Mycobacterial HflX is a ribosome splitting factor that mediates antibiotic resistance

Paulami Rudra[a,1], Kelley R. Hurst-Hess[a,1], Katherine L. Cotten[a], Andrea Partida-Miranda[a], and Pallavi Ghosh[a,b,2]

[a]Division of Genetics, Wadsworth Center, New York State Department of Health, Albany, NY 12208; and [b]School of Public Health, University at Albany, Albany, NY 12208

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Antibiotic resistance in bacteria is typically conferred by proteins that function as efflux pumps or enzymes that modify either the drug or the antibiotic target. Here we report an unusual mechanism of resistance to macrolide-lincosamide antibiotics mediated by mycobacterial HflX, a conserved ribosome-associated GTPase. We show that deletion of the *hflX* gene in the pathogenic *Mycobacterium abscessus*, as well as the nonpathogenic *Mycobacterium smegmatis*, results in hypersensitivity to the macrolide-lincosamide class of antibiotics. Importantly, the level of resistance provided by *Mab_hflX* is equivalent to that conferred by *erm41*, implying that *hflX* constitutes a significant resistance determinant in *M. abscessus*. We demonstrate that mycobacterial HflX associates with the 50S ribosomal subunits in vivo and can dissociate purified 70S ribosomes in vitro, independent of GTP hydrolysis. The absence of HflX in a *ΔMs_hflX* strain also results in a significant accumulation of 70S ribosomes upon erythromycin exposure. Finally, a deletion of either the N-terminal or the C-terminal domain of HflX abrogates ribosome splitting and concomitantly abolishes the ability of mutant proteins to mediate antibiotic tolerance. Together, our results suggest a mechanism of macrolide-lincosamide resistance in which the mycobacterial HflX dissociates antibiotic-stalled ribosomes and rescues the bound mRNA. Given the widespread presence of *hflX* genes, we anticipate this as a generalized mechanism of macrolide resistance used by several bacteria.

HflX | macrolides | *Mycobacterium abscessus* | erm41 | ribosome

Mycobacterium abscessus has emerged as an important human pathogen during the last 10 y, causing superficial and deep-tissue infections after traumatic injury and/or surgery, as well as bronchopulmonary infections in patients with chronic lung damage, such as prior tuberculosis and cystic fibrosis, resulting in a persistent decline in pulmonary functions or acute respiratory failure (1). The major threat posed by this organism is its extremely low sensitivity to most FDA-approved antibiotics, making its infections incredibly difficult to treat (2, 3). The current treatment regimen against *M. abscessus* recommends a combination of an oral macrolide in conjunction with amikacin and 1 or more of the injectables (cefoxitin, imipenem, or tigecycline) for a period of several months (2, 4). The majority of these antibiotics target the ribosome, a 2.5-MDa ribonucleoprotein enzyme composed of a 50S and 30S subunit. The binding sites for most ribosome-targeting antibiotics are primarily concentrated at 3 locations within the ribosome: the decoding site on the 30S subunit, the peptidyl transferase center (PTC), and/or the nascent peptide exit tunnel (NPET) on the 50S subunit (5). Macrolide, lincosamide, and streptogramin B antibiotics are structurally distinct but are often considered together (MLS_B antibiotics), as they have overlapping binding sites on the 50S subunit around the 23S rRNA nucleotide, A2058 (6). Macrolides are 14- to 16-member macrolactones and bind in the upper portion of the NPET between the PTC and the constriction formed by the proteins L4 and L22 (7, 8). Macrolide binding does not interfere with peptide bond formation per se, but hinders the passage of newly synthesized polypeptides, thereby interrupting translation elongation (9, 10). Lincosamides are smaller molecules that occupy the region between A2058 and the PTC in a way that overlaps with the aminoacyl moiety of the A-site tRNA, thereby preventing peptide bond formation (11).

Intrinsic resistance to macrolides is commonly attributed to 3 primary mechanisms: target modification, active efflux by ABC transporters and the Major Facilitator superfamily, and drug inactivation by esterases, lyases, and phosphorylases (12). Target modification at A2058 of the 23S rRNA by methylases confers cross-resistance to macrolide, lincosamide, and streptogramin B, commonly referred to as the MLS_B phenotype, and is the most widespread mechanism of macrolide resistance (12, 13). More recently, the Antibiotic Resistance ATP binding cassette family F (ARE ABC-F) proteins have been shown to confer macrolide resistance by ribosome protection in several Gram-positive bacteria (14, 15). While some macrolide resistance genes are constitutively expressed, the majority are inducible by low doses of antibiotics through transcriptional or translational attenuation (16, 17). In mycobacteria, the most common mechanism of macrolide resistance involves mutations in the macrolide binding site on the 23S rRNA, as well as methylation of these residues by *erm*-encoded RNA methyltransferases (18, 19). The expression of mycobacterial *erm* genes is under the control of a transcriptional activator, WhiB7, which is in turn controlled by translational attenuation in the presence of subinhibitory concentrations of structurally unrelated antibiotics (20, 21). Deletion of *whiB7* in *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and *M. abscessus* results in multidrug sensitivity to MLS_B and other ribosome-targeting antibiotics (22, 23).

Significance

The *erm41* gene is considered the primary mechanism of intrinsic resistance to macrolides in *Mycobacterium abscessus*. Here we demonstrate that the *hflX* gene plays a significant and equally important role as *erm41*. We further describe an unusual mechanism of resistance to macrolide-lincosamide antibiotics mediated by the mycobacterial HflX that likely involves the dissociation of antibiotic-stalled ribosomes. An understanding of the various mechanisms employed by bacteria for resistance to an antibiotic is critical in predicting an effective therapeutic regimen against a pathogenic isolate, and can also inform the development of novel drugs.

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[1]P.R. and K.R.H.-H. contributed equally to the work.

[2]To whom correspondence may be addressed. Email: pallavi.ghosh@health.ny.gov.

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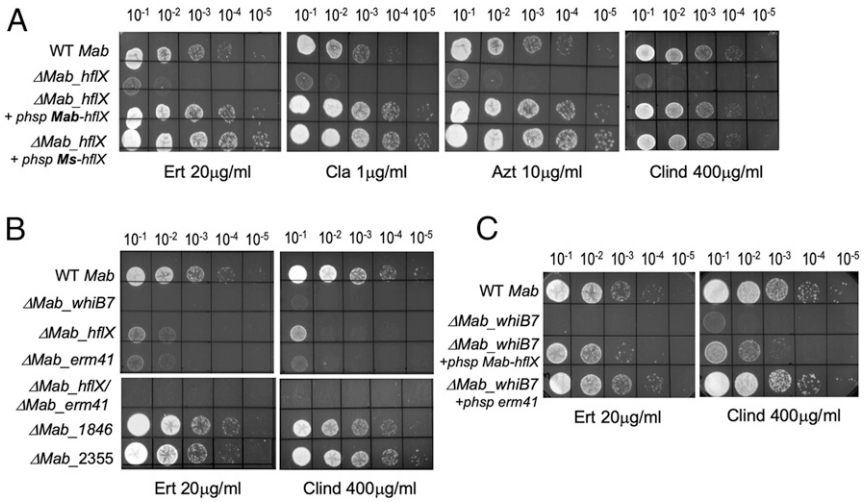

Fig. 1. Deletion of *M. abscessus hflX* confers macrolide-lincosamide sensitivity. (*A–C*) Ten-fold serial dilutions of *M. abscessus* ATCC 19977, *ΔMab_hflX, ΔMab_whiB7, ΔMab_erm41, ΔMab_hflX/ΔMab_erm41, ΔMab_1846, ΔMab_2355*, and the indicated complementing strains were grown to A_{600} of 0.7 and spotted on Middlebrook 7H10 OADC containing 20 μg/mL erythromycin, 1 μg/mL clarithromycin, 10 μg/mL azithromycin, or 400 μg/mL clindamycin.

Previously, we used genomewide transcriptomic profiling by RNAseq and identified ~80 genes in the WhiB7 regulon of *M. abscessus* and *M. smegmatis*, one of which is MAB_3042c, a homolog of the universally conserved Translation Factor (TRAFAC) family of GTPases, HflX (22). Despite its widespread distribution, knockout strains of *hflX* are viable, and the precise biological function in most organisms is unclear (24, 25). The *Escherichia coli* HflX is known to be involved in splitting of stalled ribosomes generated during heat shock into free subunits, and the *Staphylococcus aureus* HflX was shown to disassemble hibernating 100S ribosomes (26, 27). Although binding of macrolides has been shown to interfere with the GTPase activity of *E. coli* HflX, the *E. coli* HflX has not been shown to be directly involved in antibiotic resistance (28). Recently, the *hflX-r* gene from *Listeria* monocytogenes was shown to confer macrolide resistance (29). We demonstrate here that Mab-HflX (MAB_3042c) and Ms-HflX (MSMEG_2736) are required for macrolide-lincosamide resistance in *M. abscessus* and *M. smegmatis*, conferring equivalent resistance as the *erm* genes, by an *erm*-independent pathway. We also demonstrate that Ms-HflX is a ribosome splitting factor, and disruption in its ability to dissociate ribosomes results in an inability to mediate antibiotic resistance. Our results suggest that a likely mechanism of mycobacterial HflX-mediated macrolide-lincosamide resistance involves dissociation of ribosomes stalled in the presence of these antibiotics.

Results

Deletion of the HflX Homolog Confers Hypersensitivity to Macrolide-Lincosamide Antibiotics in *M. abscessus* and *M. smegmatis*. WhiB7, a transcriptional activator, is one of the earliest genes upregulated in response to ribosome-targeting antibiotics, and in turn activates the expression of ~80 genes that comprise the WhiB7 regulon (22). A few genes in this regulon have known functions such as efflux pumps, the *erm41* and *eis2* genes; the roles of most genes are, however, unknown. One such gene induced in response to ribosome targeting antibiotics as part of the WhiB7 regulon is MAB_3042c, a homolog of the universally conserved ribosome binding protein, HflX (*SI Appendix*, Fig. S1). A comparison of the WhiB7 regulon of *M. abscessus* and *M. smegmatis* showed that MAB_3042c is also one of the few genes that is shared between the WhiB7 regulons of the 2 species; MSMEG_2736, the ortholog in *M. smegmatis*, displays ~80% amino acid sequence identity to MAB_3042c (*SI Appendix*, Fig. S2) (22). Since HflX is known to bind the ribosome in several bacterial species studied, we explored whether MAB_3042c is involved in resistance to ribosome-targeting antibiotics (26, 30, 31). Isogenic deletions of MAB_3042c (*Mab_hflX*) in ATCC 19977 and MSMEG_2736 (*Ms_hflX*) in *mc²155* were constructed using phage recombineering (22, 32). The resulting deletion strains *ΔMab_hflX* and *ΔMs_hflX* were hypersensitive to the macrolides erythromycin (ERT), clarithromycin (CLA), and azithromycin (AZT) and the lincosamide clindamycin (CLIND) (Fig. 1*A*, Table 1, and *SI Appendix*, Fig. S3 and Table S1); their sensitivity to several other ribosome-targeting antibiotics remained unchanged (*SI Appendix*, Fig. S4). Constitutive expression of *hflX* driven by P_{hsp60} from a chromosomally integrated copy in the respective mutant strains restored sensitivity of the mutant to wild-type levels; in fact, the complementing strains displayed increased tolerance to macrolides and clindamycin compared with wild-type bacteria (Fig. 1*A* and *SI Appendix*, Fig. S3). Moreover, overexpression of *Ms_hflX* in *ΔMab_hflX*

Table 1. Survival of wild-type *M. abscessus* ATCC19977, *ΔMab_HflX, ΔMab_HflX* +phsp-HflX, *ΔMab_erm41*, and *ΔMab_HflX/ΔMab_erm41* in a 2-fold dilution series of antibiotics in Middlebrook 7H9/OADC medium

	Minimum Inhibitory Concentration (μg/mL)					
Antibiotic	WT *Mab*	*ΔMab_hflX*	*ΔMab_hflX* + phsp-hflX	*ΔMab_whiB7*	*ΔMab_erm41*	*ΔMab_hflX/ΔMab_erm41*
Erythromycin	2	0.25	2	0.625–0.125	0.25	0.125
Clarithromycin	1	0.125	1	0.0625	0.125	0.0625
Azithromycin	8	2	16	0.5	2	1.0
Clindamycin	200	50	200	12.5	25	25

The minimum concentration of antibiotic required to inhibit 99% of growth after 72 h is shown. Minimum inhibitory concentration values are representative of 3 independent assays.

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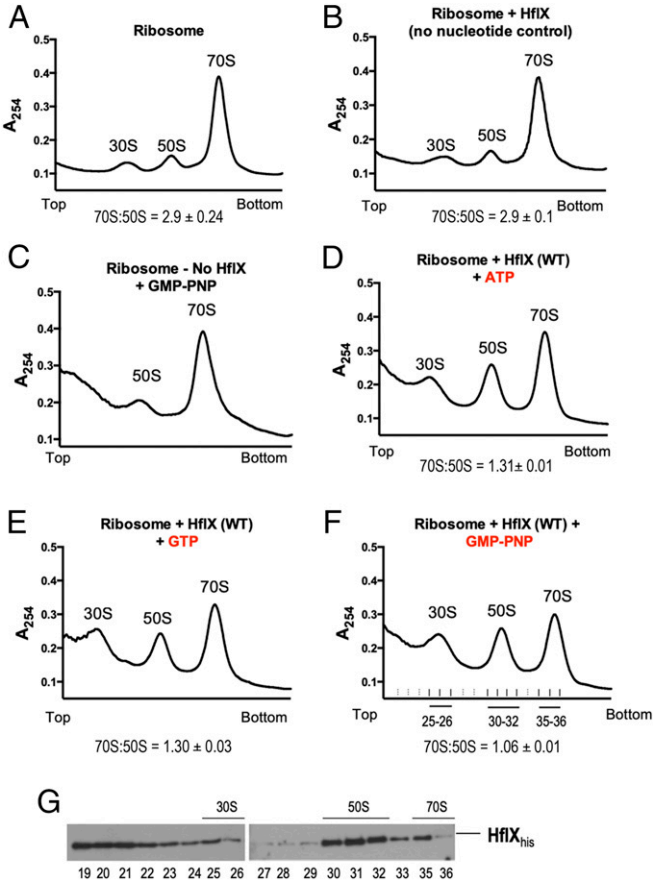

Fig. 2. Nucleotide-dependent dissociation of 70S ribosomes by Ms-HflX in vitro. (*A–F*) Dissociation of 70S ribosomes (0.2 μM) was carried out in the presence of 3.0 μM Ms-HflX$_{6his}$ in HMA-7 buffer in the presence of 1 mM GTP, ATP, or GMP-PNP at 37 °C for 45 min and examined using a 5-mL analytical 10% to 40% SDGC. Reactions lacking either nucleotide or HflX were included as controls. Percentage area under the curve was calculated for 70S and 50S peaks, using PeakChart (v. 2.08, Brandel), and expressed as a ratio of 70S:50S. Data represent mean ± SD, $n = 3$. (*G*) The samples were collected using the Brandel Teledyne ISCO gradient fractionation system, methanol-chloroform precipitated, followed by immunoblotting with anti-his antibody to determine the presence of Ms-HflX$_{6his}$ in each fraction.

also restored its antibiotic tolerance to wild-type levels, thereby suggesting a conserved function of *Mab_hflX* and *Ms_hflX* (Fig. 1*A*).

Curiously, the *M. abscessus* WhiB7 regulon contains additional known and putative effectors of macrolide resistance; specifically, the full-length *erm41* gene and homologs of ABC-F proteins, MAB_1846 and MAB_2355, transcription of which are induced on macrolide exposure. To evaluate their relative contribution to macrolide-lincosamide resistance, we constructed isogenic deletions in *erm41*, MAB_1846, and MAB_2355. Fig. 1*B* and Table 1 show that Δ*erm41* and Δ*hflX* were both equally hypersensitive to erythromycin and clindamycin; a Δ*hflX*/Δ*erm41* double-mutant and Δ*whiB7*, however, displayed significantly increased sensitivity compared with either single mutant alone. Moreover, overexpression of either *hflX* or *erm41* restored antibiotic sensitivity of the Δ*whiB7* mutant strain, suggesting that Erm41 and HflX act via independent pathways (Fig. 1*C*). Δ*MAB_2355* and Δ*MAB_1846* strains displayed mild hypersensitivity to erythromycin and clindamycin, respectively (Fig. 1*B*).

Mycobacterial HflX Dissociates 70S Ribosomes In Vitro, Independent of GTP Hydrolysis. The *E. coli hflX* has previously been shown to be inducible under heat stress, and an *E. coli* Δ*hflX* mutant is highly heat sensitive. However, the viability of Δ*Mab_hflX* and Δ*Ms_hflX* remain unchanged on exposure to elevated temperatures (*SI Appendix*, Fig. S5 *A* and *B*). The *E. coli* HflX has also been shown to be involved in splitting of stalled ribosomes generated during heat shock into free subunits (26). To determine whether mycobacterial HflX is capable of similarly dissociating 70S ribosomes in vitro, we examined the effect of adding purified Ms-HflX to 70S ribosomes, using sucrose density gradient centrifugation (SDGC). Fig. 2 *A–F* shows that Ms-HflX indeed promoted dissociation of 70S ribosomes in the presence of either GTP or ATP, consistent with previous studies showing that *E. coli* HflX can bind to both ATP and GTP (31). No ribosome dissociation was observed in the absence of added nucleotides (Fig. 2*B*); moreover, maximum splitting efficiency was observed in the presence of the nonhydrolysable GTP analog GMP-PNP (Fig. 2*F*), which implied that although nucleotide binding is necessary for ribosome splitting, GTP hydrolysis is not. The distribution of HflX in ribosomal fractions was monitored by immunoblotting using anti-his antibodies that recognized his-tagged Ms-HflX. Although HflX could be detected in the top fractions as well as 70S fractions, an enrichment was observed in the 50S subunit fractions (Fig. 2*G*).

In Vivo Association of HflX with Ribosomes from Antibiotic-Treated Cells. To determine whether HflX associates with ribosomal fractions in vivo, we constructed a strain in which *Ms-hflX* was

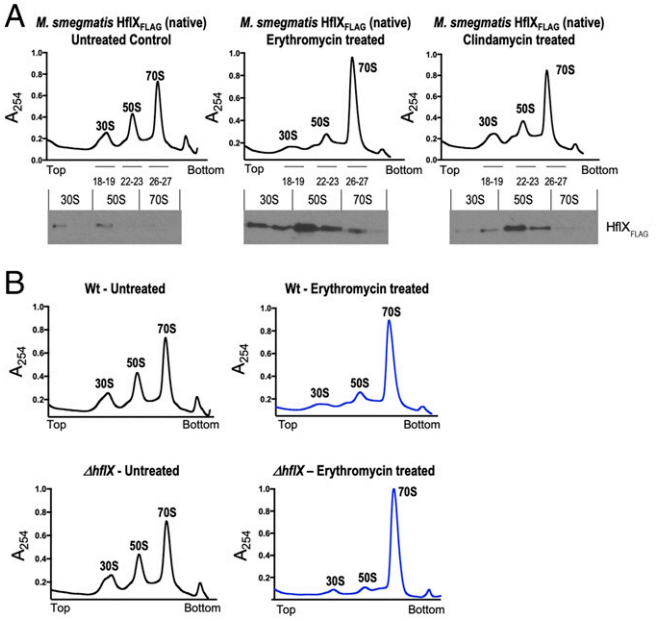

Fig. 3. (*A*) In vivo association of Ms-HflX with ribosomes. An *M. smegmatis* strain in which Ms-HflX was C-terminally tagged with the 3X-FLAG epitope at its native chromosomal location was grown to an OD of 0.7 and treated with either 20 μg/mL erythromycin or 16 μg/mL clindamycin for 1 h. Untreated cells were used as a control. A total of 50 pmoles crude ribosomes isolated from each sample were loaded on a 10-mL, 10% to 40% sucrose gradient, followed by ultracentrifugation in an SW41 rotor. Samples were collected from top to bottom on a Brandel fractionation system, and the distribution of endogenous Ms-HflX$_{FLAG}$ in ribosome fractions was analyzed by immunoblotting, using anti-FLAG antibody. (*B*) Ribosome profile of Δ*Ms_hflX* compared with wild-type bacteria. Wild-type *M. smegmatis* and Δ*Ms-HflX* strains were grown to an OD of 0.7 and treated with 20 μg/mL erythromycin for 1 h. Untreated cells were used as a control. Crude ribosomes were prepared from each sample, and equal quantities (50 pmoles) were loaded on 10-mL, 10% to 40% sucrose gradients. After ultracentrifugation, the samples were fractionated using the Brandel Teledyne gradient fractionation system, and results were normalized based on area under the curve (AUC). AUC values for WT-Untreated, WT-ERT treated, ΔhflX-untreated, and Δ*hflX*-ERT treated were 87.42, 89.66, 86.81, and 87.23, respectively. Polysome profile of erythromycin-treated samples are in blue, and untreated samples are shown in black.

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C-terminally tagged with the 3X-FLAG epitope at its native chromosomal location. The distribution of endogenous HflX in ribosome fractions obtained from *M. smegmatis* treated with either ERT or CLIND, as well as an untreated control sample, was examined by SDGC coupled with immunoblotting. Fig. 3*A* shows that Ms-$HflX_{FLAG}$ was enriched in the 50S ribosomal fractions obtained from antibiotic-treated cells, but almost undetectable in corresponding fractions obtained from bacteria untreated with antibiotics.

We next analyzed the polysome profile of ribosomes isolated from wild-type and Δ*Ms_hflX* strains when exposed to ERT, as well as in the absence of the drug, by SDGC. As seen in Fig. 3*C*, an increased accumulation of the 70S fraction was observed in the presence of ERT and likely represents ribosomes stalled in the presence of the drug. The quantity of 70S ribosome was, however, significantly ($P < 0.05$) greater in ERT-exposed Δ*Ms_hflX* cells than that observed in ERT-exposed wild-type bacteria and could reflect a decrease in dissociation of antibiotic-stalled ribosomes in the absence of HflX (Fig. 3*B* and *SI Appendix*, Fig. S6).

Ribosome Splitting Function of HflX Correlates with Its Ability to Mediate Antibiotic Resistance. Bacterial and eukaryotic HflX are 3 domain proteins composed of a unique N-terminal HflX domain (NTD), a central GTPase domain (G-domain), and a C-terminal domain (CTD) (26, 33). Interaction with the large ribosomal subunit is a conserved feature of all HflX proteins and is thought to be facilitated by the N-terminal and C-terminal domains (30, 31, 34). Moreover, the ribosome splitting function of *E. coli* HflX has previously been shown to require both the N- and C-terminal domains (26). To determine whether the ribosome-splitting function of Ms-HflX influences its ability to confer macrolide resistance, we created deletions in the N- and C-terminal domains of Ms-HflX identified based on the structure of Ms-HflX modeled on ribosome-bound *E. coli* HflX (Fig. 4 *A* and *B*). The Ms-HflX mutants containing deletions of either the N-terminal ($HflX_{236–471}$/ΔNTD) or the C-terminal ($HflX_{1–402}$/ΔCTD) domains were then assayed for ribosome splitting activity in vitro and antibiotic resistance in vivo. Fig. 4 *C–F* shows that removal of either the NTD or the CTD of Ms-HflX resulted in a defect in their ability to dissociate 70S ribosomes. Preliminary analyses show that both mutant proteins retain their ability to bind ribosomes (*SI Appendix*, Fig. S7*A*). Importantly, both HflX-ΔNTD and HflX-ΔCTD from *M. smegmatis* and *M. abscessus* were functionally defective in their ability to complement the antibiotic sensitivity of their respective Δ*hflX* mutant strains (Fig. 4*H*, Table 2, and *SI Appendix*, Fig. S7 *B* and *C*). Finally, a point mutant of Ms_HflX (*K258A/S259A*) defective in ribosome splitting was also found to be defective in complementing the antibiotic sensitivity of Δ*Ms_hflX* (Fig. 4 *G* and *H*). Together, these data indicate that the ability of mycobacterial HflX to mediate antibiotic resistance correlates directly with its ribosome splitting ability.

Macrolide Sensitivity of Mycobacterial Δ*hflX* Mutant Can Be Partially Restored by the Ribosome Recycling Factor. The ribosome recycling factor (RRF) is required for dissociation of the posttermination complex during a normal translation cycle, thereby making the subunits available for a new round of translation. Although key differences exist between the requirements of HflX and RRF, both proteins are required for recycling ribosomes that are stalled under different physiological conditions (26). We therefore tested the ability of *M. smegmatis* RRF to complement the antibiotic resistance of Δ*Ms_hflX*. As seen in Fig. 5 and *SI Appendix*, Table S1, constitutive expression of Ms-RRF partially restored antibiotic sensitivity of the mutant, supporting a role of ribosome splitting as a mechanism of antibiotic resistance. Partial complementation of HflX function by RRF in antibiotic resistance is also consistent with the notion that ribosomal substrates of HflX and RRF are likely to be fundamentally different (26).

Discussion

The *erm41* gene is considered to be the primary mechanism of intrinsic macrolide resistance in *M. abscessus* (18, 35). In the present study, we show that the *M. abscessus hflX* gene constitutes a

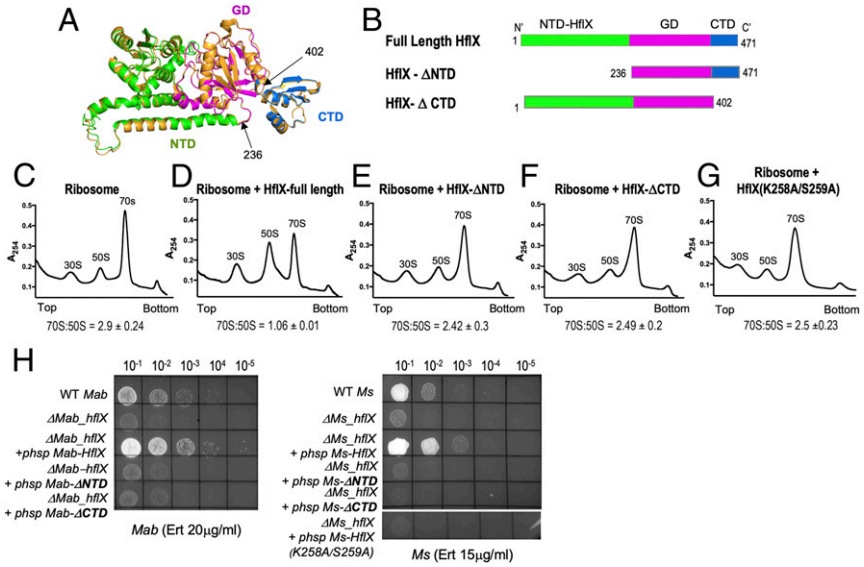

Fig. 4. Ribosome splitting function of Ms-HflX correlates with its ability to confer antibiotic resistance. (*A*) A structural model of Ms-HflX guided by the structure of *E. coli* HflX was obtained using I-TASSER and overlaid on the structure of *E. coli* HflX (orange) using PyMOL (https://pymol.org/2/). The Ms-HflX model is color coded by domains, as shown on the *Right*. (*B*) Location of truncations are indicated. (*C–G*) Dissociation of 70S ribosomes (0.2 μM) was carried out in the presence of 3.0 μM of either full-length Ms-$HflX_{6his}$, Ms-HflXΔNTD$_{6his}$, Ms-HflXΔCTD$_{6his}$ or Ms-HflX(K258A/S259A)$_{6his}$ in HMA-7 buffer containing 1 mM GMP-PNP at 37 °C for 45 min and examined using a 5-mL analytical 10% to 40% SDGC and Brandel gradient fractionation. Percentage AUC was calculated for 70S and 50S peaks, using PeakChart (v. 2.08, Brandel), and expressed as a ratio of 70S:50S. Data represent mean ± SD, $n = 3$. (*H*) Wild-type, Δ*hflX* mutant, and complementing strains containing the respective HflX-ΔNTD, HflX-ΔCTD, and HflX(K258A/S259A) at either the Bxb1 attB site of Δ*Ms_hflX* or the L5 attB site of Δ*Mab_hflX* were assayed for growth on Middlebrook 7H10 containing indicated concentrations of antibiotics. Expression of HflX-ΔNTD and HflX-ΔCTD in the complementing strain was verified using real-time PCR (*SI Appendix*, Table S2).

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Table 2. Survival of wild-type *M. abscessus* ATCC19977, *ΔMab_HflX*, and complemented strains in a 2-fold dilution series of antibiotics in Middlebrook 7H9/OADC medium

	Minimum Inhibitory Concentration (μg/mL)			
Antibiotic	WT *Mab*	*ΔMab_hflX*	*ΔMab_hflX* + phsp-hflX-ΔNTD	*ΔMab_hflX* + phsp-hflX-ΔCTD
Erythromycin	2	0.25	0.5	0.25
Clarithromycin	1	0.125	0.125	0.125
Azithromycin	8	2	2	2
Clindamycin	200	50	50	50

The minimum concentration of antibiotic required to inhibit 99% of growth after 72 h is shown. Minimum inhibitory concentration values are representative of 3 independent assays.

significant effector of macrolide-lincosamide resistance and confers equivalent levels of resistance as *erm41* via an *erm*-independent mechanism. We demonstrate that Ms-HflX strongly associates with ribosomal subunits in vivo in bacteria that are exposed to either ERT or CLIND, and an absence of HflX in the Δ*Ms_hflX* deletion strain results in an increased population of 70S ribosomes on erythromycin exposure compared with wild-type bacteria. The mycobacterial HflX is also capable of dissociating 70S ribosomes in vitro independent of GTP hydrolysis, similar to that observed in *E. coli* HflX (26). These observations led us to hypothesize that mycobacterial HflX is likely involved in dissociation of ribosomes stalled in the presence of antibiotics similar to the recycling of prematurely stalled ribosomes by *E. coli* HflX during heat shock. Nevertheless, the cryoEM structure of *E. coli* HflX reveals that the NTD binds to the 50S ribosomal subunit and protrudes into the PTC, making extensive contact with ribosomal RNA, whereas the CTD interacts with the bL12 stalk base and occupies a position distant from the PTC (26). Owing to the conservation of mycobacterial HflX with that of *E. coli*, an alternate scenario is that the mycobacterial HflX-NTD occupies a similar position within the ribosome and occludes macrolide-lincosamide binding. *SI Appendix*, Fig. S8, however, reveals that the presence of HflX neither interferes with binding of ^{3}H-ERT to ribosomes nor dissociates ^{3}H-ERT that is already bound to ribosomes. Instead, the evaluation of HflX mutants demonstrating that mutants that are defective in their ability to restore antibiotic tolerance of *ΔMs_hflX* are also defective in ribosome splitting, as well as the ability of mycobacterial RRF to restore macrolide tolerance of Δ*Ms_hflX*, together strengthen the importance of ribosome splitting in macrolide resistance (Figs. 4 and 5). Finally, the involvement of Ms-HflX-CTD, which is distant from the PTC, in conferring antibiotic tolerance reinforces the conclusion that antibiotic occlusion/ejection is unlikely to be the primary mechanism of HflX-mediated resistance to macrolides/lincosamides.

We therefore envisage a scenario in which binding of macrolides/lincosamides to ribosomes in the early stages of elongation results in accumulation of stalled, nonproductive ribosomes and a concomitant inhibition of translation. Association of HflX with antibiotic-stalled ribosomes presumably causes dissociation of the 50S and 30S subunits and rescue of the bound mRNA, which can then be translated by antibiotic-free ribosomes, thereby enabling survival in the presence of the drug. The postdissociated 50S subunit–HflX–antibiotic complex could either be sequestered from further rounds of translation due to the anti-association properties of HflX (26) or could serve as a substrate of accessory proteins that displace bound antibiotic/HflX. These possibilities are illustrated in Fig. 6. Moreover, it is likely that accessory proteins (if any) are also macrolide-lincosamide inducible and are included within the WhiB7 regulon. We speculate that MAB_1846 and MAB_2355, homologs of ABC-F proteins that displace ribosome-bound antibiotics, constitute strong candidates for accessory proteins that mediate recycling of the postdissociated 50S subunit–HflX–antibiotic complex. Although deletions in MAB_1846 or MAB_2355 alone do not significantly influence macrolide-lincosamide resistance, further investigations will be required to determine whether they act in concert with HflX, as well as to determine the identity of additional accessory proteins.

Our data in Fig. 3 shows preferential association of HflX with ribosomal fractions obtained from ERT/CLIND-treated cells; negligible levels of ribosome-bound HflX were observed in untreated cells. This difference could be attributed to a WhiB7-dependent increase in HflX expression in antibiotic-treated bacteria (*SI Appendix*, Fig. S9*A*). However, HflX levels in lysates of ERT-treated bacteria do not show an increase proportionate to the enrichment of HflX observed in ERT-treated ribosomes (~10-fold; *SI Appendix*, Fig. S9). We therefore speculate that recruitment of HflX could be facilitated by structural changes within antibiotic-bound ribosomes that are recognized either directly or indirectly by HflX. However, we cannot exclude a basal level of HflX interaction with antibiotic-free ribosomes, which is suggested by the lethal effect of multicopy expression of HflX from a strong promoter (*SI Appendix*, Fig. S10). Further investigations will be needed to elucidate the characteristics of ribosomes that serve as substrates for HflX binding, as well as the role of accessory proteins (if any) in facilitating HflX binding to ribosome.

Two classes of ribosome-associated proteins have been implicated in antibiotic resistance: the Ribosome Protection Proteins that confer tetracycline resistance and the ARE ABC-F group of proteins that confer resistance to the macrolide-lincosamide-ketolide antibiotics. Both classes of proteins show homology to the translation factors, EF-G and EF-Tu, and actively trigger the release of the antibiotic bound to the ribosome (14, 36–39). Dissociation of ribosomes stalled in the presence of antibiotics and rescue of mRNA has, however, not been described as a mechanism of antibiotic resistance until very recently, when Duval et al. (29) demonstrated that deletion of *Listeria monocytogenes hflX-r* (lmo0762) confers sensitivity to erythromycin and lincomycin and results in accumulation of 70S ribosomes in the mutant strain on antibiotic exposure. Interestingly, *L. monocytogenes* encodes 2 HflX paralogs, only 1 of which is involved in antibiotic resistance. Such a duplication event is observed in other firmicutes, as well as proteobacteria; the actinomycetes, however, encode a single copy of *hflX*. Curiously, a phylogenetic analysis shows that mycobacterial and *E. coli hflX* are more closely related to *lmo1296*, the *hflX* paralog in *L. monocytogenes* incapable of conferring resistance to macrolides/lincomycin (*SI Appendix*, Fig. S11) (29). We also note that the *E. coli* HflX is unable to complement the antibiotic sensitivity of Δ*Ms_hflX* and Δ*Mab_hflX* mutants, suggesting either that *E. coli* HflX is not involved in macrolide-lincosamide resistance or that it confers antibiotic resistance only in its native host (*SI Appendix*, Fig. S12). Functional metagenomics from antibiotic-rich environments that

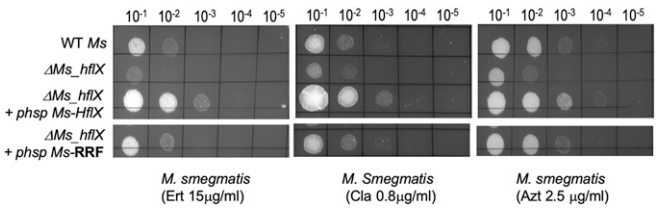

Fig. 5. Overexpression of the ribosome recycling factor (Ms-RRF) partially restores macrolide sensitivity of Δ*Ms_hflX*. Complementing strains were created by integrating either Ms_*RRF* or *Ms_hflX* at the Bxb1 attB site of Δ*Ms_hflX*. Expression of Ms-RRF in the complementing strain was verified using real-time PCR (*SI Appendix*, Table S2). Tenfold serial dilutions of wild-type *M. smegmatis*, Δ*Ms_hflX*, and the complementing strains were grown to A_{600} of 0.7 and spotted on Middlebrook 7H10 ADC containing indicated concentrations of macrolides.

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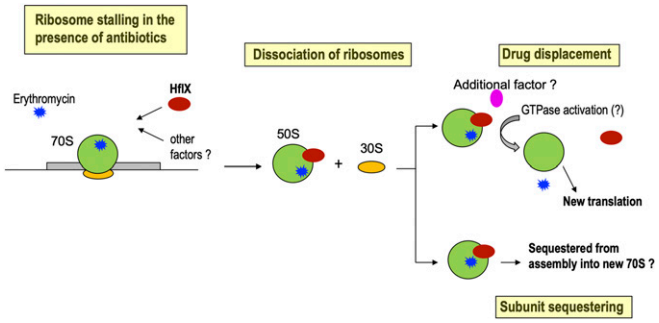

Fig. 6. Model of mycobacterial HflX-mediated antibiotic resistance.

identify *hflX* homologs as putative antibiotic resistance genes belong to both the HflX-r (*Emergencia* spp) and HflX (*Simkania* spp.) clades (40, 41). A possible explanation is that while all HflX proteins retain their ability to split 70S ribosomes into their subunits, their duplication allows each paralog to recognize ribosomes that are stalled under different physiological conditions. The ability of HflX to confer antibiotic tolerance may therefore be a generalized and widespread mechanism used by several bacteria, and not restricted only to species that contain the *hflX-r* paralog.

Materials and Methods

Referenced details of the materials and methods, including plasmids, strains, and oligonucleotides are provided in the *SI Appendix*. Proteins were purified using Ni-NTA chromatography. Antibiotic sensitivity assays were carried out either using broth dilution method or by spotting a 10-fold dilution series on plates containing the desired antibiotic concentrations. For analysis of polysome profiles on antibiotic exposure, bacteria were exposed to either ERT (20 μg/mL) or CLIND (16 μg/mL). Cells were lysed using the CryoMill (Retsch), and crude ribosomes were prepared as described (42), resuspended in HMA-8 buffer, layered on 10 mL of 10% to 40% sucrose gradients, and centrifuged using a Beckman SW 41 rotor followed by Brandel Teledyne fractionation. Ribosomes were purified from wild-type *mc²155*, Δ*Ms_hflX*, and *mc²155*:Ms_hflX-FLAG strains as described (42). For in vitro splitting assays, purified ribosomes (0.2 μM) were incubated with 15-fold molar excess of full-length Ms-HflX, point mutants, or truncated mutants (Ms-HflX ΔNTD and ΔCTD) in a 50-μL total volume in HMA-7 buffer in the presence of 1 mM GTP, ATP, or GMP-PNP. The reactions were incubated at 37 °C for 45 min and layered on 5-mL 10% to 40% sucrose gradients.

Data Availability. All data have been provided in the main article and *SI Appendix*.

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