The stringent factor Rel from *Mycobacterium abscessus* regulates metabolism, but does not promote survival in stress or antibiotics

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Abstract

The stringent response is a broadly conserved stress response system that exhibits functional variability across bacterial clades. Here, we characterize the role of the stringent factor Rel in the non-tuberculous mycobacterial pathogen, *Mycobacterium abscessus* (*Mab*). We find that Rel in *Mab* is involved in restricting transcription of anabolism and growth genes in stress, as has been observed in many other species. However, the stringent response in *Mab* does not provide a survival advantage in several stress conditions or in antibiotic treatment. According to our transcriptional profiling, Rel in *Mab* does not activate transcription of stress response or antibiotic resistance genes. Instead, Rel actually represses transcription of many antibiotic resistance genes in stress. This study implies that combinatorial therapies with stringent factor inhibitors would not potentiate antibiotic treatment against *Mab* infections.

Introduction

24

25 Bacteria must adjust their physiology to permit survival in fluctuating conditions.

26 The stringent response is a conserved signaling system that promotes survival of many
 27 species in stress and antibiotics by altering the transcription of about a quarter of the
 28 genome (1–5). In this work, we profile the role of Rel, the sole annotated stringent
 29 factor, in the non-tuberculous, rapidly-growing mycobacterium *Mycobacterium*
 30 *abscessus* (*Mab*). *Mab* is an opportunistic pathogen that both lives in the environment,
 31 and causes skin and respiratory infections which are increasingly prevalent in Cystic
 32 Fibrosis patients (6). *Mab* infections are especially difficult to treat because this species
 33 is naturally resistant to many antibiotics (7), and highly tolerant under stress to almost
 34 all antibiotics tested (8, 9). One proposed strategy to help treat such antibiotic-
 35 recalcitrant infections is to inhibit the regulatory systems, like the stringent response,
 36 that promote antibiotic tolerance (10–13).

37 The conserved aspect of the stringent response is the synthesis, upon stress, of
 38 the hyperphosphorylated guanine (p)ppGpp by either Rel/SpoT homolog proteins (RSH,
 39 or Rel) or Small Alarmone Synthases (SAS). Once made, (p)ppGpp affects transcription
 40 in different ways (14–17) and also directly modulates replication (18, 19), nucleotide
 41 metabolism (20–22), ribosome maturation (23, 24) and translation (25–27). How exactly
 42 the stringent response exerts its effects in mycobacteria is not well understood, and the
 43 *Mab* stringent response has not been studied at all.

44 Function of the stringent response varies along with the niche and lifestyle of the
 45 species (28). For example, the photosynthetic *Synechococcus elongatus* synthesizes
 46 (p)ppGpp when it is moved into the dark (29, 30), while the coprophagous *E. coli* makes

(p)ppGpp when it runs out of amino acids (31, 32) or lipids (33, 34). The physiological outputs of the stringent response also vary across species, but there are conserved themes. First, the stringent response generally downregulates genes required for growth, such as ribosome and cell wall synthesis factors, and it alters transcription of central metabolism to prioritize survival rather than construction of new cells (2, 3, 17, 35). In most species studied, activation of the stringent response inhibits growth (31, 35–40) which indirectly protects against some stresses and antibiotics that interfere with growth factors (Fig. 1). In many species, the stringent response upregulates stress response genes such as heat shock proteins, hibernation factors, and stress-specific transcription factors (3, 41, 42) and promotes survival in stress (13) (Fig. 1).

The stringent response also helps many bacteria survive through antibiotic treatment by promoting antibiotic tolerance (12)(Fig. 1). Antibiotic tolerance allows a bacterial population to survive longer during treatment. This is different from antibiotic resistance, which is the ability of a population to grow in higher concentrations of antibiotic (43). Because most antibiotics inhibit enzymes required for growth, their effectiveness is proportional to growth rates (44), and much antibiotic tolerance can be achieved simply through growth inhibition. Mutations that activate the stringent response have been shown to be responsible for antibiotic-recalcitrant infections (45, 46). Genetic manipulation of loci that decrease (p)ppGpp levels have been shown to lower tolerance to antibiotics in phylogenetically diverse species (5, 11, 47, 48).

The pathogen *Mtb* has a single stringent factor, Rel. *Mtb* induces (p)ppGpp synthesis when respiration is inhibited, in stationary phase and in total carbon and nitrogen starvation (36, 49). Rel allows long term survival of *Mtb* in nutrient and oxygen

starvation and stationary phase (36). While Rel in *Mtb* does not affect growth during early infection of macrophages (36), it promotes survival during chronic infection of mice (1) and guinea pigs (50, 51). Importantly, Rel also makes *Mtb* more tolerant to the first-line clinical antibiotic isoniazid during nutrient starvation and chronic infection in mice (11) (Fig. 1).

In this study we studied the phenotypes of the Δrel strain of *Mycobacterium abscessus*, which is lacking the sole predicted (p)ppGpp-synthesizing enzyme. We find that the *Mab* Δrel strain does not exhibit defects in survival in several different stress conditions, but has a growth defect relative to wild type. Importantly, the stringent response in *Mab* does not activate antibiotic tolerance; it actually inhibits tolerance to the clinically used antibiotic, amikacin. We transcriptionally profiled the effects of *rel* and find that it downregulates many metabolic pathways in stasis conditions, as is seen in other species. However, we do not find that the *Mab* stringent response upregulates stress response genes in any condition we tested.

Results

In order to explore the role of *rel* in regulating growth, survival and antibiotic tolerance in *Mab*, we built a strain of *Mab* ATCC19977 with a deletion of the *rel* gene (MAB_2876). In many species, the stringent response promotes survival during stresses such as stationary phase, acid stress, starvation, or oxidative stress (5, 36, 39, 52–54). To evaluate the physiological role of the stringent response in stress in *Mab*, we assayed survival of log. phase cultures, in 7H9 media, of the wild-type, Δrel and complemented

strains upon and after transfer to either carbon starvation (Fig. 2A), salt stress (Fig. 2B), oxidative stress, (Fig. 2C) or acidic media (pH 4) (Fig. 2D). We treated wild type and mutant *Mab* to growth limiting concentrations of these stressors and observed no differences in growth inhibition or survival relative to wild-type and the complemented strain. Thus, Rel in *Mab* does not regulate responses to these stresses under the conditions tested, or at least not enough to affect growth or survival. We also found that Rel does not promote survival in stationary phase in 7H9 media (Fig. 3A).

We conducted a growth curve and found that the Δrel strain grew more slowly than the wild-type and complemented strains (Fig. 3B). This result suggests that Rel in *Mab* largely functions to promote growth in low stress conditions. This is surprising, in view of the fact that in most species studied, the stringent response functions to arrest growth under stress (4). In several proteobacterial species, ppGpp⁰ strains, which have deletions of all the (p)ppGpp-synthesizing enzymes, comparable to our Δrel strain, actually grow faster than the wild-type (38, 53, 55–57). However, in the gram positive *Enterobacter faecalis* (58) and in *Mycobacterium tuberculosis* (36), the ppGpp⁰ strains also grow slowly, as we see in *Mab* (Fig.1).

Because the stringent response is a major activator of antibiotic tolerance and persistence in many species (5, 56, 58), we sought to assess how Rel contributes to antibiotic tolerance in *Mab*. First, we treated *Mab* cultures in logarithmic growth phase with the clinically used antibiotics amikacin, clarithromycin and cefoxitin (59). We found that clarithromycin alone did not kill a significant portion of any of the strains (Fig. 4A), likely due to inducible macrolide resistance that has been described (60). Cefoxitin treatment also did not have an effect. However, amikacin treatment resulted in 10-100-

fold decrease in viability of wild-type and complemented strains, but had no effect on Δrel . Typically, the stringent response promotes tolerance (5, 56, 58), but here we are seeing increased tolerance when the stringent factor Rel is missing.

Antibiotic tolerance increases in stationary phase in most bacterial species relative to log. phase (5, 61). To assess how the stringent response in *Mab* impacts stress-induced antibiotic tolerance, we repeated the antibiotic survival experiments on stationary phase cultures. In stationary phase, Rel does not affect tolerance to clarithromycin or cefoxitin, and none of the strains are killed appreciably in this condition (Fig. 4B). Similar to amikacin treatment in growth, Rel also promotes increased susceptibility in stasis. We expected to observe greater tolerance of all strains to the cell-wall targeting drug cefoxitin in stationary phase because beta-lactam susceptibility typically correlates with growth rate (44), however, we did not (Fig. 4AB).

Studies are ongoing to find drugs that would inhibit Rel proteins (11, 13), as such drugs are expected to increase susceptibility to other clinically available antibiotics. Our results indicate that Rel inhibitors, should they become available, might actually increase tolerance when administered in combination with amikacin to treat *Mab* infections, and may have no effect with clarithromycin and cefoxitin.

A major function of the stringent response in other species is to remodel the transcriptome (4). To determine the effects of Rel on transcription in *Mab*, we compared the wild-type and Δrel transcriptome in both logarithmic growth and stationary phases. We found that Rel represses many more genes than it activates in both log. and stationary phase in *Mab*.

In mid log. phase, when the Δrel strain are growing more slowly (Fig. 3B), we found 150 genes that were repressed by Rel by at least 3-fold, and only 7 genes that were activated by Rel. The only annotated upregulated genes are an efflux pump (MAB_0677) and a MFS transporter (MAB_0069). We found several *mce* family genes that were repressed by Rel (Table S2). Mce proteins are typically lipid transporters, but they also play roles in host cell entry and immune modulation (62). Notably, we also found two antibiotic resistance genes that are repressed by Rel in log. phase, MAB_4837 and MAB_2875 (Table 1). MAB_4837 is annotated as an aminoglycoside phosphotransferase; this class of enzymes inactivates aminoglycoside antibiotics. Overexpression of MAB_4837 in the Δrel strain may account for the increased tolerance to amikacin seen in that strain (Fig. 4A). MAB_2875 encodes the β -lactamase *bla*_{Mab}, which degrades β -lactams including several penams and carbapenems, but which has very poor activity against cefoxitin (63). This may explain partly why we see no difference in susceptibility to cefoxitin between our wild type and Rel mutant strains (Fig. 4).

Even though there was no apparent difference in survival between the wild-type and Δrel strains in stationary phase, we observed significant differences in transcription. We found hundreds of genes that were repressed by Rel in stationary phase, but none that were activated by Rel 3-fold or more. The two antibiotic resistance genes, MAB_4837 and MAB_2875, mentioned above were also significantly repressed by Rel in stationary phase, which may help explain why the antibiotic susceptibility results for amikacin and cefoxitin were not significantly different in log. and stationary phase. We also found many genes in the WhiB7 regulon (Table 1) which are repressed in

stationary phase, though they are mostly unaffected in log. phase. WhiB7 is a transcription factor that activates many antibiotic resistance genes and promotes resistance to many classes of antibiotics in *Mab* (64). It is notable that these antibiotic resistance genes are repressed by Rel in stasis, which would imply that the wild-type *Mab* would be more susceptible to antibiotics in this condition, which is what we see in amikacin treatment. In the case of clarithromycin and cefoxitin, increased tolerance through downregulation of target expression may counterbalance the repression of the antibiotic resistance genes, resulting in no differences in susceptibility in our assays (Fig. 4).

We also found several cell wall biosynthetic genes that are downregulated by Rel in stationary phase (Table S2). Downregulation of growth factors is typical in stringent responses across many bacterial species (3, 37, 58, 65, 66). However, unusually, the microarray experiment profiling the Rel transcriptome in *Mtb* found that several cell wall enzymes were upregulated by Rel in stationary phase (1).

We see that Rel downregulates many central metabolism genes in stationary phase. However, it is notable that not all the genes in a given pathway are downregulated by Rel equally (Fig. 5, Table S3). We hypothesize that this uneven regulation of certain pathways may allow certain metabolites to accumulate in the wild-type strain in stasis. Such metabolites may be re-directed to other pathways. We observed that several of the products of enzymes that are not downregulated, which may therefore be accumulating under these conditions, converge on the NAD synthesis pathway. None of the genes in the NAD synthesis pathway are downregulated by Rel, which implies that continued metabolism of NAD, which is a critical cofactor in many

pathways, may be important in stationary phase, and that flux toward its biosynthesis may be prioritized by the stringent response.

From our preliminary analysis, it is clear that the stringent response in *Mab* helps regulate growth and central metabolism, and affects expression of antibiotic resistance genes; however, it does not seem to upregulate specific stress responses.

Discussion

Our results show that the stringent factor Rel in *Mab* does not promote survival in many *in vitro* stress conditions (Fig.2, 3A). This is surprising because data from other species shows the stringent response is responsible for upregulating stress response genes (3, 42, 67, 68) as well as downregulating growth genes. Our transcriptomics analysis indicate that Rel in *Mab* does not upregulate stress response genes, at least during stationary phase in lab media. It is possible that stationary phase is dissimilar from any conditions that *Mab* evolved to adapt to, and therefore these data may not be physiologically relevant. However, *Mab* does not link growth arrest with activation of stress response genes by the Rel. It appears that the stringent response in *Mab* is mainly involved in downregulating metabolism for growth arrest. Other regulators must control stress response genes independently.

While our data show that the *Mab* stringent response does remodel metabolism during both growth and stationary phase (Table S3, Fig. 5), it is actually required for maximal growth rates during log. phase (Fig. 3B). This suggests that the stringent response in *Mab* may function more to modulate metabolism to promote growth under

variable conditions rather than to arrest growth in highly stressful conditions. It is interesting that ppGpp⁰ strains of Gram positive and Actinobacterial strains tend to grow slower than the wild-type strains, whereas the ppGpp⁰ Gram negative strains tend to grow faster than the wild-type (Fig. 1). Thus, the model that the function of the stringent response is to arrest growth under stress appears to be applicable mostly in the Proteobacteria. In Firmicutes and Actinobacteria, including *Mab*, the stringent response may actually be promoting growth under certain circumstances.

However, our work has not established whether the *Mab* Δrel strain completely lacks (p)ppGpp. In *Mtb*, Rel is the only (p)ppGpp synthesizing enzyme (36, 69). However, there are at least three enzymes in *Msmeg* that synthesize (p)ppGpp, and only two have been described (70). It is therefore possible that *Mab* has another enzyme that synthesizes (p)ppGpp, and the phenotypes we observe in the *Mab* Δrel strain are partly due to increased (p)ppGpp due to the loss of Rel's hydrolase function.

Rel in *Mab* may inhibit growth more in different conditions than those tested here. β -oxidation, *i.e.*, consumption of lipids as a carbon source, in *Mtb* is correlated with latency and pathogenesis (71, 72), and the stringent response inhibits growth in the presence of lipids as a carbon source (36), implying that it could be part of this regulation. Studies of *Mab* during infection show that β -oxidation genes are upregulated in macrophages and amoeba infection (73). We do not see significant differences in expression of fatty acid synthesis or degradation genes in any of the conditions we tested, but this could be because we did not add lipids to our media. The stringent response in *Mab* could possibly regulate β -oxidation or other processes in conditions in different nutrient conditions, or in infection.

We observed in our transcriptional data that Rel downregulated numerous antibiotic resistance genes in stationary phase (Table 1). This is surprising in view of the literature on the stringent response from other species, where the stringent response both promotes antibiotic tolerance (5, 11, 58) and sometimes also increases expression of antibiotic resistance genes (74, 75). *Mab* is notorious for having resistance to many clinical antibiotics and expressing many antibiotic resistance genes (64, 76, 77) which is why it is such a problematic pathogen for cystic fibrosis patients (78). Studies in *Mtb* show that the Rel-mediated stringent response activates tolerance to at least some antibiotics in that pathogen (11), and it is natural to assume that this would also hold true for *Mtb*'s close relatives (79); however, our data in *Mab* show that antibiotic tolerance in Non-tuberculous Mycobacteria (NTMs) can be regulated differently. The environmental niche of most NTMs is the soil and water systems (80), whereas *Mtb* lives exclusively in human tissues. Antibiotics have been prevalent in soil habitats for possibly a billion years (81–83), but have only been prevalent in human tissues for around 100 years (84). In addition, NTMs are likely exposed to a greater variety of environmental stresses than *Mtb*. Therefore, saprophytic NTMs like *Mab* are likely to have hard-wired the connections between stress responses and antibiotic tolerance and resistance in different ways than *Mtb*.

Our work shows that the stringent factor Rel in *Mab*, in lab media, works mainly to remodel metabolism, and does not appear to be important in stress responses and antibiotic tolerance. Future work will determine whether this stringent response regulates different genes and processes in infection.

Materials and Methods

Construction of strains. Primers 1233 – 1238 (Fig.S1A) were used to amplify a 502 bp segment upstream of *rel_{mab}* which included the start codon, a 448 bp segment downstream of *rel_{mab}* which included the stop codon, and a 788 bp ZeoR cassette. All 3 segments were stitched together by PCR to form the $\Delta rel::zeoR$ double stranded recombineering knockout construct. The Δrel_{Mab} mutant strain was generated through double stranded recombineering, as previously described (85) (Fig.S1C). Colonies from the transformation of the $\Delta rel::zeoR$ construct were PCR checked by using primers 1424-761, 1235-1236, and 762-1425 (Fig.S1A). To make the complemented strain, the *rel* gene was amplified through PCR using primers 1329-1330 and inserted into pKK216 (86) with NdeI and HindIII. This new plasmid, pCB1248, was transformed into the Δrel_{Mab} mutant strain in order to create the *rel_{Mab}* complemented strain, in which *rel* expression is driven by a constitutive promoter (BN17, Fig.S1B).

Media and culture conditions. All *M. abscessus* ATCC 19977 wild-type cultures, Δrel_{Mab} cultures, and *rel_{Mab}* complemented cultures, were started in 7H9 (Becton, Dickinson, Franklin Lakes, NJ) medium with 5 g/liter bovine serum albumin, 2 g/liter dextrose, 0.85 g/liter NaCl, 0.003 g/liter catalase, 0.2% glycerol, and 0.05% Tween 80 and shaken overnight at 37°C until log. phase. For starvation and other specific assays, Hartmans-de Bont (HdB) minimal medium was made as described previously (87).

Cultures were inoculated to an optical density of 0.05, unless otherwise stated. All CFU time points were plated on LB agar and placed in 37° C incubator for 4 days.

Δrel_{Mab} stress assays. For all stress assays, strains were prepared and grown into log. phase. Unless otherwise stated, cultures for stress assays were done in non-culture treated 24-well plates and shaken at 130rpm in 37C incubator. For carbon starvation, strains were inoculated in 30mL inkwells in HdB minimal media with no glycerol, and with Tyloxapol as a detergent. For acid stress, strains were inoculated in 7H9 medium with a pH of 4. For osmotic stress, strains were inoculated in LB medium with 1M salt (ACS Sodium Chloride, VWR Chemicals BDH). For oxidative stress, all strains were inoculated in complete HdB minimal medium, which does not contain catalase, and strains were exposed to different concentrations of tert-Butyl Hydroperoxide (Alfa Aesar). CFU time points were taken upon inoculation, at 1 hour, 3 hours, and 24 hours post-inoculation.

Δrel_{Mab} growth curve and stationary phase survival. Log-phase cultures of all strains were inoculated to OD 0.05 in 30mL inkwells in 7H9 media. Cultures were then placed in shaking incubator at 37C and 130rpm. CFU time points were then taken throughout a 12-hour period. For stationary phase survival, a second set of cultures were grown into stationary phase up to 48 hours. Initial CFU time-point was taken at 48 hour after dilution of log. phase samples to OD=0.05, with subsequent time points taken at 5, 6, 7, 8, 9, and 10 days.

299

300 **Antibiotic assays.** For the log. phase experiments, strains that had been kept in log.
301 phase in 7H9 for ~24 hours were diluted to OD=0.05 and treated with either 150 µg/mL
302 of amikacin, 200 µg/mL clarythromycin, or 80 µg/mL of cefoxitin. CFUs were measured
303 upon treatment (T=0) and 48 hours after treatment (T=48). For stationary phase, log.
304 phase cells at OD=0.05 were shaken for 48 hours and then treated as above. CFUs
305 were measured upon treatment and 72 hours after treatment.

306

307 **RNA isolation, library preparation and data analysis.** RNA from three biological
308 replicates of each strain and condition was isolated as previously described (88) with
309 some modifications. After growth for ~24 hours in either log. or stationary phase, cells
310 were transferred to 15mL conical tubes and centrifuged at 4C for 3 min at 4000rpm. Cell
311 pellets were immediately resuspended in 750µl of TriZol (Invitrogen) and lysed by bead
312 beating. RNA was purified according to protocol with the Zymogen Direct-zol RNA
313 Miniprep Plus (cat. No 2070). RNA was processed for Illumina sequencing using the
314 TRuSeq Total RNA Library Prep from Illumina, with bacterial rRNA removal probes
315 provided separately by Illumina. Sequencing was performed using Illumina NovaSeq at
316 the North Texas Genome Center at the University of Texas in Arlington.

317 Between 50-300 million pair-end reads per library were mapped to the *M.*
318 *abscessus subs. abscessus* ATCC 19977 published genome using CLC Genomic
319 Workbench software (Qiagen). To minimize the skewing effect that certain PCR
320 jackpots had on the data, we adjusted the number of reads mapped from each library so
321 that the median reads per gene was the same within an experiment. In the log. phase

samples, the median reads per gene was ~600. In the stationary phase samples, the median reads per gene was ~100. After normalization, the Reads Per Kilobase Million (RPKM) values were determined for each ORF, and the weighted proportion fold change of RPKM between the wild type and Δrel strains for each condition were calculated by CLC Workbench. The Baggerley's test was used to generate a false discovery rate corrected P-value. We then used a cut-off of 3-fold change with a false-discovery rate corrected P-value of ≤ 0.05 to identify significantly differentially regulated genes between wild type and Δrel in the different conditions. Because the median reads per gene for log. phase samples was 6 times higher than for stationary phase samples, we linearly scaled the fold-change values when comparing wild type log. to Wild-type stationary phase data to normalize for this difference in read depth.

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Table 1. Antibiotic Resistance Genes – (Under whiB7 regulon)*

Mab GENE	FC-	FDR-	FC-	FDR-	FC-	FDR-	Mab GO. Mol. function
Annotation	Δrel	corrected	Δrel	corrected	WT.stat/	corrected	
	vs.	P value	vs.WT	P value	WT.log	P value	
	WT	Δrel vs.	Stat.	Δrel vs.		WT.Log	
	LOG	WT		WT		vs.	
		LOG		Stat.		WT.Stat	
MAB_0163c	+2	1.1E-04	+40	5.1E-12	+2	1.7E-311	Aminoglycoside

							Phosphotransferase
MAB_0185c	+1	0.3	+5.4	6.4E-03	-5.4	0	Arabinosyl Transferase*
MAB_0186c	+1	0.8	+9.7	1.8E-04	-7.4	0	Arabinosyl Transferase*
MAB_1341	+1	0.45	+34	2.3E-11	-1.3	5.5E-13	Decarboxylase*
MAB_1342	+1.4	0.03	+14	0	-1	0	Acyl-CoA synthetase*
MAB_1395	+2.7	1.3E-04	+48	5.2E-10	+1	7.2E-29	Transporter*
MAB_1396	+2.5	3.7E-06	+36	0	-1	0	Multidrug MFS transporter
MAB_1846	-1.3	0.44	+28	4.2E-06	-1.4	2.9E-265	ABC transporter*
MAB_2273	+2.3	8.8E-12	+101	1.96E-08	+2.2	0	MFS transporter*
MAB_2297	+1.5	0.02	+99.2	7.1E-08	-2.1	0	Methyltransferase-erm41*
MAB_2310	+1.3	0.5	+5.7	0.03	+3.6	1.5E-50	Multidrug transporter
MAB_2355c	+2.2	1.5E-08	+17	0	+2.8	0	ABC transporter*
MAB_2396	+2.1	8.2E-03	+18	7.3E-09	+1.5	4.5E-122	Probably acetyltransferase*
MAB_2640c	+1.2	0.122	+5	5.4E-03	-2.4	0	Mmr - multidrug transport integral membrane protein
MAB_2736c	+1	0.6	+13	1.99E-10	-3.7	0	ABC transporter
MAB_2780c	+1.7	0.01	+27	1.14E-07	+3.3	0	MFS transporter*
MAB_2807	-1	0.7	+5	4.4E-03	-4.7	0	MFS transporter

MAB_2875	+5.4	9.7E-06	+38	0	+1.2	1.6E-171	Beta-lactamase
MAB_2989	+2	5.1E-04	+6.8	2.9E-05	+2.93	1.1E-222	Chloramphenicol acetyltransferase
MAB_3042c	+2.7	1.9E-12	+24	0	+1.82	0	GTpase-Hflx*
MAB_3467c	+6	2.5E-03	+21	0	+92	0	Heat shock protein*
MAB_3508c	+1.8	0.3	+31	0.08	+14	0.38	WhiB7
MAB_3762	+2	2.4E-09	+11	2.9E-10	+7.09	4.9E-143	Membrane protein*
MAB_3869c	-1.3	0.158	+6.7	5.4E-03	-1.67	0	DNA directed RNA polymerase*
MAB_4294	+1.8	3.1E-03	+28	0	+1.88	0	Aminotransferase*
MAB_4395	+2.4	0	+8	8.8E-07	+1.1	0	Aminoglycoside- 2'-N- acetyltransferase
MAB_4837	+4.6	0	+26	0	+1.84	1.9E-312	Aminoglycoside phosphotransferase

Figures

Phenotypes of ppGpp⁰ strains across clades

Clades	<div> ■ same as wt ■ worse than wt ■ better than wt </div>							
	Conditions							
Proteobacteria	Starvation	Oxidation	Acid	Growth	Stat. phase	Biofilm	Antibiotics	Infection
<i>C. crescentus</i> ^α	<small>(Gentry and Gentry, 2000)</small> ■			■				
<i>R. palustris</i> ^α					■			
<i>B. pertussis</i> ^β	<small>(Engelbrekt et al., 2015)</small> ■	<small>(Engelbrekt et al., 2015)</small> ■		<small>(Engelbrekt et al., 2015)</small> ■		<small>(Engelbrekt et al., 2015)</small> ■		
<i>V. cholerae</i> ^γ			<small>(Chen et al., 2002)</small> ■	<small>(Winkler et al., 2002)</small> ■		<small>(Winkler et al., 2002)</small> ■	<small>(Winkler et al., 2002)</small> ■	
<i>H. ducreyi</i> [†]		<small>(Murray et al., 2009)</small> ■			<small>(Murray et al., 2009)</small> ■			<small>(Murray et al., 2009)</small> ■
<i>E. coli</i> [†]				<small>(Sokol et al., 2000)</small> ■	<small>(Sokol et al., 2000)</small> ■	<small>(Sokol et al., 2000)</small> ■	■	
<i>S. typhi</i> [†]				<small>(Cheng et al., 2009)</small> ■				<small>(Cheng et al., 2009)</small> ■
<i>P. aeruginosa</i> [‡]		<small>(Engelbrekt et al., 2015)</small> ■						<small>(Engelbrekt et al., 2015)</small> ■
<i>H. pylori</i> [‡]	<small>(Sokol et al., 2000)</small> ■		<small>(Murray et al., 2009)</small> ■	<small>(Murray et al., 2009)</small> ■	<small>(Murray et al., 2009)</small> ■			<small>(Murray et al., 2009)</small> ■
Firmicutes								
<i>B. subtilis</i>		<small>(Engelbrekt et al., 2015)</small> ■		<small>(Engelbrekt et al., 2015)</small> ■	<small>(Engelbrekt et al., 2015)</small> ■			
<i>E. faecalis</i>				<small>(Sokol et al., 2000)</small> ■			<small>(Sokol et al., 2000)</small> ■	<small>(Sokol et al., 2000)</small> ■
<i>S. aureus</i>				<small>(Murray et al., 2009)</small> ■				
<i>C. difficile</i>							<small>(Sokol et al., 2000)</small> ■	
Actinobacteria								
<i>M. tuberculosis</i>	<small>(Peters et al., 2009)</small> ■	<small>(Peters et al., 2009)</small> ■		<small>(Peters et al., 2009)</small> ■	<small>(Peters et al., 2009)</small> ■	<small>(Peters et al., 2009)</small> ■	<small>(Peters et al., 2009)</small> ■	<small>(Peters et al., 2009)</small> ■
<i>M. abscessus</i>	■	■	■	■	■		■	■

alpha-proteobacteria^α beta-proteobacteria^β gamma-proteobacteria^γ epsilon-proteobacteria^ε

Figure 1. Survival or growth of ppGpp⁰ strains from different species. Summary of published phenotypic data from ppGpp⁰ strains (lacking all known factors that synthesize (p)ppGpp) across several bacterial clades in different stress conditions. Blue squares represent species in which the ppGpp⁰ phenotype was the same as the wild-type strain. Red squares indicate that the ppGpp⁰ strain either grows more slowly or survives less than wild-type strain in the indicated condition. Green squares indicate that the ppGpp⁰ strain either grows more rapidly or survives better than the wild-type strain in the indicated condition. Data for *Mycobacterium abscessus* is shown in this paper. (Sokawa *et al.*, 1975; Gentry *et al.*, 2000; Primm *et al.*, 2000; Balzer and

355 McLean, 2002; Mouery *et al.*, 2006; Nanamiya *et al.*, 2007; Lesley and Shapiro, 2008;
356 Zhou *et al.*, 2008; Abranches *et al.*, 2009; Boutte and Crosson, 2011; Potrykus *et al.*,
357 2011; Vogt *et al.*, 2011; He *et al.*, 2012; Sugisaki *et al.*, 2013; Gaca *et al.*, 2013; Holley
358 *et al.*, 2014; Oh *et al.*, 2015; Xu *et al.*, 2016; Harms *et al.*, 2017; Kim *et al.*, 2018;
359 Dasgupta *et al.*, 2019; Yin *et al.*, 2019; Schäfer *et al.*, 2020; Pokhrel *et al.*, 2020)
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A. Survival during carbon starvation

B. Survival in acidic medium

C. Survival during osmotic stress

D. Survival during oxidative stress

Figure 2. Contribution of rel_{Mab} to survival in various stresses. (A) CFU of wild-type *Mycobacterium abscessus* ATCC19977 (blue), Δrel_{Mab} mutant (red), and the complemented strain $\Delta rel L5::rel$ (green) in Hartman's du Bont medium with no glycerol

and Tyloxapol as a detergent. (B) CFU in 7H9 Middlebrook medium with a pH of 4. (C) CFU in Lennox LB with 1M of NaCl. (D) CFU in Hartmans du Bont medium with 5mM, 25mM or 60mM of tert-butyl peroxide after 24 hours. Relative CFU is calculated by taking the ratio between each CFU value and the initial CFU value at time zero. All data points are an average of three biological replicates. Error bars represent standard deviation. There are no significant differences in any of these data by a two-tailed t-test.

A. Survival in stationary phase

B. Growth in 7H9

Figure 3. Growth and stationary phase survival of Δrel_{Mab} . (A) CFU in stationary phase in 7H9 media. The 2 day time point is 48 hours after diluting log. phase cultures to OD=0.05. (B) CFU during log. phase growth in 7H9 medium. Graph is set at a log2 scale. P values are for the wild-type compared to the Δrel strain. P values for T=4, $P = 0.0415$; T=7, $P = 0.00015$; T=10, $P = 0.006$; T=12, $P = 0.00029$. P-values between wild-type and the complemented strain were not significant.

A. Antibiotic treatment in log. phase

B. Antibiotic treatment in stat. phase

Figure 4. Contribution of *rel*_{Mab} to survival in antibiotic treatment. Relative CFUs of *Mab* strain treated with either 200μg/mL of clarithromycin, 150 μg/mL of amikacin, or 80 μg/mL of cefoxitin for either (A) 48 hours in log. phase or (B) 72 hours in stationary phase. Relative CFU is calculated by taking the ratio between each CFU and the initial CFU value at time zero. The bars represent the mean of 6-9 biological replicates, the individual values are shown by the dots. Error bars represent standard deviation. Log. phase P values: (Amk150) WT vs Δ*rel* = 0.005; WT vs. Δ*rel* L5::*rel* = 0.01; Δ*rel* vs. Δ*rel* L5::*rel* = 0.004. Stationary phase P values: (AMK150) WT vs Δ*rel* = 0.00017; WT vs. Δ*rel* L5::*rel* = 0.0217; Δ*rel* vs. Δ*rel* L5::*rel* = 0.0017. Asterisks represent significance as

measured by the two-tailed student's t -test; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; n.s. = $P > 0.05$.

Figure 5. Repression of central metabolic genes by Rel. Genes in red are downregulated by Rel at least 3-fold in stationary phase, $P < 0.05$. Genes in black are not significantly regulated by Rel in stationary phase. See Table S3 for data.

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