °C in a 5% $CO_2$ atmosphere in a sealed plastic bag. Following incubation, a control growth was developed with a mixture of 20 μL of Alamar Blue solution (Trek Diagnostics, Westlake, OH) and 12 μL of sterile 10% Tween 80. The plates were re-incubated at 37 °C for 24 h. After this incubation, if the well turned pink, all of the wells received a mixture of Alamar Blue and Tween solutions in the same way as described above and were incubated for an additional 24 h. Wells with a well-defined pink color were scored as positive for growth. The MIC was defined as the lowest concentration of a sample that prevents a color change to pink. Extracts were considered active if they showed an MIC ≤ 200 μg/mL.

### Cell culture and drug exposure for microarray experiments

A frozen stock of *M. tuberculosis* strain H37Rv was inoculated into 5 mL of the Middlebrook 7H9 broth containing 0.05% Tween 80, 0.2% glycerol and 10% oleic acid, albumin, dextrose and catalase (OADC) at 37 °C for five days. Then, the culture was transferred into 200 mL of 7H9 media and incubated at 37 °C with shaking at 150 rpm until the $OD_{600}$ reached 0.7. Subsequently, 200 mL of 7H9 broth was divided into two flasks, each of which contained 100 mL of culture; the cells were harvested by centrifugation for RNA preparation. A lupulone stock solution was prepared in dimethyl sulfoxide (DMSO). Drug treatment was conducted by adding the stock solution to one of the cultures to achieve a final concentration of 10 μg/mL (MIC). Untreated paired control bacteria were grown under identical conditions to treated bacteria, with the exception that no drug was added. The final concentration of DMSO

in each culture could not exceed 0.05% (v/v) (Slayden *et al.*, 2006). Two independent 200 mL cultures were prepared to act as biological repeats. Upon completion of the predefined duration (4 h) of drug and control treatments, the bacteria were harvested by centrifugation and then stored for RNA extraction.

### RNA isolation and cDNA labeling

Bacterial cultures were centrifuged for 5 min at 2500 *g*. After removing the supernatant, the pellets were frozen on dry ice and stored at -80 °C. Total RNA was harvested using TRIzol (Invitrogen) and an RNeasy kit (Qiagen) according to the manufacturer's instructions, including a DNase digestion step. The RNA samples were redissolved to produce a final concentration of 300-500 ng/μL. For every RNA sample, 120 μL was sent to Shanghai Bio Co., Ltd. and further examined through a quality and quantity test based on electrophoresis before microarray hybridization.

Fluorescently labeled cRNA, transcribed from cDNA, was produced using a Quick Amp Kit, PLUS, Two-Color (Agilent p/n 5190-0444) in Agilent's SureHyb Hybridization Chambers. The cRNA was labeled with the fluorescent dyes Cy5 and Cy3-CTP. Double-stranded cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit according to the manufacturer's protocol (Quick Amp Kit, Agilent). T7 promoter primers were used instead of the poly-T primer provided in the kit. The Cy3- and Cy5-labeled products were purified using an RNeasy Mini Kit (Qiagen). An aliquot of 1 μL of purified cRNA was used to determine the yield and specific activity with a NanoDrop ND-1000. The amount of Cy3- or Cy5-labeled cRNA was determined by measuring the absorbance at A260 nm, A280 nm, A550 nm (Cy3) and A650 (Cy5). The specific activity (pmol dye per μg cRNA) of the cRNA can be obtained from the following calculation: specific activity = (concentration of Cy3/Cy5)/[(concentration of cRNA) * 1000] = pmol Cy3/Cy5 per μg cRNA. If the yield is < 825 ng and the specific activity is < 8.0 pmol Cy3/Cy5 per μg of cRNA, the experiment does not proceed to the hybridization step. cRNA was repeatedly prepared.

### Microarray hybridization and data analysis

*M. tuberculosis* microarray slides consisted of 4690 60-mer oligonucleotides representing 4004 open reading frames from *M. tuberculosis* strain H37Rv and 686 unique open reading frames from strain CDC1551 that are not present in the H37Rv strain's annotated gene complement. Microarray hybridization was performed in Agilent's SureHyb Hybridization Chambers using the Agilent Gene Expression Hybridization Kit. After hybridization and washing, the processed slides were scanned using an Agilent DNA microarray scanner (part number G2505B) with the settings recommended by Agilent Technologies. The resulting text files, which were extracted using Agilent Feature Extraction Software (version 10.5.1.1), were imported into Agilent GeneSpring GX software (version 11.0) for further analysis. The microarray datasets were normalized in Agilent Feature Extraction Software (mainly LOWESS normalization) and then genes marked as present were chosen for further analysis. Differentially expressed genes were identified through Volcano Plot screening. Cluster analysis was carried out by hierarchical clustering (HCL). In addition to the significance analysis of the microarrays, a fold change analysis was performed in which the ratios of the geometric means of the expression intensities of the corresponding genes in the lupulone treatment samples relative to control samples were calculated. The ratios were reported as the fold change up or down. To select differentially expressed genes, the genes were considered to be significantly differentially hybridized compared with the *M. tuberculosis* control if they displayed at least a two-fold difference in the ratio (Frota *et al.*, 2004).

### Quantitative real-time RT-PCR assays

Aliquots of the RNA preparations from the lupulone-treated and control samples used in the microarray experiments were saved for follow-up quantitative real-time RT-PCR. Quantitative real-time RT-PCRs were performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to a previously described procedure (Yu *et al.*, 2007). The primer sequences used are listed in Table 1.

## Results

### Gene expression changes of *M. tuberculosis* in response to lupulone treatment

In the study, the MIC value of lupulone against *M. tuberculosis* H37Rv (ATCC 27294) was 10 μg/mL. We analysed the microarray data and found that there were 540 genes significantly differentially expressed after 4 h of exposure to MIC concentration (10 μg/mL) of lupulone compared to untreated *M. tuberculosis* H37Rv (ATCC 27294) cells. Previous study shows that RNA preparation following 4 h of drug treatment may deliver the most meaningful results (Provvedi *et al.*, 2009). This is the reason why incubation for 4 h was chosen in our study.

Among these, 254 genes were significantly increased in expression and 286 genes were significantly inhibited. Most of these genes were classed as having an *unclassified role category not yet assigned* (20.6%), and the others were classified as involved in cell envelope (3.0%), cellular processes (2.6%), central intermediary metabolism (1.9%), conserved hypothetical protein (15.4%), DNA metabolism (2.2%), energy metabolism (6.1%), mobile and extrachromosomal element functions (2.6%), protein fate (2.0%), protein synthesis (2.8%), regulatory functions (3.9%), transport and binding proteins (3.9%), hypothetical protein

**Table 1** - Primers used in real-time RT-PCR with SYBR green probes.

| Primer | Systematic name | Sequence (5'- 3') |
|---|---|---|
| 16S rRNAfor | | GCACCGGCCAACTACGTG |
| 16S rRNArev | | GAACAACGCGACAAACCACC |
| Rv1686c for | Rv1686c | TCGGGCTCGGTCTATTGTGTA |
| Rv1686c rev | Rv1686c | GGATGAACTGTACGGCCTGAA |
| Rv2626c for | Rv2626c | GCTCAACGTCATGGAAGAACA |
| Rv2626c rev | Rv2626c | CCAAGCGGTGCTCTGAGATGA |
| *nrdZ* for | Rv0570 | GGCTGGGAGTCATGGGTTT |
| *nrdZ* rev | Rv0570 | GCTTCTTCACTGTCGTACGGAAT |
| *frdB* for | Rv1553 | TGGAGCCGATGCGAAACTT |
| *frdB* rev | Rv1553 | GAAGTCACTGATGTCGACAACGA |
| *hspX* for | Rv2031c | GACAAGGACGTCGACATTATGG |
| *hspX* rev | Rv2031c | CGCTCGGCCTTGATGGTCAGCTGA |
| *mmpS5* for | Rv0677c | CAAGGTGGTGGAGTACGAAGTTT |
| *mmpS5* rev | Rv0677c | TCGAGGTCCAGGTAGTTGATGTTG |
| *ethA* for | Rv3854c | CCCATCCTCGAGTACGTCAAGA |
| *ethA* rev | Rv3854c | CGGATATGCCTGTCGATTCCA |
| *rplN* for | Rv0714 | GGCACCCGCATTTTTGG |
| *rplN* rev | Rv0714 | CCAGCGAAATGATCTTCATAAACC |
| *htpX* for | Rv0563 | CATCCTGCGTATCCTCAATGAG |
| *htpX* rev | Rv0563 | TCGCGGTTGTAGACGTGAGA |
| *ppsA* for | Rv2931 | CCAAATCAGCACTTCGAAACC |
| *ppsA* rev | Rv2931 | CCATTCAGTTTGTGTGTCAACGA |
| *icl* for | Rv0467 | AACCAGATGAGCGCGTATGTC |
| *icl* rev | Rv0467 | CGGTGTAGCCCCGTTCTTC |

(20.0%), unknown function (7.8%) and the other categories (5.4%). The microarray-related data were submitted to Gene Expression Omnibus (GEO) under accession number GSE31732. A complete list of all of the genes differentially expressed due to lupulone treatment can be found in the Supplementary material (Table S1). Figure 2 provides a summary of the differentially regulated genes grouped by functional categories. Herein, we focused the interest mainly on specific genes that may affect the organism's survival in the presence of lupulone. These genes will be discussed in the Discussion section.

### Validation of microarray data by real-time RT-PCR

Using the same RNA samples used for the original microarray experiment against eleven selected genes (Rv1686c, Rv2626c, *nrdZ*, *frdB*, *hspX*, *mmpS5*, *ethA*, *rplN*, *htpX*, *ppsA* and *icl*), real-time quantitative RT-PCR was conducted to validate the microarray data. Overall, there was great accordance between the microarray data and the real-time RT-PCR data for all 11 genes (Table 2). When exposed to lupulone, there were 6 genes induced and 5 genes reduced. However, the numerical values of some genes (Rv1686c and *mmpS5*) determined by RT-PCR were much higher than those obtained from the microarray. In situations in which poor or no hybridization signals were generated for one of the samples tested, the n-fold induction values can be under- or overestimated as a result of the higher efficiency of the RT step (no cyanine dye incorporation) and/or the higher sensitivity of the real-time PCR (Liu *et al.*, 2005). The expression levels of other genes (Rv2626c, *icl*, *ppsA, nrdZ*, *frdB*, *hspX*, *ethA*, *rplN* and *htpX*) did not differ markedly between the microarray data and real-time RT-PCR data. In summary, the real-time RT-PCR results provided independent verification of our DNA microarray results.

## Discussion

### Induction of the genes involved in cytochrome P450 enzymes

The human pathogen *M.* tuberculosis encodes 20 cytochrome P450 (P450) enzymes. Gene essentiality for viability or host infection was demonstrated for many P450s, such as CYP128, CYP121 and CYP125 (McLean *et al.*, 2010). In the study, we found the genes *cyp140*, *cyp138*, *cyp128* and *cyp124* invovled in cytochrome P450 enzymes (CYP450s) were upregulated more than 2-fold following exposure to lupulone. CYP450s are heme-containing

**Table 2** - Real-time RT-PCR analysis of gene expression.

| Systematic name | Gene | Description | Fold change RT-PCR | Systematic name Microarray |
|---|---|---|---|---|
| Rv1686c | Rv1686c | integral membrane protein ABC transporter | +86.34 (± 2.8) [a] | +14.99 |
| Rv2626c | Rv2626c | hypothetical protein | +2.71 (± 0.24) | +3.12 |
| Rv0570 | *nrdZ* | ribonucleoside-diphosphate reductase large subunit | +2.84 (± 0.35) | +2.02 |
| Rv1553 | *frdB* | fumarate reductase iron-sulfur subunit | +3.18 (± 0.29) | +2.37 |
| Rv2031c | *hspX* | heat shock protein | +2.93 (± 0.29) | +2.48 |
| Rv0677c | *mmpS5* | hypothetical protein | +30.88 (± 1.92) | +12.04 |
| Rv3854c | *ethA* | monooxygenase | -5.61 (± 0.49) | -2.44 |
| Rv0714 | *rplN* | 50S ribosomal protein L14 | -2.06 (± 0.24) | -2.34 |
| Rv0563 | *htpX* | heat shock protein | -2.47 (± 0.21) | -2.04 |
| Rv2931 | *ppsA* | phenolpthiocerol synthesis type-I polyketide synthase | -3.64 (± 0.27) | -2.02 |
| Rv0467 | *icl* | isocitrate lyase | -3.43 (± 0.31) | -2.28 |

"+" and "-"indicated increase and reduction, respectively; a, indicated "mean ± standard deviations".

monooxygenases, well known for their roles in metabolism of fatty acids, steroids, and other lipophilic molecules (Denisov *et al.*, 2005). The *M. tuberculosis* genome sequence revealed an unexpectedly high number of CYP450s (Cole *et al.*, 2001). Among these, the second largest of the *M. tuberculosis* CYP450s is CYP128 (53,313 Da) encoded by *cyp128* that is predicted to metabolize menaquinone as a step towards its sulfation (Holsclaw *et al.*, 2008). The creation of genome-wide transposon libraries enabled the classification of CYP128 as a gene required for optimal growth of *M. tuberculosis*, and as upregulated in cell starvation (McLean *et al.*, 2007). CYP124 encoded by *cyp124* is found in pathogenic and nonpathogenic mycobacteria species, actinomycetes, and some proteobacteria, which suggests that it has an important catalytic activity (Ouellet *et al.*, 2010). It is located adjacent to a three-gene operon containing a sulfotransferase (*Sft3*, Rv2267c) that catalyzes the PAPS-dependent sulfation at the ω-position of menaquinone MK-9 DH-2 (Holsclaw *et al.*, 2008; Mougous *et al.*, 2006). The biochemical characterization of CYP124 includes identifying a series of substrates consistent with ω-hydroxylase activity and, importantly, a marked preference for lipids containing methyl branching (Johnston *et al.*, 2009). To date, gene disruption and gene deletion studies have shown that *M. tuberculosis cyp128* is an essential gene for cell growth and viability (McLean *et al.*, 2008). Cyp138 are induced at elevated temperatures (Stewart *et al.*, 2002). Some studies have reinforced the fact that *M. tuberculosis* P450s play important cellular roles and are most important in the pathogen's response to environmental stimuli and immune/chemical abuse (McLean *et al.*, 2007). The upregulation of the *M. tuberculosis* cytochrome P450 enzyme genes may be a adaptive response to environmental changes to survive. The trigger for the induced transcription of the P450s in *M. tuberculosis* following a lupulone challenge requires further study.

### PPE and PE genes differentially regulated by lupulone

When H37Rv cells were exposed to lupulone, a number of *PE* and *PPE* genes were differentially regulated, including *PPE29*, *PPE47*, *PPE67*, *PE_PGRS15*, *PE_PGRS46*, *PE_PGRS58*, *PE2*, *PE13*, *PE23*, *PE22* and *PE25*. The genes *PPE29*, *PPE47*, *PPE67*, *PE2*, *PE13*, *PE22*, *PE23*, *PE25* were inhibited following exposure to lupulone, while in contrast, the genes *PE_PGRS15*, *PE_PGRS46*, *PE_PGRS58* were upregulated. Two large protein families, the PE and PPE, named for the conserved proline and glutamate residues near the N-terminal region of the encoded proteins, contain about 100 PE members and more than 60 PPE members in the genome. Although no structure or precise function is known for any member of these families, it has been suggested that some PE_PGRS proteins have been found to associate with the cell wall (Banu *et al.*, 2002; Delogu *et al.*, 2004) and to influence interactions with eukaryotic cells (Brennan *et al.*, 2001), and some members may play a role in immune evasion (Vordermeier *et al.*, 2012). Members of the PE and PPE families also have been linked to virulence (Ramakrishnan *et al.*, 2000), and some PPE proteins have been found to be immunodominant antigens (Choudhary *et al.*, 2003). The PE/PPE gene families have been found to play critical roles in host-pathogen interactions. As previous study reported frequent natural homologous recombination events within and between PE/PPE genes (Karboul *et al.*, 2008), such a propensity for recombination could represent an ideal adaptive mechanism that ensures the creation of new recombined variant molecules in response to new selective immune pressures. Moreover, it is concluded that due to in-

dividual PE and PPE proteins failing to be expressed in a soluble form, individual PE proteins are likely protein partners for PPE proteins (Strong *et al.*, 2006). In this study, since many *PE* and *PPE* genes were differentially regulated when exposed to lupulone, the survival ability of tuberculosis may be reduced by destroying the ratio of PE and PPE.

### Downregulation of genes encoding 50s ribosomal proteins exposed to lupulone

The ribosome is the factory where protein synthesis occurs. The structure of the ribosome in bacteria and human cells differs significantly and this difference allows some antibiotics to specifically kill bacteria. Previouse study showed that the divergent properties of the mycobacterial ribosomes may be related to some exceptional properties of mycobacteria, e.g. their slow growth (Shasmal and Sengupta, 2012). In the presence of lupulone, seven genes (*rplY*, *rplX*, *rplN*, *rplJ* and *rplE*) encoding 50S ribosome protein were downregulated by 2.06 to 2.35-fold (showed in Table S1). The genes *rplY*, *rplX*, *rplN*, *rplJ*, *rplE* encoded 50S ribosomal protein L25, L24, L14, L10, L5, respectively, which belong to the rpl family. The aforementioned genes participate in 50s ribosomal protein synthesis and modification. Moreover, the gene *frr* encoding ribosome recycling factor was upregulated by 2.02-fold. The gene *infC* which are essential for the initiation of translation was also upregulated by 2.20-fold. Hence, our results suggested that the downregulation of the genes may result in reduced amounts of functional ribosomes and repressed translational capacity. The ribosome is a multiprotein complex and the protein-protein interactions of the ribosomal subunits could be attractive targets for new drug.

### Inhibition of genes ivovled in surface-exposed lipids when exposed to lupulone

Some research has demonstrated that proteins encoded by genes at the *M. tuberculosis fadD26-mmpL7 locus* (*fadD26*, *ppsA* to *ppsE*, *drrA* to *drrC*, *papA5*, *mas*, *fadD28*, and *mmpL7*) play major roles in phthiodiolone dimycocerosate (PDIM) biosynthesis and secretion (Camacho *et al.*, 1999; Rousseau *et al.*, 2004). Phthiocerol and phenolphthiocerol esterified with multiple methyl-branched long chain fatty acids belonged to surface-exposed lipids that been found to be unique to pathogenic mycobacteria. Diesters of phthiocerol and phenolphthiocerol are important virulence factors of *M. tuberculosis*. Moreover, the phthiocerol and phthiodiolone dimycocerosate esters (PDIMs) comprise a category of virulence-enhancing lipids that act as defensive, offensive, or adaptive effectors of virulence.

In our srudy, the genes *fadD26*, *ppsA*, *ppsB*, *ppsC*, *ppsD*, *ppsE*, *drrB*, *drrC*, and *papA5* were downregulated more than 2-fold when *M. tuberculosis* strains were exposed to lupulone. Type I modular polyketide synthase (PKS) encoded by the genes *ppsA-E* was responsible for the synthesis of phthiocerol and phenolphthiocerol through the elongation of a C20-C22 fatty acyl chain or an acyl chain which contained a phenol moiety with three malonyl-CoA and two methylmalonyl-CoA units. FadD26 encoded by the gene *fadD26* belongs to a family of long-chain fatty acyl-AMP ligases activating longchain fatty acids as acyl-adenylates for subsequent transfer to their cognate multifunctional polyketide synthases (Trivedi *et al.*, 2004). The ABC-type transporter DrrB and DrrC encoded by the genes *drrB* and *drrC* are both the daunorubicin-DIM-transport integral membrane protein. DrrB behaves as a functional doxorubicin efflux pump and drrC are necessary for the proper localization of dimycocerosyl phthiocerol (DIM) in the cell envelope (Choudhuri *et al.*, 2002; Camacho *et al.*, 2001). Additionally, Onwueme *et al.* (2004) have recently proposed that PapA5 is required for diesterification of phthiocerol with mycocerosate to produce PDIM. In the other study, *M. tuberculosis* ppsA-E-deficient mutants fail to synthesize DIM and are more sensitive to sodium dodecyl sulfate (SDS), which appear an increase in their outer membrane permeability (Nikaido and Vaara, 1985). The inactivation of *fadD26* in *M. tuberculosis* strains has been unable to synthesize PDIM and are attenuated in a mouse model of TB infection (Camacho *et al.*, 1999, 2001). In conclusion, downregulation of the genes may affect outer membrane or cell wall permeability of *M. tuberculosis*, this fingding may be consistnet with early reports that lupulone acts by causing membrane leakage (Teuber and Schmalreck, 1973).

### Significant regulation of the genes involved in ABC transporters

The ABC transporters genes *irtA*, *irtB*, *cysA1*, *cysT* and *cysW* were significantly downregulated following exposure to lupulone. The genes *irtA* and *irtB* encoding ATP-binding cassette transponer (ABC transporter) IrtA and IrtB (Braibant *et al.*, 2000) highly similar to the YbtPQ system of *Yersinia pestis* (Fetherson *et al.*, 1999). The genes *irtA* and *irtB* are part of the iron acquisition machinery of *M. tuberculosis* and do not participate in siderophore synthesis or secretion but are required for efficient utilization of iron from Fe-carboxymycobactin (Rodriguez and Smith, 2006). Previous study has demonstrates that inactivation of *M. tuberculosis irtA* (Rv1348) or *irtB* (Rv1349) genes results in decreased ability of *M. tuberculosis* to replicate in low-iron medium and to utilize Fe3-ExMb as the sole iron source (Ryndak *et al.*, 2010). Moreover, it is reported IrtAB is necessary for normal multiplication of *M. tuberculosis* in human Macrophages (Rodriguez and Smith, 2006). The genes *cysT* and *cysW* encoded sulfate-transport integral membrane proteins while the gene *cysA1* encoded sulfate-transport ATP-binding protein. These proteins are all ABC transporters and play important roles in sulfate acquisition. The CysTWA SubI ABC transporetr complex is responsible

for the active transport of inorganic sulfate across the mycobacterial cell membrane (Mehra and Kaushal, 2009). Sulfate assimilation is crucial for *M. tuberculosis*. It is an essential bionutrient with a key role in biosynthesis of cysteine, mycothiol and coenzyme A (Mehra and Kaushal, 2009). So our results suggest that the downregulation of the genes may affect the ability of *M. tuberculosis* to efficiently acquire iron, normal multiply, sulfate acquisition and further influence survival when exposed to lupulone.

In contrast, the ABC transporters genes Rv1686c, Rv1687c, Rv1218c, Rv1217c and Rv1739c were induced 14.99-fold, 5.08-fold, 4.30-fold, 2.74-fold and 2.25-fold respectively. It has been reported that most of the compound classes had significantly better bactericidal activity in the Δ*Rv1218c* mutant than in the wild-type H37Rv, which suggested *Rv1218c* gene product was related to effluxing these compounds from *M. tuberculosis* (Balganesh *et al.*, 2010). The genes Rv1686c and Rv1687c were also highly induced when *M. tuberculosis* in response to the 5  MIC triclosan treatments (Betts *et al.*, 2003). Moreover, when *M. tuberculosis* was under hypoxic conditions, the gene Rv1739c was also found to be upregulated (Tyagi and Saini, 2004). Hence, the induction of the genes may be adaptive response to lupulone.

## Conclusion

In summary, our results showed that lupulone has potential antimycobacterial activity and our DNA microarray analysis demonstrated that lupulone affected a number of important genes involved in different pathways in *M. tuberculosis*. These findings may have important implications for understanding the responsive mechanisms of *M. tuberculosis* to lupulone treatment.

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## Supplementary Material

Table S1: A list of genes with expression changes of at least 2.0 fold in *M. tuberculosis* H37Rv exposed to Lupulone.

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