The stringent factor Rel in *Mycobacterium abscessus* regulates metabolism and works as a (p)ppGpp hydrolase.

Augusto César Hunt-Serracín[1], Misha I. Kazi[1], Joseph M. Boll[1], Cara C. Boutte[1]

[1]Department of Biology, University of Texas, Arlington

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 found that Rel_{Mab} is not the only synthetase of (p)ppGpp in *Mab*, and that it does not provide a survival advantage in several stress conditions or in antibiotic treatment. Transcriptional data show that Rel_{Mab} is involved in regulating expression of anabolism and growth genes in stationary phase. However, it does not activate transcription of stress response or antibiotic resistance genes, and actually represses transcription of many antibiotic resistance genes. This work shows that there is an unannotated (p)ppGpp synthetase in *Mab*, and indicates that Rel_{Mab} likely functions mainly as a (p)ppGpp hydrolase.

Importance

In this study, we examined the functional roles of the stringent factor Rel in *Mycobacterium abscessus (Mab)*. In most species, stringent factors synthesize the alarmone (p)ppGpp, which globally alters transcription to promote growth arrest and survival under stress and in antibiotic treatment. Our work shows that in *Mab,* an emerging

pathogen which is resistant to many antibiotics, the stringent factor Rel is not solely responsible for synthesizing (p)ppGpp. We find that Rel_{Mab} downregulates many metabolic genes under stress, but does not upregulate stress response genes and does not promote antibiotic tolerance. This study implies that there is another critical but unannotated (p)ppGpp synthetase in *Mab,* and suggests that Rel_{Mab} inhibitors are unlikely to sensitize *Mab* infections to antibiotic treatment.

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 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 difficult to treat due to intrinsic resistance 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 regulatory systems, like the stringent response, which promotes antibiotic tolerance (10–13).

The conserved aspect of the stringent response is the synthesis, upon stress, of the hyperphosphorylated guanine (p)ppGpp. 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).

There are a handful of different protein families that synthesize (p)ppGpp across bacterial clades. The most widely conserved are the Rel/Spo Homolog or RSH proteins, which contain, from N-terminus to C-terminus, a (p)ppGpp hydrolase domain, a (p)ppGpp synthase domain, and regulatory TGS and ACT domains (28). Some RSH proteins associate with the ribosome and sense amino acid starvation by detecting when ribosomes have stalled due to an uncharged tRNA being in the A site (29, 30). Some RSH proteins detect other types of stress or nutrient deprivation via other mechanisms (31–34). Many species have only one RSH-type protein, which is competent for both (p)ppGpp hydrolysis and synthesis (28). Many species encode Small Alarmose Synthetase (SAS) and Small Alarmone Hydrolase (SAH) proteins, which contain only (p)ppGpp synthetase and hydrolase domains respectively (30, 35–38). Because SAS proteins do not have regulatory domains, they are typically controlled transcriptionally (30). There are a few other (p)ppGpp synthesizing domains that have been preliminarily studied. ToxSAS proteins are part of toxin-antitoxin systems and synthesize (p)ppGpp as a toxin - these are found in both phage and bacterial genomes (39). *Streptomyces antibioticus* has a polynucleotide phosphorylase (PNPase) which can also synthesize (p)ppGpp (40–43).

The physiological outputs of the stringent response 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, 44). Stringent inhibition of growth (44–50) indirectly protects against some stresses and antibiotics that interfere with growth factors. In many species, the stringent response upregulates stress response genes such as heat shock proteins, hibernation factors, and stress-specific transcription factors (3, 51, 52) and promotes survival in stress (13). The stringent response also helps many bacteria survive through antibiotic treatment by promoting antibiotic tolerance (12).

The stringent response has been studied in *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium smegmatis* (*Msmeg*). *Mtb* has one major (p)ppGpp synthetase, Rel_{Mtb} which makes (p)ppGpp when respiration is inhibited, in stationary phase, and in total carbon and nitrogen starvation (46, 53). Rel_{Mtb} also promotes survival of *Mtb* during nutrient and oxygen starvation, stationary phase (46) and chronic infection of mice (1) and guinea pigs (54, 55). Importantly, Rel_{Mtb} also makes *Mtb* more tolerant to the first-line clinical antibiotic isoniazid during starvation and infection in mice (11). A PNPase enzyme in *Mtb*, Rv2783, has been shown to have weak (p)ppGpp synthetase activity (56), and the *Mtb* genome contains another gene with a predicted GTP pyrophosphokinase domain, Rv1366. However, the Δrel_{Mtb} strain does not synthesize measurable (p)ppGpp in starvation conditions (46), so these other potential synthetases are either inactive or not activated in the conditions tested.

Msmeg has an RSH protein, Rel_{Msmeg}, which can both synthesize and hydrolyze (p)ppGpp (57–60). Although the Δrel_{Msmeg} strain showed defects in biofilm formation and stationary phase viability, it can still synthesize (p)ppGpp (57). The secondary (p)ppGpp synthetase, $RelZ_{Msmeg}$, has an RNaseHII domain in addition to the conserved SAS ppGpp synthetase domain (59), and also synthesizes pGpp (61). Strains missing both

synthetases have further biofilm and aggregation defects, but can still synthesize some (p)ppGpp (61), indicating that there is a third, uncharacterized synthetase in *Msmeg*.

In this study we examined the Δ*rel* strain of *Mab.* Rel_{Mab} is an RSH protein. There are no other RSH genes, and no SAS genes in the *Mab* genome. We found that the Δrel_{Mab} strain still makes (p)ppGpp, indicating that Rel_{Mab} could work primarily as a (p)ppGpp hydrolase. The Δrel_{Mab} strain does not exhibit survival defects in several stress conditions including antibiotic treatment, but has a growth defect relative to wild type. We measured transcriptional changes in Δrel_{Mab} relative to wild type and found it helps downregulate many metabolic pathways in stationary phase.

Results

In order to explore the role of rel_{Mab}, we built a strain of *Mab* ATCC19977 with a deletion of the *rel* gene (MAB_2876), which has the canonical RSH gene structure, including a (p)ppGpp hydrolase domain, a (p)ppGpp synthetase domain, a TGS domain and an ACT domain. We measured (p)ppGpp in both the wild-type *Mab* and Δrel_{Mab} strains, in logarithmic and stationary phase (Fig. 1). We found that the Δrel_{Mab} strain produces pppGpp in both growth and stasis, but only ppGpp in stationary phase. The reduced signal in stationary phase is likely due to decreased uptake and metabolism of the radiolabeled phosphate in the metabolically-depressed stationary phase cells.

In many species, *rel* orthologues promote survival during stress conditions, such as stationary phase, acid stress, starvation, or oxidative stress (5, 35, 46, 49, 62, 63). To

evaluate the physiological role of *rel* in *Mab*, we assayed survival of wild-type, Δ*rel*$_{Mab}$ and complemented strains upon and after transfer to either carbon starvation (Fig. 2A), salt stress (Fig. 2B), oxidative stress, (Fig. 2C) or acidic media (Fig. 2D). Treatment with these stressors did not induce measurable differences in growth or survival of Δ*rel*$_{Mab}$ relative to wild-type and the complemented strain. Thus, Rel$_{Mab}$ 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 Δ*rel*$_{Mab}$ does not promote survival in stationary phase (Fig. 3A). Robust survival in the Δ*rel*$_{Mab}$ in all stress conditions is likely aided by the continued high levels of (p)ppGpp under stress (Fig. 1).

Growth curves showed that Δ*rel*$_{Mab}$ had a significant growth defect relative to wild type and complemented strains (Fig. 3B). These data, along with slightly higher (p)ppGpp levels in in the Δ*rel*$_{Mab}$ strain (Fig 1) suggest that Rel$_{Mab}$ functions to promote growth in low stress conditions by hydrolyzing (p)ppGpp made by an unknown synthetase. This is reminiscent of studies in *E. coli*, where deletion of a (p)ppGpp hydrolase in a strain that contains another synthetase slows growth, but deletion of all the synthetases restores faster growth (4, 45, 64, 65).

Because the stringent response is a major activator of antibiotic tolerance and persistence in many species (5, 66, 67), we sought to assess how Rel contributes to antibiotic tolerance in *Mab.* First, we treated *Mab* cultures in logarithmic phase with amikacin, clarithromycin and cefoxitin, which are commonly prescribed to treat *Mab* infections (68). We found that clarithromycin and cefoxitin alone were ineffective against

all of the strains (Fig. 4A). However, amikacin treatment resulted in 10-100-fold decrease in viability of wild-type and complemented strains, but had no effect on Δ*rel*$_{Mab}$.

Antibiotic tolerance increases in stationary phase in most bacterial species relative to logarithmic phase (5, 69). We repeated the antibiotic survival experiments on cultures in stasis and found that Rel$_{Mab}$ does not affect tolerance to clarithromycin or cefoxitin (Fig. 4B). Similar to amikacin treatment in growth, Rel$_{Mab}$ increased susceptibility relative to wild type and the complemented strains in stasis.

A major function of the stringent response in other bacteria is to regulate transcription (4). To determine the effects of Rel$_{Mab}$ on transcription, we compared the trancriptome of wild-type and Δ*rel*$_{Mab}$ in both logarithmic and stationary phases. We found that Rel$_{Mab}$ represses many more genes than it activates (File. S1).

In logarithmic phase, when Δ*rel*$_{Mab}$ grows more slowly relative to wild type (Fig. 3B), we found 150 genes repressed by *rel*$_{Mab}$ in the wild-type strain at least 3-fold, and only 7 genes were activated. The only annotated upregulated genes are an efflux pump (MAB_0677) and a major facilitator superfamily (MFS) transporter (MAB_0069). We found several *mce* family genes that were repressed by *rel*$_{Mab}$ (Table S2). Mce proteins are typically lipid transporters, but they also play roles in host cell entry and immune modulation (70). We also found two antibiotic resistance genes that are repressed by *rel*$_{Mab}$ in logarithmic phase (Table 1).

Even though there was no apparent difference in survival between the wild-type and Δ*rel*$_{Mab}$ strains in stationary phase, we observed significant differences in transcription. It is unclear if these differences are mediated through (p)ppGpp, since we see similar levels between wild type and Δ*rel*$_{Mab}$ strains in stationary phase (Fig. 1). We

found hundreds of genes that were repressed by *rel*$_{Mab}$ in the wild type strain in stationary phase, but none that were activated by *rel*$_{Mab}$ 3-fold or more. We found many genes in the WhiB7 regulon (Table 1) which are repressed in stationary phase, though they are mostly unaffected in logarithmic phase. WhiB7 is a transcription factor that activates many antibiotic resistance genes and promotes resistance to many classes of antibiotics in *Mab* (71). It is notable that these antibiotic resistance genes are repressed by *rel*$_{Mab}$ in stasis, which would imply that 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*$_{Mab}$ in stationary phase (Table S2). Downregulation of growth factors is typical in stringent responses across many bacterial species (3, 47, 67, 72, 73). We see that *rel*$_{Mab}$ downregulates many central metabolism genes in stationary phase. However, it is notable that not all the genes in a given pathway are downregulated equally (Fig. 5, Table S3). We hypothesize that this uneven regulation of certain pathways may allow certain metabolites to accumulate and be re-directed to other pathways. In a metabolic pathway where most of the enzymes are downregulated, we expect that the product of a single gene that is not downregulated will accumulate. We note that many of the metabolites we expect to accumulate converge on the NAD synthesis pathway. In addition, none of the genes in the NAD synthesis pathway are downregulated by *rel*$_{Mab}$, which implies that

continued metabolism of NAD, which is a critical cofactor in many pathways, may be important in stationary phase.

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

Discussion

Mtb, Msmeg and *Mab* all have a stringent response, but they have evolved use of different sets of genes to synthesize the same alarmone. The *Mab* genome encodes only one annotated RSH gene, rel_{Mab} (MAB_2876), and no homologs of SAS or SAH genes. rel_{Mab} is homologous to RSH genes in *Mtb* and *Msmeg*. We found that Δrel_{Mab} could still produce (p)ppGpp, consistent with the presence of an unannotated (p)ppGpp synthase. *Mab* does not have a homolog of the SAS-RNaseHII *relZ*, which has been characterized in *Msmeg* (57, 59, 61). Despite this, *Msmeg* and *Mab* are similar in having significant (p)ppGpp synthesis in the absence of the RSH gene (Fig. 1) (53), and in exhibiting greater survival with some antibiotics (Fig. 4) (70). *Mab* does have a homolog of the PNPase which has been shown to be capable of (p)ppGpp synthesis in *Mtb* (MAB_3106c) (56).

Our results show that the stringent factor Rel_{Mab} does not promote survival during *in vitro* stress, though it does promote growth during logarithmic phase (Fig. 2, 3A). Our transcriptomics analysis corroborate these results, as they indicate that Rel_{Mab} does not upregulate stress response genes, but does alter transcription of growth metabolism

genes (Table S2, Supplemental file 1). It remains possible that increases in (p)ppGpp stimulate transcription of stress response genes in *Mab*, as is seen in other species (3, 52, 74, 75) – our experiments did not test this because Δrel_{Mab} synthesizes similar amounts of (p)ppGpp as wild-type. Our transcriptional data show that Rel_{Mab} is involved in downregulating metabolism for growth arrest (Fig. 5, S2) even though it does not increase (p)ppGpp levels (Fig. 1). This result is surprising, as transcriptional downregulation of growth-promoting metabolism in the stringent response is linked to increased (p)ppGpp in other species (3, 47, 73, 76). However, there is precedent for metabolic genes being regulated separately from stress response genes in the stringent response. In *E. coli*, some metabolic genes are repressed at a lower (p)ppGpp level than is required to induce a regulon of stress response genes (77). (p)ppGpp likely does not directly bind RNAP in mycobacteria as it does in *E. coli* (78), and it is currently unknown how it exerts its effects on transcription. Further knowledge of the functioning of the mycobacterial stringent response will be required before we can speculate about the mechanism by which (p)ppGpp could differentially affect transcription of metabolic and stress response genes in *Mab.*

Mab is notorious for having resistance to many clinical antibiotics and expressing many antibiotic resistance genes (71, 79, 80), which is why it is problematic to treat infections in cystic fibrosis patients (81). We observed in our transcriptional data that Rel_{Mab} downregulated numerous antibiotic resistance genes in stationary phase (Table 1). In other species RSH proteins promote antibiotic tolerance (5, 11, 67) and sometimes also increase expression of antibiotic resistance genes (82, 83). Our data indicate that

Rel$_{Mab}$ may function predominantly as a (p)ppGpp hydrolase (Fig. 1), thus, the slightly higher (p)ppGpp levels in Δ*rel*$_{Mab}$ could stimulate expression of antibiotic resistance genes. Studies are ongoing to find drugs that would inhibit (p)ppGpp synthesis by RSH proteins (11, 13), as such drugs are expected to increase susceptibility to clinically available antibiotics. Our results indicate that Rel$_{Mab}$ inhibitors, should they become available, are unlikely to help treat *Mab* infections. Instead, efforts should focus on identifying the other (p)ppGpp synthetase(s) in *Mab* so that it can be explored as a drug target.

Materials and Methods

Construction of strains. Primers 1233 – 1238 (Table S3) were used to amplify a 502 bp segment upstream of *Mab rel* which included the start codon, a 448 bp segment downstream of Mab *rel* which included the stop codon, and a 788 bp ZeoR cassette. All 3 segments were stitched together by PCR to form the Δ*rel*::zeoR double stranded recombineering knockout construct. Δ*rel*$_{Mab}$ was generated through double stranded recombineering, as previously described (84) (Fig. S1C). Colonies from the transformation of the Δ*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*$_{Mab}$ gene was amplified through PCR using primers 1329-1330 and inserted into pKK216 (85) with NdeI and HindIII. This new plasmid, pCB1248, was transformed into the Δ*rel*$_{Mab}$

mutant strain in order to create the complementation strain, Δ*rel*$_{Mab}$ *L5::rel*$_{Mab}$, in which *rel*$_{Mab}$ expression is driven by a constitutive promoter (BN17, Fig.S1B).

Media and culture conditions. All *M. abscessus* ATCC 19977 wild-type and mutant 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 cultures entered logarithmic phase. For starvation and other specific assays, Hartmans-de Bont (HdB) minimal medium was made as described previously (86). Cultures were inoculated to OD_{600} 0.05, unless otherwise stated. All CFU time points were plated on Luria-Bertani (LB) agar and placed in 37° C incubator for 4 days.

***Mab* (p)ppGpp extraction and detection.** *Mab* strains were grown until logarithmic phase (OD_{600} 0.5) in homemade 7H9 medium. To maximize ^{32}P-labeling, 7H9 medium with 1/25 of normal phosphate levels was used (46). Logarithmic phase *Mab* cells were pelleted and resuspended in 1mL of low phosphate medium, followed by the addition of ^{32}P-labeled orthophosphoric acid to a final concentration of 100 µCi/mL. ^{32}P-labeled cells were then incubated at 37° C and shaken at 200 rpm for 4 h. Following incubation, optical density of radioactive samples were measured, and each culture was diluted to OD_{600} 0.2. Cells were pelleted and resuspended with 100 µL of 10 mM TBST (Tris-buffer saline pH 8 with 0.05% Tyloxapol), and treated with 1 mg/mL of lysozyme in ice for 20 minutes. Equal volume 2 M Formic acid was then added to each sample, followed by a 5 minute centrifugation at max speed. Supernatant was then collected for each sample and stored in -20° C. For stationary phase cultures, a second set of log phase *Mab* strains (OD_{600}

0.5) were diluted back to OD_{600} 0.05, incubated at 37° C, and shaken at 130 rpm for 48 h. Upon reaching stationary phase, (p)ppGpp extraction was was performed as for log phase samples. Following extraction, 20 µL of extract was spotted on a PEI-cellulose TLC plate and developed in 1.5 M Potassium monophosphate buffer (pH 3.4). The plate was then air dried and placed on a phosphor screen (Molecular dynamics) overnight. The phosphor screen was scanned with a Storm 860 scanner (Amersham Biosciences) and images were analyzed with ImageQuant software (Molecular Dynamics).

Δ*rel*$_{Mab}$ stress assays. For all stress assays, strains were prepared and grown into logarithmic phase. Unless otherwise stated, cultures for stress assays were done in 24-well plates and shaken at 130 rpm at 37° C. For carbon starvation, strains were inoculated in 30 mL inkwells in HdB minimal media with no glycerol, and with Tyloxapol as a detergent. For acid stress, strains were inoculated in 7H9 medium pH 4. For osmotic stress, strains were inoculated in LB broth 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, 3, and 24 h post-inoculation.

Δ*rel*$_{Mab}$ growth curve and stationary phase survival. Log-phase cultures of all strains were inoculated to OD 0.05 in 30 mL inkwells in 7H9 media. Cultures were then placed in shaking incubator at 37° C and 130 rpm. 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 h. Initial CFU time-point was taken at 48 h after dilution of logarithmic phase samples to OD_{600} 0.05, with subsequent time points taken at 5, 6, 7, 8, 9, and 10 d.

Antibiotic assays. For the logarithmic phase experiments, strains were kept in logarithmic phase in 7H9 for *t*~24 h and diluted to OD_{600} 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 h after treatment (*t*=48). For stationary phase, logarithmic phase cells at OD_{600} 0.05 were shaken for 48 h and then treated as above. CFUs were measured upon treatment and 72 h after treatment.

RNA isolation, library preparation and data analysis. RNA from three biological replicates of each strain and condition was isolated as previously described (87) with some modifications. After growth for *t*~24 hours in either logrithmic or stationary phase, cells were transferred to 15 mL conical tubes and centrifuged at 4° C for 3 min at 1467 x g. Cell pellets were immediately resuspended in 750 µl 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* strain 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 the median reads per gene were equivalent within an experiment. In the logarithmic 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 gene, and the weighted proportion fold change of RPKM between wild type and Δrel_{Mab} for each condition were calculated by CLC Workbench. The Baggerley's test was used to generate a false discovery rate corrected P-value. We used an arbitrary cut-off of 3-fold change with a false-discovery rate corrected $P \leq 0.05$ to identify significantly differentially regulated genes between wild type and Δrel_{Mab} in each condition. Because the median reads per gene for logarithmic phase samples was 6 times higher than for stationary phase samples, we linearly scaled the fold-change values when comparing logarithmic to stationary phase data to normalize for this difference in read depth.

Real-time PCR (Supplemental data). For RNA-seq validation, real-time PCR (RT-PCR) was performed with the Kapa Biosystems Sybr Fast One-Step qRT-PCR kit. RNA was extracted in triplicates from each *Mab* strain as previously described. Primers for reverse-transcription (RT) were designed for each gene of interest by using Primer3 (Table S3). Each 20 µL RT reaction mixture contained 10 µL One-step SYBR green Master Mix, 1 µL of each primer, 4.5 µL nuclease-free water, 0.5 µL of qScript One-step RT, and 3 µL of RNA. RT-PCR was done by using Bio-Rad CFX Connect Real-time system. The relative

target levels (fold change) were calculated using the ΔΔCt method (88), with normalization of RNA targets to TetR transcription factor gene (MAB_1638).

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

***Mab* GENE Annotation**	**FC- Δ*rel*$_{Mab}$/ vs. WT LOG**	**FDR-corrected *P* value Δ*rel*$_{Mab}$ / vs. WT LOG**	**FC- Δ*rel*$_{Mab}$ / vs.WT Stat.**	**FDR-corrected *P* value Δ*rel*$_{Mab}$ / 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

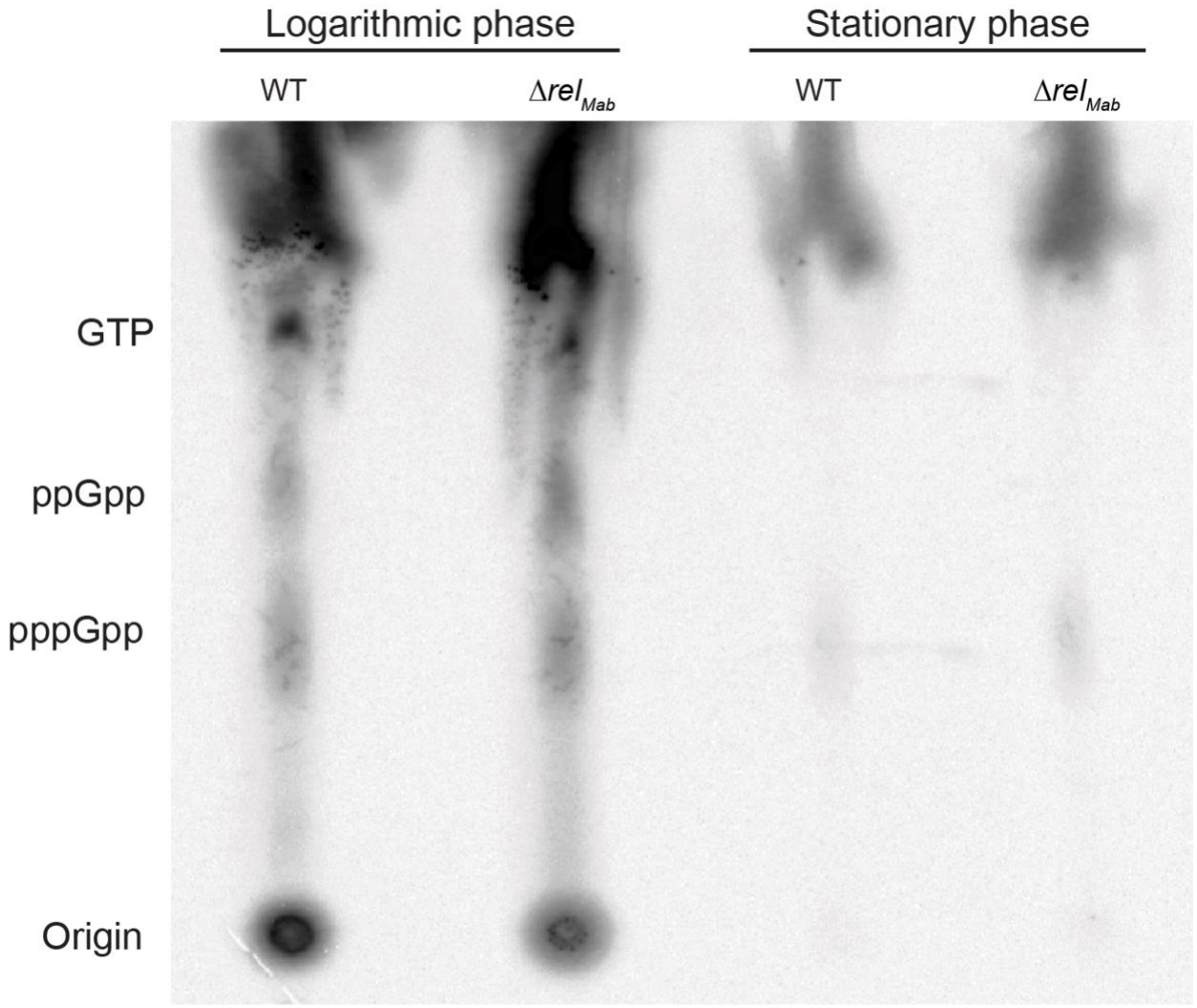
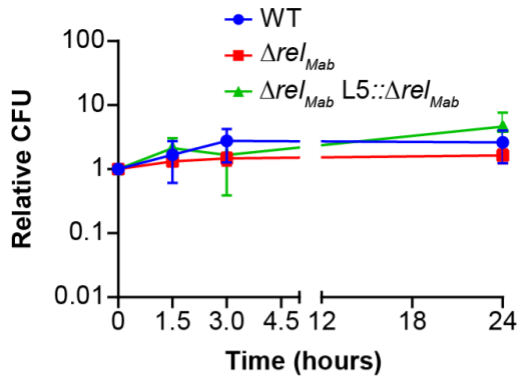
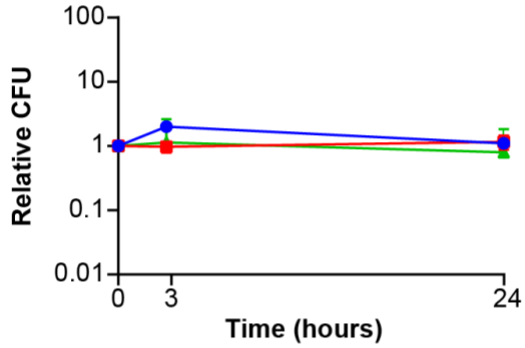


Figure 1. Thin-layer chromatography (TLC) of nucleotides extracted from *M. abscessus* strains. Guanine nucleotides extracted from ^{32}P-labeled *M. abscessus* strains from logarithmic and stationary phase. Culture ODs were normalized prior to (p)ppGpp extraction. Intensity of spots were quantified using ImageJ software (data not shown). For logarithmic phase, signal intensities for pppGpp (1.04-fold) and ppGpp (1.5-fold) were higher in Δ*rel*$_{Mab}$ than in the wild type. For stationary phase, signal intensity of pppGpp was 1.3-fold higher in Δ*rel*$_{Mab}$ than in the wild type.

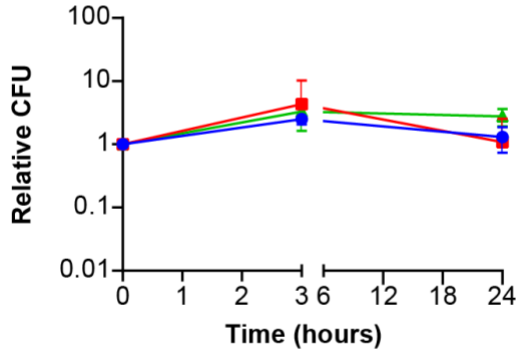
A. Survival during carbon starvation



B. Survival in acidic medium



C. Survival during osmotic stress



D. Survival during oxidative stress

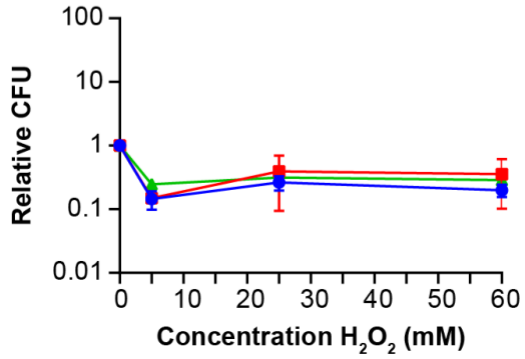


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Figure 2. Contribution of *rel*$_{Mab}$ to survival in various stresses. (A) Colony-forming units (CFU) of wild type *Mycobacterium abscessus* strain ATCC19977 (blue), Δ*rel*$_{Mab}$ (red), and the complemented strain Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ (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 student's t-test.

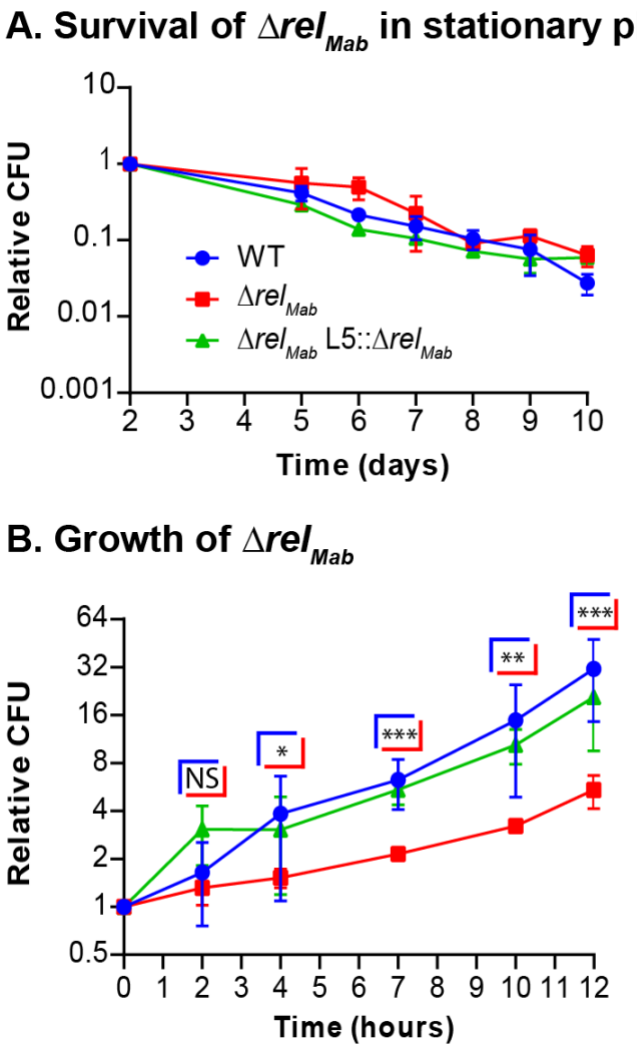

Figure 3. Growth and stationary phase survival of Δ*rel*$_{Mab}$. (A) Colony-forming units (CFU) in stationary phase in 7H9 media. The 2 day time point is 48 hours after diluting logarithmic phase cultures to OD_{600}=0.05. (B) CFU during logarithmic phase growth in 7H9 medium. Graph is set at a log2 scale. Doubling-times ± 95% confidence interval = wild type 2.15 h ± 0.08; Δ*rel*$_{Mab}$ 4.67 h ± 4.26; Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$; Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ 2.84 ± 1.85. *P*-values are for the wild-type compared to Δ*rel*$_{Mab}$. *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. *P*- values between strains in stationary phase were not significant (data not shown). 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. = $P > 0.05$.

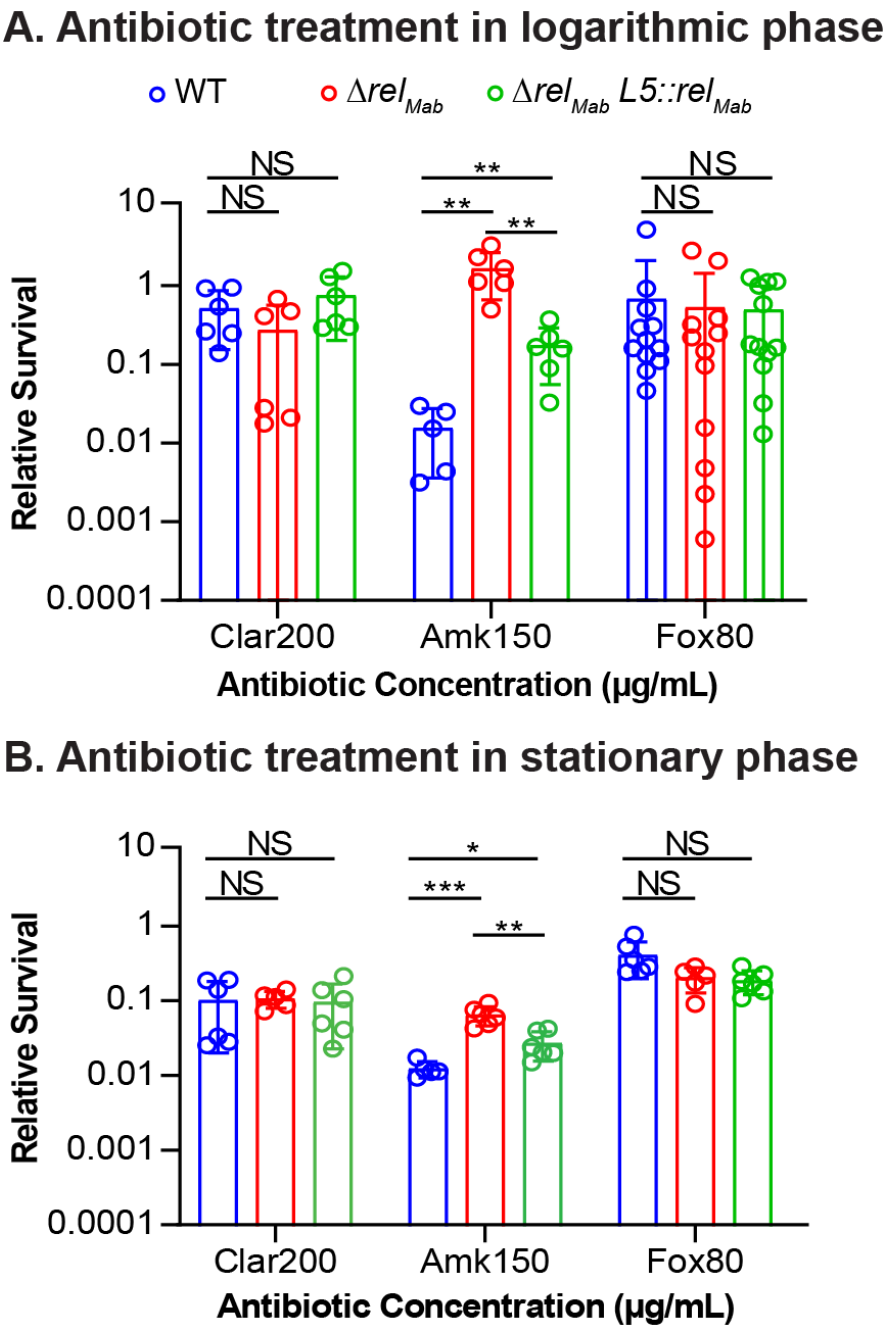

Figure 4. Contribution of *rel*$_{Mab}$ to survival in antibiotic treatment. Relative Colony-forming units (CFUs) of strains treated with either 200 µg/mL of clarithromycin (Clar), 150 µg/mL of amikacin (Amk), or 80 µg/mL of cefoxitin (Fox) for either (A) 48 h in logarithmic phase or (B) 72 h in stationary phase. Relative CFUs were calculated by taking the ratio between the CFU value after treatment 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. Logarithmic phase *P*-values: (Amk150) WT vs. Δ*rel*$_{Mab}$ = 0.005; WT vs. Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ = 0.01; Δ*rel*$_{Mab}$ vs. Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ = 0.004 . Stationary phase *P*-values: (AMK150) WT vs Δ*rel*$_{Mab}$ = 0.00017; WT vs. Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ = 0.0217; Δ*rel*$_{Mab}$ vs. Δ*rel*$_{Mab}$ L5::*rel*$_{Mab}$ = 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. = $P > 0.05$.

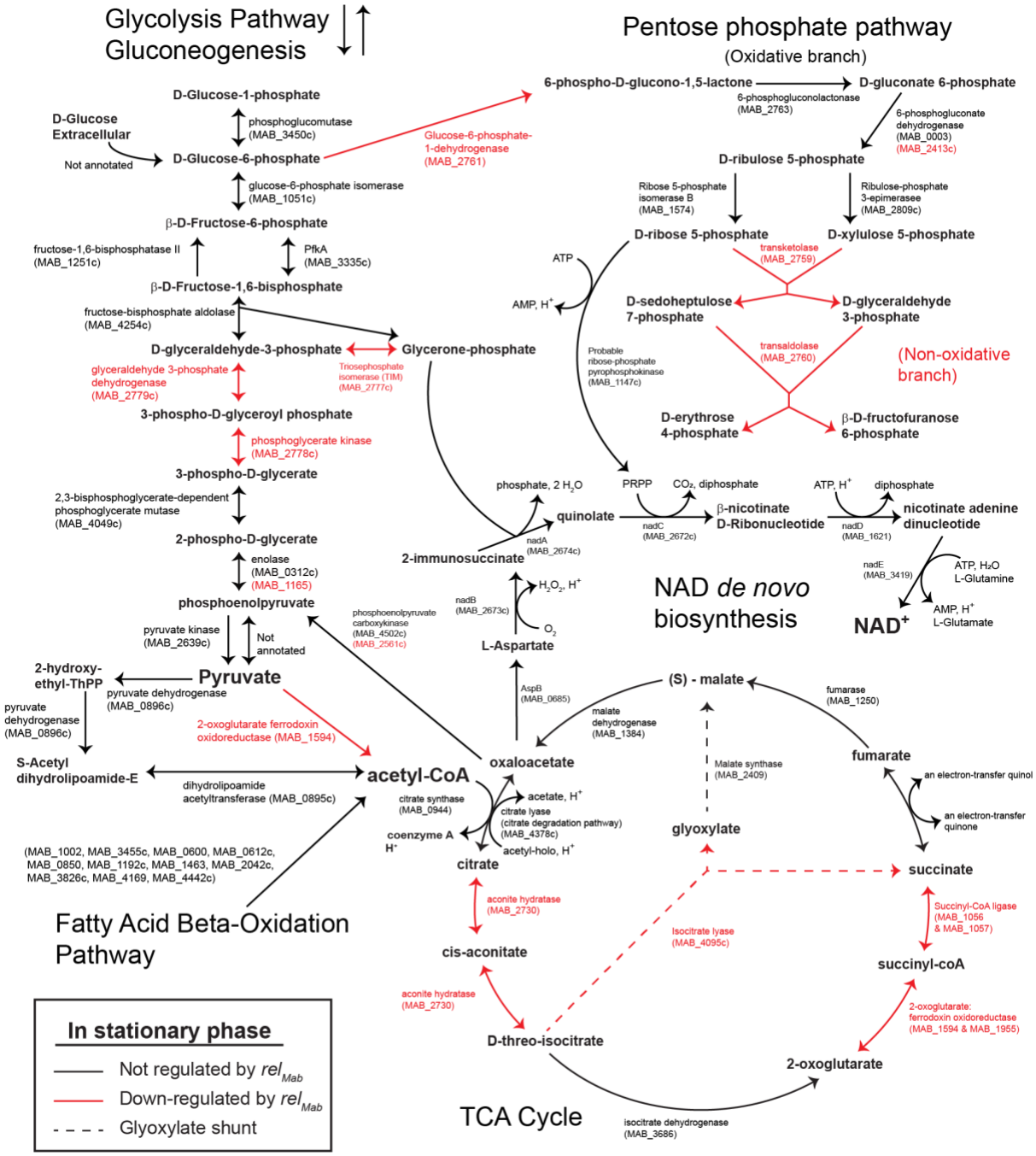

Figure 5. Repression of central metabolic genes by Rel$_{Mab}$ during stationary phase. Genes in red are downregulated by Rel$_{Mab}$ at least 3-fold in stationary phase, $P < 0.05$.

Genes in black are not significantly regulated by Rel_{Mab} in stationary phase. See Table S4 for data.

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