Supporting Information

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SI Materials and Methods

Strains, Plasmids, and Reagents. All *Escherichia coli* liquid cultures were grown in M9 minimal medium supplemented with either 0.2% glucose or 0.1% glycerol at 37 °C, and the toxin was expressed from an arabinose-inducible promoter in pBAD33-*mazF-mt6* ("mt" refers to *Mycobacterium tuberculosis*). All *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* liquid cultures were grown in 7H9 Middlebrook medium supplemented with 0.05% Tween 80, 0.5% bovine albumin, 0.2% glucose, and 0.085% NaCl, and the toxin was expressed from a tetracycline-inducible promoter in pMC1s-*mazF-mt6*. The working concentrations of ampicillin and chloramphenicol in *E. coli* were 100 and 25 μg/mL, respectively, whereas the concentration of kanamycin in *M. smegmatis* and *M. tuberculosis* was 25 μg/mL. The *mazF-mt6* (Rv1102c locus) and *mazE-mt6* (Rv1103c locus) genes were PCR-amplified with $^{5'}$NdeI/BamHI$^{3'}$ ends from *M. tuberculosis* strain H37Rv genomic DNA to create pET-21c-*mazF-mt6* (1) and pET-28a-*mazE-mt6*, respectively. To create pBAD33-*mazF-mt6*, the pET-21c-*mazF-mt6* plasmid was digested with XbaI and HindIII to include the highly efficient T7 phage ribosome binding site, and the resulting fragment was cloned into pBAD33 (1). To create pColdTF-FT-*mazF-mt6* and pMC1s-*mazF-mt6*, pET-21c-*mazF-mt6* was digested with NdeI and BamHI, and the resulting fragment was cloned into pColdTF-FT (2) and pMC1s, respectively. The *ompF* (outer membrane porin F) and *fhuF* genes were PCR-amplified from *E. coli* cultures with $^{5'}$NdeI/BamHI$^{3'}$ ends to create pET-28a-*ompF* and pET-21c-*fhuF*, respectively. Clones were confirmed by DNA sequence analysis.

RNA Isolation. Total RNA was isolated from *E. coli* strain BW25113Δ6 harboring pBAD33-*mazF-mt6*, from *M. smegmatis* strain mc^{2}155 harboring pMC1s or strain *rnj102* (RNase J deletion strain) harboring pMC1s-*mazF-mt6*, and from *M. tuberculosis* strain H37Rv, all grown to midlogarithmic phase. When *E. coli* cultures reached an OD_{600nm} value between 0.3 and 0.4, the culture was split into equal portions, and arabinose was added to one portion to a final concentration of 0.2%. When *M. smegmatis* cultures reached an OD_{600nm} value between 0.1 and 0.2, the culture was split into equal portions, and anhydrotetracycline was added to one portion to a final concentration of 100 ng/mL. RNA was extracted from *E. coli* using the hot phenol method as previously described (3) and from *M. smegmatis* and *M. tuberculosis* by resuspending cell pellets in TRIzol Reagent (Invitrogen). For *M. smegmatis* and *M. tuberculosis*, RNA was treated with TURBO DNase (Invitrogen) and further purified with an RNeasy cleanup kit (Qiagen).

Analysis of Steady-State rRNA Levels. Total RNA from *E. coli* or *M. smegmatis* (3–20 μg) was loaded onto a 1.2% (wt/vol) agarose, 2% (vol/vol) formaldehyde gel and visualized by adding ethidium bromide and exposing to UV light. The radiolabeled DNA used for Northern analysis was generated either with a random-primed DNA labeling kit (Roche) from PCR products amplified from 23S and 16S rRNA genes or with oligonucleotides that were 5′-labeled with [^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs).

In Vivo Primer Extension Analysis. Total RNA from *E. coli* or *M. smegmatis* (25 or 2.25 μg, respectively) was used in primer extension reactions using a Sequenase version 2.0 DNA sequencing kit (USB) as previously described (3).

Preparation of Recombinant MazF-mt6 and MazE-mt6. pColdTF-FT-*mazF-mt6* and pET-28a-*mazE-mt6* BL21(DE3) transformants were used to inoculate 1 L of M9 liquid medium and grown to an OD_{600nm} of 0.35 and 0.64, respectively. For pColdTF-FT-*mazF-mt6* BL21(DE3) transformants, the culture was transferred to a 15 °C water bath and incubated for 30 min before the protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and expressed for 3 h. pET-28a-*mazE-mt6* BL21(DE3) transformants were induced with 1 mM IPTG and expressed for 3.5 h. Cells were disrupted by sonication, and extracts were purified by nickel-nitrilotriacetic acid affinity chromatography (Qiagen). His_6-TF-FLAG-MazF-mt6 was cleaved with Factor Xa (New England Biolabs) to excise trigger factor from the target protein. FLAG-MazF-mt6 was further purified over an anti-FLAG resin to remove His_6-TF.

RNA Cleavage Activity of MazF-mt6. Total RNA from *M. smegmatis* strain mc^{2}155 harboring pMC1s or *M. tuberculosis* strain H37Rv (4.15 or 3.0 μg, respectively) was used. For antitoxin inhibition of RNA cleavage, 2.0 μM MazE-mt6 was preincubated with 1.0 μM MazF-mt6 for 15 min at room temperature before the RNA substrate was added. RNA was incubated with or without 0.0625, 0.125, 0.25, 0.5, or 1.0 μM MazF-mt6 or 2.0 μM MazE-mt6 in 10 mM Tris-HCl (pH 7.8) for 15 min at 37 °C.

In Vitro Primer Extension Analysis of *E. coli*, *M. smegmatis*, and *M. tuberculosis* Total RNA. For mycobacterial experiments, antitoxin rescue was performed as described above, and the same buffer and amounts of total RNA as above were incubated with or without 1.0 μM MazF-mt6 or 2.0 μM MazE-mt6 for 15 min at 37 °C. For *E. coli* experiments, 0.8 μg total RNA was used and cleavage reactions were incubated with or without 1.0 μM MazF-mt6 and with 0, 3, or 10.75 mM $MgCl_2$ for 30 min at 37 °C. Primer extension analysis was performed as above. Oligonucleotide Myco23S-2 was used for both *M. smegmatis* and *M. tuberculosis* 23S rRNA, because these rRNAs are 100% identical at that 29-nt region. The *M. smegmatis* 23S rRNA PCR product was also used to create a sequencing ladder for *M. tuberculosis*, because the sequence of *M. smegmatis* and *M. tuberculosis* 23S rRNAs are 100% identical for a >160-nt region upstream of the Myco23S-2 primer.

Ribosome Cleavage Activity of MazF-mt6. Functional *E. coli* 70S monoribosomes from a PURExpress in vitro protein synthesis kit (New England Biolabs) were incubated at a final concentration of 0.6 μM with or without 6.0, 12.0, or 24.0 μM MazF-mt6 or 12.0 μM MazE-mt6. For antitoxin inhibition of rRNA cleavage, 12.0 μM MazE-mt6 was preincubated with 6.0 μM MazF-mt6 for 15 min at room temperature before the ribosome substrate was added. To assess cleavage of rRNA in 50S and 30S ribosomal subunits vs. rRNA in 70S monosomes, the Mg^{2+} concentration was either diluted to a final concentration of 0.75 mM to dissociate 70S monosomes completely or maintained above 10 mM at every step to keep ribosomes intact to a final concentration of 10.75 mM Mg^{2+}. Cleavage reactions were incubated with 1 U/μL RNase inhibitor (New England Biolabs) in 10 mM Tris-HCl (pH 7.8) for 30 min or 4 h at 37 °C. rRNA was extracted twice with phenol-chloroform-isoamyl alcohol, extracted once with chloroform, and precipitated with ethanol. Samples were separated by 1.2% (wt/vol) agarose 2% (vol/vol) formaldehyde or 6% (wt/vol) polyacrylamide 7 M urea gel electrophoresis and visualized by

staining with ethidium bromide or SYBR Gold (Invitrogen), respectively.

Protein Synthesis Activity of MazF-mt6–Cleaved Ribosomes. Protein synthesis reactions were performed in a PURExpress Δribosome in vitro protein synthesis kit (New England Biolabs) according to the manufacturer's guidelines. As the manufacturer suggests, total reaction volumes were increased by 20% to allow more experimental flexibility. To give MazF-mt6 an opportunity to cleave rRNA in 50S subunits, the Mg^{2+} concentration in some reactions was diluted to 3.0 mM to dissociate ~70% of 70S monosomes (4). The subunits (30 pmol) were then incubated with or without 120 pmol MazF-mt6 for 60 min at 37 °C, and the Mg^{2+} concentration was increased to 13.6 mM to facilitate ribosome reassociation. In the remaining reactions, the Mg^{2+} concentration was maintained above 10 mM at every step. For all reactions, the final Mg^{2+} concentration was the recommended 13.0 mM for efficient transcription and translation. Reactions were incubated with 0.8 U/μL RNase inhibitor, 0.33 μCi/μL of [^{35}S]-Met, and 10 ng/μL of pET-28a-*ompF* or pET-21c-*fhuF*. After adding plasmid DNA to initiate transcription and translation, reactions were incubated for 2 h at 37 °C. Samples were separated by 17.5% (wt/vol) SDS/PAGE and visualized by autoradiography.

RT-PCR of mRNA from in Vitro Protein Synthesis. Transcription reactions were performed as for protein synthesis reactions but without [^{35}S]-Met and with or without 10 ng/μL of pET-28a-*ompF* or pET-21c-*fhuF*. For all transcription reactions, the Mg^{2+} concentration was lowered to 3.0 mM and raised to 13.6 mM as for protein synthesis reactions. RNA was treated with 1 U (0.067 U/μL) of TURBO DNA-free DNase (Invitrogen) for 30 min at 37 °C according to the suppliers' recommendations, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. RT was performed with 20 μg of RNA, 5 U/μL of avian myeloblastoma virus reverse transcriptase (New England Biolabs), and 10 U/μL of RNase inhibitor for 60 min at 42 °C. The resulting cDNA was used as a template for PCR, and the reactions were run on a 1% agarose gel and visualized by staining with ethidium bromide.

Ribosome Profile Analysis. Polyribosomes were isolated as described previously (3). For each of the three replicates, an equal amount of total RNA—from 8 to 13 OD_{260nm} units (320–520 μg)—was layered onto a 5–40% (wt/vol) continuous sucrose gradient in polysome profile buffer and centrifuged at 106,000 × *g* for 4 h at 4 °C. Gradients were fractionated and analyzed with continuous monitoring at 280 nm. From the resulting profiles, we determined the area of each peak and estimated the percentage of dissociated 50S and 30S subunits by dividing the area of the 50S+30S subunit peaks by the total area of 50S+30S+70S peaks.

Growth Profiles of *M. smegmatis* and *M. tuberculosis* Cultures Expressing MazF-mt6. MazF-mt6 was expressed in all *M. smegmatis* strain mc^2155 and *M. tuberculosis* strain H37Rv liquid cultures from a tetracycline-inducible promoter in pMC1s-*mazF-mt6*. *M. smegmatis* growth was compared with uninduced cultures harboring pMC1s-*mazF-mt6*, whereas *M. tuberculosis* growth was compared with cultures harboring pMC1s. MazF-mt6 was expressed in *M. smegmatis* cultures with 100 ng/mL anhydrotetracycline but induced in *M. tuberculosis* cultures with 200 ng/mL anhydrotetracycline. For *M. tuberculosis* cultures, anhydrotetracycline was added every 48 h to maintain the concentration between 12.5 and 200 ng/mL. *M. tuberculosis* cultures were inoculated with single colonies from a fresh transformation and supplemented with 30% spent culture supernatant. Spent culture supernatant was prepared from a late logarithmic phase *M. tuberculosis* H37Rv culture (i.e., OD_{600nm} of 0.8) by centrifugation at 2,000 × *g* for 10 min at 4 °C followed by filtration through a 0.22-μm filter (Millipore).

Statistical Analysis of UUMHU Frequency in *M. tuberculosis* Genes. All 4,095 annotated noncoding RNA and protein-coding genes from *M. tuberculosis* strain H37RV were retrieved from the TubercuList Web site (http://tuberculist.epfl.ch/) on August 17, 2012. These genes were divided into 11 functional categories from the genome annotation (5–7). Six genes—Rv0298, Rv0299, Rv0909, Rv0910, Rv2653c, and Rv2654c—that Cox and coworkers found to be novel functional TA systems (8) were moved into the *virulence, detoxification, and adaptation* category. The Rv2653c and Rv2654c loci were removed from the *insertion sequences and phages* group, whereas the other four genes were removed from the *conserved hypothetical protein* category. The nucleotide composition of each gene was calculated. The probability, *p*, of the degenerate MazF-mt6 cleavage motif ***UUMHU*** (in which M = C or A and H = C, U, or A; i.e., UUCCU, UUCAU, UUCUU, UUACU, UUAAU, or UUAUU) appearing anywhere in an *M. tuberculosis* gene is P = (percentage of U)^3 × [(percentage of C) + (percentage of A)] × [1 − (percentage of G)]. Let L be the length of the gene. Then the expected number, E, of motifs in the gene is $E = p(L - 4)$. Let K be the actual number of motifs in the gene. Then the probability, P, of having K or more motifs in the gene is:

$$P = 1 - \sum_{i=0}^{K-1} p^i (1-p)^{L-4-i} \frac{(L-4)!}{i!(L-4-i)!}$$

A gene with a very small P value suggests that it may have evolved to be susceptible to cleavage by MazF-mt6. Conversely, a gene with a very large P value suggests that it may have evolved to eliminate the motif from its sequence.

1. Zhu L, et al. (2006) Characterization of mRNA interferases from *Mycobacterium tuberculosis*. *J Biol Chem* 281(27):18638–18643.
2. Rothenbacher FP, et al. (2012) *Clostridium difficile* MazF toxin exhibits selective, not global, mRNA cleavage. *J Bacteriol* 194(13):3464–3474.
3. Sharp JD, et al. (2012) Growth and translation inhibition through sequence-specific RNA binding by *Mycobacterium tuberculosis* VapC toxin. *J Biol Chem* 287(16): 12835–12847.
4. van Duin J, van Dieijen G, van Knippenberg PH, Bosch L (1970) Different species of 70S ribosomes of *Escherichia coli* and their dissociation into subunits. *Eur J Biochem* 17(3): 433–440.
5. Cole ST, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393(6685):537–544.
6. Camus JC, Pryor MJ, Médigue C, Cole ST (2002) Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. *Microbiology* 148(Pt 10):2967–2973.
7. Lew JM, Kapopoulou A, Jones LM, Cole ST (2011) TubercuList—10 years after. *Tuberculosis (Edinb)* 91(1):1–7.
8. Ramage HR, Connolly LE, Cox JS (2009) Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: Implications for pathogenesis, stress responses, and evolution. *PLoS Genet* 5(12):e1000767.

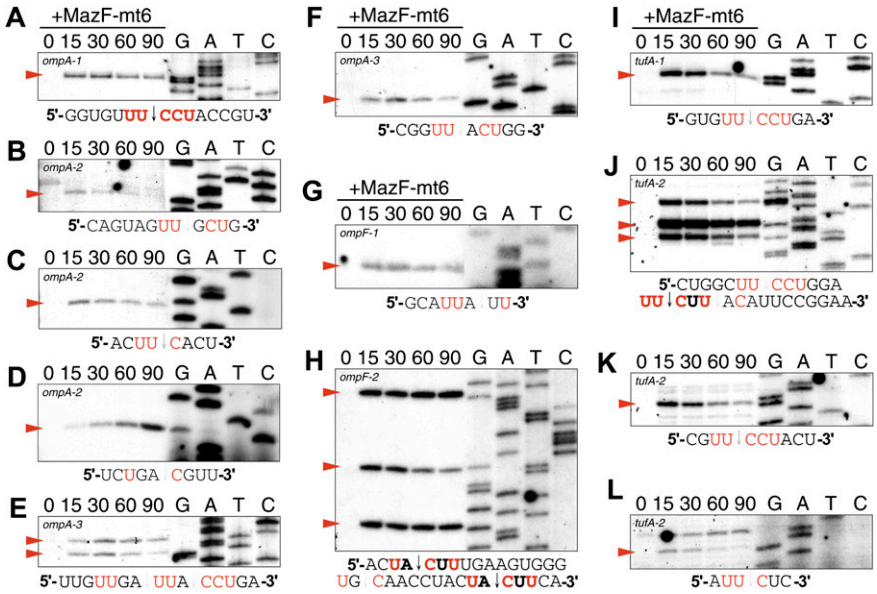

Fig. S1. MazF-mt6 expressed in *E. coli* cleaves *ompA*, *ompF*, and *tufA* mRNAs at UUCCU sequences. Primer extension analysis of sites in *ompA* (*A*–*F*), *ompF* (*G* and *H*), and *tufA* (*I*–*L*) mRNAs cleaved upon MazF-mt6 expression in *E. coli* strain BW25113Δ6. Numbered lanes indicate time of MazF-mt6 induction (in min), whereas G, A, T, and C lanes denote DNA sequencing ladders. Oligonucleotides used for sequencing and primer extension reactions are listed in the top left corner inside each box. The red arrows on the left indicate the mRNA cleavage site, and the RNA sequence surrounding each cleavage site is listed below. Bold text and black arrows indicate major mRNA cleavage sites, for which the increase in accumulated cleavage products is coupled with a corresponding decrease in the full-length transcript. Regular black text and gray arrows indicate minor cleavage sites, for which the intensity of the cleavage product is much weaker than the full-length transcript, and gray text and arrows are rare cleavage sites, for which the cleavage product is much weaker than minor products.

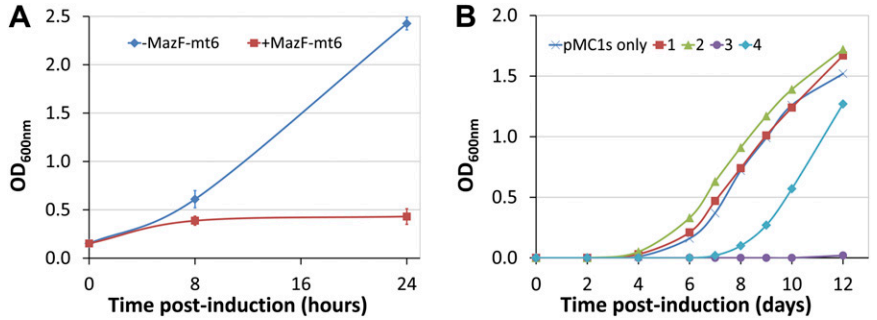

Fig. S2. Expression of MazF-mt6 arrests growth of *M. smegmatis* strain mc^{2}155 and *M. tuberculosis* strain H37Rv. (*A*) Growth profile for MazF-mt6 uninduced (blue diamonds) or induced (red squares) in *M. smegmatis* mc^{2}155 containing pMC1s-*mazF-mt6*. Data points represent the average of four independent experiments; error bars represent the SD. (*B*) Growth profile for pMC1s (dark blue ×) or four independent cultures of pMC1s-*mazF-mt6* [cultures 1 (red squares), 2 (green triangles), 3 (purple circles), or 4 (blue diamonds)] in *M. tuberculosis* H37Rv. Although expression of MazF-mt6 did not arrest growth in two of four *M. tuberculosis* cultures, its expression potently arrested growth in the other two cultures for at least 1 wk (colonies 3 and 4). This result suggests that MazF-mt6 is subject to strong selective pressure for intragenic or extragenic suppressor formation, both before induction (colonies 1 and 2) or upon prolonged induction (colony 4).

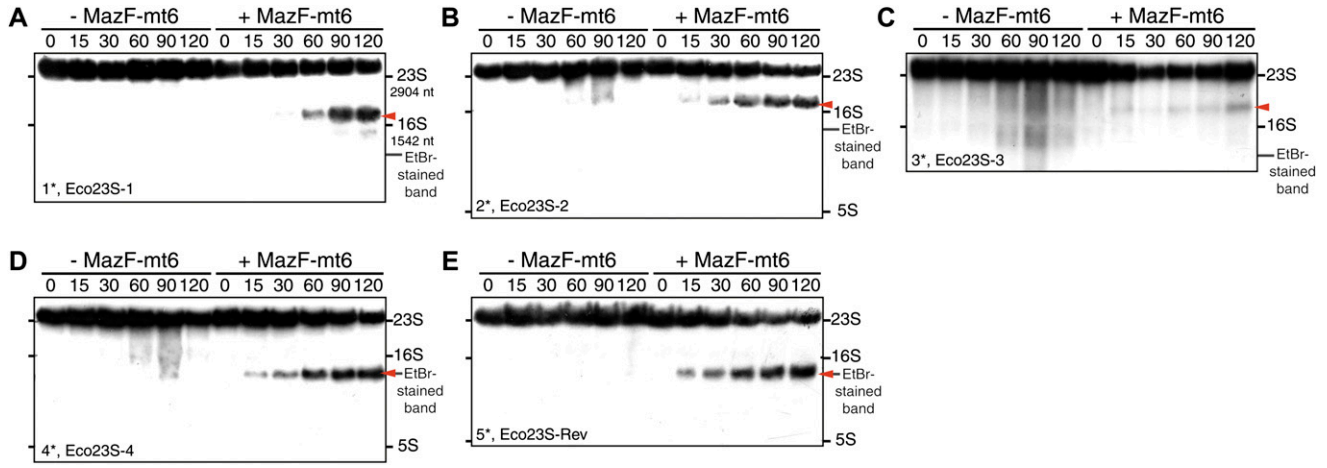

Fig. S3. Pattern of MazF-mt6–mediated cleavage in 23S rRNA is consistent with cleavage at ^{1939}UUCCU^{1943}. (*A*–*E*) Northern analysis of total RNA from *E. coli* cells expressing MazF-mt6 (*Right*) for the times shown (in min), relative to the uninduced control (*Left*). The radiolabeled DNA fragments were generated from oligonucleotides that were 5′-labeled with [^{32}P]-ATP. These oligonucleotides were designed to detect potential cleavage products at each region between the three UUCCU positions in 23S rRNA, as illustrated in Fig. 2*E*. Northern blots using each of the following oligonucleotides, from 5′ to 3′, were (*A*) *1** or Eco23S-1, (*B*) *2** or Eco23S-2, (*C*) *3** or Eco23S-3, (*D*) *4** or Eco23S-4, and (*E*) *5** or Eco23S-Rev, with each oligonucleotide name at the bottom left corner inside each box as identified in Fig. 2*E* and Table S4, respectively. All oligonucleotides upstream of ^{1939}UUCCU^{1943} (*A*–*C*) detect a "large" 23S rRNA cleavage fragment that migrates above 16S rRNA, whereas oligonucleotides downstream of that UUCCU site (*D* and *E*) detect the "small" 23S rRNA cleavage product that migrates below 16S rRNA and is easily seen on ethidium bromide-stained agarose gels (EtBr-stained band). Red arrows indicate 23S rRNA cleavage products.

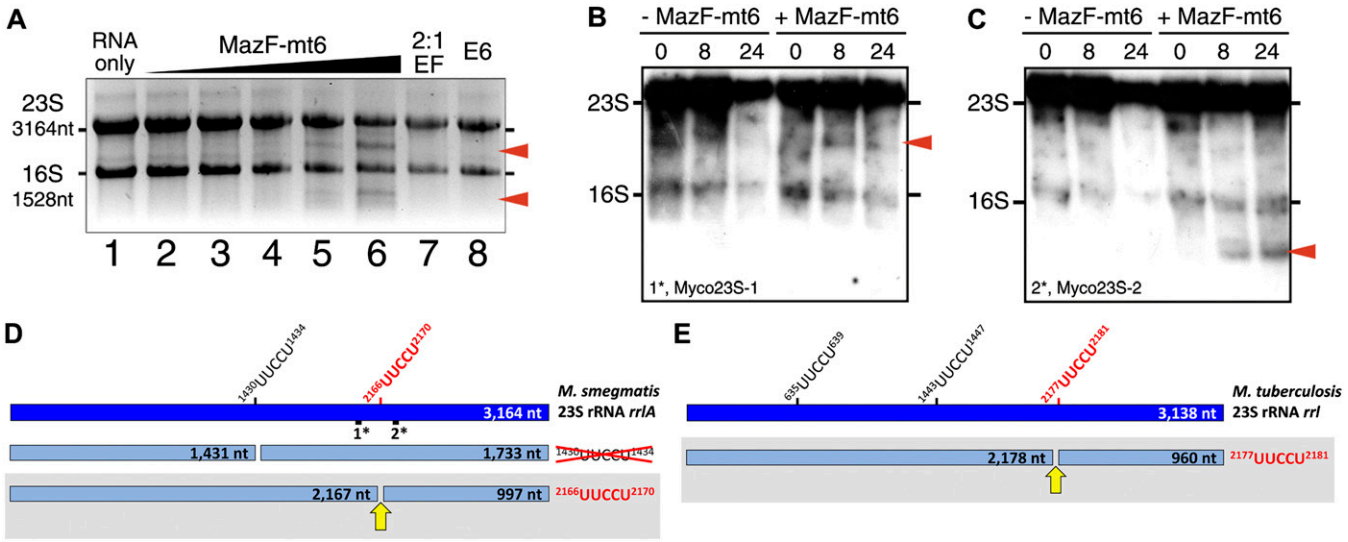

Fig. S4. MazF-mt6 also cleaves 23S rRNA in *M. smegmatis* strain Δ*rnj* and *M. tuberculosis*. (*A*) Incubation of MazF-mt6 with *M. smegmatis* total RNA generates cleavage products (red arrows) similar to those in *E. coli* (Fig. 2*A*). RNA visualized by ethidium bromide staining. A 2:1 molar ratio of antitoxin to toxin can prevent cleavage by MazF-mt6 (labeled 2:1 E:F, lane 7), whereas MazE-mt6 alone does not cleave RNA (E6, lane 8). (*B* and *C*) Northern analysis of 3 μg of total RNA/lane from *M. smegmatis* Δ*rnj* cells expressing MazF-mt6 (*Right*) for the times shown (in h), relative to the uninduced control (*Left*). The radiolabeled DNA fragments were generated from oligonucleotides that were 5′-labeled with [^{32}P]-ATP. These oligonucleotides were designed to detect potential cleavage fragments on either the 5′ or 3′ side of the expected cleavage site ^{2166}UUCCU^{2170} in *M. smegmatis* 23S rRNA, as illustrated in *D*. Northern blots using each of the following oligonucleotides, from 5′ to 3′, were (*B*) *1** or Myco23S-1 and (*C*) *2** or Myco23S-2, with each oligonucleotide name as identified in *D* and Table S4, respectively. Oligonucleotide names are at the bottom left corner inside each box. The oligonucleotide upstream of ^{2166}UUCCU^{2170} (*B*) detects the "large" 23S rRNA cleavage fragment that migrates above 16S rRNA, whereas the oligonucleotide downstream of that site (*C*) detects the "small" 23S rRNA cleavage product that migrates below 16S rRNA. Red arrows indicate 23S rRNA cleavage products. (*D*) Scale schematic of *M. smegmatis* 23S rRNA indicating the positions of the two UUCCU sites and potential cleavage products. Top blue bar, 23S rRNA. 1* and 2* represent the positions of the radioactive oligonucleotides used for individual Northern blots in *B* and *C*, respectively, to estimate the location of the MazF-mt6 cleavage site. The two lower rows of light blue bars represent the possible cleavage products for each UUCCU. Our Northern data coincided with the cleavage products represented in the bottom row of light blue bars boxed in gray, with the cleavage site shown by a yellow arrow. (*E*) Scale schematic of *M. tuberculosis* 23S rRNA indicating the positions of the three UUCCU sites. Our primer extension data (Fig. 3*D*) confirmed ^{2177}UUCCU^{2181} is the single site cleaved by MazF-mt6.

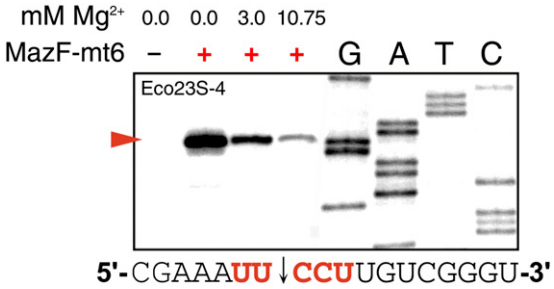

Fig. S5. MazF-mt6 cleaves 23S rRNA at 3 mM and 10.75 mM Mg^{2+}. Primer extension analysis of the ^{1939}UUCCU^{1944} site in 23S rRNA cleaved upon incubation of MazF-mt6 with *E. coli* total RNA in vitro under various Mg^{2+} concentrations. Numbered lanes indicate concentration of Mg^{2+} (mM), whereas G, A, T, and C lanes denote DNA sequencing ladders. Oligonucleotide used for sequencing and primer extension reactions is listed in the top left corner. Red arrow on the left indicates the rRNA cleavage site, and the RNA sequence surrounding the cleavage site is listed below.

Table S1. Top 20 MazF-mt6–susceptible genes in *M. tuberculosis* with degenerate UUMHU motif, sorted by *P* value

Locus	Length (nt)	Expected motif count	Actual motif count	*P* value	Gene	Cat #	Functional category	Product
Rv0355c	9,903	19.03	38	**0.000082**	*PPE8*	6	PE/PPE	PPE family protein PPE8
Rv1917c	4,380	13.84	28	**0.000525**	*PPE34*	6	PE/PPE	PPE family protein PPE34
Rv3841	546	1.02	6	**0.000640**	*bfrB*	7	Intermediate metabolism and respiration	Possible bacterioferritin BfrB
Rv3176c	957	1.50	7	**0.000931**	*mesT*	0	Virulence, detoxification, adaptation	Probable epoxide hydrolase MesT
Rv0279c	2,514	0.76	5	**0.001131**	*PE_PGRS4*	6	PE/PPE	PE-PGRS family protein PE_PGRS4
Rv0878c	1,332	2.03	8	**0.001189**	*PPE13*	6	PE/PPE	PPE family protein PPE13
Rv3157	1,662	4.84	13	**0.001493**	*nuoM*	7	Intermediate metabolism and respiration	Probable NADH dehydrogenase I NuoK, chain M
Rv3669	519	1.76	7	**0.002201**	*Rv3669*	3	Cell wall & cell processes	Probable conserved transmembrane protein
Rv2501c	1,965	2.79	9	**0.002364**	*accA1*	1	Lipid metabolism	Probable acetyl-/propionyl-CoA carboxylase, α subunit
Rv0492A	330	0.91	5	**0.002397**	*Rv0492A*	10	Conserved hypothetical proteins	Hypothetical protein
Rv0297	1,776	0.55	4	**0.002407**	*PE_PGRS5*	6	PE/PPE	PE-PGRS family protein PE_PGRS5
Rv0267	1,392	4.11	11	**0.003432**	*narU*	3	Cell wall and cell processes	Probable integral membrane nitrite extrusion protein
Rv3159c	1,773	3.60	10	**0.003961**	*PPE53*	6	PE/PPE	PPE family protein PPE53
Rv2193	612	3.05	9	**0.004115**	*ctaE*	7	Intermediate metabolism and respiration	Probable cytochrome C oxidase (subunit III) CtaE
Rv1625c	1,332	2.49	8	**0.004136**	*cya*	7	Intermediate metabolism and respiration	Membrane-anchored adenylyl cyclase Cya
Rv0278c	2,874	1.03	5	**0.004139**	*PE_PGRS3*	6	PE/PPE	PE-PGRS family protein PE_PGRS3
Rv0456B	174	0.65	4	**0.004456**	*mazE2*	0	Virulence, detoxification, adaptation	Possible antitoxin MazE-mt2
Rv2707	975	3.13	9	**0.004863**	*Rv2707*	10	Conserved hypothetical proteins	Probable conserved transmembrane protein
Rv1091	2,562	0.70	4	**0.005608**	*PE_PGRS22*	6	PE/PPE	PE-PGRS family protein PE_PGRS22
Rv0304c	6,615	15.00	26	**0.006128**	*PPE5*	6	PE/PPE	PPE family protein PPE5

The expected number of MazF-mt6 *UUMHU* (i.e., UUCCU, UUCAU, UUCUU, UUACU, UUAAU, or UUAUU) cleavage motifs for each of the 4,095 annotated noncoding RNA and protein-coding genes from *M. tuberculosis* strain H37Rv was calculated according to the length and nucleotide composition of each gene (*SI Materials and Methods*). The probability of the motif appearing anywhere in any gene was determined by comparing the actual number to the expected number of motifs. The Cat. # and Functional category columns refer to the 11 categories from the annotation of the *M. tuberculosis* H37Rv genome sequence, and color-coding for each functional category is similar to that in the TubercuList Web site (http://tuberculist.epfl.ch/). The data were sorted by lowest to highest *P* value. *P* values <0.001 are shaded pink with bold black text, whereas P values between 0.001 and 0.01 are shaded orange with bold black text.

Table S2. Top 20 MazF-mt6–susceptible genes in *M. tuberculosis* with degenerate UUMHU motif, sorted by number of actual motifs

Locus	Length (nt)	Expected motif count	Actual motif count	*P* value	Gene	Cat #	Functional category	Product
Rv0355c	9,903	19.03	38	**0.000082**	PPE8	6	PE/PPE	PPE family protein PPE8
Rv3350c	11,151	22.31	29	0.098299	PPE56	6	PE/PPE	PPE family protein PE56
Rv3343c	7,572	24.56	29	0.208947	PPE54	6	PE/PPE	PPE family protein PPE54
Rv1917c	4,380	13.84	28	**0.000525**	PPE34	6	PE/PPE	PPE family protein PPE34
Rv0304c	6,615	15.00	26	**0.006128**	PPE5	6	PE/PPE	PPE family protein PPE5
Rv3347c	9,474	21.27	20	0.637919	PPE55	6	PE/PPE	PPE family protein PPE55
Rv0405	4,209	15.19	16	0.451214	pks6	1	Lipid metabolism	Probable membrane-bound polyketide synthase Pks6
Rv3239c	3,147	7.19	15	**0.007097**	Rv3239c	3	Cell wall and cell processes	Probable conserved transmembrane transport protein
Rv2380c	5,049	8.40	15	0.024986	mbtE	1	Lipid metabolism	Peptide synthetase MbtE
Rv0101	7,539	15.05	15	0.539631	nrp	1	Lipid metabolism	Probable peptide synthetase Nrp
Rv2048c	12,456	23.25	15	0.972223	pks12	1	Lipid metabolism	Polyketide synthase Pks12
Rv2940c	6,336	10.65	14	0.186882	mas	1	Lipid metabolism	Probable multifunctional mycocerosic acid synthase
Rv3157	1,662	4.84	13	**0.001493**	nuoM	7	Intermediate metabolism and respiration	Probable NADH dehydrogenase I NuoK, chain M
Rv0987	2,568	15.15	13	0.745257	Rv0987	3	Cell wall and cell processes	Probable adhesion component ABC transporter
Rv1623c	1,458	5.20	12	**0.007212**	cydA	7	Intermediate metabolism and respiration	Probable cytochrome D ubiquinol oxidase, subunit I
Rv3795	3,297	6.45	12	0.032027	embB	3	Cell wall and cell processes	Integral membrane indolylacetylinositol arabinosyltransferase EmbB
Rv3063	2,277	6.72	12	0.041298	cstA	0	Virulence, detoxification, adaptation	Probable carbon starvation protein a homolog CstA
Rv3043c	1,722	7.45	12	0.075820	ctaD	7	Intermediate metabolism and respiration	Probable cytochrome *c* oxidase polypeptide I CtaD
Rv2946c	4,851	8.39	12	0.142118	pks1	1	Lipid metabolism	Probable polyketide synthase Pks1
Rv2524c	9,210	11.05	12	0.426907	fas	1	Lipid metabolism	Probable fatty acid synthase Fas

The expected number of MazF-mt6 *UUMHU* (i.e., UUCCU, UUCAU, UUCUU, UUACU, UUAAU, or UUAUU) cleavage motifs for each of the 4,095 annotated noncoding RNA and protein-coding genes from *M. tuberculosis* strain H37Rv was calculated based on the length and nucleotide composition of each gene (See Materials and Methods). The probability of the motif appearing anywhere in any gene was determined by comparing the actual number to the expected number of motifs. The *Cat. #* and *Functional category* columns refer to the 11 categories from the annotation of the *M. tuberculosis* H37Rv genome sequence, and color-coding for each functional category is similar to that in the TubercuList Web site (http://tuberculist.epfl.ch/). The data were sorted from highest to lowest number of actual motifs. *P* values <0.001 are shaded pink with bold black text, whereas *P* values between 0.001 and 0.01 are shaded orange with bold black text, and *P* values between 0.01 and 0.05 are shaded yellow with regular black text.

Table S3. Strains and plasmids used in this study

Bacterial strain/plasmid	Purpose	Genotype	Source
Bacterial strain, *E. coli*			
DH5α	Cloning	F^- ø80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rK^-, mK^+) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1	Takara Bio Inc.
BL21(DE3)	EXP	F^- *ompT* $hsdS_\beta(r_\beta\text{-}m_\beta)$ *dcm gal* (DE3) tonA	Novagen
BW25113Δ6	TX, NB, PE	$lacI^q$ $rrnB_{T14}$ Δ*lac*-Z_{WJ16} *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$ Δ*chpBIK* Δ*dinJ-yafQ* Δ*hipBA* Δ*mazEF* Δ*relBE* Δ*yefM-yoeB*	(1)
Bacterial strain, *M. smegmatis*			
mc^2155	TX, NB, PE, CA	Wild-type	ATCC
rnj102	NB, PE	Δ*rnj* (MSMEG_2685 locus)	(2)
Bacterial strain, *M. tuberculosis*			
H37Rv	TX, CA	Wild-type	ATCC
Plasmid, *E. coli*			
pET-21c	In vitro translation; cloning		Novagen
pET-28a	EXP; in vitro translation		Novagen
pBAD33	TX		ATCC
pColdTF-FT	EXP		pColdTF, Takaro Bio Inc.; (3)
pET-21c-*mazF-mt6*	Cloning		(4)
pBAD33-*mazF-mt6*	TX		(4)
pColdTF-FT-*mazF-mt6*	EXP		This study
pET-28a-*mazE-mt6*	EXP		This study
pET-28a-*ompF*	In vitro translation		This study
pET-21c-*fhuF*	In vitro translation		This study
Plasmid, *E. coli*-mycobacteria shuttle vectors			
pMC1s	TX		ATCC
pMC1s-*mazF-mt6*	TX		This study

EXP, protein expression; TX, toxicity; NB, Northern blot; PE, primer extension; CA, in vitro RNA cleavage assay.

1. Prysak MH, et al. (2009) Bacterial toxin YafQ is an endoribonuclease that associates with the ribosome and blocks translation elongation through sequence-specific and frame-dependent mRNA cleavage. *Mol Microbiol* 71(5):1071–1087.
2. Taverniti V, Forti F, Ghisotti D, Putzer H (2011) *Mycobacterium smegmatis* RNase J is a 5′-3′ exo-/endoribonuclease and both RNase J and RNase E are involved in ribosomal RNA maturation. *Mol Microbiol* 82(5):1260–1276.
3. Rothenbacher FP, et al. (2012) *Clostridium difficile* MazF toxin exhibits selective, not global, mRNA cleavage. *J Bacteriol* 194(13):3464–3474.
4. Zhu L, et al. (2006) Characterization of mRNA interferases from *Mycobacterium tuberculosis*. *J Biol Chem* 281(27):18638–18643.

Table S4. Oligonucleotides used in this study

Primer name	Gene	Purpose	DNA sequence
E. coli genes			
ompA-1	*ompA*	PE	5′-CAGGGTTGCTTTGTTGAAGTTGAACAG-3′
ompA-2	*ompA*	PE	5′-CAGAGCAGCCTGACCTTC-3′
ompA-3	*ompA*	PE	5′-TTAAGCCTGCGGCTGAGTTACAACGTCTTTGATACC-3′
ompF-1	*ompF*	PE	5′-AAACCAAGACGGGCATAGGTC-3′
ompF-2	*ompF*	PE	5′-TTAGAACTGGTAAACGATACCCACAGC-3′
ompF-Fwd1	*ompF*	PCR	5′-ATGATGAAGCGCAATATTCTGGCAGTG-3′
ompF-Fwd2	*ompF*	RT-PCR	5′-CTTTGGTATCGTTGGTGCTTATGGTGC-3′
ompF-Rev	*ompF*	RT, PCR	5′-TTAGAACTGGTAAACGATACCCACAGC-3′
tufA-1	*tufA*	PE	5′-GGGAAGTCGTACTGAGACAG-3′
tufA-2	*tufA*	PE	5′-CAACTTTGATGATACCGCGTTCTACACG-3′
Eco23S-Fwd	23S rRNA	PCR	5′-GGTTAAGCGACTAAGCGTACACGG-3′
Eco23S-Rev	23S rRNA	PCR, NB	5′-AAGGTTAAGCCTCACGGTTCATTAGTACC-3′
Eco23S-1	23S rRNA	NB	5′-CGTCCTTCATCGCCTCTGACTG-3′
Eco23S-2	23S rRNA	NB	5′-CTGTTTCCCATCGACTACGCCTTTC-3′
Eco23S-3	23S rRNA	NB	5′-CCGTATACGTCCACTTTCGTGTTTGC-3′
Eco23S-4	23S rRNA	NB, PE	5′-CACTGCATCTTCACAGCGAGTTCAATTTC-3′
fhuF-Fwd1	*fhuF*	PCR	5′-GGAATTC**CATATG**GCCTATCGTTCCGCACC-3′
fhuF-Rev1	*fhuF*	PCR	5′-CG**GAATTC**ATTTCAGCGTACAATCGCCACATTG-3′
fhuF-Fwd2	*fhuF*	RT-PCR	5′-CACTATGGGCACAATGGTATATCGGC-3′
fhuF-Rev2	*fhuF*	RT, PCR	5′-GATAACGCTGGCAGCAAGTGCG-3′
Mycobacteria genes			
Msmeg23S-Fwd	23S rRNA	PCR	5′-TTGTAAGTGTTTAAGGGCGCATGGTG-3′
Msmeg23S-Rev	23S rRNA	PCR	5′-GGTGCGTCAATGTTTTCTTCTTACAACGATTATG-3′
Myco23S -1	23S rRNA	NB	5′-CGTATACATCGTCTTGCGACTTCGC-3′
Myco23S -2	23S rRNA	NB, PE	5′-CGAGCATCTTTACTCGTAGTGCAATTTCG-3′
Rv1103c-Fwd	*mazE-mt6*	PCR	5′-GGAATTC**CATATG**TACCTACCCTGGGGGGTCG-3′
Rv1103c-Rev	*mazE-mt6*	PCR	5′-CG**GGATCC**TCAGTCGATGTCGAGGGCGGTAC-3′
Rv1102c-Fwd	*mazF-mt6*	PCR	5′-GGAATTC**CATATG**CGACCTATCCACATCGC-3′
Rv1102c-Rev	*mazF-mt6*	PCR	5′-CG**GGATCC**CTATGCCACCACCCAATCGAGG-3′

NdeI (**CATATG**), EcoRI (**GAATTC**), and BamHI (**GGATCC**) restriction sites within oligonucleotide DNA sequences are **bolded** and underlined. NB, Northern blot; PE, primer extension.