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Supplementary Information for

Mycobacterial HflX is a ribosome splitting factor that mediates antibiotic resistance

Paulami Rudra[1,3], Kelley R. Hurst-Hess[1,3], Katherine L. Cotton, Andrea Partida-Miranda, and Pallavi Ghosh[1, 2*]

Corresponding author : Pallavi Ghosh
Email: Pallavi.Ghosh@health.ny.gov

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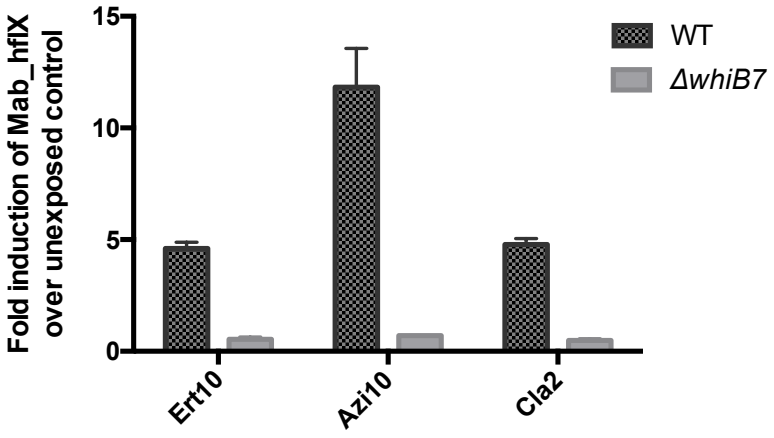


Figure S1: Antibiotic induced expression of *Mab_hflX* is WhiB7 dependent. Wild type *M. abscessus* and the *⊿Mab whiB7* strains were grown to A_{600} of 0.7, exposed to 20μg/mL of erythromycin, 10μg/mL of azithromycin or 2μg/mL of clarithromycin for 3 hours, and the amount of *Mab_hflX* transcript was determined by qPCR and plotted as fold induction over an unexposed control. Data represents mean ± SD, n=3. *SigA* was used as an internal normalization control.

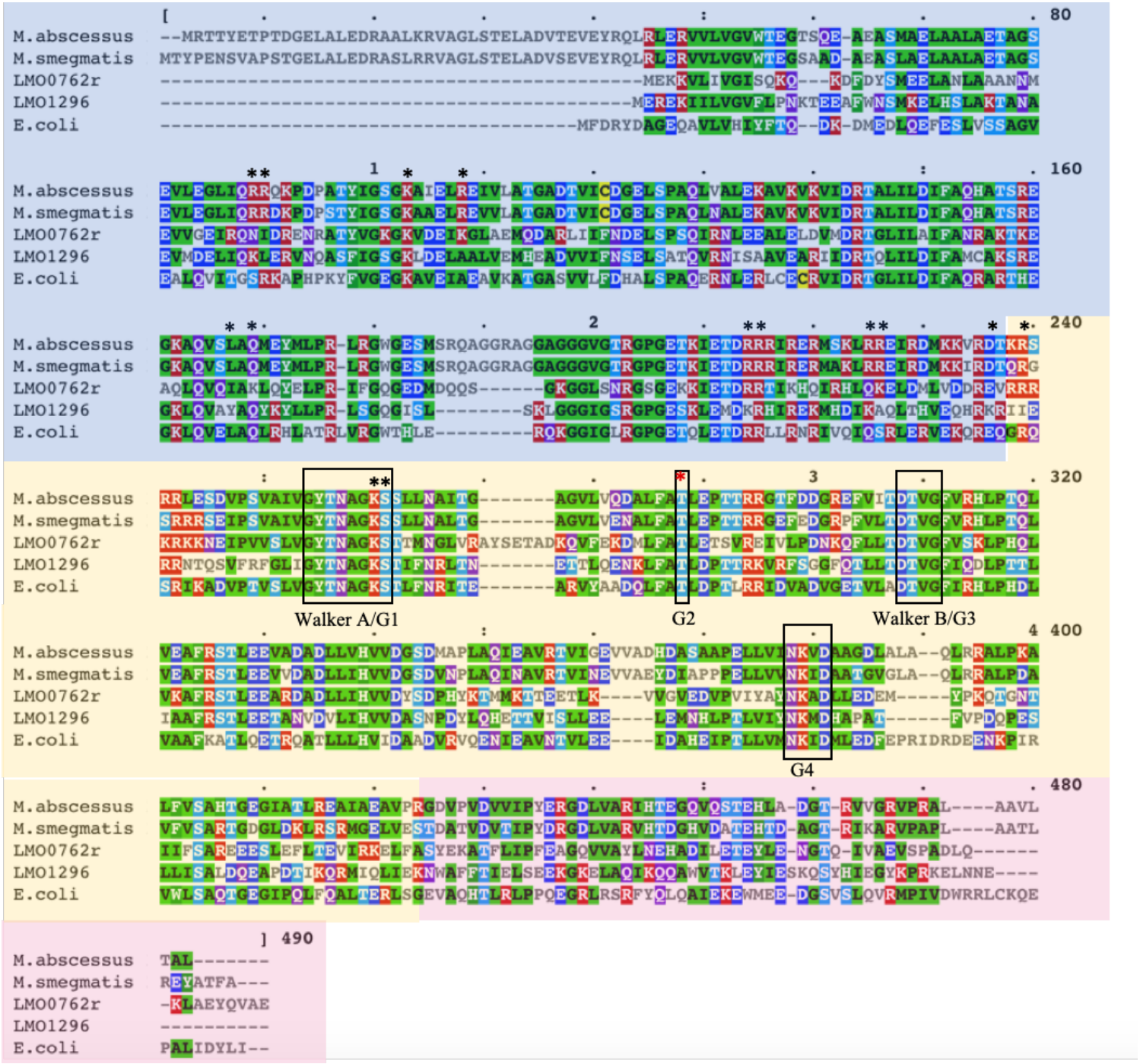


Figure S2: Sequence comparison of HflX from *M. abscessus, M. smegmatis*, *E. coli* and *L. monocytogenes.* Multiple sequence analysis was performed using CLUSTALW. The N-terminal, GTPase and C-terminal domains are indicated in blue, yellow and pink shading. Conserved P-loop GTPase domains G1-G4 are boxed. Location of point mutations created in this study are indicated using asterisks (*). None of the point mutants created besides K258A/S259A were found to be defective in conferring antibiotic resistance.

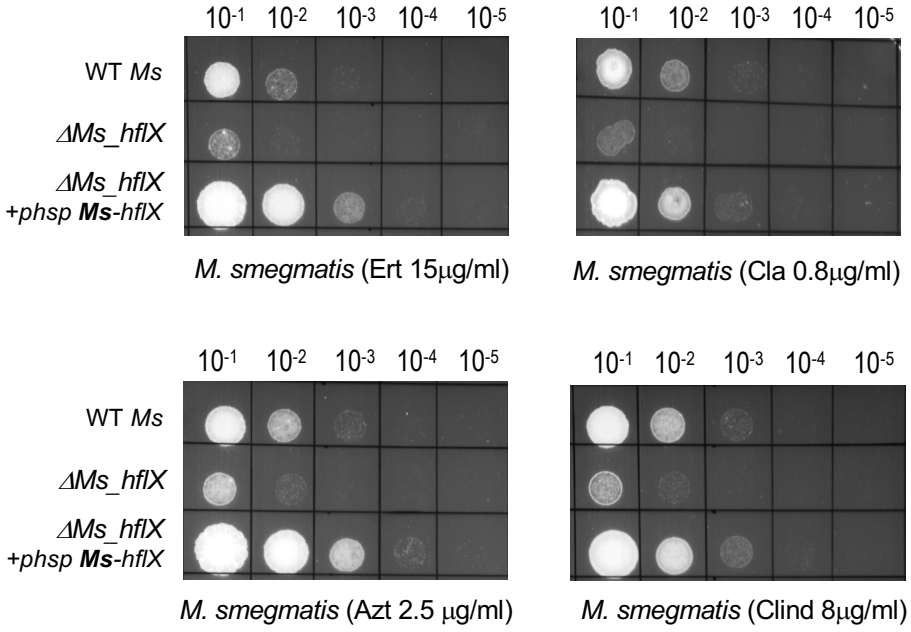


Figure S3: Deletion of *M. smegmatis hflX* confers macrolide-lincosamide sensitivity. Ten-fold serial dilutions of *M. smegmatis mc²155, ΔMs_hflX* and the complementing strain were grown to A_{600} of 0.7 and spotted on Middlebrook 7H10 ADC containing indicated concentrations of antibiotics.

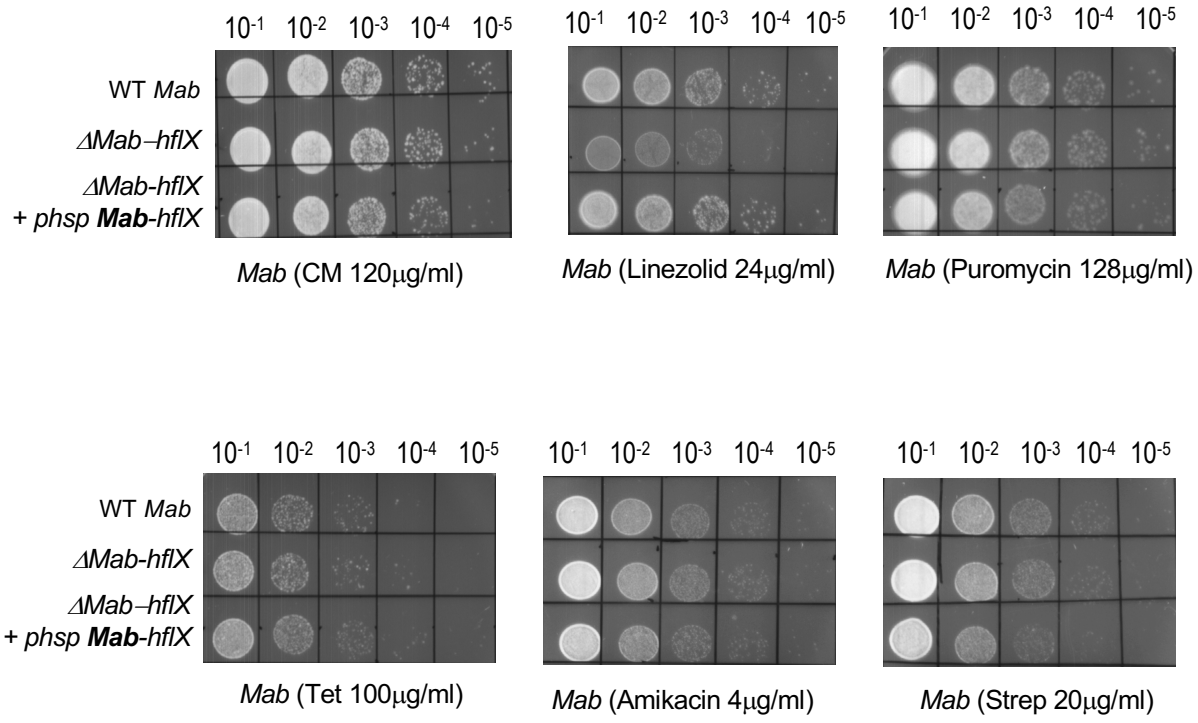
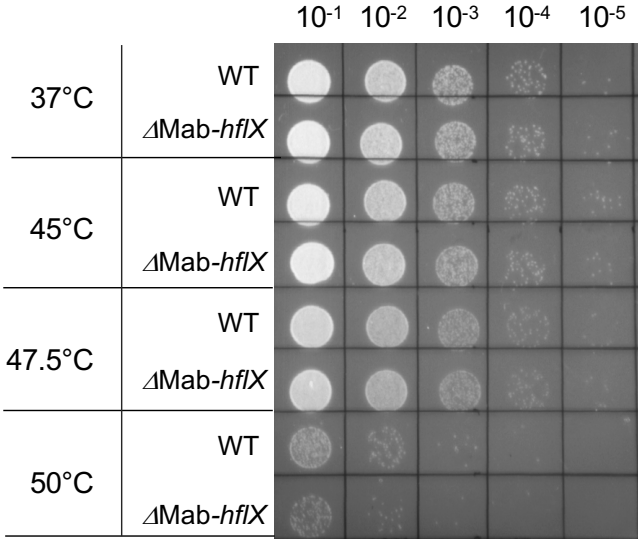


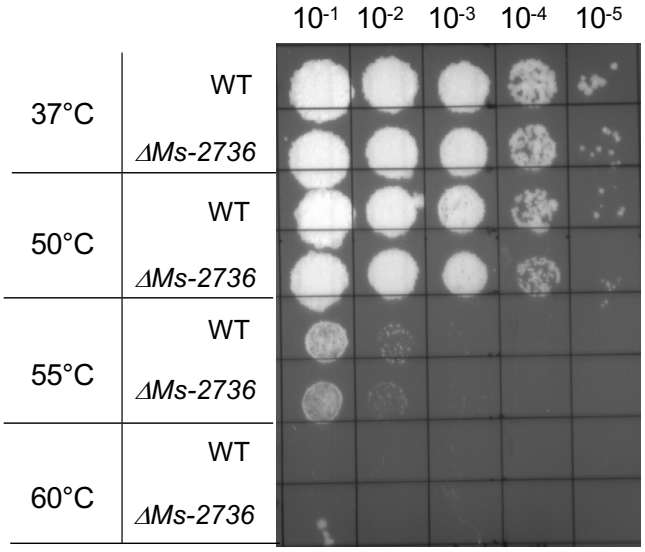
Figure S4. Sensitivity of *ΔMab_hflX* to ribosome targeting antibiotics. Ten-fold serial dilutions of *M. abscessus* ATCC 19977, *ΔMab_hflX* and the complementing strain were grown to A_{600} of 0.7 and spotted on Middlebrook 7H10 OADC containing indicated concentrations of antibiotics.

a



M. abscessus

b



M. smegmatis

Figure S5: Growth of *ΔhflX* mutants at elevated temperatures. a-b) Wild type and *ΔhflX* strains of *M. abscessus* ATCC and *M. smegmatis* were grown to an A_{600} of 0.4 at 37°C followed by exposure to elevated temperatures as indicated for a period of 2 hours. Viable bacteria were enumerated by spotting 10-fold serial dilutions on Middlebrook 7H10 ADC/OADC.

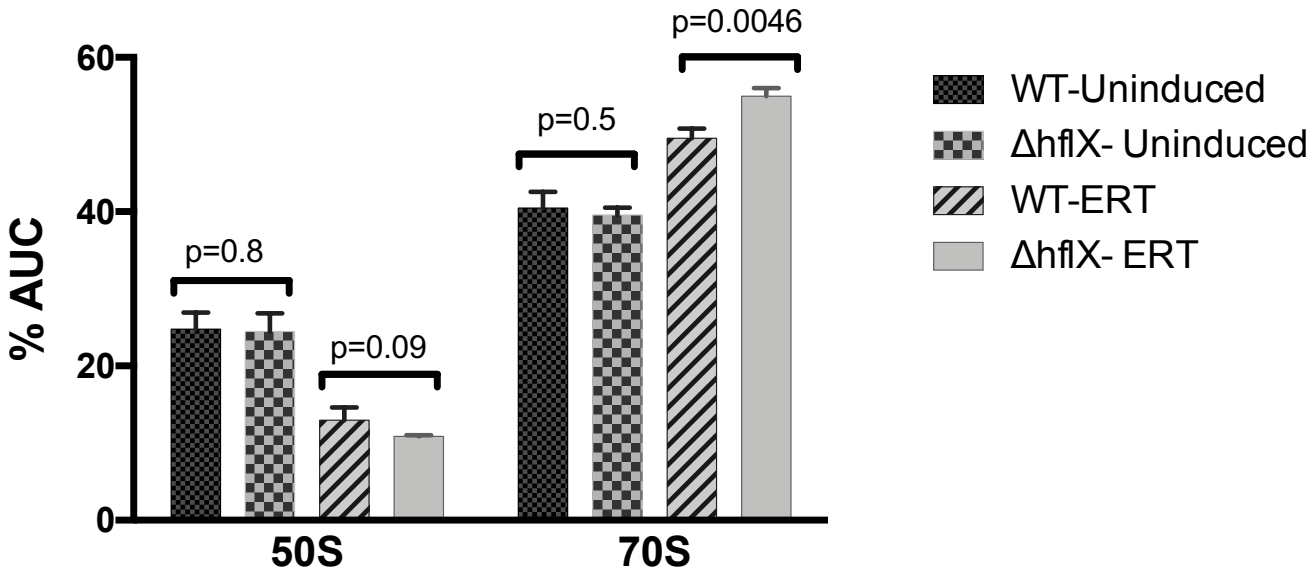


Figure S6: Area under the curve of polysome profile fractions obtained from WT and ΔhflX strains : % Area under the curve (%AUC) of 50S and 70S fractions in polysome profiles obtained from untreated and ERT-treated WT and *ΔhflX* bacteria **(Fig 3c)** were calculated using Peak Chart (v. 2.08), Brandel Inc. Data represents mean ± SD, n=3, and p values of WT and *ΔhflX* samples are indicated.

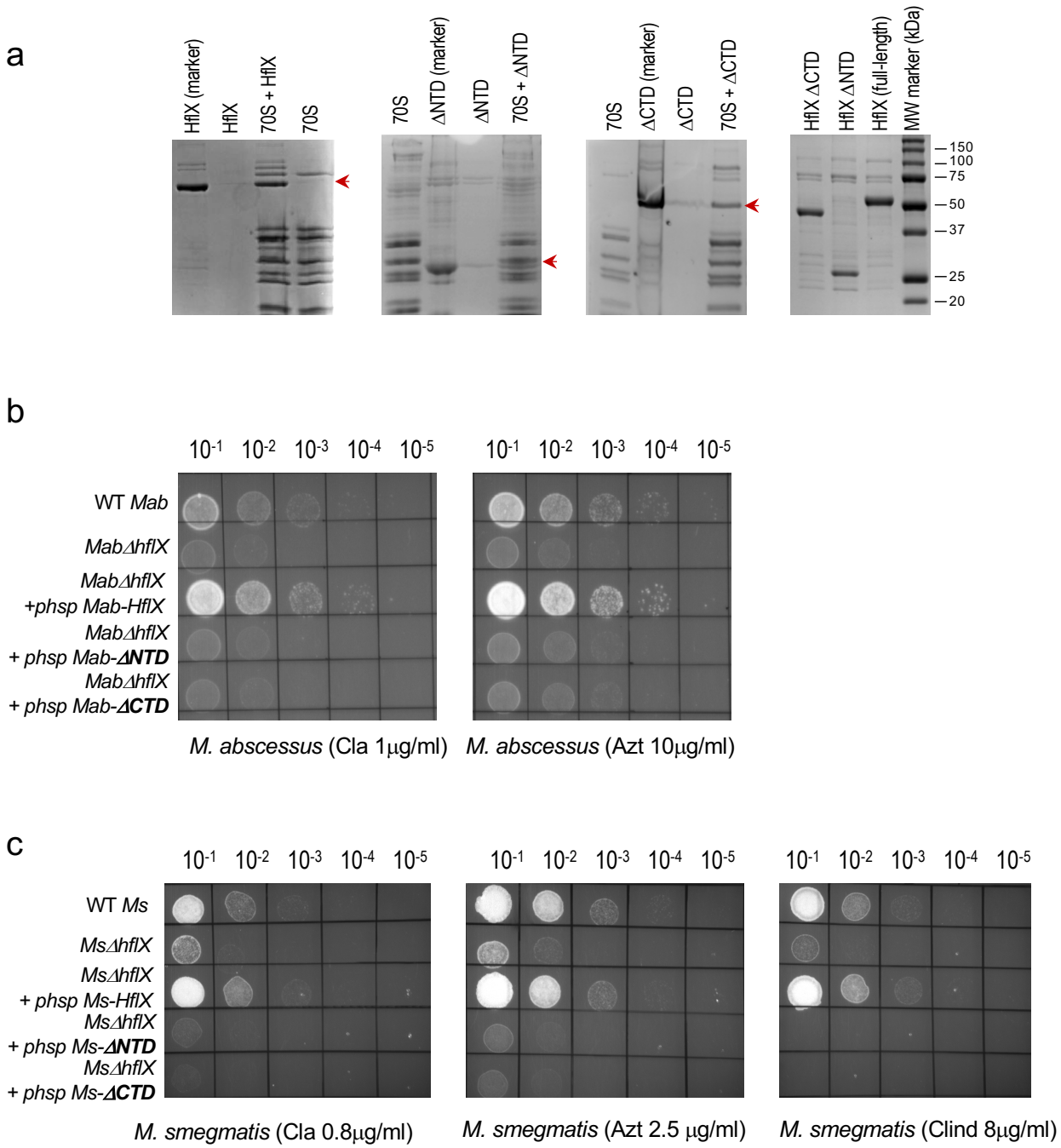


Figure S7: Behavior of truncation mutants of Ms-HflX. (a) The ability of Ms-HflX, Ms-HflX-ΔNTD and Ms-HflX-ΔCTD to associate with 70S ribosomes was studied using microfiltration through VivaSpin 500 columns (100kDa cutoff). All samples except purified HflX proteins that were used as markers (as indicated) were passed through the columns, washed with HMA-7 buffer and the filtrates were analyzed using SDS-PAGE. Location of full-length and truncated HflX proteins are indicated with a red arrow. Position of full-length and truncated domains of HflX compared to a molecular weight standard is shown. **(b-c)** Complementing strains were created by integrating the respective HflX-ΔNTD and HflX-ΔCTD at either the Bxb1 attB site of *ΔMs_hflX* or the L5 attB site of *ΔMab_hflX*. Wild type, *ΔhflX* mutant and complementing strains were assayed for growth on Middlebrook 7H10 containing indicated concentrations of antibiotics.

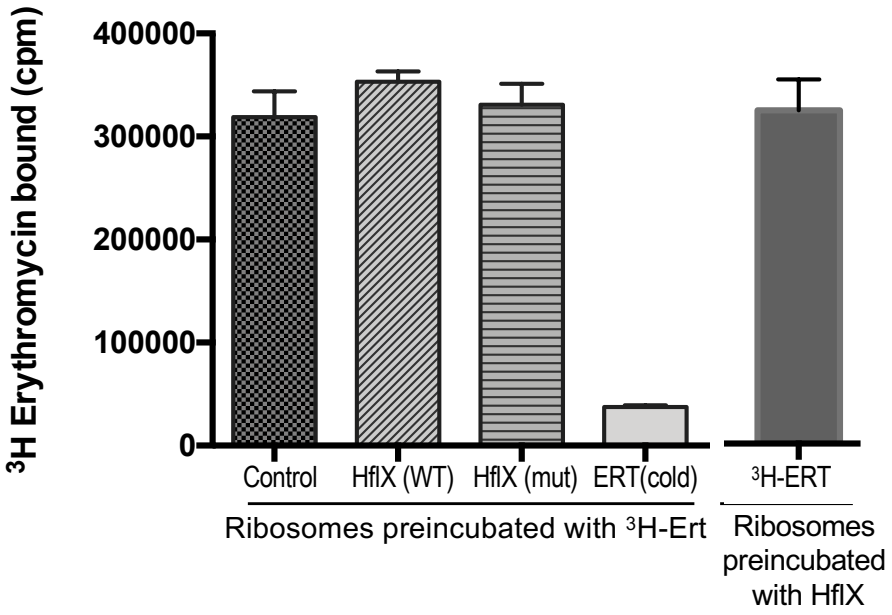
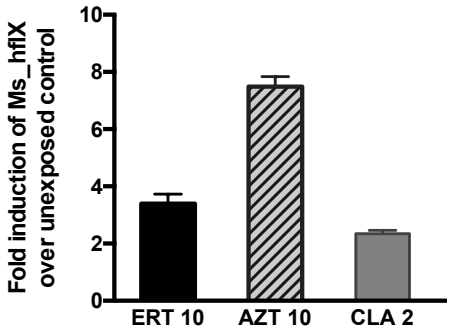


Figure S8: Ms-HflX neither prevents binding of erythromycin to ribosomes nor displaces ribosome-bound erythromycin. Purified ribosomes (0.2μM) were preincubated with 0.25 μM [^{3}H]erythromycin at 37°C for 15 mins followed by incubation with a 15-fold molar excess (3.0 μM) of Wt-HflX or an HflX(K258A/S259A) mutant protein that lacks GTPase activity. Control reactions were performed without added HflX or addition of 25 μM unlabeled erythromycin. Additionally, ribosomes were also preincubated with Wt-HflX at 37°C for 45 mins followed by incubation with 0.25 μM [^{3}H]erythromycin. Ribosome-associated [^{3}H]erythromycin was separated from free unincorporated [^{3}H]erythromycin using Biogel P30 microspin columns and quantified using a liquid scintillation counter. Results are means of 3 independent experiments and error bars represent standard deviation.

a



b

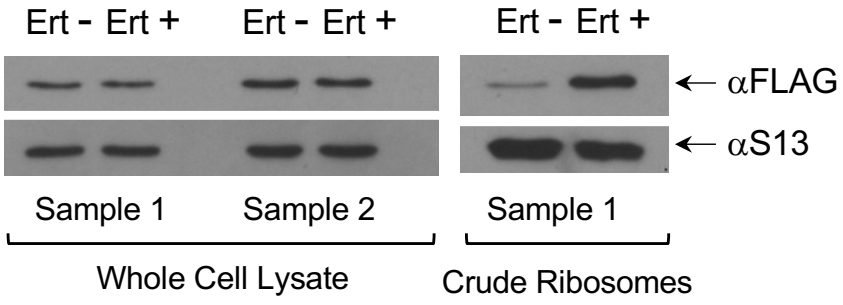


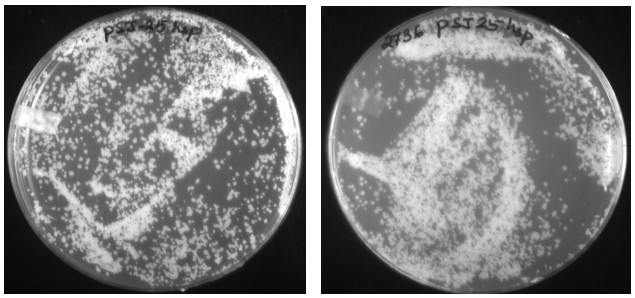
Figure S9: Levels of HflX mRNA and protein upon antibiotic exposure.
a) *M. smegmatis mc2155: hflX-FLAG* was grown to A_{600} of 0.7, exposed to 20μg/mL of erythromycin, 10μg/mL of azithromycin or 2μg/mL of clarithromycin for 3 hours, and the amount of *Ms_hflX* transcript was determined by qPCR and plotted as fold induction over an unexposed control. Data represents mean ± SD, n=3. *SigA* was used as an internal normalization control.

b) The amounts of $HflX_{FLAG}$ in whole cell lysates and crude ribosomes of ERT-exposed and unexposed control cells were determined using immunoblotting with anti-FLAG antibody. Immunoblotting with antibodies against the S13 ribosomal protein was used as a loading control. Sample 1 and 2 constitute biological replicates. The amount of HflX present in each of these lanes were quantified using Image J, v. 1.52k, and normalized to the amounts of S13 ribosomal protein. Normalized ratio of HflX-ERT$^+$/ HflX-ERT$^-$ (cell lysate) = 0.93, HflX-ERT$^+$/ HflX-ERT$^-$ (crude ribosome) = 8.02. Ratio of HflX-ERT$^+$/ HflX-ERT$^-$ (50S fraction, Fig. 3a) ~10.

a

ΔMs_hflX + pSJ25

ΔMs_hflX + pSJ25-phsp60-*Ms_hflX*

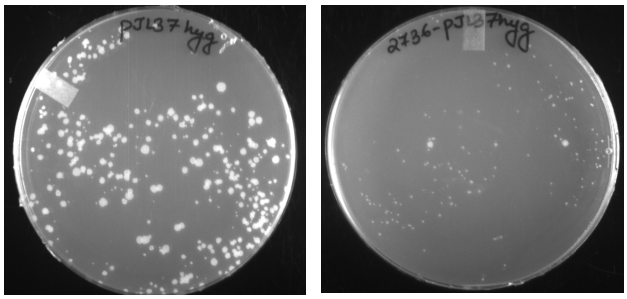


Constitutive expression from a chromosomal location (single copy)

b

ΔMs_hflX + pJL37

ΔMs_hflX + pJL37-phsp60-*Ms_hflX*

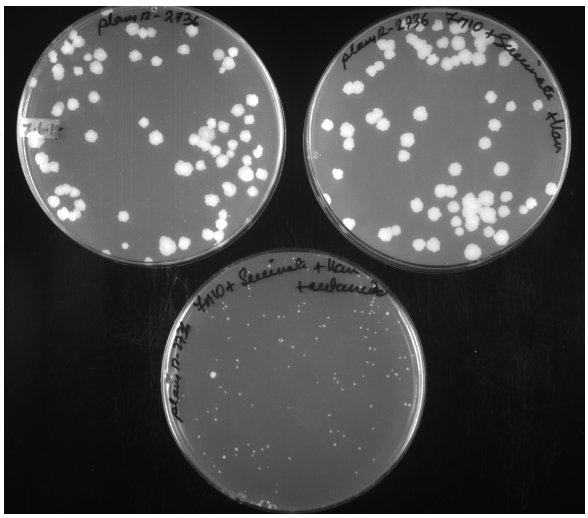


Constitutive expression from an extra- chromosomal location (multi-copy)

c

ΔMs_hflX + pAc-*Ms_hflX*

7H10 + ADC



7H10 + 0.2% succinate

Inducible expression from an extra- chromosomal location (multi-copy)

7H10 + 0.2% succinate + 0.2% acetamide

Figure S10: Overexpression of Ms-HflX on a multi-copy plasmid is deleterious for growth. (a-b) Growth of *ΔMs_hflX* strains complemented with *Ms_hflX* expressed from a constitutive promoter (*phsp60*) and located either as a single copy on the chromosome **(a)** or on a multicopy plasmid **(b)** was evaluated on Middlebrook 7H10/ADC agar. **(c)** Growth of *ΔMs_hflX* strains containing *Ms_hflX* expressed from the acetamide inducible promoter and contained on a multi-copy plasmid was evaluated on Middlebrook 7H10/ADC, Middlebrook 7H10/succinate and Middlebrook 7H10/acetamide agar.

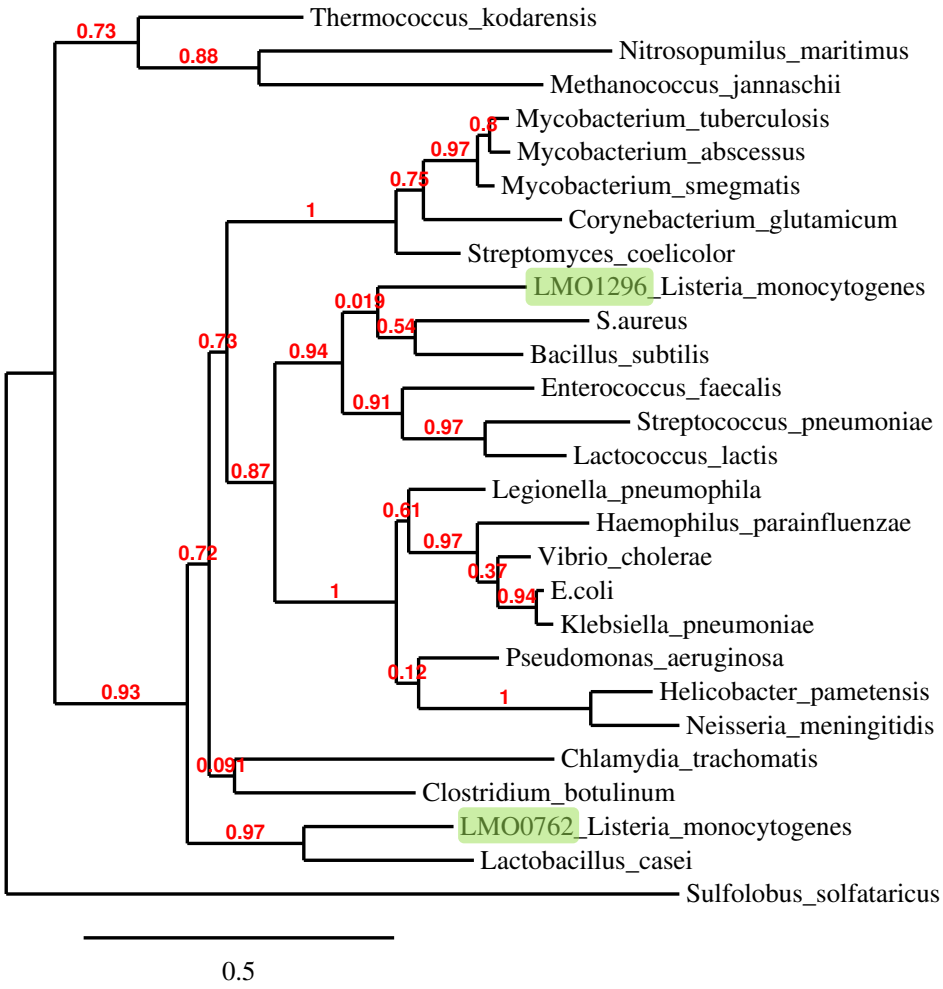


Figure S11: Maximum likelihood tree of MAB_3042c and HflX homologs

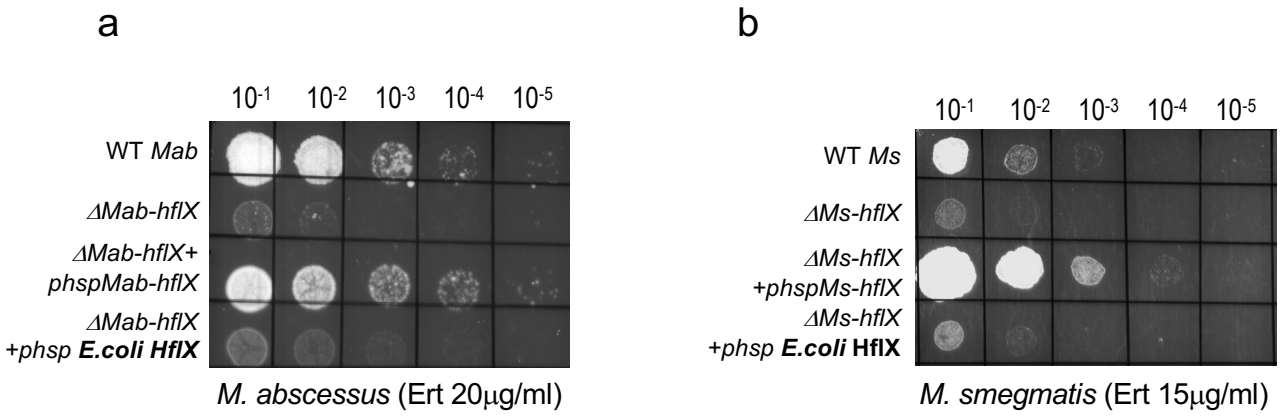


Figure S12: Lack of complementation of antibiotic sensitivity by *E. coli HflX*. Complementing strains were created by integrating *E. coli* HflX at either the Bxb1 attB site of *ΔMs_hflX* or the L5 attB site of *ΔMab_hflX*. Wild type, *ΔhflX* mutant and complementing strains were assayed for growth on Middlebrook 7H10 containing indicated concentrations of antibiotics. Expression of *E. coli hflX* in the complementing strain was verified using real time PCR (Table S2).

Table S1: Survival of wild type *M. smegmatis* mc^{2}155, *ΔMs_hflX, ΔMs_HflX* +pSJ-hflX *and ΔMs_HflX*+pSJ-MsRRF in a 2-fold dilution series of antibiotics in Middlebrook 7H9/ADC medium. The minimum concentration of antibiotic required to inhibit 99 % of growth in 48 hours is shown. Data is representative of 3 independent experiments.

Antibiotic	**Minimum Inhibitory Conc. (μg/mL)**			
	M. smegmatis WT	*M smegmatis* :*ΔhflX*	*M. smegmatis* :*ΔhflX* + pSJ-hflX	*M. smegmatis* :*ΔhflX* + pSJ-MsRRF
Erythromycin	2.5	0.625	5	1.25
Clarithromycin	0.25	0.125	0.5	0.25
Azithromycin	2	1	8	4
Clindamycin	16	4	16	16

Table S2: Indicated bacterial strains were grown to A_{600} of 0.7, exposed to 20mg/mL of erythromycin, where required. The presence of the indicated transcript was determined by qPCR. Data represents mean ΔC_T values ± SE, n=3. *SigA* was used as an internal normalization control. Fold change in expression of each probe was calculated with reference to the respective wild-type.

Strain	Probe	ΔC_T Mean	ΔC_T Standard Error	Fold increase in expression over WT
M. abscessus (WT)	*Mab*-HflX	0.36922964	0.10371712	1
M. abscessus (WT)- Ert Induced	*Mab*-HflX	-2.083200455	0.01888035	5.47
ΔMab_*hflX*	*Mab*-HflX	Undetermined		-
ΔMab_*hflX* + phsp- Mab-HflX	*Mab*-HflX	-4.935426712	0.1130803	39.5
ΔMab_*hflX* + phsp- Mab-ΔNTD	*Mab*-HflX	-3.256856203	0.08301153	12.3
ΔMab_*hflX* + phsp- Mab-ΔCTD	*Mab*-HflX	-5.382089138	0.1477447	41.6
M. abscessus (WT)	*E.coli*-HflX	Undetermined		
ΔMab_*hflX*	*E.coli*-HflX	Undetermined		
ΔMab_*hflX* + phsp- *E.coli*-HflX	*E.coli*-HflX	-3.31952095	0.1002317	Undetermined
M. smegmatis (WT)	*E.coli*-HflX	Undetermined		
ΔMsmeg_*hflX*	*E.coli*-HflX	Undetermined		
ΔMsmeg_*hflX* + phsp-*E.coli*-HflX	*E.coli*-HflX	-3.7199848	0.14934064	Undetermined
M. smegmatis (WT)	*Ms*-RRF	-0.5219765	0.08630468	1
ΔMsmeg_*hflX*	*Ms*-RRF	-0.0440108	0.10134946	
ΔMsmeg_*hflX* +*phsp*-Ms-RRF	*Ms*-RRF	-2.545543	0.12683994	5.7

Table S3: List of oligonucleotides, strains and plasmids used in the study

Construct		Primers
MAB_3042c deletion construction	A	GTT ACC TTA AGC GAG CAG TTG TTC CCC GAT GGA GTC
	B	CGG TCT AGA CCC CTA CCA GCA CAA CTC GTT CC
	C	GCG AAG CTT TCG CCG AGG CGG TGC C
	D	GCC ACT AGT GGT ATC GCC GAC GGC GAC GTG
	CHECK F	GGT CAA GGA GTT CGG TGC CGG CGA GGC
	CHECK R	TGC ACC GGG CAG CAC GTG CTG GTG C
MSMEG_2736 deletion construction	A	GGT GTC GTT CGA TCC GGC GTT GTT CCC CGA
	B	TGGTGAGGGAGATGAGGTCTGAAGTCC ACA CCC CGA CCA ACA CGA CGC GC
	C	GTTGAGGTGTGAGGTGTGCTGAAGGGA GTT GGT GGA ATC CAC CGA TGC CAC CGT CGA CG
	D	CCG AAG TTA CCG CAC CCG CCG ACA CCG
	CHECK F	CGC AGC TTC GAC GGC CTG GCC GT
	CHECK R	GTC GAC GTC AAG ACC ACC GAC ACG GGC AC
MAB_2297 deletion construction	A	GACCCGTGGCGCCACTTAAGCCCGCAC
	B	GCCCCACTGGCGTCTAGACCGTTGGC
	C	CATCCACCGCAGGAAGCTTCGTCGGTGCT
	D	CCACCGCAAGGCGATAGATCTACGCGCTGC
	CHECK F	GACCGTGAGCGGGTCTCGTTGTCGCT
	CHECK R	GTCGATCCGCAGCGCCCGC
MAB_3042c cloning into pET21a/pMH94	F	GCA TCG GTA GCC CAT ATG CGT ACC ACC TAT GAG ACG C
	R	CGC AAG CTT CTA GAG CGC GGT CAG GAC GG
MSMEG_2736 cloning into pET21a/pSJ25	F	CCT GGA AGT ACA TAT GAC CTA TCC AGA GAA TTC CGT
	R	AGC AAG CTT GTC AGG CGA ACG TGG CGT AC
E.coli MG1655 hflX cloning into pSJ25	F	GGGTTATACATATGTTTGACCGTTATGATGCTGGTGAGC
	R	CTA CAA GCT TAG ATC AGG TAA TCG ATC AAC GCC GGT TC
MSMEG_2736 -ΔCTD	R	GCG AAG CTT ACT TGT CGA GAC CGT CGC CGG TGC G
MSMEG_2736 -ΔNTD	F	GAT CCG CCA TAT GCA GCG CGG CAG CAG
MAB_3042c -ΔNTD	F	GAA GGT TCG TCA TAT GAA GCG CAG CCG CCG CCT G
MAB_3042c -ΔCTD	R	CGG TTA ACG CCT CGC GCT ACG TGG CGA TCC
MSMEG_2541(RRF) cloning into pSJ25	F	CGGGAGACGGTCATATGATCGACGAGACTCTCTTCGATGCCG
	R	ACA AGC TTG CTG ATC GGT CAC TCA GAC CTC CAG CAA CTC
MAB_2297 cloning into pMH94	F	GGT GCT GCC ATA TGT CCG GCC AAC GG
	R	CAC GAA TTC AGC GCC GCC TGA TCA CCA G

	Strain	Strain description
M. abscessus	M. abscessus subsp.abscessus ATCC 19977	Wild-type ATCC strain
	M. abscessus ΔMAB_3042c or ΔMab-hflX	Isogenic deletion of MAB_3042c (*hflX*) in type strain
	ΔMab *hflX*/pMH94hspMab-*hflX*	MAB_3042c overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3042c
	ΔMab *hflX*/pMH94hspMs-*hflX*	MSMEG_2736 overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3042c
	ΔMab *hflX* /pMH94hspEcolihflX	E.coli HflX overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3042c
	ΔMab *hflX* /pMH94hspMab *hflX*-ΔCTD	MAB_3042c – ΔCTD overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3042c
	ΔMab *hflX* /pMH94hspMab *hflX*-ΔNTD	MAB_3042c – ΔNTD overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3042c
	M. abscessus ΔMab *whiB7*	Isogenic deletion of MAB_3508c (*whiB7*) in type strain
	ΔMab *whiB7* /pMH94hspMab-whiB7	MAB_3508c overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3508c
	ΔMab *whiB7* /pMH94hspMab-erm41	MAB_2297 overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3508c
	ΔMab *whiB7* /pMH94hspMab-h*flX*	MAB_3042c overexpressed from P_{hsp60} integrated at L5 locus of ΔMAB_3508c
	M. abscessus ΔMab *1846*	Isogenic deletion of MAB_1846 (ABC-F) in type strain
	M. abscessus ΔMab 2355	Isogenic deletion of MAB_2355 (ABC-F) in type strain
	M. abscessus ΔMab_*erm41*	Isogenic deletion of MAB_2297 (erm41) in type strain
	M. abscessus ΔMab_whiB7	Isogenic deletion of *whiB7* in type strain
M. smegmatis	*M. smegmatis* mc^2155	*M. smegmatis* type strain
	M. smegmatis ΔMs *hflX*	Isogenic deletion of MSMEG_2736 in type strain
	ΔMs *hflX* /pSJ25hspMs *hflX*	MSMEG_2736 overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	ΔMs *hflX* /pSJ25hspMab *hflX*	MAB_3042c overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	ΔMs *hflX* /pSJ25hspMsRRF	MSMEG_2541 overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	ΔMs *hflX* /pSJ25hsp E.coli *hflX*	E.coli HflX overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	ΔMs *hflX* /pSJ25hsp Ms *hflX*- ΔCTD	MSMEG_2736- ΔCTD overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	ΔMs *hflX* /pSJ25hsp Ms *hflX*- ΔNTD	MSMEG_2736- ΔNTD overexpressed from P_{hsp60} integrated at Bxb1 locus of ΔMs *hflX*
	M. smegmatis: HflX $_{FLAG}$	MSMEG_2736 containing a FLAG- tag at its native chromosomal location.

Expression plasmids used in E.coli BL21(DE3)pLysS		
	pET21a + *M. smegmatis* HflX	MSMEG_2736 cloned in pET21a
	pET21a + *M. smegmatis* HflX-ΔNTD	MSMEG_2736 (aa 236-471) cloned in pET21a
	pET21a + *M. smegmatis* HflX-ΔCTD	MSMEG_2736 (aa 1-402)cloned in pET21a
	pET21a + *M. smegmatis* HflX-(K258A/S259A)	MSMEG_2736 (K258A/S259A)cloned in pET21a

Materials and Methods

Media, Strains and Plasmids

M. abscessus ATCC19977 and *M. smegmatis* were grown at 37°C in Middlebrook 7H9 (DIFCO) supplemented with 0.05% Tween 20 and 10% OADC/ADC. Gene replacement mutants as well as MSMEG_2736 with a C-terminal FLAG tag were constructed using recombineering as described previously and confirmed by sequencing (1, 2). Complementing strains were created by cloning the desired gene in pSJ25 or pMH94 under the control of the *hsp60* promoter followed by integration at the phage Bxb1/ L5 *attB* sites respectively. Point mutations in *Ms_hflX* cloned in pSJ25 and pET21a were created using the Q5 site directed mutagenesis kit (NEB). Primers for mutant construction were designed using the NEBaseChanger as recommended. All mutants were confirmed by Sanger sequencing. A list of primers and strains is included in Table S3.

Antibiotic Sensitivity Assays

Wild type and mutant strains of *M. smegmatis* and *M. abscessus* were grown to an A_{600} of 0.6-0.7. Cells were tested for their susceptibility to antibiotics by spotting a 10- fold serial dilution on Middlebrook 7H10 (DIFCO) plates containing the indicated concentration of antibiotics. Antibiotic susceptibility in liquid media was assayed by inoculating the desired strain in a two-fold dilution series of each antibiotic at an initial A_{600} of 0.0004. The cultures were incubated at 37°C and the A_{600} was measured after 72 hours for *M. abscessus* or 48 hours for *M. smegmatis.*

RNA preparation / qPCR

Wild type *M. abscessus* and the Δ*whiB7* deletion strains were exposed to ERT (10 µg/ml), CLA (2µg/ml) or AZT (10µg/ml) for 3 hours. Total RNA was prepared using the Qiagen RNA preparation kit, DNAse I treated. cDNA generated using random hexamers and Maxima reverse transcriptase (Fisher Scientific), and qRT-PCR performed using the Maxima SYBR Green qPCR Master Mix (Fisher Scientific) and MAB_3042c specific primers. Applied Biosystems 7300 Real-Time PCR System was

used with cycling conditions of: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min. Data represents mean ± SD, n=3.

Protein overexpression and purification

Wild type Ms-HflX, site-directed mutants as well as truncated mutants (ΔNTD: aa 236-471 and ΔCTD: aa 1-402) were cloned into pET21a with a C-terminal his-tag and transformed into BL21(DE3) pLysS, grown to an A_{600} of 0.5 and induced with 1mM IPTG at 30°C. The cells were lysed in a buffer containing 50mM Tris-HCl (pH 8.0), 300mM NaCl, 5% glycerol, 1mM PMSF and 0.25% sodium deoxycholate and the clarified lysate was loaded on a Ni-NTA column (Qiagen). Non-specifically bound proteins were removed by washing with lysis buffer containing 20mM imidazole and the proteins eluted with 150mM imidazole.

Ribosome preparation

Ribosomes were purified from wild type *mc²155*, Δ*Ms_hflX* and *mc²155:* Ms_hflX-FLAG strains as described previously (3, 4). Briefly, for large scale ribosome purification, WT *mc²155* was grown in 2L of Middlebrook 7H9 containing 10% ADS and 0.05% TWEEN to an OD of 0.7. The cells were harvested and lysed using The CryoMill (Retsch) in 20mM HEPES, pH 7.5, 30mM NH_4Cl and 10mM $MgCl_2$ (HMA-10 buffer). The samples were clarified three times by centrifugation and **crude ribosomes** were collected by ultracentrifugation at 42,800 rpm for 2hr 15mins using a Beckman 50.2 Ti rotor followed by resuspension in HMA-8 buffer (20mM HEPES, pH 7.5, 30mM NH_4Cl and 8mM $MgCl_2$). Crude ribosomes were treated with RNAase free DNAse I (Ambion) for 4 hours at 4°C, layered on 32 % sucrose cushion and centrifuged using a Beckman 50.2 Ti rotor at 100,000 × *g* for 16 hours at 4°C. Ribosome pellets were washed one time with HMA-8, resuspended and stored in HMA-8 at -80°C. These were used further in splitting assays. For analysis of polysome profiles upon antibiotic exposure, wild type *mc²155*, Δ*Ms_hflX* and *mc²155:* Ms_hflX-FLAG strains were grown in 500mL of Middlebrook 7H9 containing 10% ADS and 0.05% TWEEN to an OD_{600} of 0.7 and exposed to either ERT (20μg/mL) or CLIND (16μg/mL) for 1hr. Crude ribosomes were prepared as described

above, resuspended in HMA-8 buffer, layered on 10 mL of 10-40% sucrose gradients and centrifuged for 5 hours at 35,000 rpm using a Beckman SW 41 rotor followed by fractionation using the Brandel Teledyne ISCO gradient fractionation system at a flow rate of 0.75 mL/ min.

In vitro splitting assay and Western Blotting

Purified ribosomes (0.2μM) were incubated with 15 fold molar excess of full length Ms-HflX, point mutants or truncated mutants (Ms-HflX ΔNTD and ΔCTD) in a 50μl total volume in HMA-7 buffer (containing 20mM HEPES, pH 7.5, 30mM NH_4Cl and 7mM $MgCl_2$) in the presence of 1mM GTP , ATP or GMP-PNP. The reactions were incubated at 37°C for 45 mins and layered on 5mL 10-40% sucrose gradients. The gradients were centrifuged at 35,000rpm for 3 hrs at 4°C using a Beckman SW55 rotor followed by fractionation using the Brandel fractionation system. When required the individual sucrose fractions were precipitated with methanol and chloroform (5) and reconstituted in 10μl of 10mM Tris containing 8M urea. The samples were separated using 12% SDS-PAGE and transferred onto a PVDF membrane and probed with either anti-his (Thermo Fischer) or anti -FLAG (Sigma) monoclonal antibodies as required. Purified his-tagged MSMEG_HflX / ΔNTD/ΔCTD proteins were used as controls.

Microfiltration

0.2 μM of purified 70S ribosome was mixed with 2 μM of either full length or truncated mutants of Ms-HflX in HMA-7 buffer containing 1mM GMP-PNP and incubated at 37°C for 45 minutes. The samples were incubated on ice for 5 minutes before dilution in 500 μL of ice cold HMA-7 buffer and centrifuged at 10,000g in VivaSpin-500 columns with a molecular weight cut-off of 100,000 kDa (GE Healthcare). Once the volume was reduced to 25 μ, the samples were diluted again with 500 μL of ice cold HMA-7 buffer centrifuged again at 10,000g. The filtrate was collected and loaded on a 12% SDS-PAGE followed by Coomassie blue staining.

Antibiotic binding/release assay

Purified ribosomes (0.2μM) were preincubated with 0.25 μM [^{3}H]erythromycin (American Radiolabeled Chemicals Inc.) at 37°C for 15 mins followed by addition of 15-fold molar excess (3.0 μM) of Wt-HflX or an HflX(K258A/S259A) mutant protein lacking GTPase activity in HMA-7 buffer containing 1mM GTP in a total volume of 50 μL and incubation at 37°C for an additional 45 mins . Control reactions were performed without added HflX or addition of 25 μM unlabeled erythromycin. Additionally, ribosomes were preincubated with Wt-HflX at 37°C for 45 mins followed by incubation with 0.25 μM [^{3}H]erythromycin. Ribosomes were separated from free unincorporated [^{3}H]erythromycin using Biogel P30 microspin columns (Biorad). The ribosome associated- [^{3}H]erythromycin was quantified using a liquid scintillation counter.

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