Supplementary Figures

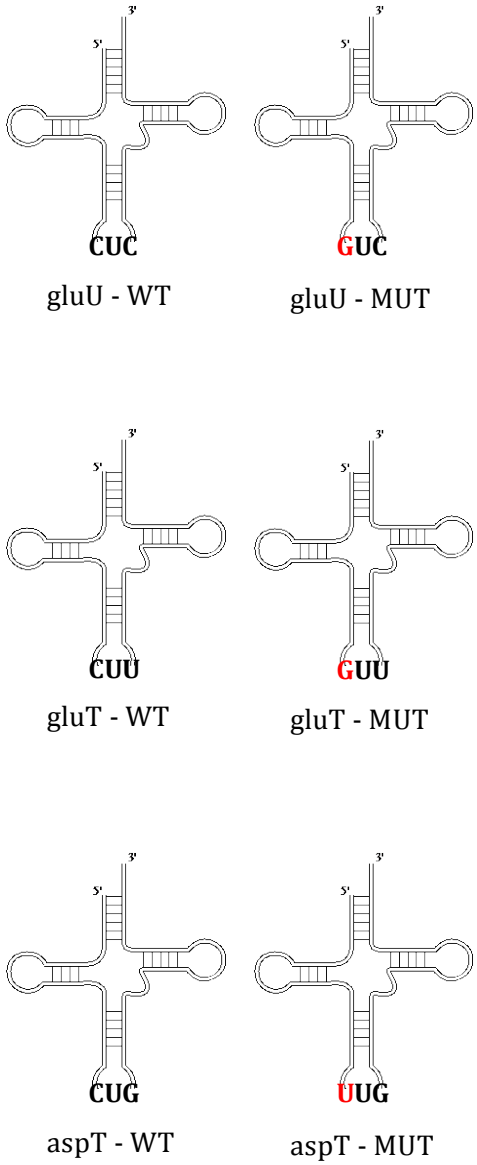

Figure S1. Schematic of tRNA anti-codon swap. The anti-codons of gluU, gluT and aspT tRNAs were mutated to that of glnU, glnT and asnT respectively.

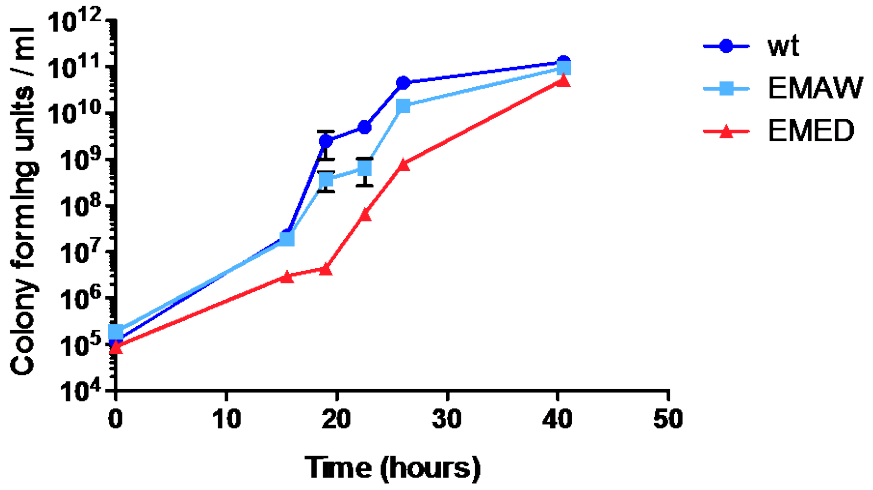

Figure S2. Growth curve of the mycobacterial strains used in this study. Cultures were performed in triplicate. Standard deviation is shown as error bars.

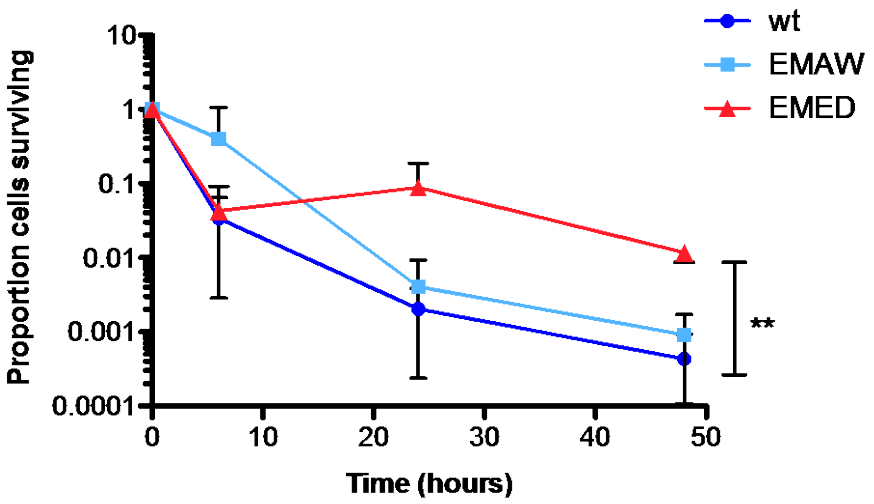

Figure S3. Stationary phase mistranslating mycobacteria are tolerant to killing by rifampicin. Stationary phase mycobacterial cultures were treated +/- Rifampicin (100µg/ml). At specified time points, aliquots were removed, diluted (to remove effective antibiotic) and plated on LB-agar with no antibiotics. Survivors were calculated and plotted. Cultures were in triplicate, and error represents SD, * $p<0.05$ by Student's t-test.

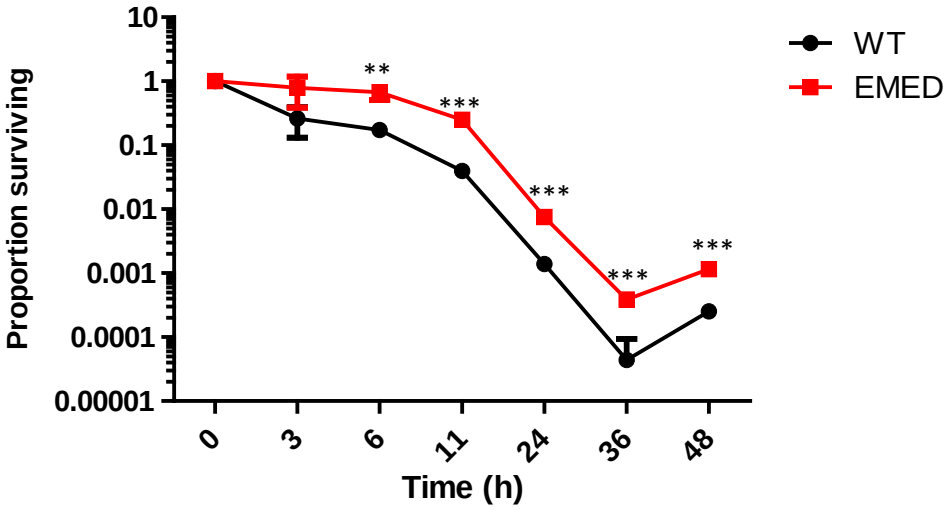

Figure S4. Log phase mistranslating mycobacteria are tolerant to killing by rifampicin. Log phase (OD=1 at time=0) bacteria were treated +/- rifampicin (70µg/ml). At specified time points, aliquots of bacteria were removed, diluted, and plated onto LB-agar without selection. Survivors were calculated and plotted. Cultures in triplicate, error bars represent SD. **= $p<0.01$, ***= $p<0.001$ by Student's t-test.

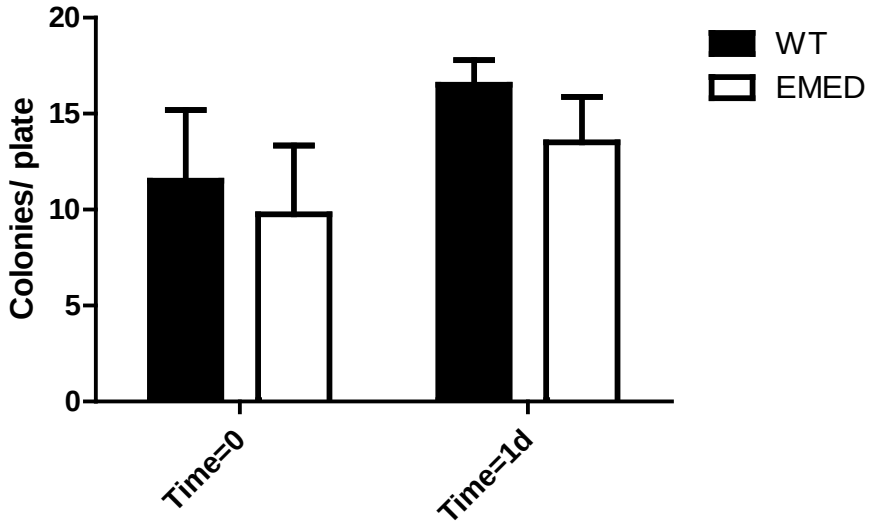

Fig. S5. The mistranslating strain EMED does not increase probability of true genetic rifampicin resistance. Stationary phase cultures of wild-type *M. smegmatis* (WT) and *M. smegmatis*-EMED (EMED) were plated on high concentration rifampicin LB-agar (250µg/ml), on which only genetically resistant colonies may arise. There was no significant difference in the number of resistant colonies that arose between wt and EMED *M. smegmatis* either at initial culture (time=0) or after 24hrs treatment with low-dose rifampicin (25µg/ml) for 24 hours (time=1d).

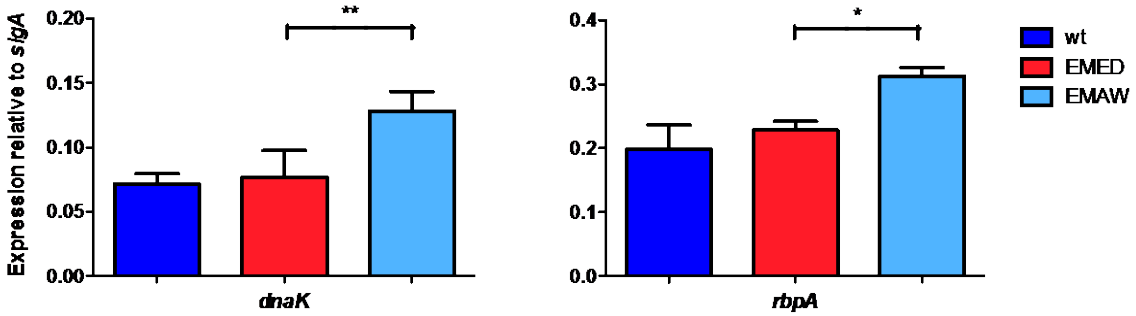

Figure S6. Expression levels of two mycobacterial stress-response genes in wild-type versus mistranslating mycobacterial strains. Real-time PCR on RNA extracted from triplicate cultures was performed, and expression of *rbpA* (right), which has been reported to be involved in rifampicin resistance, and the heat shock protein gene *dnaK* (left) measured. Mean values +/- SD are shown. Expression of the stress-response genes was consistently higher in strain *M. smegmatis* EMAW (* $p<0.05$, ** $p<0.01$, student's t-test).

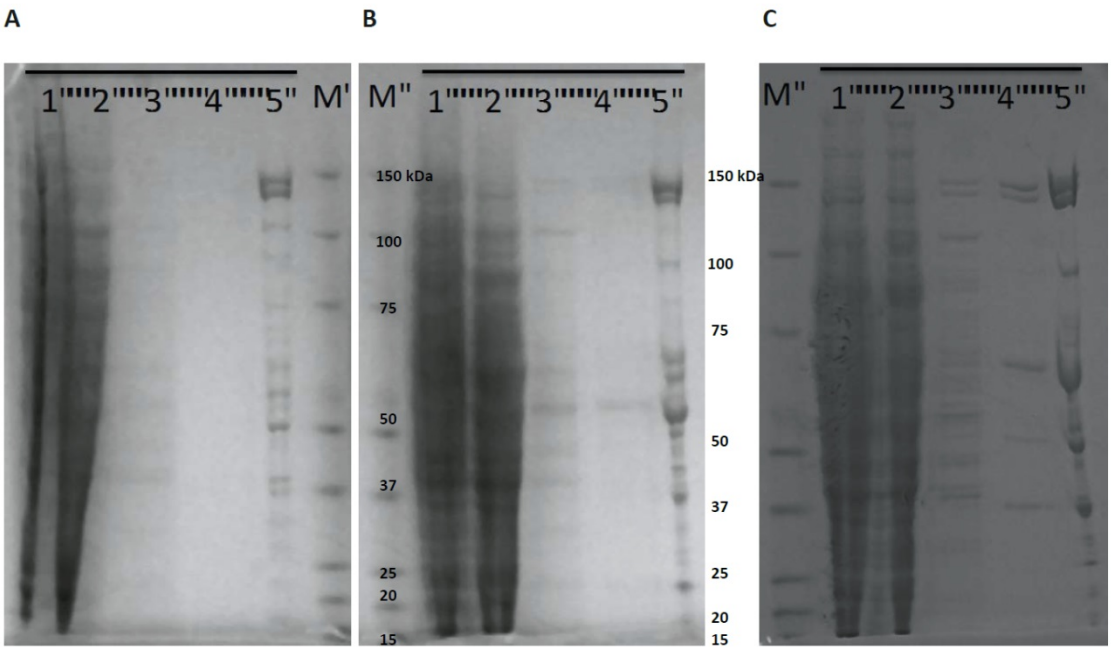

Figure S7. Samples of the purified core RNA polymerase from *M. smegmatis* strain wt (A), EMAW (B) and EMED (C) are shown on a Coomassie-stained SDS gel. The core RNA polymerase were purified as described in Materials and Methods. Lanes: 1 – cell lysate, 2 – eluate after packing column, 3 – washing, 4 – eluate before dialysis, 5 – eluate after dialysis, M – BioRad prestained marker.

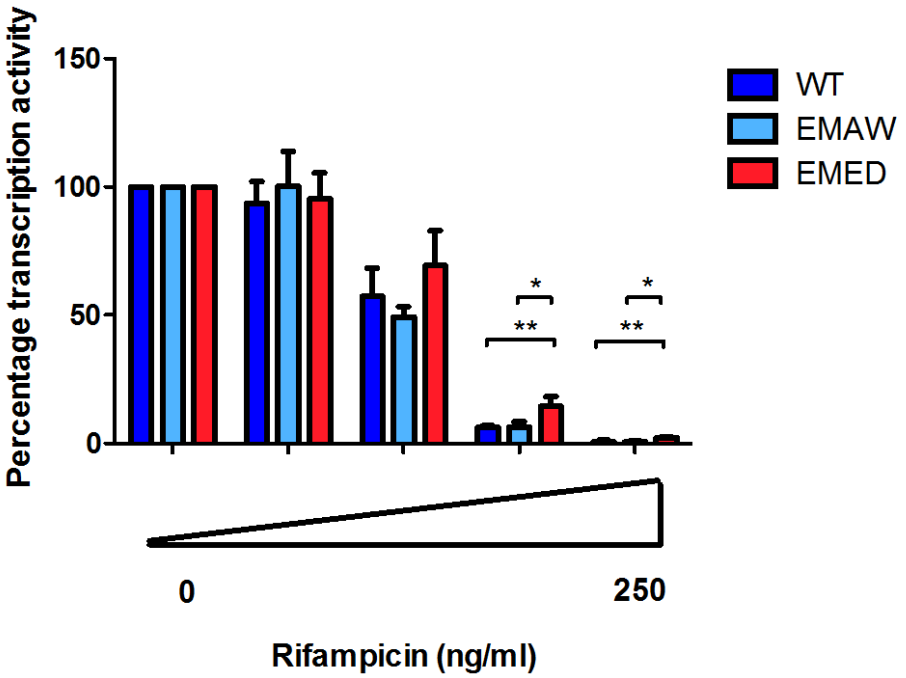

Figure S8. Relative transcription activity of RNAP purified from the three strains in varying concentrations of Rifampicin, as measured by radio-actively labelled, multi-round assay. Graph represents triplicate or quadruplicate measurements. Bands were visualised by phosphorimager, and quantified by Imagequant software. (* = $p<0.05$, ** = $p<0.01$, Student's t-test).

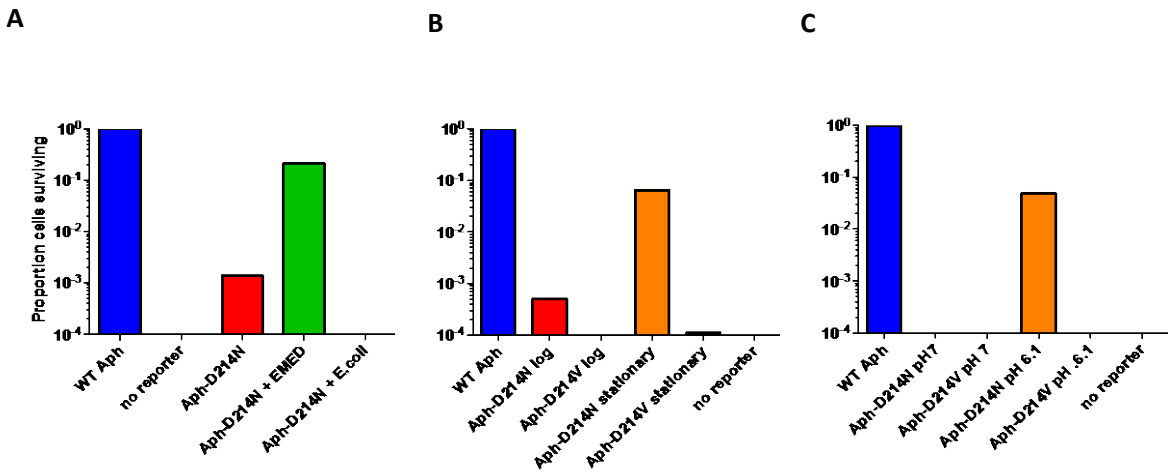

Figure S9. Measuring mycobacterial mistranslation with the gain of function antibiotic resistance enzyme, Aph. Wild-type *M. smegmatis* (A-C) or *M.* smegmatis EMED (A) were transformed with either wild-type (WT Aph), or mutated (Aph-D214N, Aph-D214V) kanamycin kinase enzyme cloned into an episomal vector. Cells were treated with kanamycin in liquid (A and B) or solid (C) medium and survivors counted. Increased survival to kanamycin represented greater rates of mistranslation. Strain *M. smegmatis* BJ1 showed enhanced survival with the Aph-D214N reporter (A) but not Aph-D214V (not shown), suggesting increased rates of Asp for Asn mistranslation. Wild-type *M. smegmatis* showed some survival in log-phase culture (A, B) representing a basal level of Asp for Asn mistranslation, which increased dramatically in stationary phase (B). Survival was not due to partial residual activity of the Aph-D214N enzyme, since transformation of the reporter into *E. coli* was not associated with increased survival to kanamycin under the same assay conditions (A). Stationary but not log phase was also associated with a small degree of Asp for Val mistranslation (measured by survival of Aph-D214V – B). Decreasing pH to 6.1 from 7 also increased mistranslation of Asp for Asn, but not Asp for Val (C).

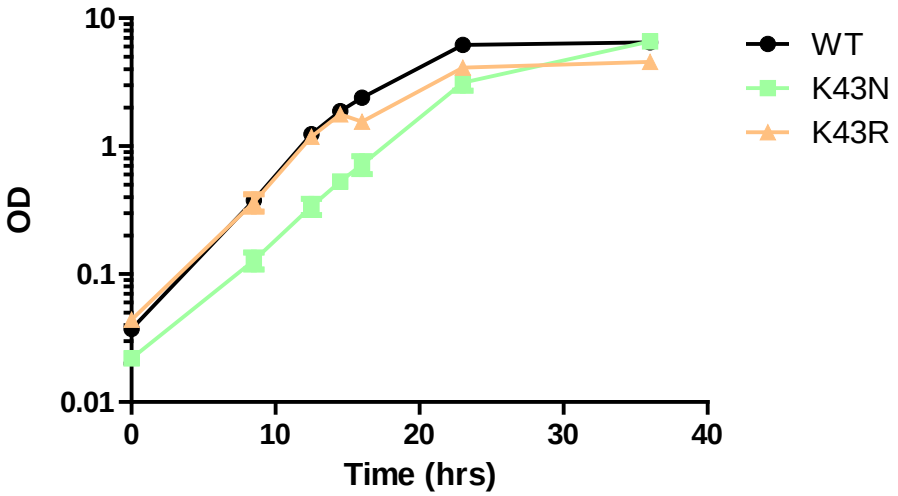

Figure S10. Growth curve of strains RpsL-K43N (K43N) and RpsL-K43R (K43R) compared with wild-type *M. smegmatis*. Cultures were performed in triplicate, error represents standard deviation.

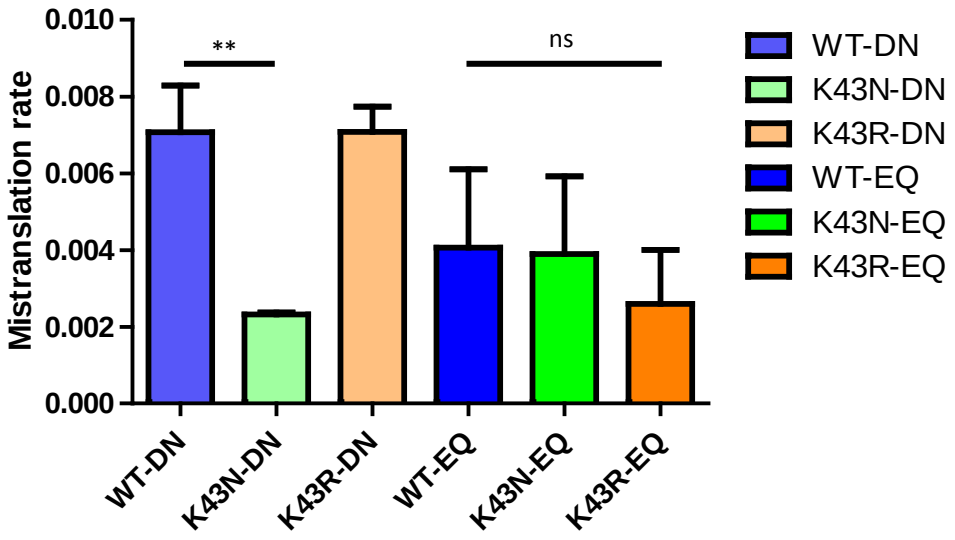

Figure S11. Mistranslation rates for strains RpsL-K43N and RpsL-K43R. K43N has significantly lower mistranslation of aspartate for asparagine (K43N-DN) than wild-type *M. smegmatis*. The mistranslation rates for strain K43R are not significantly different to wild-type mycobacteria. (**= $p<0.01$ by Student's t-test).

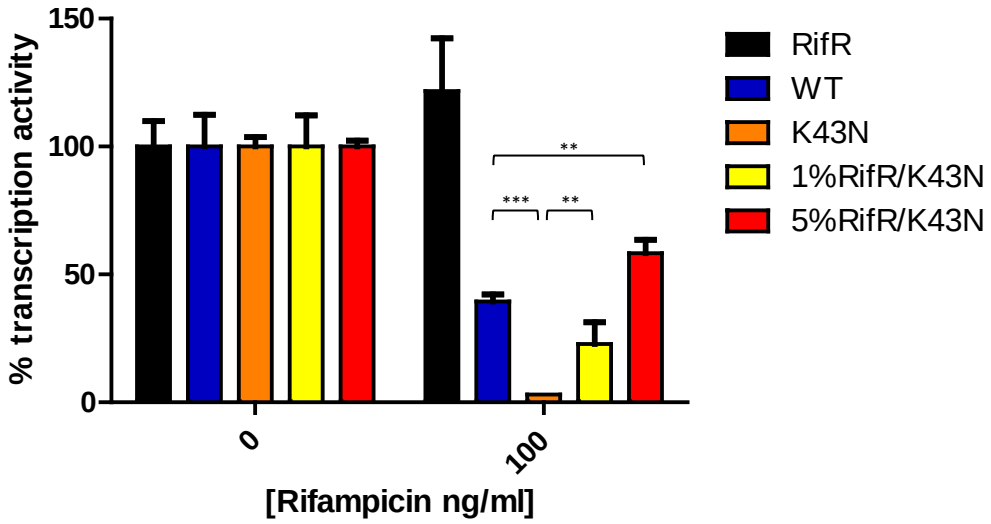

Figure S12. Modeling gain of Rifampicin resistance of RNAP by mistranslation. RNAP from WT *M. smegmatis* (WT), Rifampicin resistant *M. smegmatis* (RifR) and *M. smegmatis* – RpsL-K43N (K43N) were purified and assayed for transcription, correcting for activity. K43N RNAP was mixed with different concentrations of RifR RNAP (1% and 5% -- yellow and red bars) to measure gain of Rifampicin resistance with moderate levels of Rifampicin drug. (** $p<0.01$, *** $p<0.001$ by Student's t-test)

Strains used in this study, **Table S1**

Name	Remarks
***M. smegmatis* mc^2-155**	wild-type strain
***M. smegmatis*-EMED**	mistranslating strain: misincorporation of Glu for Gln and Asp for Asn. mc^2-155 transformed by plasmid EMED.
***M. smegmatis*-EMAW**	mistranslating strain: misincorporation of Ala for Trp. mc^2-155 transformed by plasmid EMAW.
***M. smegmatis*-RpSL-K43N**	Streptomycin resistant (K43N) strain of mc^2-155
***M. smegmatis*-RpsL-K43R**	Streptomycin resistant (K43R) strain of mc^2-155
***M. smegmatis*, P(aph)**	wild-type strain transformed with wild-type kanamycin kinase
***M. smegmatis*, P(aph-D214N)**	reporter strain transformed with mutated *aph* gene: measures Asp for Asn mistranslation
***M. smegmatis*, P(aph-D214V)**	reporter strain transformed with mutated *aph* gene: measures Asp for Val mistranslation
***M. smegmatis*, P(Ren-FF)**	reporter strain transformed with Renilla-Firefly dual luciferase construct
***M. smegmatis*, P(Ren-D120N-FF)**	reporter strain transformed with mutated Renilla-firefly dual luciferase construct: measures Asp for Asn mistranslation
***M. smegmatis*, P(Ren-D120V-FF)**	reporter strain transformed with mutated Renilla-firefly dual luciferase construct: measures Asp for Val mistranslation
***M. smegmatis*, P(Ren-E144Q-FF)**	reporter strain transformed with mutated Renilla-firefly dual luciferase construct: measures Glu for Gln mistranslation
***M. smegmatis*, P(Ren-E144V-FF)**	reporter strain transformed with mutated Renilla-firefly dual luciferase construct: measures Glu for Val mistranslation
***M. smegmatis*-EMED::P(acet)Ren-FF**	mistranslating strain transformed with Renilla-firefly dual luciferase construct integrated onto chromosome under control of an acetamide promoter

***M. smegmatis*-EMED::P(acet)Ren-D120N-FF**	mistranslating strain transformed with mutated Renilla-firefly dual luciferase construct
***M. smegmatis*-EMED::P(acet)Ren-E144Q-FF**	mistranslating strain transformed with mutated Renilla-firefly dual luciferase construct
***M. smegmatis*::*rpoC*-His_6**	strain where the wild-type *rpoC* gene is replaced by a C-terminal His_6-tagged construct (see below)
***M. smegmatis*-EMED::*rpoC*-His_6**	strain where the wild-type *rpoC* gene is replaced by a C-terminal His_6-tagged construct, transformed with plasmid EMED
***M. smegmatis*-EMAW::*rpoC*-His_6**	strain where the wild-type *rpoC* gene is replaced by a C-terminal His_6-tagged construct, transformed with plasmid EMAW
***M. smegmatis*-RpsL-K43N::*rpoC*-His_6 (K43N)**	strain where the wild-type *rpoC* gene is replaced by a C-terminal His_6-tagged construct, and then selected for the K43N mutation in *rpsL* by selection on streptomycin.
***M. smegmatis*-RpoB-H442Y::*rpoC*-*His_6* (RifR)**	Strain with his-tagged *rpoC* and subsequently selected for rifampicin resistance.

Primers used, **Table S2**

Name	Target gene	Sequence
mysA-F	*mysA* (Msmeg_2758)	aagacaccgacctggaactc
mysA-R	*mysA* (Msmeg_2758)	agcttcttcttcctcgtcctc
dnak-F	*dnaK* (Msmeg_0709)	catcgagatcgacgacaaga
dnak-R	*dnaK* (Msmeg_0709)	gatgtcctcaccgaggtagc
rbpA-F	*rbpA* (Msmeg_3858)	agccgtgagctacgagacc
rbpA-R	*rbpA* (Msmeg_3858)	cgaaaggtacgtcgaactcc
aph-D214N-F	*aph*	gggaaattaataggttgtattaacgttggacgagtcggaatcg
aph-D214N-R	*aph*	cgattccgactcgtccaacgttaatacaacctattaatttccc
aph-D214V-F	*aph*	gggaaattaataggttgtattgtcgttggacgagtcggaatcg
aph-D214V-R	*aph*	cgattccgactcgtccaacgacaatacaacctattaatttccc
Ren-D120N-F	*Renilla luc*	gatcatcttcgtgggccacaactggggcgcgtgcctggcct
Ren-D120N-R	*Renilla luc*	aggccaggcacgcgccccagttgtggcccacgaagatgatc
Ren-D120V-F	*Renilla luc*	gatcatcttcgtgggccacgtctggggcgcgtgcctggcct
Ren-D120V-R	*Renilla luc*	aggccaggcacgcgccccagacgtggcccacgaagatgatc

Ren-E144Q-F	*Renilla luc*	caaggcgatcgtccacgcccagtccgtggtcgacgtgatcg
Ren-E144Q-R	*Renilla luc*	cgatcacgtcgaccacggactgggcgtggacgatcgccttg
Ren-E144V-F	*Renilla luc*	caaggcgatcgtccacgccgtgtccgtggtcgacgtgatcg
Ren-E144V-R	*Renilla luc*	cgatcacgtcgaccacggacacggcgtggacgatcgccttg
rpoB-seq-F	*rpoB* (Msmeg_1367)	cagatccgcgtgggcctgtc
rpoB-seq-R	*rpoB* (Msmeg_1367)	cgaaccgatcagaccgatgttgg

Methods in Full

Bacterial culture and strains

Wild-type *Mycobacterium smegmatis*, strain mc^2-155 (1) was cultured in Middlebrook 7H9 medium supplemented with albumin, dextrose, catalase (ADC), glycerol and 0.05% Tween-20 unless otherwise specified. Plate culture was performed on LB-agar plates supplemented with antibiotics as indicated unless otherwise specified. For experiments performed in different pH, cells were grown in M9 minimal medium, supplemented with 0.4% glucose and 0.025% Tyloxapol (Sigma). The medium pH was buffered using HCl or NaOH. All chemicals were from Sigma unless otherwise specified.

The strains *M.smegmatis*-EMED and *M. smegmatis*-EMAW were made by transforming competent wild-type *M. smegmatis* with plasmids EMED and EMAW respectively, and plating on selective media. Reporter strains were also constructed by transformation of wild-type *M. smegmatis* with the appropriate reporter constructs.

The strains RpsL-K43N and RpsL-K43R were generated by plating *M. smegmatis* mc^2-155 onto LB-Streptomycin (10µg/ml). Streptomycin mutants were selected and the *rpsL* gene sequenced. The strains were routinely grown on 5µg/ml Streptomycin, except for the rifampicin survival experiments, when streptomycin was omitted.

Plasmids and constructs

An artificial operon of the three mycobacterial tRNAs glnU-glnT-asnT, with each tRNA flanked by 10bp of upstream and downstream sequence and flanked by PacI/EcoRV restriction sequences was constructed, with the anticodon sequence of each tRNA replaced by the sequence for gluU-gluT-aspT respectively, was synthesized by Gensript. The sequence was:

TTAATTAAGATCGGTTCCGGCCCCGTCGTCTAGCGGCCTAGGACGCCGCCCTCTG
ACGGCGGTAGCGTGGGTTCGAATCCCATCGGGGCTACAACTGCCGACTTAAGAT
TCGGTTCTGCCCCCTTCGTCTAGACGGCCTAGGACGCCGCCCTTTGAAGGCGGTA
ACGCGGGTTCGAATCCCGTAGGGGGTACCTGCGACGCATGCATAACACAGCAAG
GCCCTGTGGCGCAGTTGGTTAGCGCGCCGCCCTGTTACGGCGGAGGTCGCGGGTT
CGAGTCCCGTCAGGGTCGCCAGGACGGTGATATC

This was subcloned into vector pUVtetOR, a tetracycline regulated mycobacterial shuttle plasmid (*27*) using the PacI/EcoRV restriction sites to make plasmid EMED, and transformed into wild-type *M. smegmatis* to make strain *M. smegmatis*-EMED.

The strain *M. smegmatis*-EMAW was similarly made by synthesis of the sequence:

TTAATTAAGTGACGAAGAGGGGCCTTAGCTCAGTTGGTAGAGCACTGCCTTCCAA
AGGCAGGGGTCAGGGGTTCGAGTCCCCTAGGCTCCACAAGTGAAAATTATAA

which is the sequence of mycobacterial alaT, with the anti-codon changed to that of trpT. The sequence was cut by PacI/PsiI and subcloned into a PacI/EcoRV cut pUVtetOR vector to make plasmid EMAW, which when transformed into wild-type *M. smegmatis* made strain *M. smegmatis*-EMAW.

The *aph* kanamycin reporter was constructed from plasmid pJW3 (a kind gift from Dr. Jun-Rong Wei, Harvard School of Public Health – Genbank accession number JX456191), which has both kanamycin (*aph*) and zeocin (*ble*) markers. The *aph* gene was mutated at residue Asp214 to make the various reporters by site-directed mutagenesis (Quikchange – Stratagene), and sequences verified.
The dual-luciferase reporter was based on one used by Farabaugh and colleagues (2). The original sequence of the reporter did not express efficiently in *M. smegmatis*, so the nucleotide sequence was codon-optimised with minor modifications for mycobacterial expression and artificially synthesized (Genscript – Genbank accession number JQ606807). This was amplified in a two-step, nested PCR protocol for gateway cloning into pDONR221 (Invitrogen) according to the manufacturer's protocol. Sequence was verified, and then the construct was subcloned into pTetG by gateway cloning. Expression of the protein fusion constructs were induced by addition of 50ng/ml of anhydrotetracycline (ATc). Site-directed mutagenesis was performed by the method described by Strategene (Quikchange) according to the manufacturer's protocol, to make the various reporters. Alternatively, to measure mistranslation in strain *M. smegmatis*-EMED, where a tet-inducible system for luciferase expression could not be used, the Renilla-Firefly constructs were subcloned into the chromosomally integrating vector, pMV306, with expression under control of an acetamide promoter. Expression was induced by addition of 0.2% acetamide (final concentration) to cultures.

Antibiotic survival assays
Approximately 10^8 (rifampicin) or 10^7 (isoniazid and ciprofloxacin) bacteria were plated onto LB-agar plates containing the appropriate antibiotic and spread using sterile glass beads. Plates were wrapped in foil and incubated at 37 °C for 7 days. For liquid culture antibiotic killing/survival, cells were grown in supplemented 7H9 medium until stationary phase. Rifampicin (70-100 µg/ml) was added. Aliquots of cells were taken at various time-points, the antibiotic diluted out, and survivors plated onto non-selective medium. Survivors were counted after 5 days.

Minimum Inhibitory Concentration (MIC) measurement
Measurement of MIC in liquid culture was performed by adding 10^5 cfu bacteria into medium supplemented by doubling dilutions of antibiotic, in duplicate. The tubes were cultured until the zero drug cultures were visibly turbid. At that point, the minimum concentration of antibiotic sufficient to inhibit bacterial growth was taken as the MIC. For plate culture, ~ 10^4 cfu were plated on LB-agar plates containing different concentrations of antibiotic. The MIC_{99} was the minimum concentration of antibiotic sufficient to prevent growth of 99% of the plates bacteria (calculated from a zero antibiotic plate).

His-tagged rpoC strain construction
A recombination cassette was synthesized by GenScript, in a pUC57 plasmid containing a 200 bp region homologous to *rpoC* 3' end followed by a 6x His-tag, kanamycin resistance marker and a 200 bp region homologous to *rpoC* 3' end flanking region (see scheme below). Sequence (1330 bp):

agaacgtgatcatcggcaagctgatcccggccggtaccggtatcagccgctaccgcaacatccaggtgcagccgaccgaagaggc
ccgtgccgctgcgtacacgatcccgtcctacgaggatcagtactacagcccggacttcggccaggccaccggtgccgcggtgccgc
tggacgactacggctactcggattaccgc**CACCACCACCACCACCAC**taaGTGTCTCAAAATCTCT
GATGTTACATTGCACAAGATAAAATAATATCATCATGAACAATAAAACTGTCTGC
TTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTC
GAGGCCGCGATTAAATTCCAACatggatgctgatttatatgggtataaatgggctcgcgataatgtcgggcaatca
ggtgcgacaatctatcgcttgtatgggaagcccgatgcgccagagttgtttctgaaacatggcaaaggtagcgttgccaatgatgttaca
gatgagatggtcagactaaactggctgacggaatttatgcctcttccgaccatcaagcattttatccgtactcctgatgatgcatggttact
caccactgcgatccccggaaaaacagcattccaggtattagaagaatatcctgattcaggtgaaaatattgttgatgcgctggcagtgtt
cctgcgccggttgcattcgattcctgtttgtaattgtccttttaacagcgatcgcgtatttcgtctcgctcaggcgcaatcacgaatgaataa
cggtttggttgatgcgagtgattttgatgacgagcgtaatggctggcctgttgaacaagtctggaaagaaatgcataaacttttgccattct
caccggattcagtcgtcactcatggtgatttctcacttgataaccttatttttgacgaggggaaattaataggttgtattgatgttggacgagt
cggaatcgcagaccgataccaggatcttgccatcctatggaactgcctcggtgagttttctccttcattacagaaacggctttttcaaaaat
atggtattgataatcctgatatgaataaattgcagtttcatttgatgctcgatgagtttttctaaGCAGTAGGAAAAAGCCC
CCGGGGTGCGCAAGCGCCCCGGGGGCTTTTTCGTGTGCCGAATGTGATTACCTAG
CACTCACATTCGACGAGCGGACGGGCACGGCCGAGCCGTGCCCTGGGCGGGCTC
TCTCACCGGGCATCGCGCACGACGAGCGTGTCACGAGCGATACCGGAAGTACGG
TGCGTCTGCGCAGGCGGCGT

------ *rpoC* 3' terminal 200 bp
------ his-tag
------ stop codon
------ *aph* promoter
------ *aph* gene
------ *rpoC* flanking 200

A linear dsDNA fragment was amplified by PCR using the following primers: HisRpoC fw: AGAACGTGATCATCGGCAAG and HisRpoC rv: ACGCCGCCTGCGCAGACGCA. A *M. smegmatis* strain expressing a IVN-inducible Che9c recombinase (mc^2 pNIT Che9C::zeo^R, (3)) was transformed with 2 µg of linear His-tagged RpoC:: kan^R. The transformants were plated on 7H10 plates with 25 µg/ml kanamycin. One kan^R single recombinant was grown for 48 hours and plated for selecting the loss of pNITChe9C plasmid, in the presence of kanamycin and 10% sucrose. The clones obtained were screened for zeocin and sucrose sensitivity. A clone RH16 (zeo^S, kan^R, and $sacB^S$) was selected for further genotypic characterization by PCR using primers HisRpoC fw and HisRpoC rv, and by Western blot using an anti His-tag antibody.

Isolation of mycobacterial RNA polymerase (RNAP)

Mycobacterial RNAP was isolated from strains engineered with a His_6-tag to the C-terminus of *rpoC* (β'). Cells were harvested at late exponential growth phase, resuspended in lysis buffer (200 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol, Complete™ Protease Inhibitor Cocktail – Roche) and lysed by French Press. The cleared lysate was bound to a pre-equilibrated TALON cobalt resin (Clontech) for 4-5 hours at 4° C. The resin was washed in 20 volumes of wash buffer (50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole) and the holo-enzyme was eluted in elution buffer (50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 400 mM NaCl, 500 mM imidazole). The enzyme

was dialyzed against storage buffer (200 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol). The final concentration of the enzymes was determined by Bradford assay (Bio-Rad) and the identity of the RNAP subunits verified by SDS-PAGE (Figure 2 supplement 1) and mass spectrometry.

***In-vitro* transcription assay**

The activity isolated RNAP was measured by two methods, radio-labelled *in vitro* transcription and via rolling circle transcription assay using the Kool™ NC-45™ RNAP Activity & Inhibitor Screening kit (EpiCentre) in the absence and presence of rifampicin.

Kool™ NC-45™ assay: The supplier's protocol for end-point detection using RiboGreen was followed. The assays were performed on a Fluoroskan Ascent FL (THERMO Electron Corporation) microplate reader using 96-well black-clear bottom plates (BD Falcon). The reaction was initially incubated at 37 °C for 10 minutes in the absence of Kool NC-45 template and NTP solution to ensure the binding of RNAP and rifampicin. Then the template was added and the reaction was initiated upon the addition of the NTPs. After 1 hour, reaction was quenched in TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA). The amount of RNA synthesized was measured by the addition of 1:200 diluted RiboGreen. The excitation and emission wavelengths for RiboGreen were 480 nm and 520 nm, respectively.

Radio-labelled *in-vitro* transcription assay: Holoenzymes (formed by pre-incubating 50 nM RNAPs with 100 nM σ^A at 37°C for 10 min) were incubated in the absence or in the presence of rifampicin for 5 min. The assay was started by mixing these complexes with 5 nM linear DNA template (bears the P_R' promoter and terminator t_R' and in vitro transcription results in an 192 nt transcript) in transcription buffer (40 mM Tris-HCl, pH 7.9, 75 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM EDTA, 10 U murine RNase inhibitor, 100 µg/ml BSA) containing 200 µM GTP, ATP and CTP and 25 µM UTP (supplemented with 1 µCi/µl [α-^{32}P]-UTP). Reactions were stopped after 15 mins by adding formamide loading buffer and heating to 95°C, and RNA transcripts with similar activity were loaded and electrophoresed on 6% denaturing polyacrylamide sequencing gels. Bands were visualized by PhosphorImager and the data analyzed using ImageQuant software.

Kanamycin-survival mistranslation assay

This gain of function assay was used to semi-quantitatively measure mycobacterial mistranslation. Cells were transformed with the *aph* reporter constructs. The wild-type *aph* gene product conferred resistance to the antibiotic kanamycin and served as a positive control. Structural and biochemical studies suggested that the metal-binding residue, Asp214, was absolutely required for enzyme function (4, 5) and mutation to any other residue, including asparagine, rendered the enzyme entirely inactive. For measuring increase in mistranslation in stationary phase compared with log phase, cells were grown to stationary phase, then sub-cultured 1/20 to log phase. After allowing growth for 1 hour to allow accommodation to the new culture conditions, cells were split into +/- kanamycin (200 µg/ml) and treated for 3 hours, at 37 °C with shaking. Survivors were counted by taking

aliquots of bacteria and diluting out antibiotic before plating on non-selective medium. The proportion surviving kanamycin treatment was calculated and plotted. For the pH experiments, cells were spotted in $\log_{10}$ dilutions onto M9-glucose-agar plates, where the M9 had been buffered to different pH by addition of HCl. Cells were either plated on non-selective media or media containing 10 µg/ml of kanamycin. The proportion surviving antibiotic treatment compared with non-selective plates were counted and plotted.

Dual-luciferase assay

This assay was based on that developed by Farabaugh and colleagues (2). The sequence of the protein fusion of Renilla and firefly luciferase genes was codon-optimised for expression in *M. smegmatis* and synthesised synthetically (Genscript – Genbank JQ606807). Using structural data suggesting the critical residues for Renilla luciferase activity (6), mutations in the Renilla gene were made by site-directed mutagenesis. The resulting sequences were subcloned into a tetracycline-inducible expression shuttle plasmid, pTetG (7) using gateway cloning (Invitrogen) and transformed in competent *M. smegmatis* cells. Cells were grown to either mid-log or stationary phase. Approximately $5x10^8$ cells were harvested, pelleted, and lysed by the passive lysis buffer supplied with the dual-luciferase assay kit (Promega). Luciferase activity was measured by the dual luciferase assay kit according to the manufacturer's instructions using a Fluoroskan Ascent FL luminometer using integration times of 1 second for measurements. Mistranslation rates were calculated as per Farabaugh *et al* (2).

Quantitative Real-time PCR

RNA was isolated from 25 ml early log phase cultures in triplicate using standard techniques and diluted to 500 ng/µl. Reverse transcription reaction of 1 µg of total RNA from each sample was performed using a High Capacity cDNA Reverse Transcription Kit (Invitrogen) with random primers according to the manufacturer's protocol. Aliquots of cDNA (25 ng) were used as templates for quantitative real-time PCR with SYBR green PCR Master Mix (Applied Biosystems) containing a 1x final concentration of real-time master mix and 10 pmol each of the forward and reverse primers (see below) in a StepOnePlus Real Time PCR System (Applied Biosystems). Amplification of *mysA* cDNA served as an internal control and analyses were carried out for triplicate samples of RNA. Changes in the expression levels were calculated by the relative (ΔC_T) quantification method. The differences in the expression patterns were analyzed for significance by using Student's paired *t* test.

References cited in the Supplementary Methods

1. Snapper SB, Melton RE, Mustafa S, Kieser T, & Jacobs WR, Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. (Translated from eng) *Mol Microbiol* 4(11):1911-1919 (in eng).

2. Kramer EB & Farabaugh PJ (2007) The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. (Translated from eng) *RNA* 13(1):87-96 (in eng).
3. Wei JR, *et al*. (2011) Depletion of antibiotic targets has widely varying effects on growth. (Translated from eng) *Proc Natl Acad Sci U S A* 108(10):4176-4181 (in eng).
4. Boehr DD, Thompson PR, & Wright GD (2001) Molecular mechanism of aminoglycoside antibiotic kinase APH(3')-IIIa: roles of conserved active site residues. (Translated from eng) *J Biol Chem* 276(26):23929-23936 (in eng).
5. Hon WC, *et al*. (1997) Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. (Translated from eng) *Cell* 89(6):887-895 (in eng).
6. Loening AM, Fenn TD, & Gambhir SS (2007) Crystal structures of the luciferase and green fluorescent protein from Renilla reniformis. (Translated from eng) *J Mol Biol* 374(4):1017-1028 (in eng).
7. Siegrist MS, *et al*. (2009) Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. (Translated from eng) *Proc Natl Acad Sci U S A* 106(44):18792-18797 (in eng).