

Supplementary Table S1. Collection parameters of multiple reaction monitoring (MRM).

Bio-transformed Products	RT (min)	Ionization channel	Electrospray ionization
H ₂ PtePAS	3.193	331.0 -> 178.0	ESI+
H ₂ PtePAS-Glu	3.181	460.0 -> 178.0	ESI+

Supplementary Table S2. Mutation analysis of *ribD* in *ThyA* or *DfrA* deficient *M. tuberculosis* isolates.

genome_ID	<i>thyA</i> _CDS	<i>dfrA</i> _CDS	<i>ribD</i> _promoter	<i>ribD</i> _CDS	Reference
ERR502929	deletion	deletion	WT	WT	[22]
ERR751453	deletion	deletion	WT	WT	
ERR775373	deletion	deletion	WT	WT	
ERR779910	deletion	deletion	WT	WT	
SRR16588749	deletion	deletion	WT	WT	[11]
SRR1952721	deletion	deletion	WT	WT	
SRR2328057	deletion	deletion	WT	WT	[27]
SRR2333215	deletion	deletion	WT	WT	
GCA_000177835.2	Leu63fs	WT	WT	WT	NCBI database
GCA_000652475.1	Thr22fs	WT	WT	WT	
GCA_000657935.1	Gln165*	WT	WT	WT	
GCA_000657955.1	Gln165*	WT	WT	WT	
GCA_000657995.1	Gln165*	WT	WT	WT	
GCA_000658015.1	Gln165*	WT	WT	WT	
GCA_000669315.1	Gln165*	WT	WT	WT	
GCA_000669395.1	Gln165*	WT	WT	WT	
GCA_000669555.1	Gln165*	WT	WT	WT	
GCA_000673575.1	Gln165*	WT	WT	WT	
GCA_000673735.1	Gln165*	WT	WT	WT	
GCA_000673755.1	Glu139*	WT	WT	WT	
GCA_000674395.1	Trp80*	WT	WT	WT	
GCA_000674475.1	Gln165*	WT	WT	WT	
GCA_000674535.1	Arg99*	WT	WT	WT	
GCA_000674575.1	Gln165*	WT	WT	WT	
GCA_000677115.1	Trp133*	WT	WT	WT	
GCA_000678495.1	Gln111*	WT	WT	WT	
GCA_000679475.1	Leu63fs	WT	WT	WT	
GCA_000804245.1	Glu139*	WT	WT	WT	
GCA_003392965.1	Ile69fs	WT	WT	WT	
GCA_014900815.1	Tyr4fs	WT	WT	WT	
GCA_021026275.1	Ser180fs	WT	WT	WT	

Fs, frameshift; *, stop transcription

Supplementary Table S3. Plasmids, strains, and primers used in this study.

Category	Name	Characterization/Sequence (5'-3')	Source
Plasmids			
	pMAL-c2X	<i>E. coli</i> expression plasmid under control of Tac promoter with N-terminal MBP tag linked with a factor Xa cleavage site, Ap ^R	New England BioLabs
	pMAL-c2X:: <i>folC</i>	pMAL-c2X with <i>M. tuberculosis</i> H37Rv <i>folC</i> inserted downstream of the Tac promoter, Ap ^R	This study
	pET28a	<i>E. coli</i> expression plasmid under control of T7 promoter with N-terminal His tag, Km ^R	Novagen
	pET28a:: <i>folP1</i>	pET28a with <i>M. tuberculosis</i> H37Rv <i>folP1</i> inserted downstream of the T7 promoter, Km ^R	This study
	pMV261	Non-integrated shuttle plasmid under control of <i>hsp60</i> promoter, Km ^R	Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
	pMV261:: <i>thyA</i>	pMV261 with <i>M. tuberculosis</i> H37Rv <i>thyA</i> inserted downstream of the <i>hsp60</i> promoter, Km ^R	This study
	pMV261:: <i>thyX</i>	pMV261 with <i>M. tuberculosis</i> H37Rv <i>thyX</i> inserted downstream of the <i>hsp60</i> promoter, Km ^R	This study
	pMV261:: <i>folP1</i>	pMV261 with <i>M. tuberculosis</i> H37Rv <i>folP1</i> inserted downstream of the <i>hsp60</i> promoter, Km ^R	This study
	pMV261:: <i>folC</i>	pMV261 with <i>M. tuberculosis</i> H37Rv <i>folC</i> inserted downstream of the <i>hsp60</i> promoter, Km ^R	This study
	pMV261:: <i>dfrA</i>	pMV261 with <i>M. tuberculosis</i> H37Rv <i>dfrA</i> inserted downstream of the <i>hsp60</i> promoter, Km ^R	This study
	p0004s	Homologous arms construction plasmid, HygB ^R	Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
	phAE159	Phage transduction plasmid, Ap ^R	Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
Strains			
	<i>E. coli</i> BL21 (DE3)	Host for protein expression	New England BioLabs
	BL21 (DE3) pMAL-c2X:: <i>folC</i>	<i>E. coli</i> BL21 (DE3) transformed with pMAL-c2X:: <i>folC</i>	This study

BL21 (DE3) pET28a:: <i>folP1</i> <i>E. coli</i> HB101	<i>E. coli</i> BL21 (DE3) transformed with pET28a:: <i>folP1</i> Host for plasmid amplification	This study Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
<i>M. smegmatis</i> mc ² 155	High-efficiency transformation strain	Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
H37Ra	<i>M. tuberculosis</i> H37Ra avirulent strains	Prof. William R Jacobs Jr, Albert Einsterin College of Medicine
H37Ra Δ <i>thyA</i>	<i>M. tuberculosis</i> H37Ra with <i>thyA</i> gene deletion	This study
H37Ra pMV261	<i>M. tuberculosis</i> H37Ra transformed with pMV261	This study
H37Ra Δ <i>thyA</i> pMV261	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261	This study
H37Ra Δ <i>thyA</i> pMV261:: <i>thyA</i>	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261:: <i>thyA</i>	This study
H37Ra Δ <i>thyA</i> pMV261:: <i>thyX</i>	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261:: <i>thyX</i>	This study
H37Ra Δ <i>thyA</i> pMV261:: <i>folP1</i>	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261:: <i>folP1</i>	This study
H37Ra Δ <i>thyA</i> pMV261:: <i>folC</i>	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261:: <i>folC</i>	This study
H37Ra Δ <i>thyA</i> pMV261:: <i>dfrA</i>	<i>M. tuberculosis</i> H37Ra Δ <i>thyA</i> transformed with pMV261:: <i>dfrA</i>	This study
H37Ra pMV261:: <i>thyA</i>	<i>M. tuberculosis</i> H37Ra transformed with pMV261:: <i>thyA</i>	This study
H37Ra pMV261:: <i>thyX</i>	<i>M. tuberculosis</i> H37Ra transformed with pMV261:: <i>thyX</i>	This study
H37Ra pMV261:: <i>folP1</i>	<i>M. tuberculosis</i> H37Ra transformed with pMV261:: <i>folP1</i>	This study
H37Ra pMV261:: <i>folC</i>	<i>M. tuberculosis</i> H37Ra transformed with pMV261:: <i>folC</i>	This study
H37Ra pMV261:: <i>dfrA</i>	<i>M. tuberculosis</i> H37Ra transformed with pMV261:: <i>dfrA</i>	This study
F461	Clinical isolate of <i>M. tuberculosis</i> H37Rv with mutation <i>thyA</i> (²³⁵ Arg→Pro)	Chongqing Public Health Medical Center
F461 pMV261:: <i>folC</i>	<i>thyA</i> (²³⁵ Arg→Pro) transformed with pMV261:: <i>folC</i>	This study

folC-BamHI-F	TTAAGGATCCATGAATTCGACGAATTCCGG	This study
folC-HindIII-R	TTACAAGCTTTTATTGCGGATCACGACC	This study
folP1-EcoRI-F	ATAGAATTCATGAGTCCGGCGCCCGTGCA	This study
folP1-HindIII-R	ATAAAGCTTTTAGCCATCGCGTTCTATCCTTTCC	This study
dfrA-BamHI-F	TTAAGGATCCATGGTGGGGCTGATCTGGGCT	This study
dfrA-HindIII-R	TAATAAGCTTTTATGAGCGGTGGTAGCTGTAC	This study
thyA-BamHI-F	TTAAGGATCCATGACGCCATACGAGGACCTGCTGC	This study
thyA-HindIII-R	TACTAAGCTTTTATACCGCGACTGGAGCTTTGATCG	This study
thyX-BamHI-F	TTAAGGATCCATGGCCGAGACCGCGCCGCTG	This study
thyX-HindIII-R	TACTAAGCTTTTAGGCTTCGGTCGCCAACGGGCT	This study
pMV261-JDFP	GTGGCAGCGAGGACAACCTTG	This study
pMV261-JDRP	GATGCCTGGCAGTCGATCGTAC	This study
thyAko-LFP	TTTTTTTTCCATAAATTGGCCGGGTAGCACTCGATGCGGTGCG	This study
thyAko-LRP	TTTTTTTTCCATTCTTGGTCGAGCACGAAGCGCAGCAGGTCCT	This study
thyAko-RFP	TTTTTTTTCCATAGATTGGATCCGCATCCGGCGATCAAAGCT	This study
thyAko-RRP	TTTTTTTTCCATCTTTTGGGTAATACAAGTGCCGGCGTTTGCC	This study
thyA-IDF	ATCGGGATCGACGTCGCGGCGCCT	This study
thyA-IDR	AGATCACCCCGGGTGGACACGGCGAT	This study

Supplementary Figures and Figure Legends

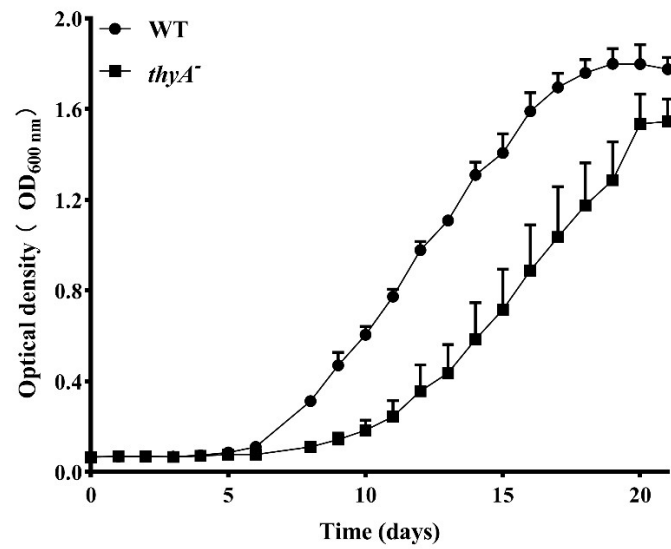


Figure S1. Growth curves of H37Ra (WT) and H37Ra Δ *thyA* (*thyA*⁻) in liquid culture at 37°C. The OD₆₀₀ was measured by using a SynergyH1 Hybrid reader (BioTek, USA). Data represent the means of three biological replicates, and error bars denote the standard deviations.

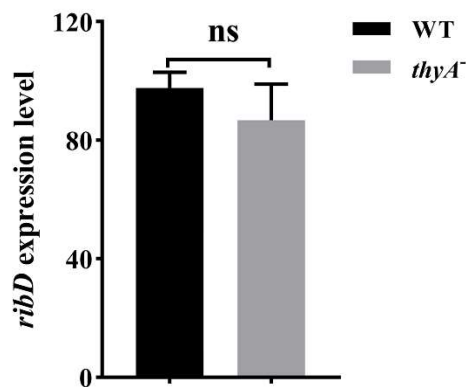


Figure S2. Comparison of the transcriptional level of the *ribD* gene during the exponential phase in H37Ra (WT) and H37Ra Δ *thyA* (*thyA*⁻) by RNA-seq. ns, no significance.