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DNA Microarray Gene Expression Profile of *Mycobacterium tuberculosis* when Exposed to Osthole

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Abstract

Tuberculosis (TB), affecting one-third of the global population, kills an estimated two to three million people every year. The development of drug resistance is becoming a serious threat to any attempt to control this disease, which underscores the need for new agents targeting *Mycobacterium tuberculosis* (*M. tuberculosis*). Osthole (7-methoxy-8-isopentenoxycoumarin) is a coumarin derivative present in many medicinal plants. Previous studies have shown that osthole possesses antimycobacterial effects, however, the action mechanism of osthole is unclear. In the study, we used a commercial oligonucleotide microarray to determine the overall transcriptional response of *M. tuberculosis* H37Rv triggered by exposure to osthole. Analysis of the microarray data revealed that a total of 478 genes were differentially regulated by osthole. Of these, 241 genes were upregulated, and 237 genes were downregulated. Some of the important genes that were significantly regulated are related to different pathways such as fumarate reductase, class I peroxidase, cell wall, nitrate respiration, and protein synthesis. Real-time quantitative RT-PCR was performed for chosen genes to validate the microarray results. To our knowledge, this genome-wide transcriptomics approach has produced the first insights into the response of *M. tuberculosis* when exposed to osthole.

Key words: *Mycobacterium tuberculosis*, antimycobacterial activity, microarray, osthole, transcriptome

Introduction

Mycobacterial diseases are still among the world's leading infection problems. The World Health Organization (WHO) estimates that nearly one third of the global population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) (Raviglione, 2003). Pathogenic mycobacteria initiate long-term infection in the lungs by entering host macrophages and spreading rapidly. Whilst the currently available antimicrobials are often effective at eradicating the infection, there are issues with patient drug compliance and the emergence of drug-resistant *M. tuberculosis* (Ormerod 2005). In addition, the rising incidence of HIV infection, especially in parts of Africa, has further promoted mortality related to tuberculosis (Nunn *et al.*, 2005). Although effective therapeutic regimens exist, the prevalence of *M. tuberculosis* strains resistant to antimicrobial agents for treatment of tuberculosis (TB) and the emergence of multidrug resistance urgently requires additional compounds targeted at new pathways.

Plants and other natural materials may prove to be valuable sources of useful new antimycobacterial drugs. Osthole (7-methoxy-8-isopentenoxycoumarin, chemical formula shown in Fig. 1), a coumarin derivative, has been extracted from many medicinal plants, such as *Cnidium monnieri* and *Angelica pubescens*. It is used in traditional Chinese medicine as tonics and aphrodisiacs. Previous studies have shown that osthole possesses antimycobacterial, antiproliferation, vasorelaxation,

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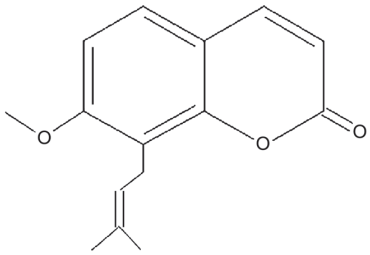

Fig. 1. Chemical formula of 7-methoxy-8-isopentenoxycoumarin.

antihepatitis, anti-inflammatory, antiaggregatory, and antiallergic effects (Figueroa *et al*., 2007; Huang *et al*., 1996; Liu *et al*., 1998; Matsuda *et al*., 2002; Yang *et al*., 2003). In our preliminary experiments, we found that osthole showed good antimycobacterial activity. However, few studies have reported on the mechanisms underlying the effects of antimycobacterial compounds (Betts *et al*., 2003).

DNA microarray technology has been used to understand biochemical pathways, to discover gene functions and to discover drug targets (Yu *et al*., 2007; Yu *et al*., 2008; Yu *et al*., 2010; Liang *et al*., 2011). Here, we describe the use of a microarray to generate the transcriptional response profile of *M. tuberculosis* following exposure to osthole to indicate the pathways induced by drug treatment, and a subset of the microarray results was verified by real-time RT-PCR.

Experimental

Materials and Methods

Bacterial strain and reagents. 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) were 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. Osthole was obtained from National Institute for the control of Pharmaceutical and biological products (Beijing, China). Stock solutions of osthole were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Antimycobacterial susceptibility test. The activity of osthole against the aforementioned *M. tuberculosis* strain was tested using a microplate Alamar Blue assay (MABA) according to the method of Franzblau *et al.* (Franzblau *et al*., 1998), as modified by Jiménez-Arellanes *et al* (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 osthole tested ranged from 1 to 512 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 minimal inhibitory concentration (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 treatment with for microarray experiments. A frozen stock of *M. tuberculosis* strain H37Rv was inoculated into Middlebrook 7H9 broth containing 0.05% Tween 80, 0.2% glycerol and 10% oleic acid, albumin, dextrose and catalase (OADC) and incubated at 37°C for five days. Then, the culture was transferred into 200 ml of 7H9 media and incubated at 37°C with shaking 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 osthole 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 16 µg/ml (1/2 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 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 osthole 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 twofold difference in the ratio (Frota *et al.*, 2004).

Quantitative real-time reverse transcription RT-PCR. Aliquots of the RNA preparations from the osthole-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 I.

Results

Gene transcription responses to osthole exposure. In this experiment, the MIC value of osthole against *M. tuberculosis* H37Rv (ATCC 27294) was 32 µg/ml. Whole-genome microarrays were employed to analyze gene expression in *M. tuberculosis* H37Rv when exposed to a subinhibitory (1/2 MIC) concentration of osthole for 4 h. Overall, there were 478 genes differentially regulated by osthole. Among these, 241 exhibited a significant increase in traIscription and 237 exhibited a significant decrease in transcription. The microarray-related data were submitted to Gene Expression Omnibus (GEO) under accession number GSE32076. The genes which were differentially expressed in response to osthole and their biological roles according to their functional class are shown in the supplementary material attached to PJM Vol. 62, No 1 (Table SI on line at http://www.pjm.microbiology.pl), and some genes which are high differently expressed in response to osthole were listed in Table II.

Of the genes that showed a significant response to osthole, most were classified as having an unclassified role category not yet assigned (24.7%) and the others were classified as involved in biosynthesis of cofactors, prosthetic groups, and carriers (2.1%), cell envelope (5.6%), cellular processes (2.1%), central intermediary metabolism (4.4%), conserved hypothetical proteins (14.2%), DNA metabolism (2.3%), energy metabolism (6.3%), hypothetical proteins (13.0%), protein synthesis (2.7%), regulatory functions (5.0%), transport and binding proteins (4.2%), unknown function (7.1%)

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

Primer	Systematic name	Sequence (5'-3')	Reference
16S rRNAfor		GCACCGGCCAACTACGTG	Grassi et al.
16S rRNArev		GAACAACGCGACAAACCACC	Grassi et al.
mmpS5 for	Rv0677c	CAAGGTGGTGGAGTACGAAGTTT	This study
mmpS5 rev	Rv0677c	TCGAGGTCCAGGTAGTTGATGTTG	This study
infC for	Rv1641	AGACCGTCGTCAAAGAACAAAAG	This study
infC rev	Rv1641	TTGGTCTCGTAATCGTGATCGT	This study
nirA for	Rv2391	CTGGACCGGTGACGACAAC	Liang J et al.
nirA rev	Rv2391	CAACGCACCCGCATCA	Liang J et al.
cysH for	Rv2392	TGCGATCGAGTCCGTCTATG	Liang J et al.
cysH rev	Rv2392	TGTGTGCTCCGGAGTGACAT	Liang J et al.
tgs1 for	Rv3130c	GATTCGCTGCGTACGCTAGTG	This study
tgs1 rev	Rv3130c	ACACGGTTATCGGTCTTGCTC	This study
sigE for	Rv1221	TCCGGTCGGTCCAGAATTAC	This study
sigE rev	Rv1221	TTGGTGGTGATGCGGTGTAG	This study
groEL2 for	Rv0440	TCGAGGAGTCCAACACCTTTG	This study
groEL2 rev	Rv0440	TCACGAAGTACCCCGAGATGTA	This study
mpt83 for	Rv2873	TTGGTATGTGGCGGAGTTCA	This study
mpt83 rev	Rv2873	AGCACCGTATCGATCATGTA	This study
frdD for	Rv1555	GCAACCCGATCACCAAGCTTGTT	This study
frdD rev	Rv1555	CATGGTCGAGCACGAACCGGAAC	This study
frdA for	Rv1552	ACGAGCACAACAAAGGAAGGA	This study
frdA rev	Rv1552	AGGTGCCGCAGGTCTAGATAGA	This study
cadI for	Rv2641	GAGGCCGCAATCACGTTCTA	This study
cadI rev	Rv2641	TATCCGGGCTTGCGTTTG	This study

Table II
Selected genes which are high differently expressed in response to osthole.

Functional category	Systematic name	Gene	Product or putative function	Fold change
Biosynthesis of cofactors, prosthetic groups, and carriers	Rv3398c	*idsA1*	Multifunctional dimethylallyltransferase % 2Ffarnesyl diphosphate synthetase % 2F farnesyltranstransferase	3.96
Cell envelope	Rv0676c	*mmpL5*	Transmembrane transport protein mmpl5	9.98
	Rv0450c	*mmpL4*	Transmembrane transport protein mmpl4	3.06
Central intermediary metabolism	Rv3397c	*phyA*	Phytoene synthase	4.16
Energy metabolism	Rv0136	*cyp138*	Cytochrome P450 138	6.38
	Rv2391	*nirA*	Ferredoxin-dependent nitrite reductase NIRA	3.03
Protein fate	Rv3418c	*groES*	Co-chaperonin groes	–3.06
	Rv3417c	*groEL*	Chaperonin groel	–3.06
Regulatory functions	Rv0135c	–	Transcriptional regulatory protein	4.22
	Rv0452	–	Transcriptional regulatory protein	3.97
	Rv1685c	–	Hypothetical protein	10.28
Transport and binding proteins	Rv1686c	–	Integral membrane protein ABC transporter	13.20

"+" increase and "–" reduction

and other categories (6.3%) as shown in Fig. 2. In this study, our interest was focused mainly on specific genes that could affect the organism's survival when exposed to osthole, and these were mainly involved in different pathways, such as fumarate reductase, class I peroxidase, nitrate respiration, and genes involved in the cell wall and protein synthesis. These genes will be discussed in the "Discussion" section.

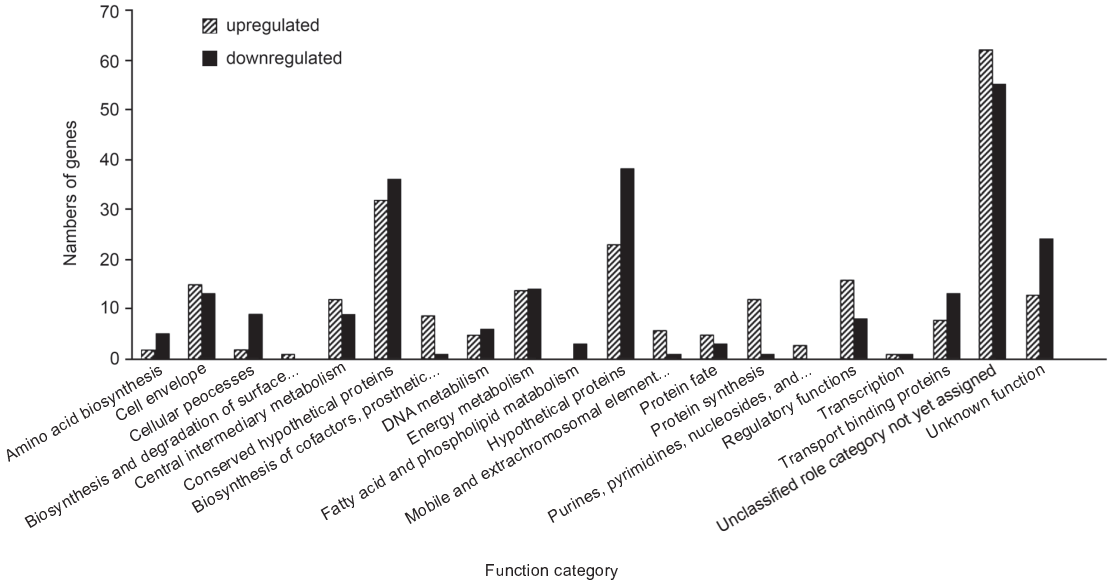

Fig. 2. The genes that showed a significant response to osthole.

Validating microarray data by real-time RT-PCR. Eleven genes were selected (*i.e. mmpS5*, *infC*, *nirA*, *cysH*, *tgs1*, *sigE*, *groEL2*, *mpt83*, *frdD*, *frdA* and *cadI*) to verify the microarray data by the technique of real-time quantitative RT-PCR. In summary, there was a good correlation between the microarray data and the real-time RT-PCR data (Table III). Among the eleven genes, six genes exhibited upregulation and five genes exhibited downregulation upon exposure to osthole. It is notable that for two genes, *mmpS5* and *groEL2* the real-time RT-PCR results showed greater n-fold changes than the microarray results, which may be reflective of the greater dynamic range of real-time RT-PCR analysis. For nine genes (*infC*, *nirA*, *cysH*, *tgs1*, *sigE*, *mpt83*, *frdD*, *frdA* and *cadI*), the expression levels did not differ markedly between the real-time RT-PCR data and the microarray data.

Discussion

Inhibition of the fumarate reductase genes in the presence of osthole. In our study, the genes *frdA*, *frdB*, *frdC* and *frdD* encoding fumarate reductase were

Table III
Real-time RT-PCR analysis of gene expression.

Systematic name	Gene	Description	Fold change	
			RT-PCR	Microarray
Rv0677c	*mmpS5*	hypothetical protein	+78.03	+16.90
Rv1641	*infC*	translation initiation factor IF-3	+5.83	+2.55
Rv2391	*nirA*	ferredoxin-dependent nitrite reductase NIRA	+2.31	+3.03
Rv2392	*cysH*	phosphoadenosine phosphosulfate reductase	+3.08	+2.72
Rv3130c	*tgs1*	triacylglycerol synthase	+3.64	+2.53
Rv1221	*sigE*	RNA polymerase sigma factor	+2.64	+2.31
Rv0440	*groEL2*	chaperonin GroEL	−19.35	−4.29
Rv2873	*mpt83*	cell surface lipoprotein mpt83 %28lipoprotein P23%29	−2.12	−2.45
Rv1555	*frdD*	fumarate reductase subunit D	−3.01	−2.23
Rv1552	*frdA*	fumarate reductase subunit A	−4.11	−2.16
Rv2641	*cadI*	cadmium inducible protein	−2.38	−2.03

"+" increase and "−" reduction.

downregulated by 2.16-fold, 2.06-fold, 2.11-fold and 2.23-fold respetively when exposed to osthole. Fumarate reductase (FRD) is an important enzyme in fumarate respiration, which is an alternative means of acquiring energy by utilizing fumarate as the terminal electron acceptor when oxygen or nitrate is absent. As previously reported, *frdA* has been found to be upregulated in *M. tuberculosis* interacting with the macrophage versus *M. tuberculosis* grown in broth cultures (Schnappinger *et al.*, 2003) as well as in studies that examined the behaviour of *M. tuberculosis* grown under carbon starvation (Betts *et al.*, 2002). The transcriptome in stationary-phase *M. tuberculosis* H37Rv cultures was found to increase *frdB* and *frdC* transcripts versus bacteria in exponential growth (Raman *et al.*, 2001). Additionally, investigations into the respiratory behaviour of *Mycobacterium phlei* found that FRD activity increased fourfold when bacteria were grown under low-oxygen conditions (Gillespie *et al.*, 1988). With regard to other pathogenic organisms, FRD has been considered a target in the treatment of *Helicobacter pylori*, as it was found to be an essential gene in the establishment of *H. pylori* colonization of the mouse stomach (Wang *et al.*, 2000). Furthermore, FRD has been reported to be a successful target in the treatment of protozoan and helminth infections using a variety of compounds (Chen *et al.*, 2001; Omura *et al.*, 2001). Our results suggest the FRD may be a novel target in *M. tuberculosis* when exposed to osthole and the mechanism involved needs further study.

Genes involved in nitrate respiration were inhibited when exposed to osthole. Nitrate respiration is an important physiological process that allows bacteria to generate sufficient energy to permit anaerobic growth. Lastly, nitrate respiration was found equally important in protecting *M. tuberculosis* against reactive nitrogen species which are likely to be encountered by the pathogen in the infected host (Tan *et al.*, 2010). During nitrate respiration, nitrate is reduced to nitrite in the cytoplasm by a membrane-bound nitrate reductase (Nishimura *et al.*, 2008). Nitrate reductase, encoded by *narGHJI*, is a membrane-bound molybdenum-containing complex and is typical for a respiratory enzyme (Malm *et al.*, 2009). The membrane-bound nitrate reductase is a three-subunit protein, with the transmembrane γ subunit (NarI) anchoring the cytoplasmically exposed α subunit (NarG) and β subunit (NarH) to the membrane. In our study, on exposure to osthole, the genes *narJ*, *narI*, *narH* and *narG* were all downregulated by 2.08-fold, 2.28-fold, 2.20-fold, and 2.15-fold, respectively. The expression of *narKGHJI* operon is regulated by anaerobiosis and nitrate (Nishimura *et al.*, 2007).

Assimilation of nitrogen into mycobacterial metabolism is essential for the survival of *M. tuberculosis* in vitro and in vivo (Malm *et al.*, 2009). It is suggested that *narGHJI* mediates nitrate assimilation in *M. tuberculosis* (Malm *et al.*, 2009). Though nitrate can not be utilized as a nitrogen source, *M. tuberculosis* can utilize nitrite as a source of nitrogen for growth. The *narG* mutant showed no nitrate reductase activity in whole culture or in cell-free assays (Sohaskey and Wayne, 2003). In the absence of the NarJ protein, the balanced overproduction of subunits α, β and γ from a multicopy plasmid does not result in the formation of either fully active α-β or α-β-γ complex (Dubourdieu and De Moss, 1992). The downregulation of the genes may inhibit the activity and biosynthesis of nitrate reductase, which affect the nitrate respiration and attenuate the ability of assimilation of nitrogen.

Increased transcription of the gene cluster related to the biosythesis of polyacyltrehalose. In the study, the genes *pks3*, *papA3* and *mmpL10* were upregulated by 2.34-fold, 2.06-fold and 2.27-fold, respectively, when exposed to osthole. These genes reside in the gene cluster *pks3/4-papA3-mmpL10*, which is involved in the biosynthesis of polyacyltrehalose (PAT). PAT, a pentaacylated, trehalose-based glycolipid, is a cell wall molecule unique to pathogenic *M. tuberculosis*. In *M. tuberculosis*, PapA3 encoded by *papA3* is an acyltransferase essential for the biosynthesis of PAT and was selective for trehalose; no activity was observed with other structurally related disaccharides. Disruption of the *papA3* gene from *M. tuberculosis* resulted in the loss of PAT from bacterial lipid extracts (Hatzios *et al.*, 2009). Pks3 is a polyketide synthase, which is involved in the synthesis of mycolipanolic and mycolipenic acids, the trimethyl branched fatty acids present in the PAT of *M. tuberculosis* (Matsuda *et al.*, 2002). In some strains, including the sequenced H37Rv strain, there is an intervening stop codon in *pks3/4* that results in two separate open reading frames (termed *pks3* and *pks4*) (Dubey *et al.*, 2002). Strains containing this mutation do not synthesize PAT (Domenech *et al.*, 2005), indicating that an intact *pks3/4* gene is essential for the biosynthesis of this glycolipid. Within the same gene cluster resides *mmpL10* encoding a putative lipid transporter, which transports PAT or its precursor to the cell surface. Previous studies reported that the genes from the PAT biosynthetic gene cluster were upregulated under various conditions of environmental stress, including phagosomal acidification and nutrient starvation (Rohde *et al.*, 2007). This region was also upregulated in *M. bovis* after acid shock, reaching maximum expression after 24 h (Golby *et al.* 2007). Hence, the upregulation of the genes may be an adaptive response to environmental changes for survival of the organism. The trigger for the induced transcription of the gene cluster in *M. tuberculosis* following osthole challenge requires further study.

Upregulation of genes involved in the ribosome proteins in osthole. Following exposure to osthole, the genes *rpsH* and *rpsS* encoding 30S ribosome proteins

were upregulated by 2.03-fold and 2.28-fold; the genes encoding 50S ribosomal proteins were also upregulated from 2.03-fold to 2.21-fold, including *rplW*, *rplT*, *rplO*, *rplD* and *rplB*. The initiation factors *infC*, which is essential for the initiation of translation, was upregulated by 2.55-fold and the levels of expression of the gene *rho*, which encodes transcription termination factor, was also increased by 2.06-fold. A recent study showed that exposure of *Clostridium difficile* to three subinhibitory concentrations of antibiotics (amoxicillin, clindamycin, and metronidazole) resulted in a general upregulation of the transcription and translation machinery, including significant upregulation of 25 ribosomal protein-encoding genes (in addition to four located elsewhere) (Emerson *et al*., 2008). Moreover, Dennis demonstrated that when experiencing low levels of translation inhibition, cells can compensate by upregulation of the synthesis of ribosomal proteins, but as inhibition increases, this compensation fails to keep pace (Dennis, 1976). In the study, these genes encode essential components of the protein synthesis pathway. Their upregulation may be a response to the translation inhibition caused by osthole targeting the ribosome or its associated translation factors, which aims at surviving antimicrobial challenge.

The downregulation of the genes related to arginine biosynthesis. In many microorganisms, L-arginine is used as a source of both carbon and nitrogen, and the transport and metabolism of L-arginine has been shown to be essential for *Mycobacterium tuberculosis* (Gordhan *et al*., 2002). In the study, the genes *argR*, *argJ*, *argG*, *argF* and *argD*, residing in the arginine biosynthetic gene cluster (*argCJBDFRGH*), were inhibited by 2.37-fold, 2.37-fold, 2.47-fold, 2.36-fold and 2.38-fold respectively. As a previous study reported, deletion of gene *argR* in *Legionella pneumophila* does affect the transcript level of genes predicted to encode the terminal steps of L-arginine biosynthesis from L-ornithine (Hovel-Miner *et al*., 2010). Interestingly, the mutant of *Streptomyces clavuligerus* with an *argJ* disruption is auxotrophic, which was suggested to be a polar effect on the transcription of arginine biosynthesis genes located downstream of *argJ* (de la Fuente *et al*., 2004). In *M. tuberculosis*, the *argF* mutant strains required exogenous L-arginine for growth *in vitro* and were defective in *argG* function. The mutant strains exerted polar effects on *argR* and *argG,* and also exhibited a very strong attenuation in virulence, which was reflected in a reduction in c.f.u. values and survival in immunocompetent mice (Gordhan *et al*., 2002). In *Listeria monocytogenes*, the insertional mutation of *argD* led to a reduced replication rate in Caco-2 cells (Joseph *et al*., 2006). Moreover, in *Corynebacterium glutamicum*, genes encoding proteins of the L-arginine biosynthesis pathway (*argB*, *argC*, *argD*, *argF*, *argG*, *argH*, *argJ*, *argR* and *argS*) showed a decreased expression in ammonium-limited chemostat cultures (Silberbach *et al*., 2005).

Hence, the downregulation of the genes may affect the biosynthesis of the L-arginine, which destroyed the survival ability of *M. tuberculosis* in the presence of osthole.

These results show that osthole has potential antimycobacterial activity. Our DNA microarray analysis demonstrated that osthole affected a number of important genes involved in different metabolic pathways in *M. tuberculosis*. These findings will pave the way for exploring the responsive mechanisms of *M. tuberculosis* to osthole treatment.

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