

# Essential Roles for *Mycobacterium tuberculosis* Rel beyond the Production of (p)ppGpp

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In *Mycobacterium tuberculosis*, the stringent response to amino acid starvation is mediated by the *M. tuberculosis* Rel ( $Rel_{Mtb}$ ) enzyme, which transfers a pyrophosphate from ATP to GDP or GTP to synthesize ppGpp and pppGpp, respectively. (p)ppGpp then influences numerous metabolic processes.  $Rel_{Mtb}$  also encodes a second, distinct catalytic domain that hydrolyzes (p)ppGpp into pyrophosphate and GDP or GTP.  $Rel_{Mtb}$  is required for chronic *M. tuberculosis* infection in mice; however, it is unknown which catalytic activity of  $Rel_{Mtb}$  mediates pathogenesis and whether (p)ppGpp itself is necessary. In order to individually investigate the roles of (p)ppGpp synthesis and hydrolysis during *M. tuberculosis* pathogenesis, we generated  $Rel_{Mtb}$  point mutants that were either synthetase dead ( $Rel_{Mtb}^{H344Y}$ ) or hydrolase dead ( $Rel_{Mtb}^{H80A}$ ). *M. tuberculosis* strains expressing the synthetase-dead  $Rel_{Mtb}^{H344Y}$  mutant did not persist in mice, demonstrating that the  $Rel_{Mtb}$  (p)ppGpp synthetase activity is required for maintaining bacterial titers during chronic infection. Deletion of a second predicted (p)ppGpp synthetase had no effect on pathogenesis, demonstrating that  $Rel_{Mtb}$  was the major contributor to (p)ppGpp production during infection. Interestingly, expression of an allele encoding the hydrolase-dead  $Rel_{Mtb}$  mutant,  $Rel_{Mtb}^{H80A}$ , that is incapable of hydrolyzing (p)ppGpp but still able to synthesize (p)ppGpp decreased the growth rate of *M. tuberculosis* and changed the colony morphology of the bacteria. In addition,  $Rel_{Mtb}^{H80A}$  expression during acute or chronic *M. tuberculosis* infection in mice was lethal to the infecting bacteria. These findings highlight a distinct role for  $Rel_{Mtb}$ -mediated (p)ppGpp hydrolysis that is essential for *M. tuberculosis* pathogenesis.

At least 30% of the world's population is infected with latent *Mycobacterium tuberculosis*, which will reactivate in some individuals and cause an estimated 1.4 million deaths a year (1). Significant obstacles in controlling the epidemic result from the chronic nature of *M. tuberculosis* infection, which necessitates prolonged treatment and generates a large reservoir of latently infected people. This health crisis is exacerbated by the alarming emergence of drug-resistant strains. The inadequacies of present tuberculosis therapies demand the discovery of new agents to treat *M. tuberculosis* infection, which requires insight into the pathways used by the pathogen to survive in the host. During infection, the host restrains mycobacteria from proliferating by imposing an arsenal of defenses, including oxidative stress, hypoxia, acid stress, genotoxic stress, cell surface stress, and starvation (reviewed in reference 2). Despite this onslaught of attacks, *M. tuberculosis* is able to persist for the lifetime of the host, indicating that this pathogen has substantial molecular mechanisms to resist host-inflicted damage.

One way bacteria resist stress is via the stringent response. The stringent response is a conserved global stress response in bacteria that involves the production of the hyperphosphorylated guanine nucleotides ppGpp and pppGpp [collectively called (p)ppGpp]. (p)ppGpp metabolism is controlled by Rel/SpoT homolog proteins (RSHs), named for their sequence similarity to RelA and SpoT enzymes in *Escherichia coli* (3). The activity of RelA homologs is best characterized during amino acid starvation when an uncharged tRNA enters the A site of the ribosome and stimulates RelA-mediated (p)ppGpp production (4). (p)ppGpp then regulates a number of cellular processes, including transcription, replication, cell surface morphology, and nucleotide levels (reviewed in references 5 and 6).

In *M. tuberculosis*, the stringent response is initiated by the *M. tuberculosis* Rel ( $Rel_{Mtb}$ ) enzyme, which transfers the 5'- $\beta,\gamma$ -py-

rophosphate from ATP to the 3'-OH of GDP or GTP to synthesize ppGpp and pppGpp, respectively (7). The (p)ppGpp synthetase domain is in the middle of the  $Rel_{Mtb}$  protein, and a mutation of a conserved histidine (H344) or glycine (G241) residue in this domain abolishes synthetase activity (8, 9). The C terminus of  $Rel_{Mtb}$  is not required for catalytic activity but is necessary for association with the  $Rel_{Mtb}$  activating complex, which consists of ribosomes, mRNA, and tRNAs and regulates (p)ppGpp synthesis (10, 11).  $Rel_{Mtb}$  also hydrolyzes (p)ppGpp into diphosphate pyrophosphate ( $PP_i$ ) and GDP or GTP via an N-terminal catalytic HD superfamily domain that is distinct from the (p)ppGpp synthetase domain (9). HD superfamily members are phosphohydrolases with conserved histidine and aspartate residues that are involved in the coordination of divalent cations, which is essential for their activity (12). Alanine substitution of the conserved histidine (H80) or aspartate (D81) residue in  $Rel_{Mtb}$  abolishes hydrolase activity *in vitro* without affecting (p)ppGpp synthesis (9). The crystal structure of the  $Rel_{Mtb}$  homolog in *Streptococcus dysgalactiae* subsp. *equisimilis*,  $Rel_{Seq}$ , indicates that at any given time only one enzymatic function is active, thereby preventing simultaneous synthesis and hydrolysis of (p)ppGpp (13).

*M. tuberculosis* mutants deleted for the entire  $rel_{Mtb}$  gene ( $\Delta rel_{Mtb}$ ) are compromised for long-term survival in culture (7) and are unable to persist in mouse models of infection (14, 15). In *Mycobacterium smegmatis*, deletion of  $rel_{Msm}$  causes changes in

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cellular and colony morphology, decreased long-term survival, and increased sensitivity to nutrient starvation and hypoxia (16). However, it is still unclear which catalytic activities of Rel<sub>Mtb</sub> contribute to these diverse phenotypes and what role (p)ppGpp has in these processes. In addition to Rel<sub>Mtb</sub>, *M. tuberculosis* also encodes a homolog to a group of RSHs called small alarmone synthetases (SAS), which encode the catalytic domain to produce (p)ppGpp but not the hydrolase domain (3). The role of the *M. tuberculosis* SAS, Rv1366, in maintaining (p)ppGpp levels, responding to stress, and virulence has yet to be investigated. In many other bacteria, SAS activity is toxic in the absence of a functional (p)ppGpp hydrolase (17–21). If this were the case in *M. tuberculosis*, Rv1366-mediated (p)ppGpp synthesis in the  $\Delta rel_{Mtb}$  strain could be the cause for attenuation of this strain. We have addressed these questions regarding the roles of (p)ppGpp metabolism by studying strains of *M. tuberculosis* that express alleles of Rel<sub>Mtb</sub> that are defective in individual catalytic activities and strains deficient in the *rv1366* gene.

## MATERIALS AND METHODS

***M. tuberculosis* strains and media.** All *M. tuberculosis* strains were derived from Erdman and were grown planktonically at 37°C in 7H9 (broth) or 7H10 (agar) (Difco) medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 (broth). Biofilm cultures were grown in 24-well dishes at 37°C by inoculating 1.5 ml of Sauton's medium with 150  $\mu$ l of saturated planktonic culture. The 24-well dish was placed in a tightly sealed Tupperware dish for 3 weeks, at which point the Tupperware was opened and incubated for another week before photographing. This is similar to previously described conditions used to form *M. tuberculosis* biofilms (22).

The  $\Delta rel_{Mtb}$  strain was made by infecting *M. tuberculosis* Erdman with a specialized transducing phage (phAE87) containing homology to *M. tuberculosis* nucleotides 2907267 to 2907826 and 2910191 to 2910844, which replaced all but the first two codons and the stop codon of the endogenous *rel\_{Mtb}* gene with a hygromycin resistance cassette. The  $\Delta rv1366$  strain was made by infecting wild-type (WT) *M. tuberculosis* Erdman with phAE87 containing homology to *M. tuberculosis* nucleotides 1537706 to 1538406 and 1539203 to 1539884, which replaced all but the first 6 codons and the last 2 codons of the endogenous *rv1366* gene with a hygromycin resistance cassette flanked by two *loxP* sites. The deletion of *rv1366* in this strain was confirmed by EcoRI digestion of genomic DNA and Southern blot analysis using a radiolabeled fragment containing *M. tuberculosis* nucleotides 1537706 to 1538406 as the probe. The hygromycin resistance cassette was cured from the  $\Delta rv1366$  strain by transforming the strain with an unstable episomal plasmid (pmsg381) that expresses the Cre recombinase. Loss of the hygromycin resistance cassette was confirmed by both PCR and plating in the presence or absence of hygromycin. The *rel\_{Mtb}* gene was deleted from the  $\Delta rv1366$  strain to make the  $\Delta rel_{Mtb}$   $\Delta rv1366$  strain by infecting the  $\Delta rv1366$  strain, which had been cured of the hygromycin resistance cassette, with the phAE87 phage that replaces the endogenous *rel\_{Mtb}* gene with a hygromycin resistance cassette. The deletion of the *rel\_{Mtb}* gene in this strain was confirmed by Southern blot analysis of SmaI-digested genomic DNA using a radiolabeled fragment containing *M. tuberculosis* nucleotides 2910191 to 2910844 as a probe.

To express the *rel\_{Mtb}*<sup>WT</sup>, *rel\_{Mtb}*<sup>H344Y</sup>, *rel\_{Mtb}*<sup>H80A</sup>, *rel\_{Mtb}*<sup>H344Y-H80A</sup>, or *rv1366* allele from the *attB* site of the gene deletion strains, the  $\Delta rel_{Mtb}$ ,  $\Delta rv1366$ , or  $\Delta rel_{Mtb}$   $\Delta rv1366$  strain was transformed with a pMSG430 plasmid that integrates into the *attB* site of the genome, confers kanamycin resistance, and expresses the respective allele from a constitutive pmc1tetO promoter. Control strains were made by transforming each deletion mutant with an empty pMSG430 vector. The *rel\_{Mtb}* and *rv1366* genes from each transformant were sequenced to confirm the presence of the correct sequence. There is only a single copy each of the *rel\_{Mtb}* and *rv1366* genes in all strains of mycobacteria used in this paper.

To achieve conditional expression of *rel\_{Mtb}*<sup>H80A</sup>, the  $\Delta rel_{Mtb}$  strain was cotransformed with pMSG430-*rel\_{Mtb}*<sup>H80A</sup> and pTES-2MOX (kindly provided by Dirk Schnappinger and Sabine Ehrh, Cornell University, NY), which confers streptomycin resistance and expresses the WT tetracycline repressor (TetR). This generated the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain, wherein the WT TetR allows for induced expression of the *rel\_{Mtb}*<sup>H80A</sup> allele in the presence of tetracycline (Tet) analogs. A control strain was constructed by cotransforming WT *M. tuberculosis* Erdman with empty pMSG430 and pTES-2MOX.

***M. smegmatis* strains and media.** All *M. smegmatis* strains were isogenic to strain mc<sup>2</sup>155 and were grown at 37°C in LB (broth and agar) or 7H10 (agar) supplemented with 0.5% dextrose, 0.5% glycerol, and 0.05% Tween 80 (broth). The  $\Delta rel_{Msm}$  strain was made by infecting mc<sup>2</sup>155 with phAE87 containing homology to mc<sup>2</sup>155 nucleotides 3027905 to 3028545 and 3030884 to 3031534, which deleted all but the first 4 codons and the last 14 codons of the *rel\_{Msm}* gene. The *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain was made as described for *M. tuberculosis*. The *M. smegmatis* control strain was made by cotransforming the  $\Delta rel_{Msm}$  strain with pMSG430-*rel\_{Mtb}*<sup>WT</sup> and pTES-2MOX.

**Antibiotics.** Twenty  $\mu$ g/ml kanamycin, 50  $\mu$ g/ml hygromycin, 20  $\mu$ g/ml streptomycin, and 50 ng/ml of anhydrotetracycline (ATc) were used for both mycobacterial species.

**Mouse infections.** Before infection, exponentially replicating *M. tuberculosis* bacteria were washed in phosphate-buffered saline (PBS) plus 0.05% Tween 80 and sonicated to disperse clumps. Eight- to 9-week-old female C57BL/6 mice (Jackson Laboratory) were exposed to  $8 \times 10^7$  CFU of the appropriate strain in an inhalation exposure system (Glas-Col), which delivers  $\sim 100$  bacteria to the lung per animal. Bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto 7H10 agar plates in the absence or presence of kanamycin and streptomycin. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 3 weeks prior to counting colonies. When indicated, the standard diet was replaced with doxycycline-containing mouse chow (2,000 ppm; Research Diets). All procedures involving animals were conducted by following the National Institutes of Health guidelines for housing and care of laboratory animals and were performed in accordance with institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of The Washington University in St. Louis School of Medicine (protocol 20100190; Analysis of Mycobacterial Pathogenesis). Washington University is registered as a research facility with the U.S. Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care. The Animal Welfare Assurance is on file with OPRR-NIH. All animals used in these experiments were subjected to no or minimal discomfort. All mice were euthanized by CO<sub>2</sub> asphyxiation, which is approved by the Panel on Euthanasia of the American Veterinary Association.

**qRT-PCR.** RNA was extracted from exponentially growing mycobacteria using TRIzol (Invitrogen), cDNA was prepared using Superscript III (Invitrogen), and quantitative reverse transcription-PCR (qRT-PCR) was performed using the Bio-Rad Sso Advanced SYBR green kit. Levels of *rel\_{Mtb}* and *rel\_{Msm}* transcript were normalized to *sigA* transcript levels as previously described (23).

**Assaying nucleotide and metabolite levels using LC-MS/MS.** *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>H80A</sup> and control strains were grown to exponential phase and treated with 50 ng/ml ATc for 6 h, and  $5.9 \times 10^9$  CFU per sample was collected and washed in PBS. ATP, GTP, ppGpp, and pppGpp were assayed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. For the nucleotide extraction, 10  $\mu$ l of 0.1 mM internal standard Br-ATP was spiked in each sample. Six hundred  $\mu$ l of cold 50% ethanol was added to each sample, and samples were homogenized with TissueLyser II for 5 min at a frequency of 20 Hz/s. After centrifugation at  $16,000 \times g$  for 5 min at 4°C, the supernatants were transferred to new tubes and the pellets were reextracted as previously described. The second supernatant was combined with the first one and dried down. The pellets were dissolved in water for LC-MS/MS analysis.

TABLE 1 Mass spectrometry of nucleotide standards<sup>a</sup>

Q1 MS	Q3 MS	Time (ms)	Name	DP (V)	CE (V)
506.2	408.0	50	ATP1	-70	-30
506.2	158.8	50	ATP2	-70	-35
506.2	78.8	50	ATP3	-70	-80
522.2	423.9	50	GTP1	-70	-30
522.2	158.8	50	GTP2	-70	-40
522.2	78.8	50	GTP3	-70	-80
602.2	504.0	50	ppGpp1	-70	-35
602.2	423.9	50	ppGpp2	-70	-45
602.2	158.8	50	ppGpp3	-70	-60
682.2	584.0	50	pppGpp1	-70	-35
682.2	504.0	50	pppGpp2	-70	-45
586.0	273.0	50	Br-ATP1	-55	-60
586.0	159.0	50	Br-ATP2	-55	-50
586.0	79.0	50	Br-ATP3	-55	-95

<sup>a</sup> DP, declustering potential; CE, collision energy.

The LC-MS/MS system used is composed of a Shimadzu LC system with two Shimadzu solvent delivery pumps (model LC10AD), a Shimadzu integrated controller (SCL10Avp), a Valco two-position diverter valve, and a LEAP CTC PAL autosampler with a 50- $\mu$ l sample loop. This LC system is interfaced with an AB Sciex 4000 QTRAP mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source. Source parameters were set as the following: curtain gas, 10 arbitrary units (AU); source gas 1, 50 AU; source gas 2, 50 AU; collision activated dissociation, high; interface heater, on; temperature, 650°C; ionspray voltage, -4,500. Both quadrupoles (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.2) was used to control sample acquisition and data analysis. The 4000 QTRAP mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. Each compound was detected using 3 multiple reaction monitoring (MRM) transitions that were previously optimized using standards described in Table 1, with the exception of pppGpp, for which no commercial standard is available. Only 2 MRM transitions for pppGpp were used based on the ppGpp standard.

For LC separation, a Synergi 2.5-U Hydro-RP 100A column (100 by 2 mm; Phenomenex) was used at a flow rate of 0.11 ml/min. The gradient started at 100% solvent A (10 mM tributylamine in 5% methanol, pH 4.8), was held for 2 min, and then moved to 20% solvent B (100% methanol) in 2 min. The gradient then increased to 50% solvent B in 33 min. It was finally ramped up to 90% solvent B in 1 min and then ramped back to initial conditions (100% solvent A) in 1 min and reequilibrated for an additional 7 min. For quantification, a series of standard samples containing different concentrations of compounds was prepared. Calibration curves for all of the compounds were achieved by integration of the peak area under the curve of the standards and then normalized to the internal standard Br-ATP.

## RESULTS

**Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis is required for WT rates of growth on solid media and formation of biofilms.** Deletion of *M. tuberculosis* rel<sub>Mtb</sub> results in slightly slower growth, compromised long-term survival in cultures (7), and an inability to persist in mouse models of infection (14, 15). rel<sub>Mtb</sub> encodes two different enzymatic activities, (p)ppGpp synthesis and hydrolysis, and it is unknown if the phenotypes of the  $\Delta$ rel<sub>Mtb</sub> strain result exclusively from the loss of (p)ppGpp production. A Rel<sub>Mtb</sub><sup>H344Y</sup> point mutant is unable to synthesize (p)ppGpp but retains hydrolase activity *in vitro* (9). To determine whether Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis is specifically important for mycobacteria, we constructed *M. tuberculosis* strains deleted for the endogenous rel<sub>Mtb</sub> gene and constitutively expressing either the rel<sub>Mtb</sub><sup>WT</sup> allele or the

rel<sub>Mtb</sub><sup>H344Y</sup> synthetase-dead mutant from the attB site of the genome. As previously reported, the *M. tuberculosis*  $\Delta$ rel<sub>Mtb</sub> mutant grew more slowly than a strain complemented with the rel<sub>Mtb</sub><sup>WT</sup> allele, which is particularly evident on 7H10 solid media (Fig. 1A) (7). Expression of the Rel<sub>Mtb</sub><sup>H344Y</sup> mutant did not fully complement this growth defect, demonstrating that (p)ppGpp synthesis by Rel<sub>Mtb</sub> is necessary for optimal growth on solid media. A lower growth rate was defined by smaller colony size on 7H10 plates, as illustrated in Fig. 1A at the 10<sup>-3</sup> and 10<sup>-4</sup> dilutions. In addition to the slow growth on solid media, we also observed that loss of Rel<sub>Mtb</sub>-mediated (p)ppGpp synthetase activity resulted in delayed formation of biofilms and pellicles in static liquid cultures (Fig. 1B). This illustrates another *in vitro* condition that requires Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis. Biofilms may serve as a model for persister cell formation, in that they harbor a larger number of drug-tolerant cells than planktonic *M. tuberculosis* cultures (22). Thus, this defect in biofilm formation by the  $\Delta$ rel<sub>Mtb</sub> mutant may be due to the interference of the same pathways necessary to persist in the mouse infection model.

**(p)ppGpp synthesis by Rel<sub>Mtb</sub> is required for chronic infection in mice.** *M. tuberculosis* infection in mice begins with an initial phase of unrestricted bacterial growth termed acute infection. The onset of adaptive immunity stops the bacterial titers from increasing at 3 to 4 weeks postinfection, but bacteria are still able to persist for the lifetime of the mouse. *M. tuberculosis*  $\Delta$ rel<sub>Mtb</sub> aerosol infection of mice results in normal initial bacterial growth but impaired chronic infection, resulting in less severe gross pathology of the disease (Fig. 2) (14). To determine whether Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis was specifically required for chronic infection, we infected C57BL/6 mice with *M. tuberculosis*  $\Delta$ rel<sub>Mtb</sub> expressing rel<sub>Mtb</sub><sup>WT</sup>, rel<sub>Mtb</sub><sup>H344Y</sup>, or no rel<sub>Mtb</sub> allele from the attB site. *M. tuberculosis* strains expressing the Rel<sub>Mtb</sub><sup>H344Y</sup> mutant phenocopied the null mutant by not persisting in the murine lungs and spleens and causing less tissue pathology than control strains, demonstrating that Rel<sub>Mtb</sub> (p)ppGpp synthetase activity is required for chronic *M. tuberculosis* infection (Fig. 2).

**Rv1366 activity does not contribute to phenotypes of a  $\Delta$ rel<sub>Mtb</sub> mutant and is dispensable for pathogenesis in the mouse model of infection.** The SAS homolog Rv1366 is a second predicted (p)ppGpp synthetase in *M. tuberculosis* and could con-

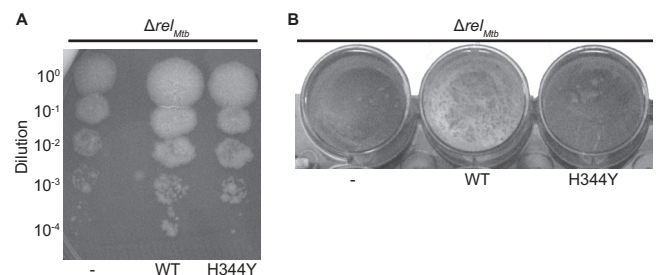
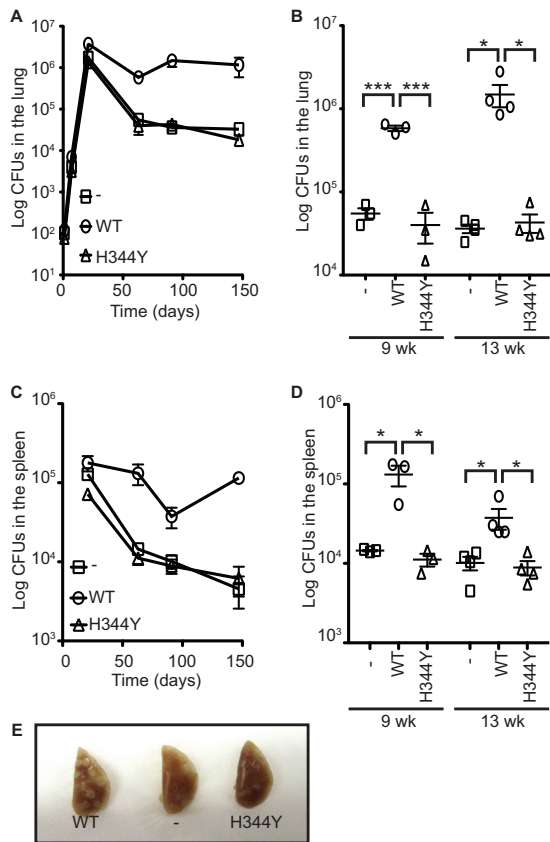
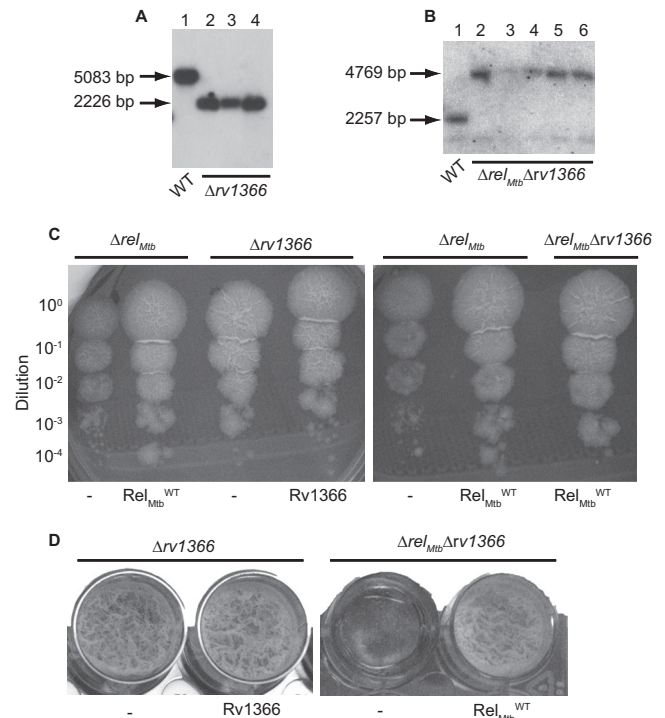


FIG 1 Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis is necessary for optimal *M. tuberculosis* growth and biofilm formation *in vitro*. *M. tuberculosis*  $\Delta$ rel<sub>Mtb</sub> strains containing a vector that constitutively expresses Rel<sub>Mtb</sub><sup>WT</sup> (WT) or Rel<sub>Mtb</sub><sup>H344Y</sup> (H344Y) or containing an empty vector (-) integrated at the attB site in the genome were normalized to the same OD<sub>600</sub> and either diluted and plated on 7H10 plates (A) or grown in Sauton's medium in static, biofilm-forming conditions in a 24-well dish (B). (A) 7H10 plates were incubated for 3 weeks at 37°C. (B) Biofilms were incubated for 4 weeks at 37°C. The parent strain used is labeled above each photo, and the protein expressed from the attB site is labeled below the photo.



**FIG 2**  $Rel_{Mtb}$ -mediated (p)ppGpp synthesis is necessary for chronic *M. tuberculosis* infection in mice. C57BL/6 mice were infected with *M. tuberculosis*  $\Delta rel_{Mtb}$  strains containing a vector that constitutively expresses  $Rel_{Mtb}^{WT}$  (WT; open circles) or  $Rel_{Mtb}^{H344Y}$  (H344Y; open triangles) or containing an empty vector (-; open squares) integrated at the *attB* site in the genome. Shown are bacterial titers from lungs (A and B) and spleens (C and D) at various time points. Each time point is the means  $\pm$  standard errors of the means (SEM) of data from 3 to 4 mice per strain. (B and D) Significance of differences compared to the  $Rel_{Mtb}^{WT}$ -expressing strain at 9 and 13 weeks postinfection was determined by calculating *P* values by Student's *t* tests; one asterisk indicates significance with a *P* value of  $<0.05$ , and three asterisks indicates a significance with a *P* value of  $<0.005$ . (E) Gross pathology of representative left lungs from each strain at 13 weeks postinfection shows more lesions in the  $Rel_{Mtb}^{WT}$ -expressing strain.

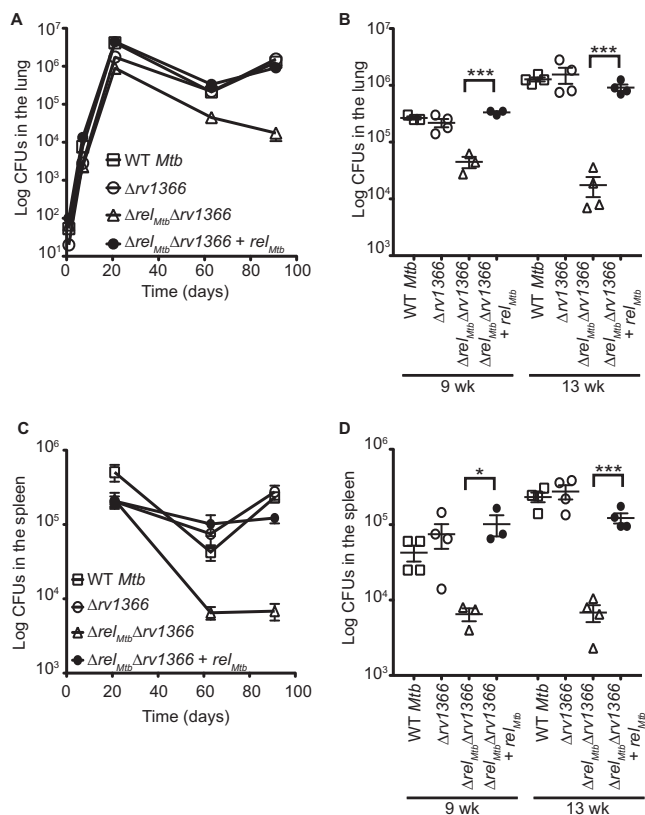
tribute to (p)ppGpp metabolism; however, it has yet to be characterized. To investigate the function of Rv1366, we constructed an *M. tuberculosis* strain deleted for the predicted (p)ppGpp synthetase *rv1366* ( $\Delta rv1366$  strain) and an *M. tuberculosis* double mutant of  $rel_{Mtb}$  and *rv1366* ( $\Delta rel_{Mtb} \Delta rv1366$  strain), which were both confirmed by Southern blot analysis (Fig. 3A and B), and monitored their growth rate and ability to form biofilms. The  $\Delta rv1366$  mutant did not have a growth defect compared to controls on either solid media or under biofilm conditions (Fig. 3C and D). Additionally, the  $\Delta rel_{Mtb} \Delta rv1366$  double mutant phenocopied the  $\Delta rel_{Mtb}$  single mutant on both solid media (data not shown) and under biofilm conditions (Fig. 3D). Expression of  $Rel_{Mtb}^{WT}$  was also able to fully complement the growth and biofilm phenotypes in the  $\Delta rel_{Mtb} \Delta rv1366$  double mutant (Fig. 3C and D). These data demonstrate that Rv1366 does not provide a function necessary for growth in nutrient-rich media or under biofilm conditions regardless of the presence of  $Rel_{Mtb}$ -mediated



**FIG 3** Rv1366 is not necessary for *M. tuberculosis* growth or biofilm formation *in vitro*. (A) Southern blot analysis of WT *M. tuberculosis* Erdman (lane 1) and the  $\Delta rv1366$  strain (lanes 2 to 4) using EcoRI-digested genomic DNA and a probe that spans nucleotides 1537706 to 1538406 of the *M. tuberculosis* genome. EcoRI digestion of WT *M. tuberculosis* yields a band at 5,083 bp. EcoRI digestion of  $\Delta rv1366$  results in a 2,226-bp band due to the replacement of the *rv1366* gene with a hygromycin resistance cassette. (B) Southern blot analysis of WT *M. tuberculosis* Erdman (lane 1) and the  $\Delta rel_{Mtb} \Delta rv1366$  strain (lanes 2 to 6) using SmaI-digested genomic DNA and a probe that spans nucleotides 2910191 to 2910844 of the *M. tuberculosis* genome. SmaI digestion of WT *M. tuberculosis* yields a band at 2,257 bp. SmaI digestion of the  $\Delta rel_{Mtb} \Delta rv1366$  strain results in a 4,769-bp band due to the replacement of the  $rel_{Mtb}$  gene with a hygromycin resistance cassette. (C and D) *M. tuberculosis*  $\Delta rel_{Mtb}$ ,  $\Delta rv1366$ , and  $\Delta rel_{Mtb} \Delta rv1366$  strains containing a vector that constitutively expresses  $Rel_{Mtb}^{WT}$  or Rv1366 or containing an empty vector (-) integrated at the *attB* site in the genome were normalized to the same  $OD_{600}$  and either diluted and plated on 7H10 plates (C) or grown in Sauton's media in static, biofilm-forming conditions in a 24-well dish (D). (C) 7H10 plates were incubated for 3 weeks at 37°C. (D) Biofilms were incubated for 4 weeks at 37°C. The parent strain used is labeled above each photo, and the protein expressed from the *attB* site is labeled below the photo.

(p)ppGpp synthesis. We then examined whether Rv1366 was necessary during *M. tuberculosis* infection of mice by infecting C57BL/6 mice with the  $\Delta rv1366$  mutant and the  $\Delta rel_{Mtb} \Delta rv1366$  double mutant. The  $\Delta rv1366$  single mutant was not attenuated during infection in either the lung or the spleen, and the  $\Delta rel_{Mtb} \Delta rv1366$  double mutant phenocopied the  $\Delta rel_{Mtb}$  mutant and was fully complemented by expression of only  $Rel_{Mtb}^{WT}$  (Fig. 4). These data show that Rv1366 is not necessary for *M. tuberculosis* growth or survival *in vitro* or *in vivo* in the models tested.

In *Bacillus subtilis*, the phenotypes of the  $\Delta relA$  strain are attributed in part to the absence of (p)ppGpp hydrolase activity in the presence of two SAS homologs, such that suppressors of the *B. subtilis*  $\Delta relA$  mutant have inactivating mutations in the SAS genes (17). Therefore, we wanted to confirm that the *M. tuberculosis* *rv1366* gene was intact in the  $\Delta rel_{Mtb}$  strain. We sequenced *rv1366*



**FIG 4** Rv1366 is not necessary for *M. tuberculosis* pathogenesis in mice. C57BL/6 mice were infected with the WT *M. tuberculosis* Erdman strain (open squares), the  $\Delta rv1366$  strain (open circles), the  $\Delta rel_{Mtb} \Delta rv1366$  strain containing an empty vector integrated at the *attB* site in the genome ( $\Delta rel_{Mtb} \Delta rv1366$ ; open triangles), or the  $\Delta rel_{Mtb} \Delta rv1366$  strain containing a vector that constitutively expresses Rel<sub>Mtb</sub><sup>WT</sup> from the *attB* site in the genome ( $\Delta rel_{Mtb} \Delta rv1366 + rel_{Mtb}$ ; closed circles). Shown are bacterial titers from lungs (A and B) and spleens (C and D) at various time points. Each time point is the means  $\pm$  SEM of data from 3 to 4 mice per strain. (B and D) Significance of differences compared to the  $\Delta rel_{Mtb} \Delta rv1366$  strain at 9 and 13 weeks postinfection was determined by calculating *P* values by Student's *t* tests; one asterisk indicates significance with a *P* value of <0.05, and three asterisks indicates significance with a *P* value of <0.005.

along with 159 nucleotides upstream and 182 nucleotides downstream of the *rv1366* gene in WT *M. tuberculosis* and  $\Delta rel_{Mtb}$  strains. The nucleotide sequences were identical between strains and were the same as the sequence deposited in NCBI for the *M. tuberculosis* H37Rv strain. Therefore, the role of Rv1366 in *M. tuberculosis* remains unknown, but its function is not redundant with (p)ppGpp production by Rel<sub>Mtb</sub> under the conditions examined.

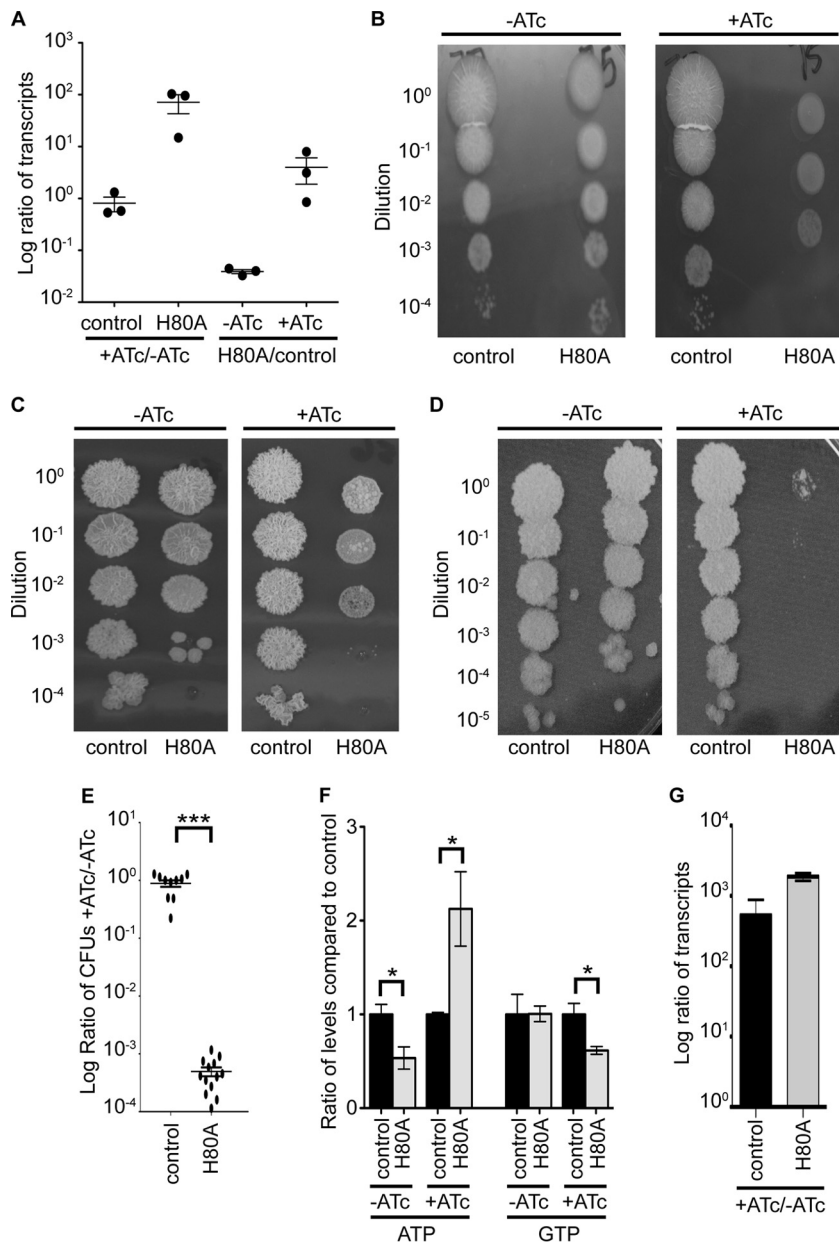
**Rel<sub>Mtb</sub> hydrolase activity is critical for mycobacterial physiology when (p)ppGpp synthesis is intact.** In addition to (p)ppGpp synthesis, Rel<sub>Mtb</sub> is the only known enzyme in *M. tuberculosis* that is capable of hydrolyzing (p)ppGpp for turnover and renewal of GTP and GDP by removal of pyrophosphate. In order to investigate the roles of the Rel<sub>Mtb</sub> hydrolase activity during growth and survival, we attempted to generate strains of *M. tuberculosis* expressing a single copy of Rel<sub>Mtb</sub> that was hydrolase dead but retained (p)ppGpp synthetase activity (Rel<sub>Mtb</sub><sup>H80A</sup>) or was inactive for both synthesis and hydrolysis (Rel<sub>Mtb</sub><sup>H80A-H344Y</sup>). Although we were able to obtain the *M. tuberculosis* Rel<sub>Mtb</sub><sup>H80A-H344Y</sup>-expressing strain, we did not recover clones expressing the *rel<sub>Mtb</sub><sup>H80A</sup>* allele. This suggested that the

retention of the Rel<sub>Mtb</sub> (p)ppGpp synthetase activity is toxic in the absence of a functional hydrolase. To investigate this further, we used a tetracycline-inducible expression system (24) and engineered an *M. tuberculosis*  $\Delta rel_{Mtb}$  strain to induce expression of the *rel<sub>Mtb</sub><sup>H80A</sup>* allele from a cassette at the *attB* site in the presence of tetracycline analogs, such as anhydrotetracycline (ATc), due to the expression of a tetracycline repressor (TetR) from an episomal plasmid. We named this strain Tet-Rel<sub>Mtb</sub><sup>H80A</sup>. We also constructed a control strain by inserting an empty vector into the *attB* site of WT *M. tuberculosis* and transforming this strain with the same TetR-expressing plasmid as that in Tet-Rel<sub>Mtb</sub><sup>H80A</sup>. *rel<sub>Mtb</sub>* expression from its endogenous locus in the control strain is not affected by ATc (Fig. 5A). Induction of *rel<sub>Mtb</sub><sup>H80A</sup>* transcription in the presence of ATc in the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain was confirmed by quantitative real-time PCR (qRT-PCR) (Fig. 5A).

We plated the *M. tuberculosis* control and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strains onto 7H10 agar in the absence or presence of ATc and found that although the strains had equivalent survival rates under both conditions, the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain exhibited smaller, rounder, and smoother colonies than control bacteria in the presence of ATc (Fig. 5B). This demonstrates that inducing expression of Rel<sub>Mtb</sub><sup>H80A</sup>, which will synthesize (p)ppGpp but will not hydrolyze (p)ppGpp, has an impact on *M. tuberculosis* physiology. Thus, (p)ppGpp turnover is required to maintain normal colony morphology. There was a mild effect on colony morphology in the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain in the absence of ATc (Fig. 5B), suggesting that even low levels of *rel<sub>Mtb</sub><sup>H80A</sup>* expression in the absence of inducer due to the inherent leakiness of the TetR system was enough to affect bacterial physiology. This low level of *rel<sub>Mtb</sub><sup>H80A</sup>* transcription in the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain in the absence of ATc was detectable by qRT-PCR and was close to 100-fold lower than in induced conditions (Fig. 5A).

We also engineered *M. smegmatis*  $\Delta rel_{Msm}$  strains to induce expression of either the WT *rel<sub>Mtb</sub>* allele (Tet-Rel<sub>Mtb</sub><sup>WT</sup>) or the *rel<sub>Mtb</sub><sup>H80A</sup>* allele (Tet-Rel<sub>Mtb</sub><sup>H80A</sup>) in the presence of ATc. Similar to the results from *M. tuberculosis* experiments, when we plated *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>WT</sup> and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> on 7H10 agar in the presence of ATc, the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain exhibited smaller, smoother colonies than the Tet-Rel<sub>Mtb</sub><sup>WT</sup> strain (Fig. 5C). This demonstrated that the role of (p)ppGpp turnover in colony morphology is conserved in *M. tuberculosis* and *M. smegmatis*. Interestingly, when we plated the *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>WT</sup> and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strains on LB agar in the absence or presence of ATc, the induced expression of the *rel<sub>Mtb</sub><sup>H80A</sup>* mutant allele inhibited growth (Fig. 5D and E) and all of the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> colonies that grew were no longer responsive to the presence of ATc, as determined by plating and qRT-PCR. Therefore, in order to grow on nutrient-rich LB media, the *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain must mutate the Tet-inducible system and permanently repress the expression of *rel<sub>Mtb</sub><sup>H80A</sup>* to survive. This proved that inhibition of the Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity in the presence of a functional (p)ppGpp synthetase is toxic to *M. smegmatis* in nutrient-rich LB media and links nutrient availability with the importance of (p)ppGpp turnover. (p)ppGpp production has been associated with colony morphology in mycobacteria (16, 25), but this is the first time in mycobacteria that the hydrolysis of (p)ppGpp has been shown to be required for maintenance of WT colony morphology and growth in nutrient-rich conditions.

(p)ppGpp levels were measured in the *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>WT</sup> and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strains by liquid chromatography-



**FIG 5**  $Rel_{Mtb}$ -mediated (p)ppGpp hydrolysis is required for growth *in vitro* and maintenance of ATP and GTP levels when (p)ppGpp is being produced. (A and B) An *M. tuberculosis*  $\Delta rel_{Mtb}$  strain containing an episomal vector expressing the WT TetR that induces expression of  $rel_{Mtb}^{H80A}$  from a cassette integrated at the *attB* site in the genome in the presence of ATc (Tet- $Rel_{Mtb}^{H80A}$  strain, designated H80A here) and a WT *M. tuberculosis* strain containing an empty vector in the *attB* site and transformed with the same TetR-expressing plasmid (control strain). (A) Transcript levels in exponential-growth cultures in liquid 7H9 media.  $rel_{Mtb}^{H80A}$  transcripts from the cassette at the *attB* site were detected in the Tet- $Rel_{Mtb}^{H80A}$  strain, and transcripts from the endogenous  $rel_{Mtb}$  gene were detected in the control strain. The first comparison shows the ratio of transcript levels in the presence compared to the absence of ATc in each strain (+ATc/-ATc), and it illustrates the induction of  $rel_{Mtb}^{H80A}$  in the Tet- $Rel_{Mtb}^{H80A}$  strain when exposed to ATc and the unresponsiveness of the  $rel_{Mtb}$  gene to ATc in the control strain. The second comparison shows the ratio of transcript levels in the Tet- $Rel_{Mtb}^{H80A}$  strain compared to the control strain (H80A/control) under each condition to illustrate the low level of transcription of  $rel_{Mtb}^{H80A}$  in the absence of ATc compared to that of induced cultures. (B) *M. tuberculosis* Tet- $Rel_{Mtb}^{H80A}$  and control strains were diluted and plated on 7H10 plates in the absence or presence of ATc. (C to G) *M. smegmatis*  $\Delta rel_{Mtb}$  strains containing an episomal vector expressing the WT TetR that turns on expression of  $rel_{Mtb}^{WT}$  (Tet- $Rel_{Mtb}^{WT}$  strain; designated control) or  $rel_{Mtb}^{H80A}$  (Tet- $Rel_{Mtb}^{H80A}$  strain; designated H80A here) from cassettes integrated at the *attB* site in the genome in the presence of ATc. (C and D) *M. smegmatis* strains were diluted and plated on 7H10 (C) and LB (D) plates in the absence or presence of ATc. (E) Graphic representation of the number of *M. smegmatis* CFU that grew in the presence compared to the absence of ATc when grown on LB, where all Tet- $Rel_{Mtb}^{H80A}$  strain bacteria that grew in the presence of ATc on LB were suppressors and were no longer responsive to ATc treatment. Data are means  $\pm$  SEM from 13 replicates. (F) ATP and GTP levels in *M. smegmatis* strains grown in the absence or presence of ATc for 6 h to an  $OD_{600}$  of  $\sim 0.6$  in LB liquid media were measured by LC-MS/MS. The ratio of levels in the Tet- $Rel_{Mtb}^{H80A}$  strain (H80A) to those in the Tet- $Rel_{Mtb}^{WT}$ -expressing strain (control) under the same conditions is graphed. Data are the means  $\pm$  SEM from 4 to 6 replicates. (E and F) The significance of differences was determined by calculating *P* values by Student's *t* tests; one asterisk indicates significance with a *P* value of  $< 0.05$ , and three asterisks indicates a significance with a *P* value of  $< 0.005$ . (G) Log ratio of transcript levels in the presence of ATc compared to the absence of ATc, illustrating the induction of  $rel_{Mtb}^{WT}$  and  $rel_{Mtb}^{H80A}$  transcripts within 6 h of ATc treatment in Tet- $Rel_{Mtb}^{WT}$  and Tet- $Rel_{Mtb}^{H80A}$  strains, respectively. Data are the means  $\pm$  SEM from 3 replicates.

tandem mass spectrometry (LC-MS/MS), but because of the low levels of (p)ppGpp in the samples, the measurements observed were not confidently reproduced. However, the same LC-MS/MS approach was used to monitor ATP and GTP levels in LB liquid cultures of *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>WT</sup> and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> in the presence and absence of ATc (Fig. 5F). We found that following induction of *rel*<sub>Mtb</sub><sup>H80A</sup> expression in *M. smegmatis* for 6 h, GTP levels were significantly decreased compared to those of strains expressing Rel<sub>Mtb</sub><sup>WT</sup>. Conversely, ATP levels during induction of *rel*<sub>Mtb</sub><sup>H80A</sup> expression were actually higher than those of control strains. The increased transcription of the *rel*<sub>Mtb</sub><sup>H80A</sup> gene within 6 h of ATc treatment in the *M. smegmatis* Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain was confirmed by qRT-PCR (Fig. 5G). These data demonstrate that inhibition of (p)ppGpp hydrolysis impacts not only (p)ppGpp levels but also ATP and GTP levels within the bacteria.

**Inhibition of Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity is lethal for acute and chronic *M. tuberculosis* infection in mice.** We next investigated whether inhibition of (p)ppGpp hydrolysis, while keeping (p)ppGpp synthesis intact, would compromise *M. tuberculosis* pathogenesis. We tested this by infecting mice with either the control *M. tuberculosis* strain that expresses *rel*<sub>Mtb</sub> from its native locus or the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain that allows for conditional expression of the *rel*<sub>Mtb</sub><sup>H80A</sup> allele in the presence of doxycycline. We then determined bacterial titers in the lungs and spleens during both acute and chronic infection (Fig. 6). In the absence of doxycycline, control and Tet-Rel<sub>Mtb</sub><sup>H80A</sup> *M. tuberculosis* strains displayed similar kinetics and virulence in mice (Fig. 6A and B). Since both strains express TetR from a plasmid that also confers streptomycin resistance and contain a kanamycin resistant cassette integrated into the *attB* site that, in the case of Tet-Rel<sub>Mtb</sub><sup>H80A</sup>, carries the *rel*<sub>Mtb</sub><sup>H80A</sup> allele, we could monitor the retention of the Tet-inducible system during infection. By plating the lung and spleen homogenates on plates containing streptomycin and kanamycin, we showed that the Tet-inducible system was intact for at least 10 weeks postinfection in both strains in the absence of doxycycline (Fig. 6C). It was surprising that the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain was able to maintain high titers during chronic infection, since the levels of *rel*<sub>Mtb</sub><sup>H80A</sup> transcripts were a log lower than those in the control strain (Fig. 5A), and we have shown that the absence of *rel*<sub>Mtb</sub> expression leads to lower titers during chronic infection (Fig. 2). This indicated that the low-level leaky expression of the *rel*<sub>Mtb</sub><sup>H80A</sup> allele in the absence of doxycycline was able to result in enough (p)ppGpp synthesis to phenocopy WT *M. tuberculosis* instead of the  $\Delta$ *rel*<sub>Mtb</sub> strain (Fig. 2) but not too much (p)ppGpp synthesis to confer toxicity in the absence of a functional hydrolase. In order to confirm that the low levels of *rel*<sub>Mtb</sub><sup>H80A</sup> transcription allowed for synthesis of (p)ppGpp in the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain in the absence of inducer, we analyzed the ability of this strain to form biofilms, which is a phenotype we had previously shown requires (p)ppGpp synthesis (Fig. 1B). In the absence of ATc, when only low levels of *rel*<sub>Mtb</sub><sup>H80A</sup> transcription are occurring, the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> strain was able to form biofilms similar to those observed in WT Rel<sub>Mtb</sub>-expressing strains, further supporting that the low level of *rel*<sub>Mtb</sub><sup>H80A</sup> transcription in the absence of inducer is enough to allow for adequate (p)ppGpp synthesis to confer normal bacterial physiology (Fig. 6D).

Separate groups of mice were infected with either control or Tet-Rel<sub>Mtb</sub><sup>H80A</sup> *M. tuberculosis* strains and administered doxycycline starting on day 1 (acute infection) (Fig. 6E and F) or day 63 (chronic infection) (Fig. 6G to I) postinfection to determine the

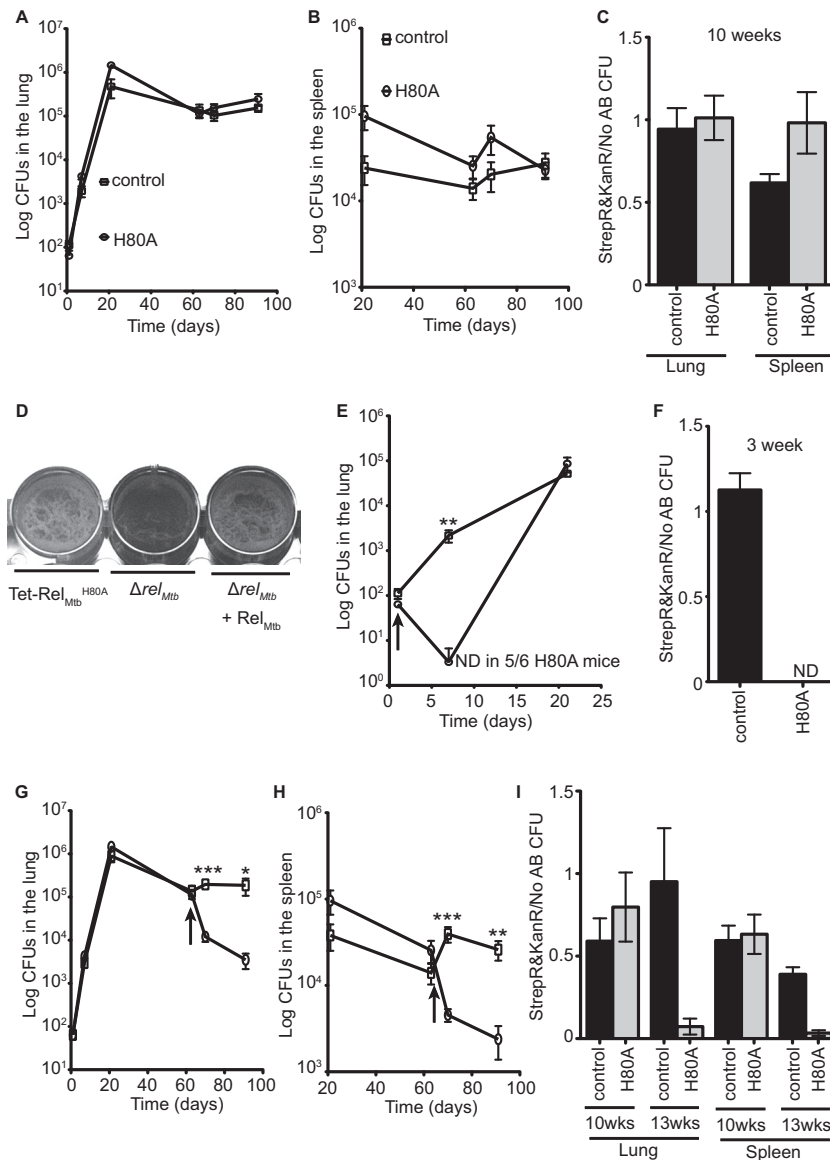
effect of inhibiting Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity during acute and chronic infection in mice. After 1 week of inducing Rel<sub>Mtb</sub><sup>H80A</sup> expression with doxycycline at day 1 postinfection in Tet-Rel<sub>Mtb</sub><sup>H80A</sup>, the bacteria were unable to replicate and decreased in titers to the point that 5 out of 6 animals tested had no detectable *M. tuberculosis* in the lungs, with a limit of detection of 20 CFU. Additionally, none of the surviving Tet-Rel<sub>Mtb</sub><sup>H80A</sup> cells at day 21 retained the Tet-inducible expression system, as determined by the lack of colony growth from lung homogenates on plates containing streptomycin and kanamycin (Fig. 6F). In contrast, the control strain that expressed the endogenous *rel*<sub>Mtb</sub> allele was not affected by doxycycline treatment at this stage of infection (Fig. 6E) and retained the Tet-inducible expression system (Fig. 6F). These data demonstrate that even though *M. tuberculosis* was capable of growth on 7H10 media during inhibition of Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity (Fig. 5B), this catalytic activity is essential for acute infection in mice.

During chronic infection, expression of Rel<sub>Mtb</sub><sup>H80A</sup> caused a 2-log decrease of titers in the lungs (Fig. 6G) and a 1-log decrease in the spleens (Fig. 6H) over a 4-week period. In the 4 weeks following induction of Rel<sub>Mtb</sub><sup>H80A</sup> expression during chronic infection, 93% of the Tet-Rel<sub>Mtb</sub><sup>H80A</sup> bacteria lost the plasmids encoding the Tet-inducible system and most likely reverted to *rel*<sub>Mtb</sub> null by losing the allele and linked kanamycin resistant cassette at the *attB* site (Fig. 6I). This would explain why the bacterial titers did not recover after loss of the Tet-inducible system, since an *M. tuberculosis*  $\Delta$ *rel*<sub>Mtb</sub> strain is attenuated for chronic infection (Fig. 2 and 6G and H). These data demonstrate that inhibition of the Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity in both acute and chronic infection compromises *M. tuberculosis* survival and presents the Rel<sub>Mtb</sub> hydrolase domain as a promising drug target. In addition, suppression of the toxic effect of inhibiting the Rel<sub>Mtb</sub> (p)ppGpp hydrolase activity by losing Rel<sub>Mtb</sub>-mediated (p)ppGpp synthetase activity still leaves the bacteria unable to persist in the mouse model of infection.

## DISCUSSION

A previous study reported that an *M. tuberculosis*  $\Delta$ *rel*<sub>Mtb</sub> mutant is attenuated for chronic infection in mice (14). In the same study, microarray analyses revealed that more than a quarter of the genome was differentially expressed in the  $\Delta$ *rel*<sub>Mtb</sub> strain in both nutrient-rich and depleted conditions, suggesting that there are extensive metabolic alterations in strains lacking *rel*<sub>Mtb</sub>. The transcripts affected included virulence factors, cell wall biosynthetic enzymes, heat shock proteins, and secreted antigens, any of which may contribute to the attenuation *in vivo* (14). In addition, WT *M. tuberculosis*, but not the  $\Delta$ *rel*<sub>Mtb</sub> mutant, downregulated the cellular translation machinery during nutrient starvation, which is consistent with Rel<sub>Mtb</sub>'s role in the stringent response and may also be necessary for *M. tuberculosis* virulence (14).

Rel<sub>Mtb</sub> is a large protein encoding two distinct catalytic activities. Despite its importance for pathogenesis, it was still unknown what function(s) of Rel<sub>Mtb</sub> contribute to its role *in vivo*. We have dissected the roles of the two enzymatic activities encoded by Rel<sub>Mtb</sub> during both growth in culture and pathogenesis in mice. By studying a point mutant that specifically abolishes (p)ppGpp synthesis by Rel<sub>Mtb</sub>, we conclude that (p)ppGpp production by Rel<sub>Mtb</sub> is required for efficient rates of growth and biofilm formation in culture, as well as for maintaining titers during chronic infection in the mouse model of infection. It is worth noting that we do not



**FIG 6**  $Rel_{Mtb}$  (p)ppGpp hydrolase activity is essential for acute and chronic *M. tuberculosis* infection of mice. Bacterial titers in the lungs (A, E, and G) and spleens (B and H) of C57BL/6 mice infected with either control or Tet- $Rel_{Mtb}^{H80A}$  (designated H80A in the figure) *M. tuberculosis* strains. The parent strain for the control is WT *M. tuberculosis* Erdman, and the parent strain for Tet- $Rel_{Mtb}^{H80A}$  is *M. tuberculosis*  $\Delta rel_{Mtb}$ . Both strains express TetR from a plasmid that confers streptomycin resistance and contain a kanamycin resistance cassette integrated into the *attB* site that, in the case of Tet- $Rel_{Mtb}^{H80A}$ , carries the  $rel_{Mtb}^{H80A}$  allele. The strain symbols in panel A are the same for all panels. (A to C) Mice were given normal mouse chow throughout infection. (E and F) Mice were administered doxycycline-containing mouse chow starting at day 1 postinfection (designated by the arrow). (G to I) Mice were administered doxycycline-containing mouse chow starting at day 63 postinfection (designated by the arrow). (C, F, and I) The ratio of CFU from the lungs or spleens grown on 7H10 plates containing streptomycin and kanamycin compared to 7H10 containing no antibiotics (No AB). ND denotes when no colonies were recovered after plating 5% of the lung homogenate (limit of detection, 20 CFU). Data are means  $\pm$  SEM of 6 Tet- $Rel_{Mtb}^{H80A}$  strain-infected mice and 3 control strain-infected mice per time point from two replicate experiments. (E, G, and H) The significance of differences were determined by calculating *P* values by Student's *t* tests; two asterisks indicate significance with a *P* value of  $<0.01$ , and three asterisks indicate significance with a *P* value of  $<0.005$ . (D) *M. tuberculosis* Tet- $Rel_{Mtb}^{H80A}$ ,  $\Delta rel_{Mtb}$ , and  $\Delta rel_{Mtb}$  complemented with WT  $rel_{Mtb}$  were normalized to the same OD $_{\lambda 600}$  and grown in Sauton's media under static, biofilm-forming conditions in a 24-well dish. Biofilms were incubated for 4 weeks at 37°C.

have an antibody specific for  $Rel_{Mtb}$ ; therefore, we cannot confirm that the  $Rel_{Mtb}^{H344Y}$  protein is stably expressed and that the phenotypes are not due to decreased  $Rel_{Mtb}$  protein rather than loss of synthetase activity. However, there are multiple lines of evidence that support that it is the loss of synthetase activity, and not protein instability, that results in these phenotypes. First, our group and others are able to purify the mutant protein easily, and it is

stable *in vitro* with no loss in hydrolase activity, suggesting that the protein can fold normally and other domains can function in the presence of the H344Y substitution (9) and data not shown). Second, data presented here indicate that lower levels of  $Rel_{Mtb}$  would not phenocopy complete loss of the  $rel_{Mtb}$  allele. For instance, infection of mice with the Tet- $Rel_{Mtb}^{H80A}$  strain in the absence of doxycycline, where  $rel_{Mtb}^{H80A}$  transcript levels are a log lower than



transcripts from the allele in WT *M. tuberculosis* (Fig. 5A), indicated that even this low level of synthetase-active Rel<sub>Mtb</sub> expression is able to sustain WT titers during chronic infection of mice, unlike the  $\Delta rel_{Mtb}$ - and Rel<sub>Mtb</sub><sup>H344Y</sup>-expressing strains (Fig. 2 and 6).

(p)ppGpp has been shown to directly impact many processes in other bacteria, including transcription of the translation machinery (26–29), GTP and ATP levels (30–33), DNA replication (34–37), and metabolism (38–40). In general, the many effects of (p)ppGpp are complex and seem to vary greatly among different organisms. Any number of these mechanisms may be conserved in mycobacteria, and determining which functions of (p)ppGpp make its production necessary for chronic *M. tuberculosis* infection requires further study. Since it has been shown that (p)ppGpp accumulates in mycobacteria during starvation (7, 23, 25), oxidative stress (23), and stationary phase (7), these would be good conditions to begin to investigate a molecular mechanism for (p)ppGpp in *M. tuberculosis*.

Our experiments also demonstrate that the production of (p)ppGpp is not the only role of Rel<sub>Mtb</sub> during pathogenesis. By expressing a *rel<sub>Mtb</sub>* point mutant that retains the ability to synthesize (p)ppGpp but is unable to hydrolyze (p)ppGpp, our experiments have highlighted a critical role for Rel<sub>Mtb</sub>-mediated hydrolysis of (p)ppGpp in colony morphology, survival, and virulence. In contrast to (p)ppGpp synthesis, which is necessary for chronic but not acute infection, hydrolysis of (p)ppGpp is required for all stages of *M. tuberculosis* infection in mice, suggesting a role for this catalytic activity in general homeostasis. We predict that expression of the Rel<sub>Mtb</sub><sup>H80A</sup> hydrolase mutant leads to increased abundance of (p)ppGpp, which may directly impact cellular pathways in an uncontrolled manner that is detrimental for the bacteria. In addition, we identify a dysregulation of ATP and GTP levels during *rel<sub>Mtb</sub>*<sup>H80A</sup> expression, which also may contribute to the lethality of expressing this allele. A recently published study on *B. subtilis* highlighted a role for (p)ppGpp in directly inhibiting GTP biosynthesis to maintain GTP levels within a range that supports viability (33). To investigate whether the phenotypes caused by *rel<sub>Mtb</sub>*<sup>H80A</sup> expression in *M. tuberculosis* are due to the low levels of GTP, we attempted to increase GTP production by adding guanosine to the media, as was previously done in *B. subtilis* (33). Unfortunately, guanosine supplementation did not restore WT GTP levels, colony morphology, or growth rate during Rel<sub>Mtb</sub><sup>H80A</sup> expression in mycobacteria. However, this may be due to inability of the nucleoside to access the mycobacterial cytosol.

Evidence of the importance of controlling (p)ppGpp levels specifically by hydrolysis activity comes from studies of small alarmone synthetase (SAS) homologs in *B. subtilis* (17, 18), *Streptomyces mutans* (19), *Enterococcus faecalis* (20), and *Vibrio cholerae* (21). SAS proteins encode the catalytic domain to produce (p)ppGpp but not the domain to hydrolyze it. Expression of a *V. cholerae* or *S. mutans* SAS homolog in *E. coli* is toxic in the absence of SpoT-mediated (p)ppGpp hydrolysis (19, 21), supporting the theory that production of (p)ppGpp in the absence of a functional hydrolase is deleterious. In addition, deletion of the respective SAS homologs largely abolishes the slow-growth phenotypes of  $\Delta relA$  strains of *S. mutans*, *E. faecalis*, and *B. subtilis* (17–20), which, like *M. tuberculosis*  $\Delta rel_{Mtb}$ , lack the only known enzyme capable of hydrolyzing (p)ppGpp. These data suggest that in *S. mutans*, *E. faecalis*, and *B. subtilis*, the slow growth of the  $\Delta relA$  mutant is due

to the lack of (p)ppGpp hydrolase activity in the presence of (p)ppGpp synthesis.

With these data in mind, we have also reported the first investigations into the *M. tuberculosis* SAS homolog, *rv1366*. Interestingly, another important finding from our experiments is that a  $\Delta rv1366$  strain exhibited WT rates of growth in liquid shaking cultures, on plates, and in biofilms as well as WT levels of virulence in the mouse model of infection. This indicates that under the conditions we tested and during pathogenesis in the mouse model, Rel<sub>Mtb</sub> is the main producer of (p)ppGpp. Deletion of *rv1366* had no effect on the phenotypes of the  $\Delta rel_{Mtb}$  strain, demonstrating that, unlike in other bacteria, the presence of an intact *M. tuberculosis* SAS homolog allele was not a source of toxicity in strains lacking Rel<sub>Mtb</sub>-mediated (p)ppGpp hydrolysis. Therefore, the question regarding the role of Rv1366 in *M. tuberculosis* remains unanswered. Murdeshwar et al. recently reported a study of the *M. smegmatis* SAS homolog, which they term MS\_RHII-RSD because, unlike the *M. tuberculosis* homolog, MS\_RHII-RSD encodes an amino-terminal RNase HII domain (41). The authors show that a small amount of (p)ppGpp is produced in an *M. smegmatis*  $\Delta rel_{Msm}$  mutant that is dependent on MS\_RHII-RSD; however, the importance of this activity for survival has not been explored. Interestingly, the authors were unable to detect (p)ppGpp synthetase activity *in vitro* using the region of MS\_RHII-RSD with homology to Rv1366, raising the possibility that MS\_RHII-RSD has evolved functions distinct from those of Rv1366.

In summary, our data point toward a Goldilocks model of (p)ppGpp levels in mycobacteria, where too little (p)ppGpp is harmful for the bacteria in certain situations, like chronic infection, and too much is always deleterious. Recent antibiotic development efforts aim to target Rel<sub>Mtb</sub> (p)ppGpp synthetase activity as a therapeutic option (42, 43). We propose that tipping the balance of (p)ppGpp levels in the other direction by inhibiting (p)ppGpp hydrolysis is also an attractive approach to targeting *M. tuberculosis*. Our data imply that if we were to inhibit (p)ppGpp hydrolysis with an antibiotic, mutants that develop resistance by abolishing (p)ppGpp production would be unable to persist in the host. Our findings would also apply to other bacteria that require a balance of (p)ppGpp production.

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