Rv0494 Mediates Tolerance to Antibiotics in *Mycobacterium Tuberculosis*

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Abstract

Background: Fatty acid metabolism plays an important role in the survival and pathogenesis of *Mycobacterium tuberculosis*. During dormancy, lipids are considered to be the main source of energy. The previous studies find that Rv0494 is a starvation-inducible, lipid-responsive transcriptional regulator. However, the role of Rv0494 in bacterial persister survival has not been studied.

Methods: We constructed *Rv0494* deletion mutant and assessed the susceptibility of the mutant to various antibiotics conditions in persister assays.

Results: We found that mutations in *Rv0494* caused a defect in persister survival as shown by their increased susceptibility to Isoniazid.

Conclusions: We conclude that Rv0494 is important for persister survival and may serve as a good target for developing new antibiotics that kill persister bacteria for improved treatment of persistent bacterial infections.

Background

Persisters are assumed to be non-replicating or slow-growing bacteria that are not killed by antibiotics, can return to a growth state upon removal of antibiotics, and are sensitive to the same antibiotics (1). Persisters may be medically important and pose a major challenge to the treatment of many bacterial infections such as tuberculosis and may be responsible for latent and persistent infections, long-term treatment and relapse after treatment (2). The mechanisms of persister formation are so complex that not well understood.

Bacteria have evolved various survival mechanisms under stressful conditions. The success of *Mycobacterium tuberculosis* as a pathogen lies in its ability to survive asymptomatically within the host for long periods of time and to reactivate when host immunity is compromised. During infection, *M. tuberculosis* is exposed to harsh environmental conditions such as hypoxia, low pH and nutrient deprivation (3–5). Mycobacteria utilize their resources by efficiently coordinating gene expression according to prevailing conditions. The correct use of promoters in concert with transcriptional regulators plays an important role in mycobacterial physiology.

FadR acts as a sensor of the fatty acid level in bacteria. FadRs of *Escherichia coli*, *Vibrio vulnificus* and *Corynebacterium glutamicum* have been extensively studied and are reported to play important roles in cell physiology and virulence (6–10). A FadR homologue in *M. tuberculosis*, Rv0494, is a starvation-inducible, auto-regulatory FadR-like regulator (11). Fatty acid metabolism plays an important role in the survival and pathogenesis of *M. tuberculosis*, lipids are assumed to be the major source of energy during persistence. Therefore, we assume that FadR plays a very important role in the persistence of *M. tuberculosis*.

In this study, we constructed the Rv0494 deletion mutant in *M. tuberculosis* H_{37}Rv, and exposed the mutants to various antibiotics to exploring whether Rv0494 is important for persister survival. Our results demonstrate that Rv0494 is indeed involved in persister survival and tolerance to antibiotics.

Methods

Bacterial strains and growth conditions

M. smegmatis mc^2 155 was grown in Middlebrook 7H9 broth or Middlebrook 7H10 Agar. *M. tuberculosis* H_{37}Rv was grown in Middlebrook 7H9 supplemented with OADC/ADC or Middlebrook 7H10 supplemented with OADC. Antibiotics were used at the following concentrations: Ampicillin (100 μg mL^{-1}), hygromycin (75 μg mL^{-1}), kanamycin (25 μg mL^{-1}), isoniazid (4 μg mL^{-1}) and rifampin (8 μg mL^{-1}). All oligonucleotides and plasmids used in this study are listed in Tables 1 and 2.

Table 1
Primers used in this study

Primer	Sequence 5'-3'
LFP	TTTTTTTTCCATAAATTGGGGGTGGTTTCGCCGACGG
LRP	TTTTTTTTCCATTTCTTGGTCTCGCTCGGGCGGCAGG
RFP	TTTTTTTTCCATAGATTGGGCGATCAACGGGGCGGTG
RRP	TTTTTTTTCCATCTTTTGGCGGGCTTCGTGGTCTAAC
LYZFP	GATGCGGAAGCGAACGAAC
LYZRP	GTGGACCTCGACGACCCTAG
RYZFP	TGGATCTCTCCGGCTTCACC
RYZRP	CCCAATCGCACGAAAACA
Rv0494FP	GATCCAGCTGCAGAATTCTTGGTTGAGCCAATGAACCA
Rv0494RP	ACCGCGACCGCGCTTAGaAGCTTATCGATGTCGACG

Table 2
Plasmids used in this study

Plasmid/strain	Description
phAE159	Ampicillin-resistant vector, used for construction of Rv0494 knockout mutants
p0004s	Hygromycin-resistant vector, used for construction of homologous arms
pMV361	Kanamycin-resistant mycobacterial overexpression plasmid
E.coli DH5α	Used for conventional plasmid amplification
E.coli HB101	Used for conventional plasmid construction

Construction of *Rv0494* knockout mutants and complementation of the mutants

The *Rv0494* knockout mutants was constructed as described previously (12–14). Amplicons between 640 and 940 bases flanking the gene were PCR generated with primer sets (LFP/LRP and RFP/RRP (Table 1)) to generate gene-specific LHS and RHS. Plasmid p0004s was digested with Van91I, This fragment was ligated in one step to Van91I-digested LHS and RHS fragments corresponding to the gene *Rv0494*. The ligation mix was transformed in *E.coli* DH5α, and the clones were confirmed by sequencing. Thus the p0004s-AES plasmid was constructed.

After cleaving phAE159 and p0004s-AES, respectively, using PacI enzyme, the fragments were ligated. The ligation mix was transformed in *E.coli* HB101, single colonies growing on hygromycin -resistant plates were picked into LB + $Hyg^{150\ \mu g/ml}$ broth. The plasmids were extracted and identified using PacI restriction endonuclease digestion. Thus the phAE159-AES phasmid was constructed.

The phasmid phAE159-AES was transformed into *M. smegmatis* mc^2 155 to obtain phages that could be transfected with *M. tuberculosis* $H_{37}Rv$. Phages were transfected with *M. tuberculosis* $H_{37}Rv$ and then screened for positive clones using Middlebrook 7H10 + OADC + $Hyg^{75\mu g/ml}$. The primer sets (LYZFP/LYZRP and RYZFP/RYZRP (Table 1)) were then used to verify that the mutants were constructed.

Complementation of the *Rv0494* knock-out mutants was performed utilizing the plasmid vector pMV361. A functional wild type copy of *Rv0494* was amplified by primers Rv0494FP and Rv0494RP. PCR products were digested with restriction enzymes EcoRI and HindIII and cloned into pMV361. The recombinant pMV361 containing *Rv0494* (pMV361-Rv0494) was verified by DNA sequencing. The resulting constructs were transformed along with the empty vector pMV361 into mutant for complementation.

Susceptibility to antibiotics in exposure assays

The susceptibilities of stationary phase Rv0494 mutants, complemented strains and the parent strain *M. tuberculosis* $H_{37}Rv$ to various antibiotics, including isoniazid (4 μg mL^{-1}) and rifampin (8 μg mL^{-1}), were evaluated in drug exposure experiments on Middlebrook 7H10 supplemented with OADC. The stationary

phase cultures (diluted 1:100 with Middlebrook 7H9) were exposed to different antibiotics, where undiluted cultures were used for incubation without shaking at 37°C for various times, after which the cultures were plated for CFU determination on Middlebrook 7H10 + OADC plates.

Results And Discussion

Construction of phasmid

The LHS and RHS products were recovered by Van91I digestion and ligated with Van91I digested plasmid p0004s and transformed into *E. coli* DH5α to screen positive clones and sequenced. The positive clones obtained by identification were further cleaved by PacI and recovered, and ligated with the plasmid phAE159, also PacI cleaved, packaged and transformed into *E. coli* HB101 cells to screen for phasmid, and the results of PacI cleavage validation were shown in Fig. 1.

Phage-based mutant construction

After transform the phasmid phAE159-AES into *M. smegmatis* mc^2 155, we obtain the phages that could be transfected with *M. tuberculosis* H_{37}Rv. Finally, the Δ*Rv0494* strain was successfully completed using the phage we constructed. PCR was used to confirm the successful construction of the knockout strain (Fig. 2, 3).

Reduced persister levels of the *Rv0494* mutants in antibiotic exposure assays

To determine the persister levels of the *Rv0494* mutant, the stationary phase cultures of the mutants and wild-type strain *M. tuberculosis* H_{37}Rv were exposed to no antibiotics as control (Fig. 4a) and various antibiotics, including isoniazid (4 μg mL^{-1}) (Fig. 4b)and rifampin (8 μg mL^{-1})(Fig. 4c), and the survival of the bacteria was monitored at different time points. Overall, the results showed that *Rv0494* mutant was more susceptible than the wild-type strain *M. tuberculosis* H_{37}Rv to isoniazid and that complementation of the *Rv0494* mutant restored the level of persisters to close to wild-type levels in the antibiotic exposure assay (Fig. 4). It is worth noting that, this result did not occur in the rifampicin exposure experiment.

Conclusions

Different phenotypes emerged after treatment of the mutants with different antibiotics. The rifampicin-treated mutant showed no significant persisters reduction, whereas, the mutant showed a significant persisters reduction after isoniazid treatment, suggesting that Rv0494 may play an important role in maintaining the persister in response to isoniazid stress conditions. Suhail Yousuf's(11) research suggests that Rv0494 is a starvation-induced, auto-regulated fadR-like regulator, whereas this study was conducted after the strain entered the stationary phase with antibiotic stress applied, and it is expected that the absence of Rv0494 during this period resulted in the absence of its regulatory role, leading to the reduction of the persisters, and further studies are needed to address this hypothesis. Since FadR likely to

be involved in persistence in other bacteria, our findings support the idea that FadR could serve as a novel target for the development of new antibiotics that target persister bacteria for improved treatment of persistent bacterial infections.

Abbreviations

ADC: Albumin, dextrose and catalase medium; AES: Allelic exchange substrates; CFU: Colony forming units; FadR: Fatty acid metabolism regulator; LHS: Left homology sequence; MUT: Mutation; OADC: Oadc oleic acid, albumin, dextrose and catalase medium; PCR: Polymerase chain reaction; RHS: Right homology sequence; WT: Wild type;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JL was responsible for the initial study concept. JL and XJ all contributed to the study design. CD carried out the data collection. HK conducted the thematic analysis. DP drafted the initial paper with input from all authors. CY edited the initial paper. The remaining authors critically reviewed it and made revisions. All authors read and approved the final manuscript.

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Figures

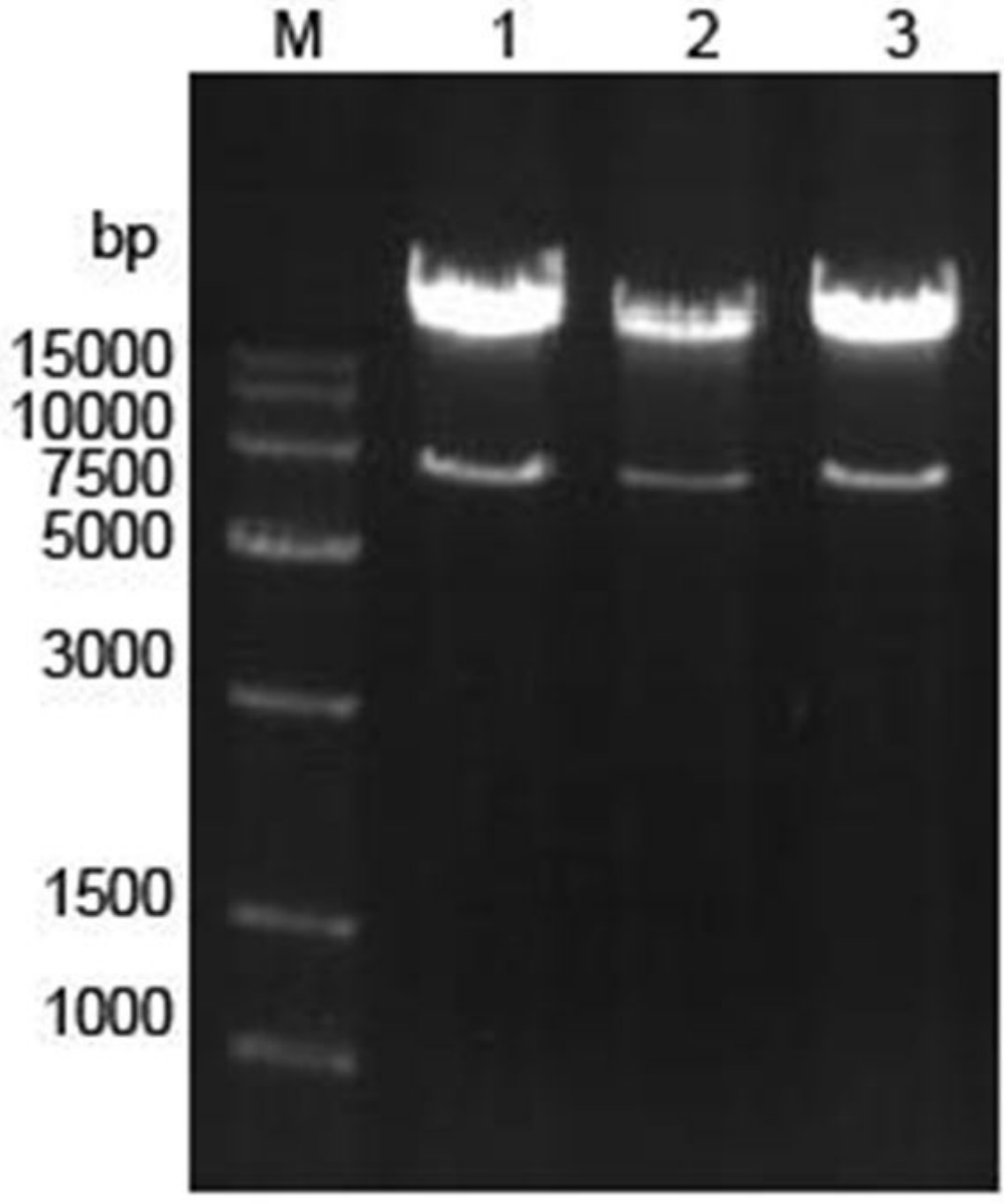

Figure 1

Confirm the completion of phasmid construction after digestion of phasmid using PacI enzyme. Lane M: DNA Ladder, Lane 1-3: Results of phasmid digestion.

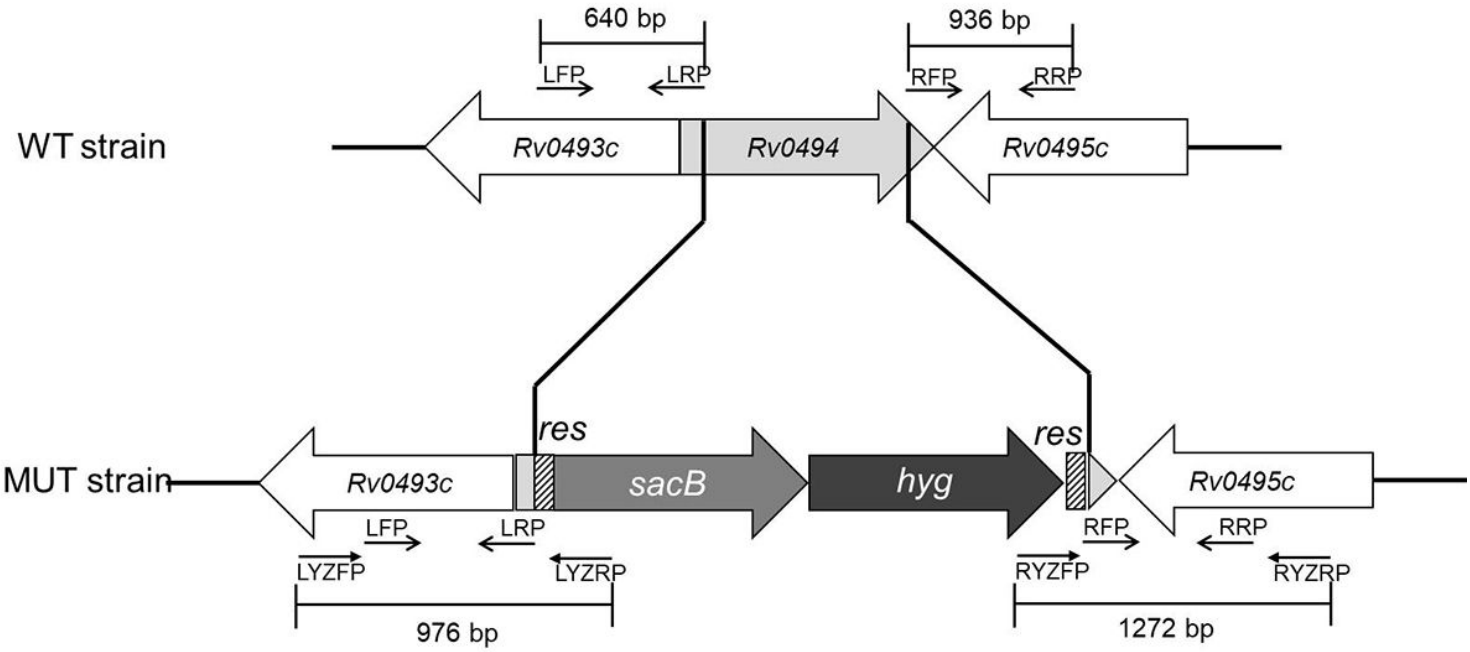

Figure 2

Design of primers for gene knockdown and validation. The knockdown validation primers were designed 100-200 bp upstream of the LFP primer match (LYZFP) and 100-200 bp downstream of the RRP primer match (RYZRP), with the LYZRP primer designed on sacB and the RYZFP primer designed on hyg.

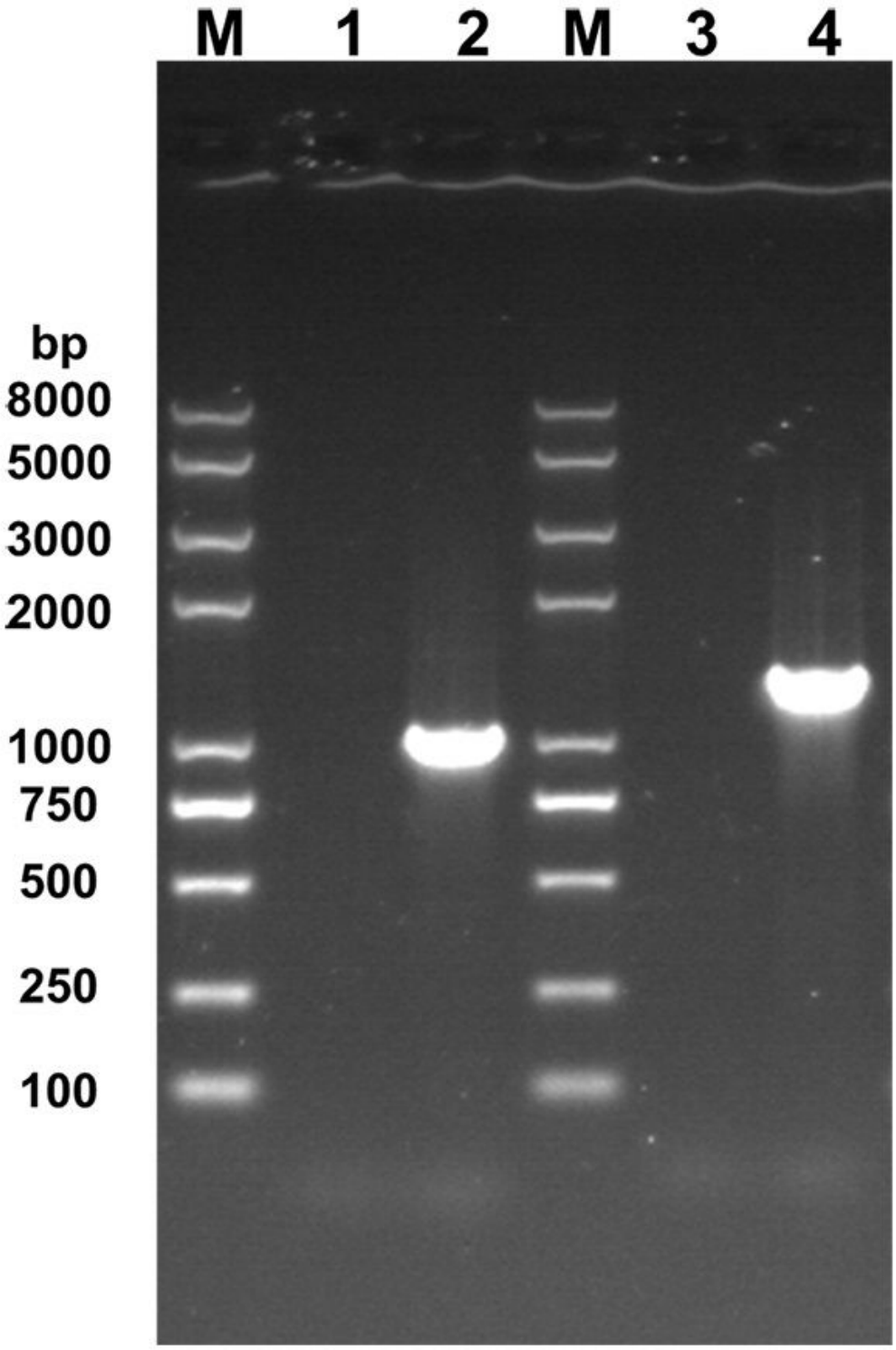

Figure 3

PCR results show successful knockdown of Rv0494 gene in Mycobacterium tuberculosis H37Rv strain. Lane M: DNA Ladder, Lane 1 and Lane 2: Use primer pairs LYZFP/LYZRP to amplification WT strain and MUT strain genome. Lane 3 and Lane 4: Use primer pairs RYZFP/RYZRP to amplification WT strain and MUT strain genome. The PCR was validated using both primer pairs LYZFP/LYZRP and RYZFP/RYZRP and using the knockout strain genome (MUT strain) as the template to amplify DNA fragments of 976 bp

and 1272 bp in size, while no target DNA fragments were amplified when the control was using the wild-type strain genome (WT strain) as the template and the same primer pairs were used for PCR.

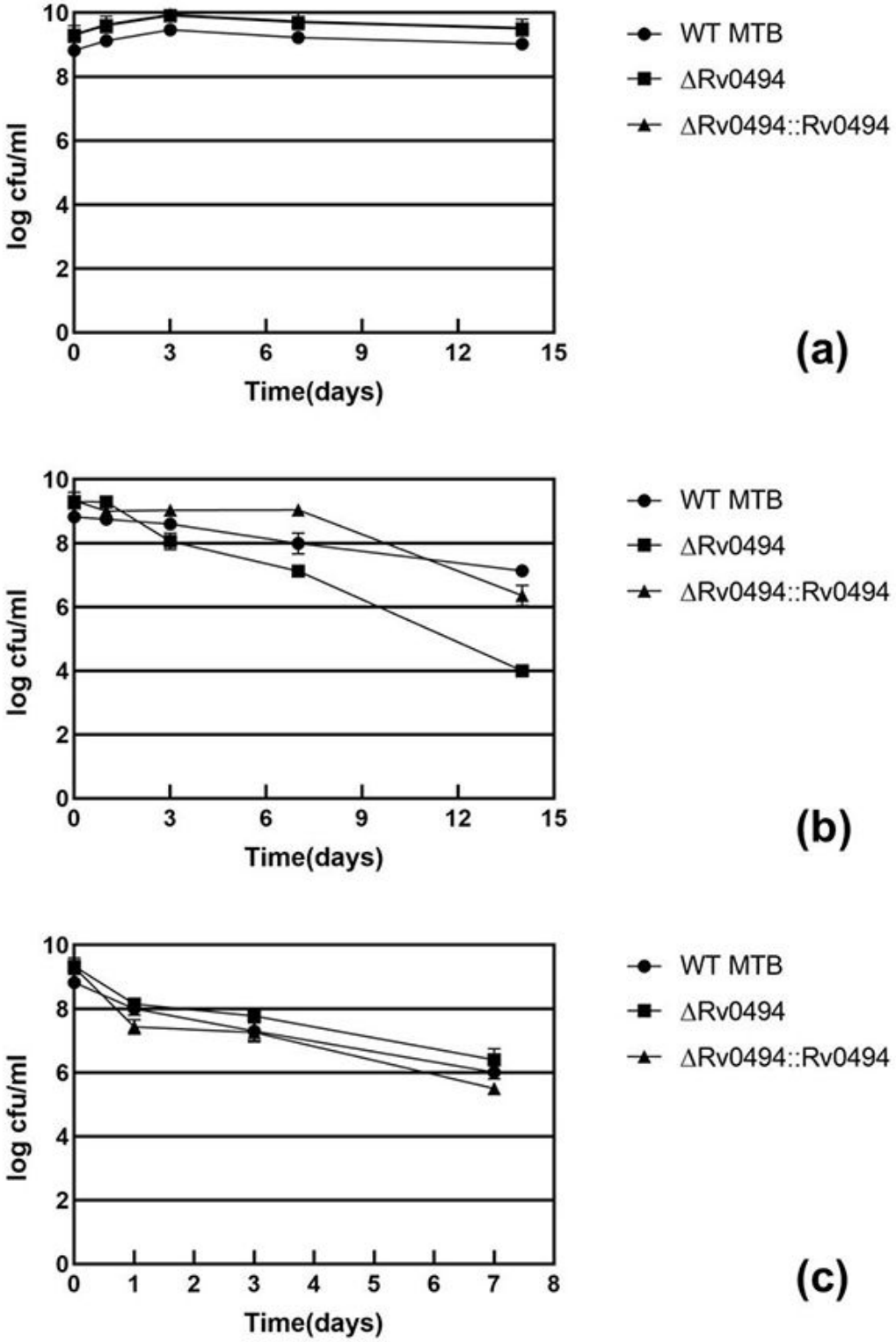

Figure 4

Rv0494 mutant have defective persister levels in antibiotic exposure assays. Stationary phase cultures of ΔRv0494, it complemented strains and the parent strain M. tuberculosis H37Rv were exposed to no antibiotic (a), isoniazid (4 μg mL-1) (b), rifampin (8 μg mL-1) (c) for various times. Aliquots of cultures

were taken at different timepoints and the dilutions were plated for CFU determination on Middlebrook 7H10 Agar plates. The vertical axis represents CFU values on a log scale and the horizontal axis represents time of antibiotic exposure indays.