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 chosen 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 activity, 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.*, 2002).
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 Phyttonicides, 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 discover 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).

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 1:100 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% CO₂ 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 OD₆₀₀ 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 RNaseasy 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 RNaseasy 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
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 involved in cytochrome P450 enzymes (CYP450s) were upregulated more than 2-fold following exposure to lupulone. CYP450s are heme-containing enzymes that play a key role in drug metabolism and resistance. The induction of these genes following exposure to lupulone suggests a potential role in resistance to lupulone. Further study is needed to determine the exact mechanisms of induction and the role of these genes in the drug resistance of M. tuberculosis.

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

| Primer     | Systematic name | Sequence (5’- 3’) |
|------------|-----------------|-------------------|
| 16S rRNAfor|                 | GCACCCGCGCAACTACGTG |
| 16S rRNArev|                 | GAAACAACGCCAACAACC |
| Rv1686c for| Rv1686c         | TCGGCGCTGGTCTATTTGTA |
| Rv1686c rev| Rv1686c         | GGAATCTGGTACGCAGCTGAA |
| Rv2626c for| Rv2626c         | GCCTCGGCTGCTGAGGACAA |
| Rv2626c rev| Rv2626c         | CCAAGCGGTGCTGAGATGA |
| nrdZ for   | Rv0570          | GCTCTCTCAGTGTCAGGGAAT |
| nrdZ rev   | Rv0570          | GCTCTCTCAGTGTCAGGGAAT |
| frdB for   | Rv1533          | TGGAGCCGATGCGCAAAC |
| frdB rev   | Rv1533          | TGGAGCCGATGCGCAAAC |
| hspX for   | Rv2031c         | GACAAGAGACGTCGACATTAGG |
| hspX rev   | Rv2031c         | CGCTCGGCTGCTGAGGACAA |
| mmpS5 for  | Rv0677c         | CAAAGTGGTGGAGTACGAAG |
| mmpS5 rev  | Rv0677c         | CAAAGTGGTGGAGTACGAAG |
| ethA for   | Rv3854c         | CCCATCTCGAGTACGCAAGA |
| ethA rev   | Rv3854c         | CCCATCTCGAGTACGCAAGA |
| rplN for   | Rv0714          | GGCACCCGCAATTTGG |
| rplN rev   | Rv0714          | GGCACCCGCAATTTGG |
| htpX for   | Rv0563          | CATCTCGGTACTCCTAAGG |
| htpX rev   | Rv0563          | TCGGCGTGGTACGTCAGG |
| ppsA for   | Rv2931          | CCAATCGACCTTCGAAA |
| ppsA rev   | Rv2931          | CCAATCGACCTTCGAAA |
| icl for    | Rv0467          | AACCGATGAGGCGGTAGTGC |
| icl rev    | Rv0467          | AACCGATGAGGCGGTAGTGC |
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 non-pathogenic 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 ε-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.

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

| Systematic name | Gene     | Description                              | Fold change RT-PCR | Systematic name | Gene     | Description                              | Fold change Microarray |
|-----------------|----------|------------------------------------------|--------------------|-----------------|----------|------------------------------------------|------------------------|
| Rv1686c         | Rv1686c  | integral membrane protein ABC transporter| +86.34 (± 2.8)      | +14.99         |
| Rv2626c         | Rv2626c  | hypothetical protein                     | +2.71 (± 0.24)     |                |
| Rv0570          | nddZ     | ribonucleoside-diphosphate reductase large subunit | +2.84 (± 0.35)   |                |
| Rv1553          | frdB     | fumarate reductase iron-sulfur subunit    | +3.18 (± 0.29)     |                |
| Rv2031c         | hspX     | heat shock protein                       | +2.93 (± 0.29)     |                |
| Rv0677c         | mmpS5    | hypothetical protein                     | +30.88 (± 1.92)    |                |
| Rv3854e         | 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          | ppxA     | phenolphthiocerol 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”.

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 have also 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 recombinant variant molecules in response to new selective immune pressures. Moreover, it is concluded that due to in-
individual 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. Previous 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 Sen-gupta, 2012). In the presence of lupulone, seven genes (rplY, rplX, rplN, rplJ and rplE) encoding 50S ribosomal protein were downregulated by 2.06 to 2.35-fold (showed in Table S1). The genes rplY, rplX, rplN, rplJ, rplF 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 involved 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 dimycocer- osate esters (PDIMs) comprise a category of virulence-enhancing lipids that act as defensive, offensive, or adaptive effectors of virulence.

In our study, 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 transport 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, mycolthiol 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 ΔRv1218c 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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**References**

Ates O, Muselli M, Ongen G, Topal-Sarikaya A (2008) Interleukin-10 and tumor necrosis factor-alpha gene polymorphisms in tuberculosis. J Clin Immunol. 28:232-236.

Balganesh M, Kuruppath S, Marcel N, Sharma S, Nair A, Sharma U (2010) Rv1218c, an ABC Transporter of *Mycobacterium tuberculosis* with Implications in Drug Discovery. Antimicrob Agents Chemother 54:5167-5172.

Banu S, Honoré N, Saint-Joanis B, Philpott D, Prévost MC, Cole ST (2002) Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? Mol Microbiol 44:9-19.

Betts JC, McLaren A, Lennon MG, Kelly FM, Lukey PT, Blakemore SJ, Duncan K (2003) Signature gene expression profiles discriminate between isoniazid-, thiolactomycin-, and triclosan-treated *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47:2903-2913.

Braitman M, Giot P, Content J (2000) The ATP binding cassette (ABC) transport systems of *Mycobacterium tuberculosis*. *FEBS*. Microbiol Rev 24:449-467.

Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, Jacobs WR (2001) Jr. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect Immun 69:7326-7333.

Camacho LR, Constant P, Raynald C, Lanecelle MA, Tricas JA, Gicquel B, Daffe M, Guilhot C (2001) Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. J Biol Chem 276:19845-19854.

Camacho LR, Ensergueix D, Perez E, Gicquel B, Guilhot C (1999) Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Mol Microbiol 34:257-267.

Cantrell CL, Franzblau SG, Fischer NH (2001) Antimycobacterial plant terpenoids. Planta Med 67:685-694.

Chin YC, Chang NC, Anderson HH (1949) Factors influencing the antibiotic activity of lupulon. J Clin Invest 28:909-915.

Choudhary RK, Mukhopadhyay S, Chakhaiyar P, Sharma N, Murthy KJ, Katoch VM, Hasnain SE (2003) PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. Infect Immun. 71:6338-6343.

Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P (2002) Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drdA and drdB of *Mycobacterium tuberculosis*. Biochem J 367:279-285.

Cole ST, Eiglemeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honoré N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG (2001) Massive gene decay in the leprosy bacillus. Nature 409:1007-1011.

Delogu G, Pusceddu C, Bua A, Fadda G, Brennan MJ, Zanetti S (2004) Rv1818c-encoded PE_PGRS protein of *Mycobacterium tuberculosis* is surface exposed and influences bacterial cell structure. Mol Microbiol 52:725-733.

Denisov IG, Makris TM, Sligar SG, Schlichting I (2005) Structure and chemistry of cytochrome P450. Chem. Rev 105:2253-2277.
Erdmann WF, Phytoncides I (1951) Lupulone and humulone; their antibacterial action and their use in tuberculous infections. Pharmazie 6:442-451.

Fetherson JD, Bertolino VJ, Perry RD (1999) YbtP and YbtQ two ABC transporters required for iron uptake in Yersinia pestis. Mol Microbiol 32:289-299.

Frantzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH (1998) Rapid low-technology MIC determination with clinical, Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36:362-366.

Frota CC, Hunt DM, Buxton RS, Rickman L, Hinds J, Kremer K, van Soolingen D, Colston MJ (2004) Genome structure in the vole bacillus, Mycobacterium microti, a member of the Mycobacterium tuberculosis complex with a low virulence for humans. Microbiology 150:1519-1527.

Holcslaw CM, Sogi KM, Gilmore SA, Schelle MW, Leavell MD, Bertozzi CR, Leary JA (2008) Structural characterization of a novel sulfated menaquinone produced by stf3 from Mycobacterium tuberculosis. ACS Chem Biol 3:619-624.

Jiménez-Arellanes A, Meckes M, Ramírez R, Torres J, Luna-Herrera J (2003) Activity against multidrug-resistant Mycobacterium tuberculosis in Mexican plants used to treat respiratory diseases. Phytother Res 17:903-908.

Johnston JB, Kells PM, Podust LM, Ortiz de Montellano PR (2009) Biochemical and structural characterization of CYP124: A methyl-branched lipid o-hydroxylase from Mycobacterium tuberculosis. Proc Natl Acad Sci USA. 106:20687-20692.

Karboul A, Mazza A, Gey van Pittius NC, Ho JL, Brousseau R, Mardassi H (2008) Frequent homologous recombination events in Mycobacterium tuberculosis PE/PPE multigene families: potential role in antigenic variability. J Bacteriol 190:7838-7846.

Lewis JC, Aldergon G, Carson JF, Reynolds DM, Macalay WD (1949) Lupulon and humulon, antibiotic constituents of hops. J Clin Invest 28:916-919.

Liang J, Zeng F, Guo A, Liu L, Guo N, Li L, Jin J, Wu X, Liu M, Zhao D, Li Y, Jin Q, Yu L (2011) Microarray analysis of the chelerythrine-induced transcriptome of Mycobacterium tuberculosis. Curr Microbiol. 62:1200-1208.

Liu TT, Lee RE, Barker KS, Lee RE, Wei L, Homayouni R, Rogers PD (2005) Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in Candida albicans. Antimicrob Agents Chemother 49:2226-2236.

McLean KJ, Belcher J, Driscoll MD, Fernandez CC, Le Van D, Bui S, Golovanova M, Munro AW (2010) The Mycobacterium tuberculosis cytochromes P450: physiology, biochemistry & molecular intervention. Future Med Chem. 2:1339-1353.

McLean KJ, Carroll P, Lewis DG, Dunford AJ, Seward HE, Neeli R, Chessman MR, Marsollier L, Douglas P, Smith WE, Rosenkranz I, Cole ST, Leys D, Parish T, Munro AW (2010) The Mycobacterium tuberculosis cytochrome P450: physiology, biochemistry & molecular intervention. Future Med Chem. 2:1339-1353.

McLean KJ, Dunford AJ, Neeli R, Driscoll MD, Munro AW (2007) Structure, function and drug targeting in Mycobacterium tuberculosis cytochrome P450 systems. Arch Biochem Biophys 464:228-240.

Mehra S, Kaualhal D (2009) Functional Genomics Reveals Extended Roles of the Mycobacterium tuberculosis Stress Response Factor. J Bacteriol 191:3965-3980.

Mougdou JD, Senaratne RH, Petzold CJ, Jain M, Lee DH, Schelle MW, Leavell MD, Cox JS, Leary JA, Riley LW, Bertozzi CR (2006) A sulfated metabolite produced by stf3 negatively regulates the virulence of Mycobacterium tuberculosis. Proc Natl Acad Sci USA 103:4258-4263.

Nikaido H, Vaara M (1985) Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49:1-32.

Onwueme KC, Ferreras JA, Buglino J, Lima CD, Quadri LE (2004) Mycobacterial polyketide-associated proteins are acyltransferases: proof of principle with Mycobacterium tuberculosis PpaA5. Proc Natl Acad Sci USA 101:4608-4613.

Ouellet H, Johnston JB, Ortiz de Montellano PR (2010) The Mycobacterium tuberculosis cytochrome P450 system. Arch Biochem Biophys 493:82-95.

Proveddi R, Boldrin F, Falciani F, Palù G, Manganelli R (2009) Global transcriptional response to vancomycin in Mycobacterium tuberculosis. Microbiology 155:1093-1102.

Ramakrishnan L, Federspiel NA, Falkow S (2000) Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science. 288:1436-1439.

Rodrigue GM, Smith I (2006) Identification of an ABC transporter required for iron acquisition and virulence in Mycobacterium tuberculosis. J Bacteriol 188:424-430.

Rousseau C, Winter N, Pivert E, Bordat Y, Neyrolles O, Avé P, Huere M, Gicquel B, Jackson M (2004) Production of phthiocerol dimycocerosates protects Mycobacterium tuberculosis from the cidal activity of reactive nitrogen intermediates produced by macrophages and modulates the early immune response to infection. Cell Microbiol 6:277-287.

Ryndak MB, Wang S, Smith I, Rodriguez GM (2010) The Mycobacterium tuberculosis High-Affinity Iron Importer, IrtA, Contains an FAD-Binding Domain. J Bacteriol 192:861-869.

Sacks LE, Humphreys EM (1951) Antagonistic effect of serum on bacteriostatic action of lupulone. Proc Soc Exp Biol Med 76:234-238.

Shasmal M, Sengupta J (2012) Structural diversity in bacterial ribosomes: mycobacterial 70S ribosome structure reveals novel features. PLoS One. 7:31742.

Sairusa GR, Haas GJ, Matthews PD, Smith RJ, Buhr RJ, Dale NM, Wise MG (2008) Antimicrobial activity of lupulone against Clostridium perfringens in the chicken intestinal tract jejunum and caecum. J Antimicrob Chemother 61:853-858.

Slayden RA, Knudson DL, Belisle JT (2006) Identification of cell cycle regulators in Mycobacterium tuberculosis by inhibition of septum formation and global transcriptional analysis. Microbiology 152:1789-1797.

Stewart GR, Wernisch L, Stabler R, Mangan JA, Hinds J, Laing KG, Young DB, Butcher PD (2002) Dissection of the heat-shock response in Mycobacterium tuberculosis using mutants and microarrays. Microbiology 148:3129-3138.

Strong M, Sawaya MR, Wang S, Phillips M, Cascio D, Eisenberg D (2006) Toward the structural genomics of complexes: Crystal structure of a PE/PPE protein complex from Myco-
bacterium tuberculosis. Proc Natl Acad Sci USA 103:8060-8065.

Tagashira M, Watanabe M, Uemitsu N (1995) Antioxidative activity of hop bitter acids and their analogues. Biosci Biotechnol Biochem 59:740-742.

Teuber M, Schmalreck AF (1973) Membrane leakage in Bacillus subtilis 168 induced by the hop constituents lupulone, humulone, isohumulone and humulinic acid. Arch Microbiol 94:159-171.

Trivedi OA, Arora P, Sridharan V, Tickoo R, Mohanty D, Gokhale RS (2004) Enzymic activation and transfer of fatty acids as acyl-adenylates in mycobacteria. Nature 428:441-445.

Tyagi JS, Saini DK (2004) Did the loss of two-component systems initiate pseudogene accumulation in Mycobacterium leprae? Microbiology 150:4-7.

Vordermeier HM, Hewinson RG, Wilkinson RJ, Wilkinson KA, Gideon HP, Young DB, Sampson SL (2012) Conserved immune recognition hierarchy of mycobacterial PE/PPE proteins during infection in natural hosts. PLoS One. 7, e40890. doi: 10.1371/journal.pone.0040890.

Youm J, Saier MH Jr (2012) Comparative analyses of transport proteins encoded within the genomes of Mycobacterium tuberculosis and Mycobacterium leprae. Biochim Biophys Acta. 1818:776-97.

Yu L, Guo N, Liu B, Tang X, Jin J, Cui Y, Deng X (2010) Allicin-induced global gene expression profile of Saccharomyces cerevisiae. Appl Microbiol Biotechnol 88:219-229.

Yu L, Xiang H, Fan J, Wang D, Yang F, Guo N, Jin Q, Deng X (2008) Global transcriptional response of Staphylococcus aureus to rhein, a natural plant product. J Biotechnol 135:304-308.

Yu L, Zhang W, Wang L, Yang J, Liu T, Peng J, Leng W, Chen L, Li R, Jin Q (2007) Transcriptional profiles of the response to ketoconazole and amphotericin B in Trichophyton rubrum. Antimicrob Agents Chemother 51:144-153.

Zhao F, Nozawa H, Daikonnya A, Kondo K, Kitanaka S (2003) Inhibitors of nitric oxide production from hops (Humulus lupulus L.). Biol Pharm Bull 26:61-65.

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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