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

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

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

Function of the stringent response varies along with the niche and lifestyle of the species (28). For example, the photosynthetic *Synechococcus elongatus* synthesizes (p)ppGpp when it is moved into the dark (29, 30), while the copraphagous *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 survives 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 Δ*rel* strain of *Mycobacterium abscessus,* which is lacking the sole predicted (p)ppGpp-synthesizing enzyme. We find that the *Mab* Δ*rel* 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, Δ*rel* and complemented

93 strains upon and after transfer to either carbon starvation (Fig. 2A), salt stress (Fig. 2B),
94 oxidative stress, (Fig. 2C) or acidic media (pH 4) (Fig. 2D). We treated wild type and
95 mutant *Mab* to growth limiting concentrations of these stressors and observed no
96 differences in growth inhibition or survival relative to wild-type and the complemented
97 strain. Thus, Rel in *Mab* does not regulate responses to these stresses under the
98 conditions tested, or at least not enough to affect growth or survival. We also found that
99 Rel does not promote survival in stationary phase in 7H9 media (Fig. 3A).
100 We conducted a growth curve and found that the Δ*rel* strain grew more slowly
101 than the wild-type and complemented strains (Fig. 3B). This result suggests that Rel in
102 *Mab* largely functions to promote growth in low stress conditions. This is surprising, in
103 view of the fact that in most species studied, the stringent response functions to arrest
104 growth under stress (4). In several proteobacterial species, ppGpp^0 strains, which have
105 deletions of all the (p)ppGpp-synthesizing enzymes, comparable to our Δ*rel* strain,
106 actually grow faster than the wild-type (38, 53, 55–57). However, in the gram positive
107 *Enterobacter faecalis* (58) and in *Mycobacterium tuberculosis* (36), the ppGpp^0 strains
108 also grow slowly, as we see in *Mab* (Fig.1).
109 Because the stringent response is a major activator of antibiotic tolerance and
110 persistence in many species (5, 56, 58), we sought to assess how Rel contributes to
111 antibiotic tolerance in *Mab.* First, we treated *Mab* cultures in logarithmic growth phase
112 with the clinically used antibiotics amikacin, clarithromycin and cefoxitin (59). We found
113 that clarithromycin alone did not kill a significant portion of any of the strains (Fig. 4A),
114 likely due to inducible macrolide resistance that has been described (60). Cefoxitin
115 treatment also did not have an effect. However, amikacin treatment resulted in 10-100-

116 fold decrease in viability of wild-type and complemented strains, but had no effect on
117 Δ*rel* . Typically, the stringent response promotes tolerance (5, 56, 58), but here we are
118 seeing increased tolerance when the stringent factor Rel is missing.
119 Antibiotic tolerance increases in stationary phase in most bacterial species
120 relative to log. phase (5, 61). To assess how the stringent response in *Mab* impacts
121 stress-induced antibiotic tolerance, we repeated the antibiotic survival experiments on
122 stationary phase cultures. In stationary phase, Rel does not affect tolerance to
123 clarithromycin or cefoxitin, and none of the strains are killed appreciably in this condition
124 (Fig. 4B). Similar to amikacin treatment in growth, Rel also promotes increased
125 susceptibility in stasis. We expected to observe greater tolerance of all strains to the
126 cell-wall targeting drug cefoxitin in stationary phase because beta-lactam susceptibility
127 typically correlates with growth rate (44), however, we did not (Fig. 4AB).
128 Studies are ongoing to find drugs that would inhibit Rel proteins (11, 13), as such
129 drugs are expected to increase susceptibility to other clinically available antibiotics. Our
130 results indicate that Rel inhibitors, should they become available, might actually
131 increase tolerance when administered in combination with amikacin to treat *Mab*
132 infections, and may have no effect with clarithromycin and cefoxitin.
133 A major function of the stringent response in other species is to remodel the
134 transcriptome (4). To determine the effects of Rel on transcription in *Mab*, we compared
135 the wild-type and Δ*rel* transcriptome in both logarithmic growth and stationary phases.
136 We found that Rel represses many more genes than it activates in both log. and
137 stationary phase in *Mab.*

In mid log. phase, when the Δ*rel* 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 Δ*rel* 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 Δ*rel* 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 ppGpp^0 strains of Gram positive and Actinobacterial strains tend to grow slower than the wild-type strains, whereas the ppGpp^0 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* Δ*rel* 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* Δ*rel* 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.

Δ*rel*$_{Mab}$ 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.

Δ*rel*$_{Mab}$ 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.

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

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

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

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

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335

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

337

***Mab* GENE Annotation**	**FC-Δ*rel* vs. WT LOG**	**FDR-corrected *P* value Δ*rel* vs. WT LOG**	**FC-Δ*rel* vs.WT Stat.**	**FDR-corrected *P* value Δ*rel* vs. WT Stat.**	**FC-WT.stat/ WT.log**	**FDR-corrected *P* value WT.Log vs. WT.Stat**	**Mab GO. Mol. function**
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

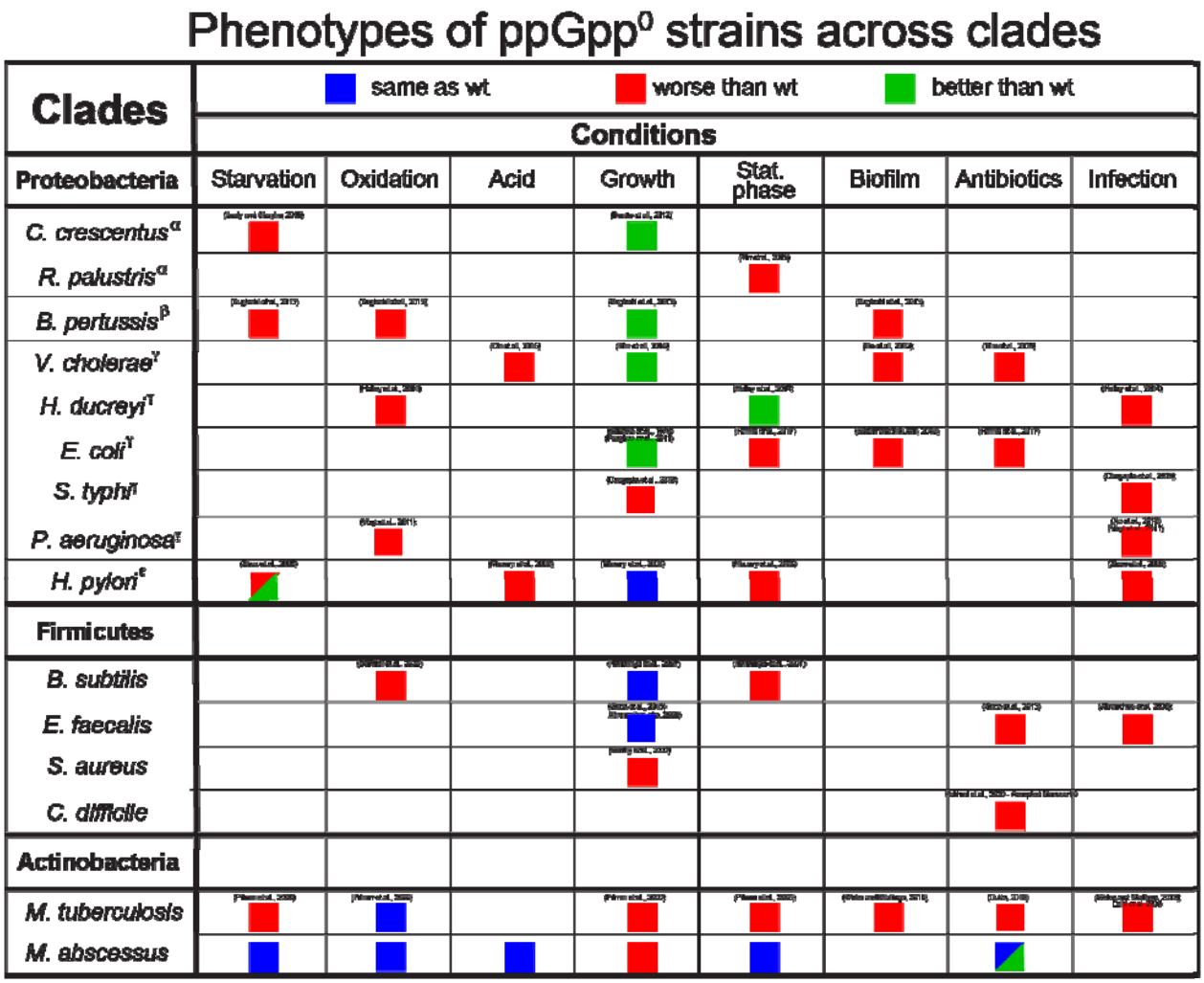

Figure 1. Survival or growth of ppGpp^0 strains from different species. Summary of published phenotypic data from ppGpp^0 strains (lacking all known factors that synthesize (p)ppGpp) across several bacterial clades in different stress conditions. Blue squares represent species in which the ppGpp^0 phenotype was the same as the wild-type strain. Red squares indicate that the ppGpp^0 strain either grows more slowly or survives less than wild-type strain in the indicated condition. Green squares indicate that the ppGpp^0 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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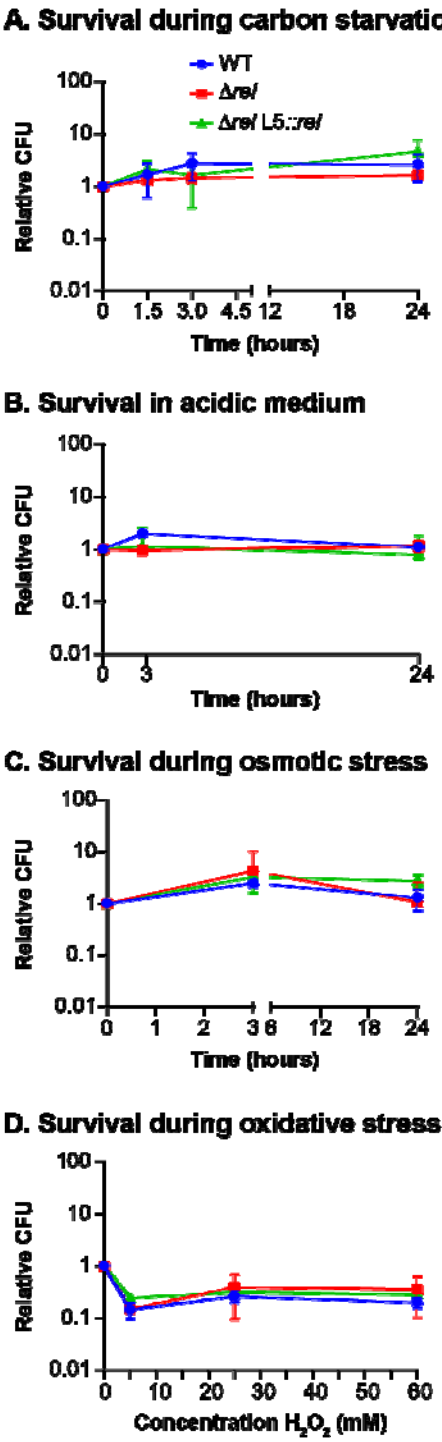
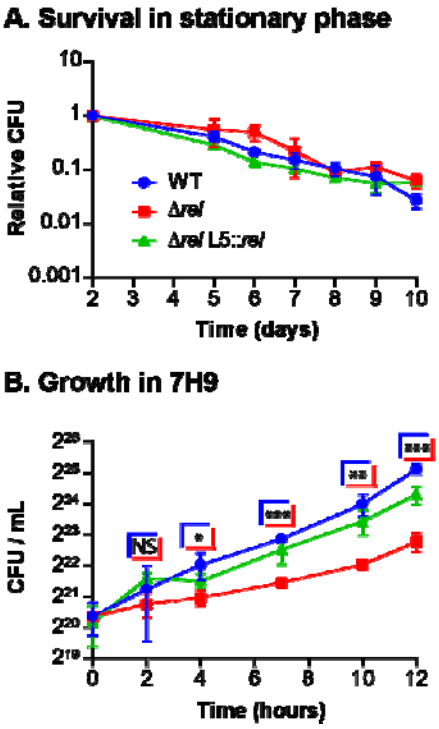


Figure 2. Contribution of *rel*$_{Mab}$ to survival in various stresses. (A) CFU of wild-type *Mycobacterium abscessus* ATCC19977 (blue), ▲*rel*$_{Mab}$ mutant (red), and the complemented strain ▲*rel* L5::*rel* (green) in Hartman's du Bont medium with no glycerol

366 and Tyloxapol as a detergent. (B) CFU in 7H9 Middlebrook medium with a pH of 4. (C)
367 CFU in Lennox LB with 1M of NaCl. (D) CFU in Hartmans du Bont medium with 5mM,
368 25mM or 60mM of tert-butyl peroxide after 24 hours. Relative CFU is calculated by
369 taking the ratio between each CFU value and the initial CFU value at time zero. All data
370 points are an average of three biological replicates. Error bars represent standard
371 deviation. There are no significant differences in any of these data by a two-tailed t-test.
372



373

374 **Figure 3. Growth and stationary phase survival of ▲*rel*$_{Mab}$**. (A) CFU in stationary
375 phase in 7H9 media. The 2 day time point is 48 hours after diluting log. phase cultures
376 to OD=0.05. (B) CFU during log. phase growth in 7H9 medium.Graph is set at a log2
377 scale. P values are for the wild-type compared to the Δ*rel* strain. P values for T=4, *P* =
378 0.0415; T=7, *P* = 0.00015; T=10, *P* =0.006; T=12, *P* =0.00029. *P* -values between wild-
379 type and the complemented strain were not significant.

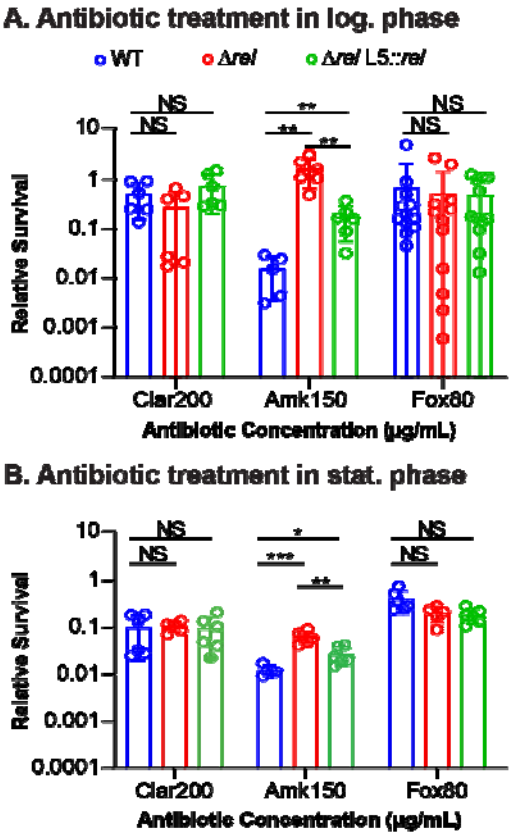


Figure 4. Contribution of *rel*$_{Mab}$ to survival in antibiotic treatment. Relative CFUs of *Mab* strain treated with either 200µg/mL of clarithromycin, 150 µg/mL of amikacin, or 80 µg/mL of cefoxitin for either (A) 48 hours in log. phase or (B) 72 hours in stationary phase. Relative CFU is calculated 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

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

393 0.001; n.s. = $P > 0.05$.

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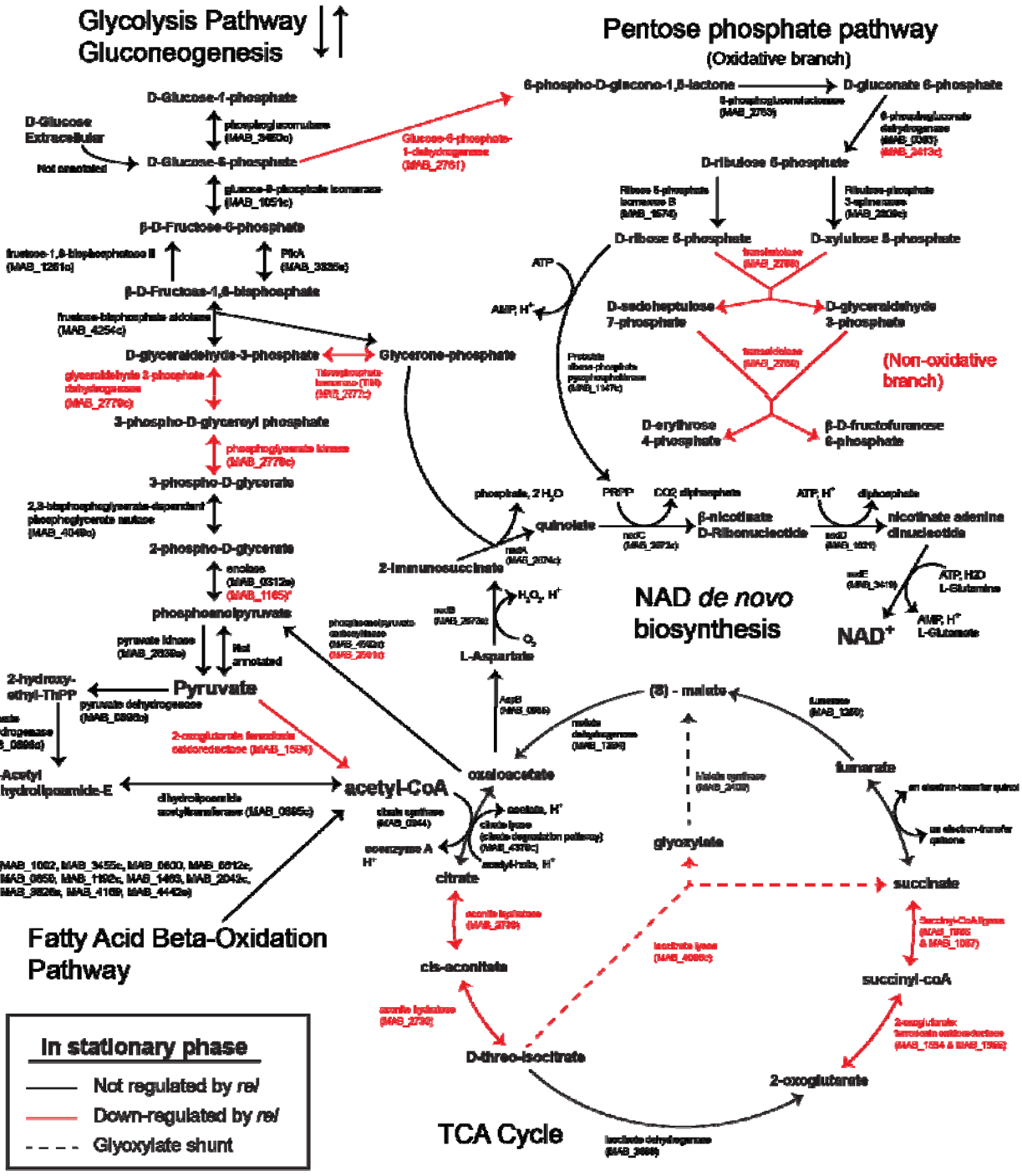

Figure 5. Repression of central metabolic genes by Rel. Genes in red are downregulated by Rel at least 3-fold in stationary phase, $P < 0.05$. Genes in black are not significantly regulated by Rel in stationary phase. See Table S3 for data.

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415 **References**

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418 1. Dahl JL, Kraus CN, Boshoff HIM, Doan B, Foley K, Avarbock D, Kaplan G, Mizrahi
419 V, Rubin H, Barry CE. 2003. The role of RelMtb-mediated adaptation to stationary
420 phase in long-term persistence of Mycobacterium tuberculosis in mice.
421 Proceedings of the National Academy of Sciences 100:10026–10031.

422 2. Traxler MF, Summers SM, Nguyen H-T, Zacharia VM, Hightower GA, Smith JT,
423 Conway T. 2008. The global, ppGpp-mediated stringent response to amino acid
424 starvation in Escherichia coli. Molecular Microbiology 68:1128–1148.

425 3. Boutte CC, Crosson S. 2011. The complex logic of stringent response regulation
426 in Caulobacter crescentus: starvation signalling in an oligotrophic environment.
427 Molecular Microbiology 80:695–714.

428 4. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. 2015. Recent
429 functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev
430 Microbiol 13:298–309.

5. Harms A, Fino C, Sørensen MA, Semsey S, Gerdes K. 2017. Prophages and Growth Dynamics Confound Experimental Results with Antibiotic-Tolerant Persister Cells. mBio 8.

6. Johansen MD, Herrmann J-L, Kremer L. 2020. Non-tuberculous mycobacteria and the rise of Mycobacterium abscessus. Nat Rev Microbiol https://doi.org/10.1038/s41579-020-0331-1.

7. Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. 2012. Mycobacterium abscessus: a new antibiotic nightmare. Journal of Antimicrobial Chemotherapy 67:810–818.

8. Clary G, Sasindran SJ, Nesbitt N, Mason L, Cole S, Azad A, McCoy K, Schlesinger LS, Hall-Stoodley L. 2018. *Mycobacterium abscessus* Smooth and Rough Morphotypes Form Antimicrobial-Tolerant Biofilm Phenotypes but Are Killed by Acetic Acid. Antimicrobial Agents and Chemotherapy 62.

9. Hunt-Serracin AC, Parks BJ, Boll J, Boutte C. 2019. Biofilm-associated Mycobacterium abscessus cells have altered antibiotic tolerance and surface glycolipids in Artificial Cystic Fibrosis Sputum Media. Antimicrobial Agents and Chemotherapy AAC.02488-18.

10. Wexselblatt E, Oppenheimer-Shaanan Y, Kaspy I, London N, Schueler-Furman O, Yavin E, Glaser G, Katzhendler J, Ben-Yehuda S. 2012. Relacin, a Novel Antibacterial Agent Targeting the Stringent Response. PLOS Pathogens 8:e1002925.

11. Dutta NK, Klinkenberg LG, Vazquez M-J, Segura-Carro D, Colmenarejo G, Ramon F, Rodriguez-Miquel B, Mata-Cantero L, Porras-De Francisco E, Chuang Y-M, Rubin H, Lee JJ, Eoh H, Bader JS, Perez-Herran E, Mendoza-Losana A, Karakousis PC. 2019. Inhibiting the stringent response blocks *Mycobacterium tuberculosis* entry into quiescence and reduces persistence. Science Advances 5:eaav2104.

12. Hobbs JK, Boraston AB. 2019. (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. ACS Infect Dis 5:1505–1517.

13. Pacios O, Blasco L, Bleriot I, Fernandez-Garcia L, Ambroa A, López M, Bou G, Cantón R, Garcia-Contreras R, Wood TK, Tomás M. 2020. (p)ppGpp and Its Role in Bacterial Persistence: New Challenges. Antimicrob Agents Chemother 64:e01283-20, /aac/64/10/AAC.01283-20.atom.

14. Barker MM, Gaal T, Josaitis CA, Gourse RL. 2001. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro1 1Edited by R. Ebright. Journal of Molecular Biology 305:673–688.

15. Barker MM, Gaal T, Gourse RL. 2001. Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP11Edited by R. Ebright. Journal of Molecular Biology 305:689–702.

16. Krásný L, Gourse RL. 2004. An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation. EMBO J 23:4473–4483.

17. Krásný L, Tišerová H, Jonák J, Rejman D, Šanderová H. 2008. The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in Bacillus subtilis. Molecular Microbiology 69:42–54.

18. Maciąg M, Kochanowska M, Łyżeń R, Węgrzyn G, Szalewska-Pałasz A. 2010. ppGpp inhibits the activity of Escherichia coli DnaG primase. Plasmid 63:61–67.

19. Kraemer JA, Sanderlin AG, Laub MT. 2019. The Stringent Response Inhibits DNA Replication Initiation in *E. coli* by Modulating Supercoiling of *oriC*. mBio 10:e01330-19, /mbio/10/4/mBio.01330-19.atom.

20. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, Rendon S, Chen R, Tu BP, Wang JD. 2012. Direct Regulation of GTP Homeostasis by (p)ppGpp: A Critical Component of Viability and Stress Resistance. Molecular Cell 48:231–241.

21. Wang B, Dai P, Ding D, Del Rosario A, Grant RA, Pentelute BL, Laub MT. 2019. Affinity-based capture and identification of protein effectors of the growth regulator ppGpp. 2. Nature Chemical Biology 15:141–150.

22. Wang B, Grant RA, Laub MT. 2020. ppGpp Coordinates Nucleotide and Amino-Acid Synthesis in E. coli During Starvation. Molecular Cell S1097276520305487.

490 23. Fan H, Hahm J, Diggs S, Perry JJP, Blaha G. 2015. Structural and Functional
491 Analysis of BipA, a Regulator of Virulence in Enteropathogenic *Escherichia coli*. J
492 Biol Chem 290:20856–20864.

493 24. Corrigan RM, Bellows LE, Wood A, Gründling A. 2016. ppGpp negatively impacts
494 ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive
495 bacteria. Proc Natl Acad Sci USA 113:E1710–E1719.

496 25. Rojas A-M, Ehrenberg M, Andersson SGE, Kurland CG. 1984. ppGpp inhibition of
497 elongation factors Tu, G and Ts during polypeptide synthesis. Mol Gen Genet
498 197:36–45.

499 26. Milon P, Tischenko E, Tomsic J, Caserta E, Folkers G, La Teana A, Rodnina MV,
500 Pon CL, Boelens R, Gualerzi CO. 2006. The nucleotide-binding site of bacterial
501 translation initiation factor 2 (IF2) as a metabolic sensor. Proceedings of the
502 National Academy of Sciences 103:13962–13967.

503 27. Mitkevich VA, Ermakov A, Kulikova AA, Tankov S, Shyp V, Soosaar A, Tenson T,
504 Makarov AA, Ehrenberg M, Hauryliuk V. 2010. Thermodynamic Characterization
505 of ppGpp Binding to EF-G or IF2 and of Initiator tRNA Binding to Free IF2 in the
506 Presence of GDP, GTP, or ppGpp. Journal of Molecular Biology 402:838–846.

507 28. Boutte CC, Crosson S. 2013. Bacterial lifestyle shapes stringent response
508 activation. Trends in Microbiology 21:174–180.

509 29. Surányi G, Korcz A, Pálfi Z, Borbély G. 1987. Effects of light deprivation on RNA
510 synthesis, accumulation of guanosine 3’(2’)-diphosphate 5’-diphosphate, and

protein synthesis in heat-shocked Synechococcus sp. strain PCC 6301, a cyanobacterium. Journal of Bacteriology 169:632–639.

30. Hood RD, Higgins SA, Flamholz A, Nichols RJ, Savage DF. 2016. The stringent response regulates adaptation to darkness in the cyanobacterium *Synechococcus elongatus*. Proceedings of the National Academy of Sciences 113:E4867–E4876.

31. Cashel M, Gallant J. 1969. Two Compounds implicated in the Function of the RC Gene of Escherichia coli. Nature 221:838.

32. Winther KS, Roghanian M, Gerdes K. 2018. Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site. Molecular Cell 70:95-105.e4.

33. Battesti A, Bouveret E. 2006. Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Molecular Microbiology 62:1048–1063.

34. Germain E, Guiraud P, Byrne D, Douzi B, Djendli M, Maisonneuve E. 2019. YtfK activates the stringent response by triggering the alarmone synthetase SpoT in Escherichia coli. Nat Commun 10:5763.

35. Schofield WB, Zimmermann-Kogadeeva M, Zimmermann M, Barry NA, Goodman AL. 2018. The Stringent Response Determines the Ability of a Commensal Bacterium to Survive Starvation and to Persist in the Gut. Cell Host & Microbe 24:120-132.e6.

531 36. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE. 2000. The
532 Stringent Response of Mycobacterium tuberculosis Is Required for Long-Term
533 Survival. Journal of Bacteriology 182:4889–4898.

534 37. Eymann C, Homuth G, Scharf C, Hecker M. 2002. Bacillus subtilis functional
535 genomics: global characterization of the stringent response by proteome and
536 transcriptome analysis. JB 184:2500–2520.

537 38. Potrykus K, Murphy H, Philippe N, Cashel M. 2011. ppGpp is the major source of
538 growth rate control in E. coli. Environmental Microbiology 13:563–575.

539 39. Weiss LA, Stallings CL. 2013. Essential Roles for Mycobacterium tuberculosis Rel
540 beyond the Production of (p)ppGpp. Journal of Bacteriology 195:5629–5638.

541 40. Pulschen AA, Sastre DE, Machinandiarena F, Asis AC, Albanesi D, Mendoza D
542 de, Gueiros-Filho FJ. 2017. The stringent response plays a key role in Bacillus
543 subtilis survival of fatty acid starvation. Molecular Microbiology 103:698–712.

544 41. Murch AL, Skipp PJ, Roach PL, Oyston PCF. 2017. Whole genome
545 transcriptomics reveals global effects including up-regulation of Francisella
546 pathogenicity island gene expression during active stringent response in the
547 highly virulent Francisella tularensis subsp. tularensis SCHU S4. Microbiology
548 163:1664–1679.

549 42. Schäfer H, Beckert B, Frese CK, Steinchen W, Nuss AM, Beckstette M, Hantke I,
550 Driller K, Sudzinová P, Krásný L, Kaever V, Dersch P, Bange G, Wilson DN,

551 Turgay K. 2020. The alarmones (p)ppGpp are part of the heat shock response of
552 Bacillus subtilis. PLoS Genet 16:e1008275.

553 43. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between
554 resistance, tolerance and persistence to antibiotic treatment. Nature Reviews
555 Microbiology 14:320–330.

556 44. Tuomanen E, Cozens R, Tosch W, Zak O, Tomaz A. 1986. The Rate of Killing of
557 Escherichia coli by P-Lactam Antibiotics Is Strictly Proportional to the Rate of
558 Bacterial Growth. Journal of General Microbiology 132:1297–1304.

559 45. Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE,
560 Rhee H-W, Hong J-I, Hartland EL, Stinear TP, Howden BP. 2010. Two Novel
561 Point Mutations in Clinical Staphylococcus aureus Reduce Linezolid Susceptibility
562 and Switch on the Stringent Response to Promote Persistent Infection. PLoS
563 Pathog 6:e1000944.

564 46. Honsa ES, Cooper VS, Mhaissen MN, Frank M, Shaker J, Iverson A, Rubnitz J,
565 Hayden RT, Lee RE, Rock CO, Tuomanen EI, Wolf J, Rosch JW. 2017. RelA
566 Mutant Enterococcus faecium with Multiantibiotic Tolerance Arising in an
567 Immunocompromised Host. mBio 8:mBio.02124-16, e02124-16.

568 47. Korch SB, Henderson TA, Hill TM. 2003. Characterization of the hipA7 allele of
569 Escherichia coli and evidence that high persistence is governed by (p)ppGpp
570 synthesis. Molecular Microbiology 50:1199–1213.

571 48. Geiger T, Kästle B, Gratani FL, Goerke C, Wolz C. 2014. Two Small (p)ppGpp
572 Synthases in Staphylococcus aureus Mediate Tolerance against Cell Envelope
573 Stress Conditions. J Bacteriol 196:894–902.

574 49. Stallings CL, Stephanou NC, Chu L, Hochschild A, Nickels BE, Glickman MS.
575 2009. CarD Is an Essential Regulator of rRNA Transcription Required for
576 Mycobacterium tuberculosis Persistence. Cell 138:146–159.

577 50. Klinkenberg LG, Lee J, Bishai WR, Karakousis PC. 2010. The Stringent Response
578 Is Required for Full Virulence of *Mycobacterium tuberculosis* in Guinea Pigs. J
579 INFECT DIS 202:1397–1404.

580 51. Prusa J, Zhu DX, Stallings CL. 2018. The stringent response and Mycobacterium
581 tuberculosis pathogenesis. Pathogens and Disease; Oxford 76.

582 52. Nanamiya H, Kasai K, Nozawa A, Yun C-S, Narisawa T, Murakami K, Natori Y,
583 Kawamura F, Tozawa Y. 2007. Identification and functional analysis of novel
584 (p)ppGpp synthetase genes in Bacillus subtilis: Novel (p)ppGpp synthetase genes
585 in B. subtilis. Molecular Microbiology 67:291–304.

586 53. Sugisaki K, Hanawa T, Yonezawa H, Osaki T, Fukutomi T, Kawakami H,
587 Yamamoto T, Kamiya S. 2013. Role of (p)ppGpp in biofilm formation and
588 expression of filamentous structures in Bordetella pertussis. Microbiology
589 159:1379–1389.

590 54. Oh YT, Lee K-M, Bari W, Raskin DM, Yoon SS. 2015. (p)ppGpp, a Small
591 Nucleotide Regulator, Directs the Metabolic Fate of Glucose in *Vibrio cholerae*. J
592 Biol Chem 290:13178–13190.

593 55. Boutte CC, Henry JT, Crosson S. 2012. ppGpp and Polyphosphate Modulate Cell
594 Cycle Progression in Caulobacter crescentus. Journal of Bacteriology 194:28–35.

595 56. Kim J-S, Liu L, Fitzsimmons LF, Wang Y, Crawford MA, Mastrogiovanni M, Trujillo
596 M, Till JKA, Radi R, Dai S, Vázquez-Torres A. 2018. DksA–DnaJ redox
597 interactions provide a signal for the activation of bacterial RNA polymerase.
598 Proceedings of the National Academy of Sciences 115:E11780–E11789.

599 57. Sokawa Y, Sokawa J, Kaziro Y. 1975. Regulation of stable RNA synthesis and
600 ppGpp levels in growing cells of Escherichia coli. Cell 5:69–74.

601 58. Gaca AO, Kajfasz JK, Miller JH, Liu K, Wang JD, Abranches J, Lemos JA. 2013.
602 Basal Levels of (p)ppGpp in Enterococcus faecalis: the Magic beyond the
603 Stringent Response. mBio 4:e00646-13.

604 59. Novosad SA, Beekmann SE, Polgreen PM, Mackey K, Winthrop KL. 2016.
605 Treatment of Mycobacterium abscessus Infection. Emerg Infect Dis 22:511–514.

606 60. Nash KA, Brown-Elliott BA, Wallace RJ. 2009. A Novel Gene, erm(41), Confers
607 Inducible Macrolide Resistance to Clinical Isolates of Mycobacterium abscessus
608 but Is Absent from Mycobacterium chelonae. AAC 53:1367–1376.

609 61. Cabral DJ, Wurster JI, Belenky P. 2018. Antibiotic Persistence as a Metabolic
610 Adaptation: Stress, Metabolism, the Host, and New Directions. Pharmaceuticals
611 (Basel) 11.

612 62. Fenn K, Wong CT, Darbari VC. 2020. Mycobacterium tuberculosis Uses Mce
613 Proteins to Interfere With Host Cell Signaling. Front Mol Biosci 6:149.

614 63. Soroka D, Dubee V, Soulier-Escrihuela O, Cuinet G, Hugonnet J-E, Gutmann L,
615 Mainardi J-L, Arthur M. 2014. Characterization of broad-spectrum Mycobacterium
616 abscessus class A -lactamase. Journal of Antimicrobial Chemotherapy 69:691–
617 696.

618 64. Hurst-Hess K, Rudra P, Ghosh P. 2017. Mycobacterium abscessus WhiB7
619 Regulates a Species-Specific Repertoire of Genes To Confer Extreme Antibiotic
620 Resistance. Antimicrobial Agents and Chemotherapy 61.

621 65. Brockmann-Gretza O, Kalinowski J. 2006. Global gene expression during
622 stringent response in Corynebacterium glutamicum in presence and absence of
623 the rel gene encoding (p)ppGpp synthase. BMC Genomics 7:230.

624 66. Traxler MF, Summers SM, Nguyen H-T, Zacharia VM, Hightower GA, Smith JT,
625 Conway T. 2008. The global, ppGpp-mediated stringent response to amino acid
626 starvation in Escherichia coli. Molecular Microbiology 68:1128–1148.

627 67. Vercruysse M, Fauvart M, Jans A, Beullens S, Braeken K, Cloots L, Engelen K,
628 Marchal K, Michiels J. 2011. Stress response regulators identified through

genome-wide transcriptome analysis of the (p)ppGpp-dependent response in Rhizobium etli. Genome Biol 12:R17.

68. Yang H, Yu M, Lee JH, Chatnaparat T, Zhao Y. 2020. The stringent response regulator (p) ppGpp mediates virulence gene expression and survival in Erwinia amylovora. BMC Genomics 21:261.

69. Njire M, Wang N, Wang B, Tan Y, Cai X, Liu Y, Mugweru J, Guo J, Hameed HMA, Tan S, Liu J, Yew WW, Nuermberger E, Lamichhane G, Liu J, Zhang T. 2017. Pyrazinoic Acid Inhibits a Bifunctional Enzyme in Mycobacterium tuberculosis. Antimicrob Agents Chemother 61:e00070-17, e00070-17.

70. Petchiappan A, Naik SY, Chatterji D. 2019. RelZ-mediated stress response in *Mycobacterium smegmatis*: pGpp synthesis and its regulation. J Bacteriol JB.00444-19, jb;JB.00444-19v1.

71. Rodríguez JG, Hernández AC, Helguera-Repetto C, Aguilar Ayala D, Guadarrama-Medina R, Anzóla JM, Bustos JR, Zambrano MM, González-y-Merchand J, García MJ, Del Portillo P. 2014. Global Adaptation to a Lipid Environment Triggers the Dormancy-Related Phenotype of Mycobacterium tuberculosis. mBio 5:e01125-14.

72. Wilburn KM, Fieweger RA, VanderVen BC. 2018. Cholesterol and fatty acids grease the wheels of Mycobacterium tuberculosis pathogenesis. Pathogens and Disease 14.

649 73. Dubois V, Pawlik A, Bories A, Le Moigne V, Sismeiro O, Legendre R, Varet H,
650 Rodríguez-Ordóñez M del P, Gaillard J-L, Coppée J-Y, Brosch R, Herrmann J-L,
651 Girard-Misguich F. 2019. Mycobacterium abscessus virulence traits unraveled by
652 transcriptomic profiling in amoeba and macrophages. PLoS Pathog 15:e1008069.

653 74. Koskiniemi S, Pränting M, Gullberg E, Näsvall J, Andersson DI. 2011. Activation
654 of cryptic aminoglycoside resistance in Salmonella enterica: Activation of cryptic
655 resistance. Molecular Microbiology 80:1464–1478.

656 75. Aedo S, Tomasz A. 2016. Role of the Stringent Stress Response in the Antibiotic
657 Resistance Phenotype of Methicillin-Resistant Staphylococcus aureus. Antimicrob
658 Agents Chemother 60:2311–2317.

659 76. Jayasingam S, Zin T, Ngeow Y. 2017. Antibiotic resistance in Mycobacterium
660 Abscessus and Mycobacterium Fortuitum isolates from Malaysian patients. Int J
661 Mycobacteriol 6:387.

662 77. Luthra S, Rominski A, Sander P. 2018. The Role of Antibiotic-Target-Modifying
663 and Antibiotic-Modifying Enzymes in Mycobacterium abscessus Drug Resistance.
664 Front Microbiol 9.

665 78. Bar-On O, Mussaffi H, Mei-Zahav M, Prais D, Steuer G, Stafler P, Hananya S,
666 Blau H. 2015. Increasing nontuberculous mycobacteria infection in cystic fibrosis.
667 Journal of Cystic Fibrosis 14:53–62.

668 79. Parker H, Lorenc R, Ruelas Castillo J, Karakousis PC. 2020. Mechanisms of
669 Antibiotic Tolerance in Mycobacterium avium Complex: Lessons From Related
670 Mycobacteria. Front Microbiol 11.

671 80. Halstrom S, Price P, Thomson R. 2015. Review: Environmental mycobacteria as a
672 cause of human infection. International Journal of Mycobacteriology 4:81–91.

673 81. Hall BG, Barlow M. 2004. Evolution of the serine β-lactamases: past, present and
674 future. Drug Resistance Updates 7:111–123.

675 82. Perry J, Waglechner N, Wright G. 2016. The Prehistory of Antibiotic Resistance.
676 Cold Spring Harb Perspect Med 6:a025197.

677 83. Waglechner N, McArthur AG, Wright GD. 2019. Phylogenetic reconciliation
678 reveals the natural history of glycopeptide antibiotic biosynthesis and resistance.
679 Nat Microbiol 4:1862–1871.

680 84. Durand GA, Raoult D, Dubourg G. 2019. Antibiotic discovery: history, methods
681 and perspectives. International Journal of Antimicrobial Agents 53:371–382.

682 85. van Kessel JC, Hatfull GF. 2007. Recombineering in Mycobacterium tuberculosis.
683 Nat Methods 4:147–152.

684 86. Kieser KJ, Boutte CC, Kester JC, Baer CE, Barczak AK, Meniche X, Chao MC,
685 Rego EH, Sassetti CM, Fortune SM, Rubin EJ. 2015. Phosphorylation of the
686 Peptidoglycan Synthase PonA1 Governs the Rate of Polar Elongation in
687 Mycobacteria. PLOS Pathogens 11:e1005010.

688 87. Hartmans S, de Bont JAM, Stackebrandt E. 2006. The Genus Mycobacterium--
689 Nonmedical, p. 889–918. *In* Dworkin, M, Falkow, S, Rosenberg, E, Schleifer, K-H,
690 Stackebrandt, E (eds.), The Prokaryotes. Springer New York, New York, NY.

691 88. Shell SS, Prestwich EG, Baek S-H, Shah RR, Sassetti CM, Dedon PC, Fortune
692 SM. 2013. DNA Methylation Impacts Gene Expression and Ensures Hypoxic
693 Survival of Mycobacterium tuberculosis. PLoS Pathog 9:e1003419.

694 89. Gentry D, Li T, Rosenberg M, McDevitt D. 2000. The rel Gene Is Essential for In
695 Vitro Growth of Staphylococcus aureus. J Bacteriol 182:4995–4997.

696 90. Balzer GJ, McLean RJC. 2002. The stringent response genes relA and spoT are
697 important for Escherichia coli biofilms under slow-growth conditions 48:6.

698 91. Mouery K, Rader BA, Gaynor EC, Guillemin K. 2006. The Stringent Response Is
699 Required for Helicobacter pylori Survival of Stationary Phase, Exposure to Acid,
700 and Aerobic Shock. JB 188:5494–5500.

701 92. Lesley JA, Shapiro L. 2008. SpoT Regulates DnaA Stability and Initiation of DNA
702 Replication in Carbon-Starved Caulobacter crescentus. Journal of Bacteriology
703 190:6867–6880.

704 93. Zhou YN, Coleman WG, Yang Z, Yang Y, Hodgson N, Chen F, Jin DJ. 2008.
705 Regulation of Cell Growth during Serum Starvation and Bacterial Survival in
706 Macrophages by the Bifunctional Enzyme SpoT in Helicobacter pylori. JB
707 190:8025–8032.

708 94. Abranches J, Martinez AR, Kajfasz JK, Chávez V, Garsin DA, Lemos JA. 2009.
709 The Molecular Alarmone (p)ppGpp Mediates Stress Responses, Vancomycin
710 Tolerance, and Virulence in Enterococcus faecalis. JB 191:2248–2256.

711 95. Vogt SL, Green C, Stevens KM, Day B, Erickson DL, Woods DE, Storey DG.
712 2011. The Stringent Response Is Essential for Pseudomonas aeruginosa
713 Virulence in the Rat Lung Agar Bead and Drosophila melanogaster Feeding
714 Models of Infection. Infect Immun 79:4094–4104.

715 96. He H, Cooper JN, Mishra A, Raskin DM. 2012. Stringent Response Regulation of
716 Biofilm Formation in Vibrio cholerae. Journal of Bacteriology 194:2962–2972.

717 97. Holley C, Gangaiah D, Li W, Fortney KR, Janowicz DM, Ellinger S, Zwickl B, Katz
718 BP, Spinola SM. 2014. A (p)ppGpp-Null Mutant of Haemophilus ducreyi Is
719 Partially Attenuated in Humans Due to Multiple Conflicting Phenotypes. Infect
720 Immun 82:3492–3502.

721 98. Xu X, Yu H, Zhang D, Xiong J, Qiu J, Xin R, He X, Sheng H, Cai W, Jiang L,
722 Zhang K, Hu X. 2016. Role of ppGpp in Pseudomonas aeruginosa acute
723 pulmonary infection and virulence regulation. Microbiological Research 192:84–
724 95.

725 99. Kim HY, Go J, Lee K-M, Oh YT, Yoon SS. 2018. Guanosine tetra- and
726 pentaphosphate increase antibiotic tolerance by reducing reactive oxygen species
727 production in Vibrio cholerae. J Biol Chem 293:5679–5694.

728 100. Dasgupta S, Das S, Biswas A, Bhadra RK, Das S. 2019. Small alarmones
729 (p)ppGpp regulate virulence associated traits and pathogenesis of *Salmonella*
730 *enterica* serovar Typhi. Cellular Microbiology 21.

731 101. Yin L, Ma H, Nakayasu ES, Payne SH, Morris DR, Harwood CS. 2019. Bacterial
732 Longevity Requires Protein Synthesis and a Stringent Response. mBio
733 10:e02189-19, /mbio/10/5/mBio.02189-19.atom.

734 102. Pokhrel A, Poudel A, Castro KB, Celestine MJ, Oludiran A, Rinehold AJ, Resek
735 AM, Mhanna MA, Purcell EB. 2020. The (p)ppGpp synthetase RSH mediates
736 stationary phase onset and antibiotic stress survival in *Clostridioides difficile*. J
737 Bacteriol JB.00377-20, jb;JB.00377-20v1.

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