Jundishapur J Microbiol. 2018 February; 11(2):e58591. doi: 10.5812/jjm.58591.

Published online 2018 February 7. **Research Article**

The Effect of Ubiquitin Like Protein-Proteasome System on the Drug Resistance of Isoniazid Mono-Resistant *Mycobacterium tuberculosis*

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Received 2017 March 10; **Revised** 2017 July 27; **Accepted** 2017 August 20.

Abstract

Background: Tuberculosis (TB) is one of the most widespread and lethal infectious diseases worldwide. The emergence of drug-resistant TB has hampered effective TB treatment and control. Prokaryotic ubiquitin-like Protein-Proteasome System (PPS) contributes to the survival of *Mycobacterium tuberculosis* in the host. However, whether PPS effects drug resistance of isoniazid mono-resistant *Mycobacterium tuberculosis* (INH-MTB) is still unknown.
Objectives: This study aimed at exploring the effect of PPS on drug resistance of INH-MTB strain.
Methods: In this study, over-expression of strains and deletion of mutant strains were constructed using electroporation. The researchers identified these constructed strains by Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) or PCR. The Minimum Inhibitory Concentration (MIC) of isoniazid in INH-MTB strain and its derivative PPS mutant strains were determined using the Resazurin micro-titre assay.
Results: The MIC of isoniazid was 8 μg/mL higher in INH-MTB with *Pup* over-expression strain than that in INH-MTB. The MIC of isoniazid was 4.82 μg/mL, 4.98 μg/mL, 4.99 μg/mL, and 4.9 μg/mL lower in INH-MTB with deletion of *Pup*, *Dop*, *PafA* or *Mpa* strains than that in INH-MTB, respectively. The differences had statistical significance ($P < 0.05$). The MIC of isoniazid was 1.03 μg/mL higher in INH-MTB with *PafA* over-expression strain than that in INH-MTB. The MIC of isoniazid was 1.03 μg/mL and 0.68 μg/mL lower in INH-MTB with *Dop*, *Mpa* over-expression strains than that in INH-MTB, respectively. The differences had no statistical significance ($P > 0.05$).
Conclusions: These results show that PPS effects the drug resistance of the INH-MTB strain.

Keywords: Isoniazid, Drug Resistance, *Mycobacterium Tuberculosis*, *Pup*

1. Background

Tuberculosis (TB) is a top infectious killer worldwide, and is responsible for more deaths than human immunodeficiency virus (HIV) and malaria, resulting in 400,000 deaths, annually. In 2016, the world health organization (WHO) estimated that 580 000 people worldwide develop Multidrug-Resistant (MDR) TB. India, China, and the Russian Federation accounted for 45% of the 580 000 cases. Although isoniazid (INH) is an essential element of all first-line treatment regimens for TB (1), INH resistance is an obstacle to the treatment of TB (2, 3). In order to achieve the ambitious target set in the End TB Strategy, these highest TB burden countries need to depend on advances in TB prevention and care.

According to broad analyses, mutations in the *katG* gene and *inhA* gene are the cause of the majority of INH resistance in clinical isolates (4), yet other mutations carry the explanation fora subset of INH resistance cases that lack the 2 most common canonical mutations (5, 6). These results contribute to making a broader search in the mechanisms of INH resistance.

In 2008, the first bacterial protein modifier, prokaryotic ubiquitin-like protein (*Pup*) was identified in *Mycobacterium tuberculosis* (MTB) (7). Functionally analogous to ubiquitin, conjugation with *Pup* serves as a signal for degradation by the *mycobacterial* proteasome (8). Protein-proteasome system (PPS) is a posttranslational regulatory mechanism (9). In the system, *Pup* could tag a variety of functional proteins and mediate these proteins degraded by proteasome, with the help of *Dop* (deamidase of *Pup*), *PafA* (*Pup* ligase), and *Mpa*. These proteins involve inter-

mediary metabolism, lipid metabolism, and detoxification/virulence (10). However, whether PPS influences drug resistance of isoniazid mono-resistant *M. tuberculosis* (INH-MTB) is still unknown.

2. Objectives

The purpose of this study was to clarify whether PPS effects drug resistance of INH-MTB strain.

3. Methods

3.1. Ethics Statement

The protocol of this study was reviewed and approved by the ethical and research committee of Shihezi University of Medical Sciences with the following approval number, 1380/5/18/3/4/5735. Written informed consent was provided by all participants.

3.2. Strains and Growth Conditions

Ten INH-MTB strains were attained from sputum of 10 separate patients with TB infection at the first Affiliated Hospital of Shihezi University, following the guidelines of the centers for disease control and prevention (11). The Key Laboratory of Xinjiang Endemic and Ethnic Diseases provided these strains of *M. tuberculosis* for the experiments. The INH-MTB strains were grown at 37°C in minimal medium containing 40 mM K_2HPO_4, 22 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 0.4% glycerol, and 0.05% Tween-80. All chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The derivative PPS mutants were grown at 37°C with kanamycin (10μg/mL) (Invitrogen, USA) in that minimal medium.

3.3. The Construction of Over-expression Strains and Deletion Mutant Strains

The recombinant shuttle plasmids and deletion mutation vectors used in the current study (carrying a resistance gene of kanamycin, respectively) had been constructed and identified by previous experiments. According to the manufacturer's instructions, the INH-MTB strains were disposed through a series of processes to damage its cell walls to integrate with recombinant shuttle plasmid or mutation vector. The 20-μL INH-MTB strain was added to electrode cups, which contained a 2-μL plasmid or vector. Then the plasmid or vector was transformed to INH-MTB strain by using the optimal electro-porational parameters (2000 - 2500kv/cm) to form different kinds of over-expression or deletion mutation strains. These strains were then incubated at 37°C in a minimal medium containing kanamycin (10 μg/mL). Samples of each strain were taken for RNA extraction and RT-qPCR or PCR analysis.

3.4. RNA Extraction

The over-expression or deletion mutant strains were grown at 37°C until they reached an exponential phase. Total RNA was isolated from each strain using a Fast RNA Pro Blue kit (Qiagen, Germany), according to the manufacturer's recommendations. Quantity and quality of the purified RNA was measured using a Nano Drop 2000 spectrophotometer (Thermo Scientific, USA).

3.5. Quantitative Real-time Polymerase Chain Reaction

Relative gene expression levels of *Pup*, *Dop*, *PafA* or *Mpa* were analysized by RT-qPCR in these over-expressed strains, respectively. The employed primers (Shanghai Bioengineering, China) are described in The The housekeeping sigma factor gene SigA was used as an internal control for the normalization of mRNA levels (12). The RT-qPCR procedure was performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Australia). Each sample was provided with 3 repeated parallel holes. The relative expression of each gene was determined by comparison of the relative quantity of the respective mRNA in INH-MTB strain to its derivative PPS mutant strains using the comparative quantification $2^{-\Delta\Delta Ct}$ method (13).

Table 1. Primer Sequences of *Pup* Gene, *Dop* Gene, *PafA* Gene, *Mpa* Gene and Reference Gene *SigA*

Primer	Sequence (5' - 3')	Amplification Product, bp
Pup (F)	AAAGGCCATGAGGAAGCAG	160
Pup (R)	GTCGATTTCGTCGAGCAG	
Dop (F)	ACCAGCCCACGCAATAGAT	164
Dop (R)	TAGCCACTCGGTCCAGGTA	
PafA (F)	CTACCTGTTTCGCCGTGTG	173
PafA (R)	GGTCTTCCAGCACCCATTC	
Mpa (F)	CAACGGCGACAAGGAAGT	106
Mpa (R)	CTCCAGCACCGATTTGATG	
SigA (F)	TCGAGGTGATCAACAAGCTG	254
SigA (R)	CTGCAGCAAAGTGAAGGACA	

3.6. Polymerase Chain Reaction

The PCR were carried out with 2 × Taq PCR MasterMix (TianGen, China). Primers (Shanghai Bioengineering, China) are shown in Table 2. The INH-MTB strain was considered as a control group and Δ*Pup* strain as an experimental group, for which primers *Pup-F* and *Pup-R* were used to amplify a 213-bp specific fragment of the *Pup* gene. In the experimental group, primers *Pup-N-F* and *Km-R* were used to amplify a 1601-bp specific fragment of *Pup-N-Km*;

and primers *Km-F* and *Pup-C-R* were used to amplify a 1591-bp specific fragment of *Km-Pup-C*. The INH-MTB strain was considered as a control group and Δ*Dop* strain as an experimental group, for which primers *Dop-F* and *Dop-R* were used to amplify a 1683-bp specific fragment of the *Dop* gene. In the experimental group, primers *Dop-N-F* and Km-R were used to amplify a 1515-bp specific fragment of *Dop-N-Km*; and primers Km-F and *Dop-C-R* were used to amplify a 1509-bp specific fragment of *Km-Dop-C*.

Table 2. Primers Used in Our Study for the Identification of Deletion Mutant Strains

Primer	Sequence (5'→3')
Pup-N-F	GAACACCCGCTGTAGACCTATC
Pup-C-R	CACGCCCGCTGTCTTTCT
Mpa-N-F	CCTGGCTGACGGTGTATG
Mpa-C-R	AGGGTTACACGGATGTTTCGG
Dop-N-F	GAACACCCGCTGTAGACCTATC
Dop-C-R	GGCTGGTTGGTGTCGCAGATA
PafA-N-F	GAGGGCTATCGCATCAACCG
PafA-C-R	CATGAACACCACCTCGCCCG
Km-F	GCCACCTGGGATGAATGTC
Km-R	CGGTCATTTCGAACCCCAA
Pup-F	ATGGCGCAAGAGCAGACCAAGCGCGTC
Pup-R	GTCACTGTCCGCCCTTTTGGACGT
Mpa-F	ATGGGTGAGTCAGAGCGTTCTCAGGTCTT
Mpa-R	CTACAGGTACTGGCCGAGGTTGGAC
Dop-F	ATGTTCTGGGTCGGCGGGCCTTCGCTGGG
Dop-R	TTAGCGAGGCTCAGCGGTCAGT
PafA-F	GTGCAGCGTCGAATCATGGGCATCGTCGT
PafA-R	CTACATGCTCGCGATCAGCCGCTT

Isoniazid mono-resistant *M. tuberculosis* strain was considered as a control group and Δ*PafA* strain as an experimental group, for which primers *PafA-F* and *PafA-R* were used to amplify a 1377-bp specific fragment of the *PafA* gene. In the experimental group, primers pafA-N-F and *Km-R* were used to amplify a 1584-bp specific fragment of *PafA-N-Km*; and primers *Km-F* and *PafA-C-R* were used to amplify a 1584-bp specific fragment of *Km-PafA-C*. Isoniazid mono-resistant *M. tuberculosis* strain was considered a control group and Δ*Mpa* strain as an experimental group, for which primers *Mpa-F* and *Mpa-R* were used to amplify a 1848-bp specific fragment of the *Mpa* gene. Primers *Mpa-N*-F and *Km-R* were used to amplify a 1604-bp specific fragment of *Mpa-N-Km*; and primers *Km-F* and *Mpa-C-R* were used to amplify a 1602-bp specific fragment of *Km-Mpa-C*.

3.7. Minimum Inhibitory Concentration Determination of Strains

To determine the minimum inhibitory concentrations (MICs) of INH-MTB strains and its derivative PPS mutants, a Microplate Alamar Blue Assay (MABA) was performed, as described elsewhere (14, 15). The results were obtained after 7 to 14 days for *M. tuberculosis*. The final drug concentration range of INH (Aladdin, China) was determined by preliminary experiments. At the time of testing, 2-fold serial dilutions were prepared to achieve the desired concentrations. The MICs were defined as the lowest concentration of INH that reduced the viability of the culture by at least 90%, as determined by fluorescence measurements at room temperature in top-reading mode. The excitation wavelength and emission wavelength were 530nm and 590 nm, respectively (16). The high-level INH resistance was defined as the strains with MIC $\geq$ 1 μg/mL (17). The assays were performed in septuplicate.

3.8. Statistical Analysis

Statistical analysis was performed with the SPSS 17.0 software. The LSD-t test was used for comparisons between groups. A P value of < 0.05 was considered statistically significant.

4. Results

4.1. Real-Time PCR Results

The amplification curves of over-expression strain are shown in Figure 1. The results of RT- qPCR showed that the mRNA expression level of *Pup*, *Dop*, *PafA* and *Mpa* was lower in INH-MTB than that in its 4 kinds of over-expression strains (four kinds of over-expression strains were named rINH-MTB: *Pup*, rINH-MTB: *Dop*, rINH-MTB: *PafA* and rINH-MTB: *Mpa*) (Figure 2). The mRNA expression level of each gene in INH-MTB was not different with that in the INH-MTB (pMV361) strain (Figure 2). These results showed that 4 kinds of over-expression strains were constructed successfully and that the plasmid of pMV361 had no effect on the mRNA expression level. The MIC of isoniazid was not different between the INH-MTB strain and INH-MTB (pMV361) strain (data did not show here). Therefore, the researchers selected INH-MTB as a control group in the subsequent experiments.

4.2. PCR Results

The researchers detected the expression of *Pup*, *Dop*, *PafA* and *Mpa* in four kinds of deletion mutant strains by PCR (4 kinds of deletion mutant strains were named

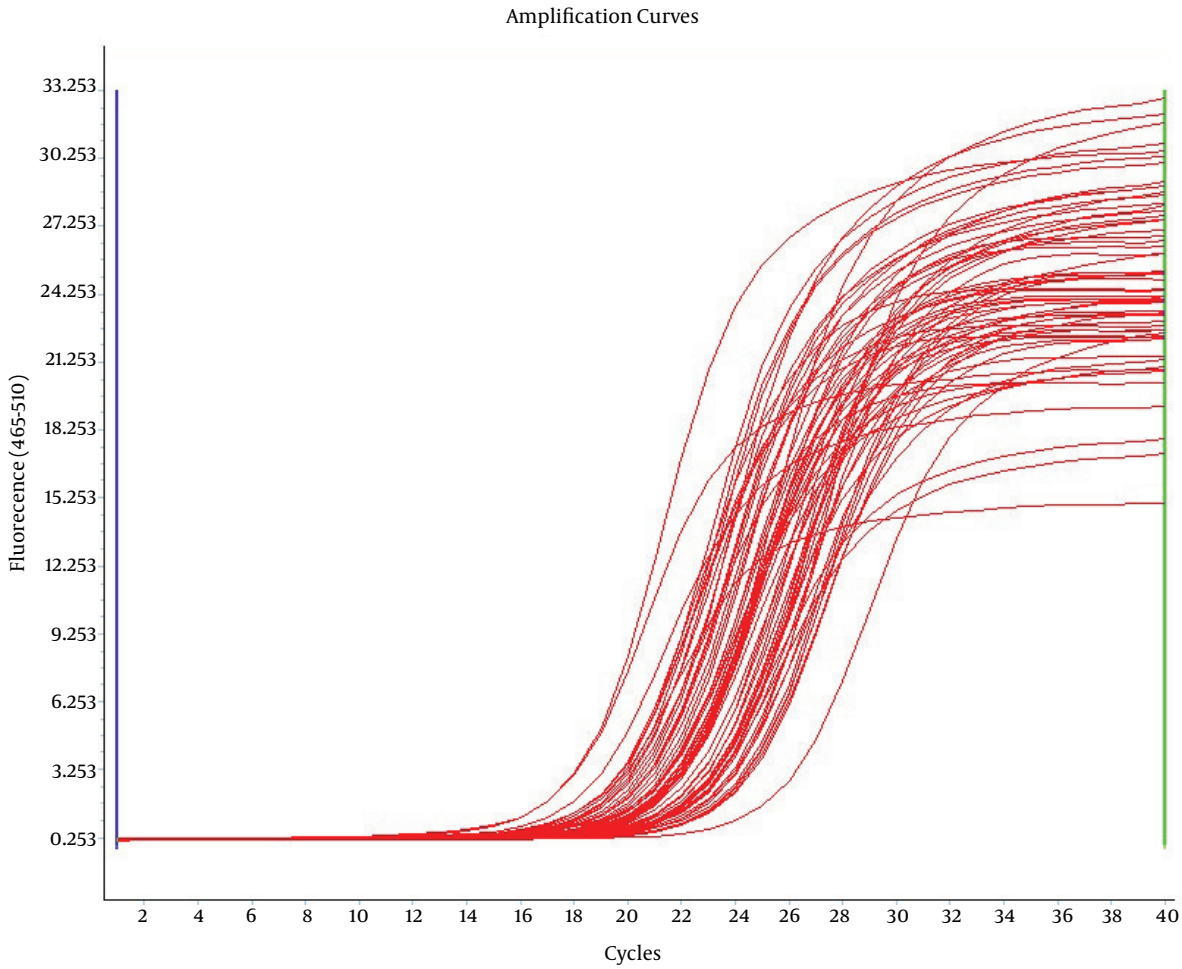
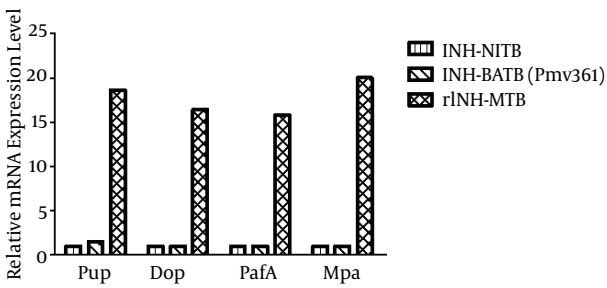


Figure 1. Amplification Curves of Over-Expression Strains

Figure 2. Analysis of Over-Expression Strains Gene Expression



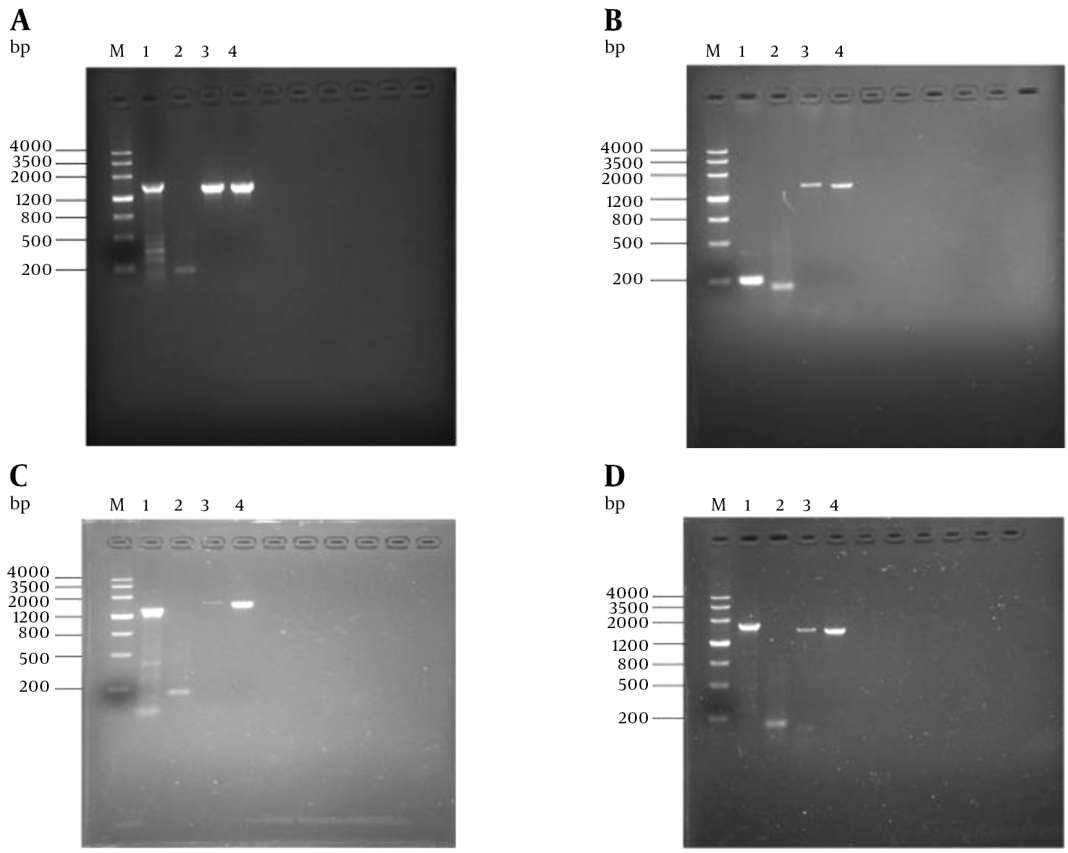
Note; INH-MTB: isoniazid monoresistant *Mycobacterium tuberculosis*; INH-MTB (PMV361): isoniazid monoresistant M. tuberculosis with a PMV361; rINH-MTB: *Pup*, *Dop*, *PafA* and *Mpa* over-expression in isoniazid monoresistant *M. tuberculosis*, respectively.

INH-MTBΔ*Pup*, INH-MTBΔ*Dop*, INH-MTBΔ*PafA*, and INH-MTBΔ*Mpa*). They found that the gene could not be detected in its deletion mutant strain (Figure 3). These results showed that 4 kinds of deletion mutant strains were constructed successfully.

4.3. The Over-Expression of Pup Gene Increased the MIC of INH

The MIC of INH was 1.03 μg/mL lower in INH-MTB than that in rINH-MTB: *PafA* strain; the MIC of INH was 1.03 μg/mL and 0.68 μg/mL higher in INH-MTB than that in rINH-MTB: *Dop* and rINH-MTB: Mpa, respectively. The differences had no statistical significance ($P > 0.05$) (Figure 4A). The MIC of INH was 8 μg/mL lower in INH-MTB than that in rINH-MTB: *Pup* ($P < 0.05$) (Figure 4A).

Figure 3.



A, PCR verification of electrophoregram of INH-MTBΔ*Pup*. Note, M:DNA Marker; lane 1 was the Pup fragment in INH-MTB strain; lane 2 was the Pup fragment in INH-MTBΔ*Pup* strain; lane 3 was the Pup-N-Km fragment in INH-MTBΔPup strain; lane 4 was the *Km-Pup-C* fragment in INH-MTBΔ*Pup* strain. B, PCR verification of electrophoregram of INH-MTBΔ*Dop*. Note, M: DNA Marker; lane 1 was the *Dop* fragment in INH-MTB strain; lane 2 was the *Dop* fragment in INH-MTBΔ*Dop* strain; lane 3 was the *Dop-N-Km* fragment in INH-MTBΔDop strain; lane 4 was the Km-Dop-C fragment in INH-MTBΔDop strain. C, PCR verification of electrophoregram of INH-MTBΔ*PafA*. Note, M: DNA Marker; lane 1 was the *PafA* fragment in INH-MTB strain; lane 2 was the *PafA* fragment in INH-MTBΔ*PafA* strain; lane 3 was the *PafA-N-Km* fragment in INH-MTBΔ*PafA* strain; lane 4 was the *Km-PafA-C* fragment in INH-MTBΔ*PafA* strain. D, PCR verification of electrophoregram of INH-MTBΔ*Mpa*. Note, M:DNA Marker; lane 1 was the *Mpa* fragment in INH-MTB strain; lane 2 was the Mpa fragment in INH-MTBΔ*Mpa* strain; lane 3 was the *Mpa-N-Km* fragment in INH-MTBΔ*Mpa* strain; lane 4 was the *Km-Mpa-C* fragment in INH-MTBΔ*Mpa* strain.

4.4. The Deletion of Pup Gene, Dop Gene, Pafa Gene and Mpa Gene Could Reduce the MIC of INH

The MIC of INH were 4.82 μg/mL, 4.98 μg/mL, 4.99 μg/mL, and 4.9 μg/mL higher in INH-MTB than that in INH-MTBΔ*Pup*, INH-MTBΔ*Dop*, INH-MTBΔ*PafA*, and INH-MTBΔ*Mpa*, respectively. The differences had statistical significance ($P < 0.05$) (Figure 4B).

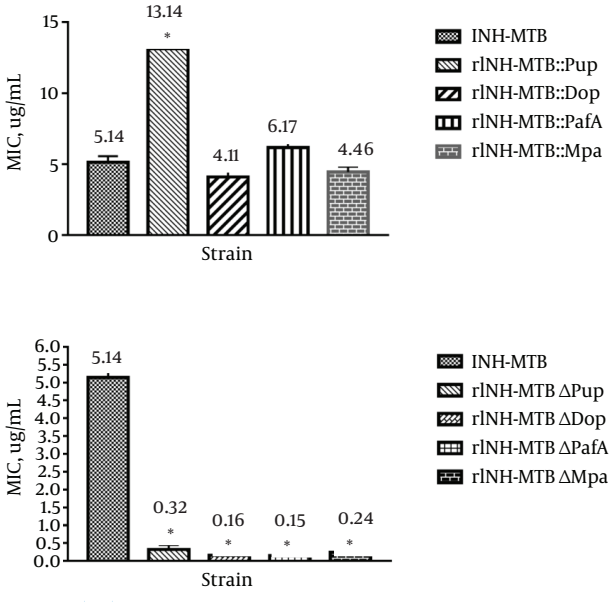
5. Discussion

The current study showed that the MIC of INH in rINH -MTB: *Dop* did not change when compared with that in INH-MTB strain. This result indicated that Dop-over-expression had no effect on the drug resistance of the INH-MTB strain. The researchers speculated that *Dop* protein was partly degraded in order to maintain the functional stability of PPS.

Elharar et al. (9) found that exponential phase cells harboring *Dop* over-expression presented a 7-fold increase at the *Dop* mRNA level, compared with cells containing an empty vector. However, a barely detectable increase was noted when the Dop protein level was assessed, suggesting that *Dop* over-expression had been counterbalanced, potentially at the proteolytic level (9). Meanwhile, this study also found that low *Dop* protein levels promoted efficient pupylation, yet high *Dop* protein levels shifted the balance between the 2 activities of *Dop* to facilitate the process of de-pupylation (9). High Dop levels would lead to futile cycle between pupylation and de-pupylation.

The deletion of *Dop* gene and *pafA* gene could reduce

Figure 4.



A, The MIC of INH in INH-MTB strain and its over-expression strains. Note, INH-MTB strain as a control group;*Compared with the control group $P < 0.05$. B, The MIC of INH in INH-MTB strain and its deletion mutant strains. Note, INH-MTB strain as a control group;*Compared with the control group $P < 0.05$.

the MIC of INH. The deletion of *Dop* or *PafA* interdicted the process of pupylation of substrate protein, leading to the accumulation of *FabD* (10, 18). The *FabD* involved in the synthesis of fatty acids and it was a crucial constituent of *Mycobacterial* cell wall. However, these abnormal *FabD* might hinder the synthesis of normal *Mycobacterial* cell wall, increasing the permeability of anti-tuberculosis drugs.

Delley et al. (19) found that in M. smegmatis (Msm) with a *Dop* deletion mutant, pupylation was severely impaired and proteasomal substrates were increased. Festa et al. (10) found that Δ*PafA* strain could cause the accumulation of substrate proteins in MTB. These results suggest that *PafA* or *Dop* are necessary to finish the degradation of substrate proteins. The MIC of INH in INH-MTB strain did not change when compared with that in rINH-MTB: *Mpa* strain and rINH-MTB: *PafA* strain. The *Mpa* protein could be pupylated and then degraded by proteasome. However, when *PafA* was knocked out, *Mpa* would accumulate in bacterial body, suggesting that the pupylation of *Mpa* could make itself be degraded (20).

Posttranslational modification of target proteins with the help of *Pup*, not only made a substrate be degraded by the proteasome but also regulated the enzyme activity of PPS itself (19). It has been reported that *PafA* could be polypupylated by itself and *PafA* accumulated in the strain with the deletion of *Mpa* or *prcBA* (21). The results suggest that the pupylation of Mpa or PafA intends to maintain the stability of PPS.

The current study showed that the MIC of INH in INH-MTB strain was lower than that in the INH-MTBΔ*Mpa* strain and INH-MTBΔ*Pup* strain. The *Mpa* contributed to the survival of MTB in a host; the virulence of Δ*Mpa* strain was reduced in the infection model of mice (22). The *Pup* made the target proteins to attach an unordered label to facilitate the degradation of target proteins. The researchers speculated that the deletion of *Mpa* or *Pup* would lead to the accumulation of target proteins, which decreased the tolerance of bacteria to all kinds of stress.

This study showed that the MIC of INH was lower in the INH-MTB strain than that in the rINH-MTB: *Pup* strain. They speculated that the increase of *Pup* protein accelerated the speed of degradation of harmful proteins in MTB, enhancing the adaptability of MTB. Ino1 could not be detected in *Pup*-over-expression Msm, speculating that the speed of elimination of Ino1 was expedited (23).

6. Conclusions

In summary, the experiments of this study clarify that MTB PPS affect drug resistance of the INH-MTB strain. This study provides new information for the treatment of drug-resistant TB.

Acknowledgments

The researchers are grateful to Dr. Feng Li (School of Basic Medical Sciences, Shihezi University, China) for critically reading the manuscript. Le Zhang is acknowledged for providing technical help.

Footnotes

Authors' Contribution: Study concept and design, Shuai Zhang, Shun Wen Zhang, and Wan Jiang Zhang; analysis and interpretation of data, Shuai Zhang, Jiang Dong Wu, and Jie Zhang; drafting of the manuscript, Jiang Tao Dong and Shun Wen Zhang; critical revision of the manuscript for important intellectual content, Shuai Zhang, Fang Wu, and Wan Jiang Zhang; statistical analysis, Jiang Dong Wu and Hui Yun Zhu; administrative, technical, and material support: Fang Wu and Hui Yun Zhu; study supervision, Fang Wu and Wan Jiang Zhang.

Financial Disclosure: The authors declared that they have no conflict of interest.

Funding/Support: This study was supported by the national natural science foundation (Grant NO: 81260261).

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