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ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria

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The stringent response is a survival mechanism utilized by bacteria to deal with stress. It is coordinated by the nucleotides guanosine tetra- and pentaphosphate ((p)ppGpp), which interact with target proteins in order to promote bacterial survival. Although this response has been well characterized in proteobacteria, very little is known about the effectors of this signaling system in Gram-positive species. Here we report on the identification of seven target proteins for the stringent response nucleotides in the Gram-positive bacterium *Staphylococcus aureus*. We demonstrate that the GTP synthesis enzymes HprT and Gmk bind with a high affinity leading to an inhibition of GTP production. In addition, we identified five putative GTPases - RsgA, RbgA, Era, HflX and ObgE as (p)ppGpp target proteins. We show that RsgA, RbgA, Era, HflX are functional GTPases and that their activity is promoted in the presence of ribosomes, but strongly inhibited by the stringent response nucleotides. By characterizing the function of RsgA *in vivo*, we ascertain that this protein is involved in ribosome assembly, with an *rsgA* deletion strain, or a strain inactivated for GTPase activity, displaying decreased growth, a decrease in the amount of mature 70S ribosomes and an increased level of tolerance to antimicrobials. We additionally demonstrate that the interaction of ppGpp with cellular GTPases is not unique to the Staphylococci, as homologues from *Bacillus subtilis* and *Enterococcus faecalis* retain this ability. Taken together, this study reveals ribosome inactivation as a new mechanism through which the stringent response functions in Gram-positive bacteria.

ribosome | stringent response | tolerance | ppGpp | Staphylococcus aureus

The stringent response is a complex mechanism utilized by all bacteria to deal with cell stresses including amino acid deprivation, carbon source starvation, fatty acid depletion and osmotic stress (1-3). This response, first characterized over 40 years ago, is coordinated by the rapid synthesis of the nucleotides guanosine tetra- and pentaphosphate, collectively termed (p)ppGpp (2). Once produced, these alarmones are responsible for controlling a cellular switch resulting in the downregulation of active growth and an upregulation of genes involved in the stress response (4). Additionally these nucleotides have been shown to be vital for controlling the transition of bacteria into stationary phase, biofilm formation, sporulation, virulence, antibiotic tolerance and persister cell formation (5-9).

In proteobacteria, it has long been established that after enduring stress, (p)ppGpp is synthesized by both the monofunctional enzyme RelA and the bifunctional enzyme SpoT, a protein that also contains (p)ppGpp hydrolase activity (4). RelA associates with ribosomes and synthetase activity is triggered upon an accumulation of uncharged tRNA sensed by the ribosome during amino acid depletion (10-13). The synthetase activity of SpoT, on the other hand, is induced by other stresses such as fatty acid depletion (1). Once synthesized the major effect of (p)ppGpp production is an alteration in gene transcription, where stable RNAs (rRNA and tRNA), as well as cell proliferation genes are downregulated and genes involved in the stress and starvation response are upregulated (4). In Gram-negative bacteria this transcriptional shift is mediated by (p)ppGpp interacting with the RNA polymerase (RNAP), which in combination with the transcription factor DksA, modulates gene expression on a transcriptional level (3, 14). Aside from the RNAP, there are at least 15 other direct (p)ppGpp target proteins in *Escherichia coli*, such as the translation elongation factors EFG and EF-Tu, the DNA primase DnaG and a number of amino acid decarboxylases that are involved in the acid stress response (15-17). These nucleotides also play major roles in controlling bacterial persistence in Gram-negative bacteria by activating toxin-antitoxin systems and triggering slow growth (9), leading to cells that persist in the host following antibiotic treatment.

In *Staphylococcus aureus*, as well as other Gram-positive species, (p)ppGpp is synthesized by RSH, a bifunctional RelA/SpoT homologue that contains both a synthetase and hydrolase domain (18, 19). The genome of *S. aureus* also encodes two other monofunctional synthetases, RelP and RelQ, and transcription of these enzymes increases when cells are exposed to cell wall-targeting antimicrobials (20, 21). Recent work on *S. aureus* has shown that the ability to switch on the stringent response is essential for its virulence and is required for the organism to cause chronic infections (22-25).

In contrast to the situation in proteobacteria, very little is known about the binding targets for (p)ppGpp in Gram-positive species. These nucleotides do not interact with the RNAP (26)

Significance

When bacteria encounter stresses such as nutrient deprivation they react by switching on the stringent response, the effects of which are mediated by two nucleotides collectively referred to as (p)ppGpp. These nucleotides function by binding to target proteins leading to bacterial cells shutting down active growth and entering a state that promotes survival. In *Staphylococcus aureus* relatively little is known about the target proteins with which these nucleotides interact. In this work, a genome-wide nucleotide-protein interaction screen was used to identify protein targets of (p)ppGpp in order to fully establish the pathways these nucleotides control in Gram-positive bacteria. In doing so we identify several previously unknown targets with roles in ribosomal assembly, cell growth and antimicrobial tolerance.

Reserved for Publication Footnotes

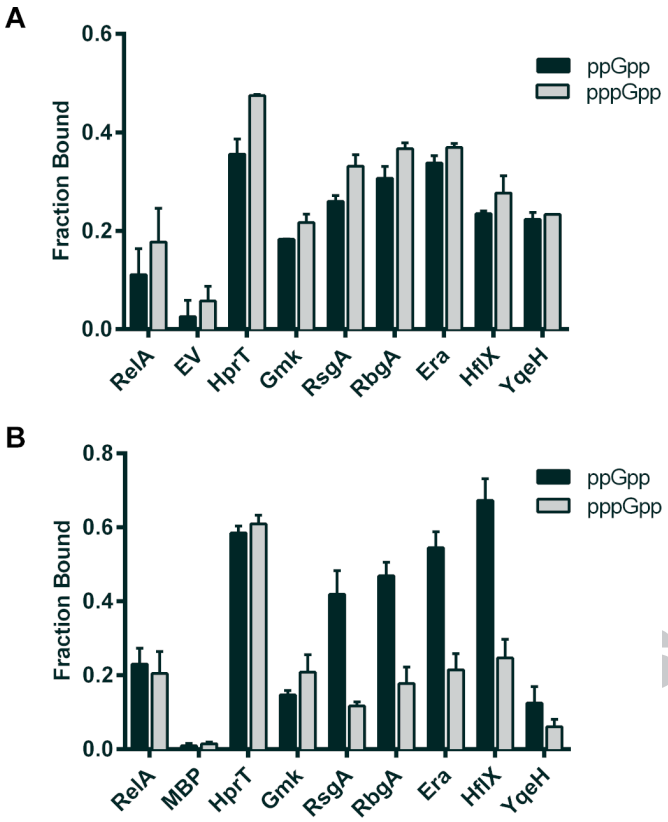

Fig. 1. Confirmation of the interactions between (p)ppGpp and target proteins. (*A*) DRaCALA with ^{32}P-labeled (p)ppGpp and whole cell lysates prepared from *E. coli* strains overexpressing the different target proteins. (*B*) DRaCALA with purified recombinant proteins and ^{32}P-labeled (p)ppGpp. All experiments were carried out in quadruplicate with the data plotted using the GraphPad Prism software.

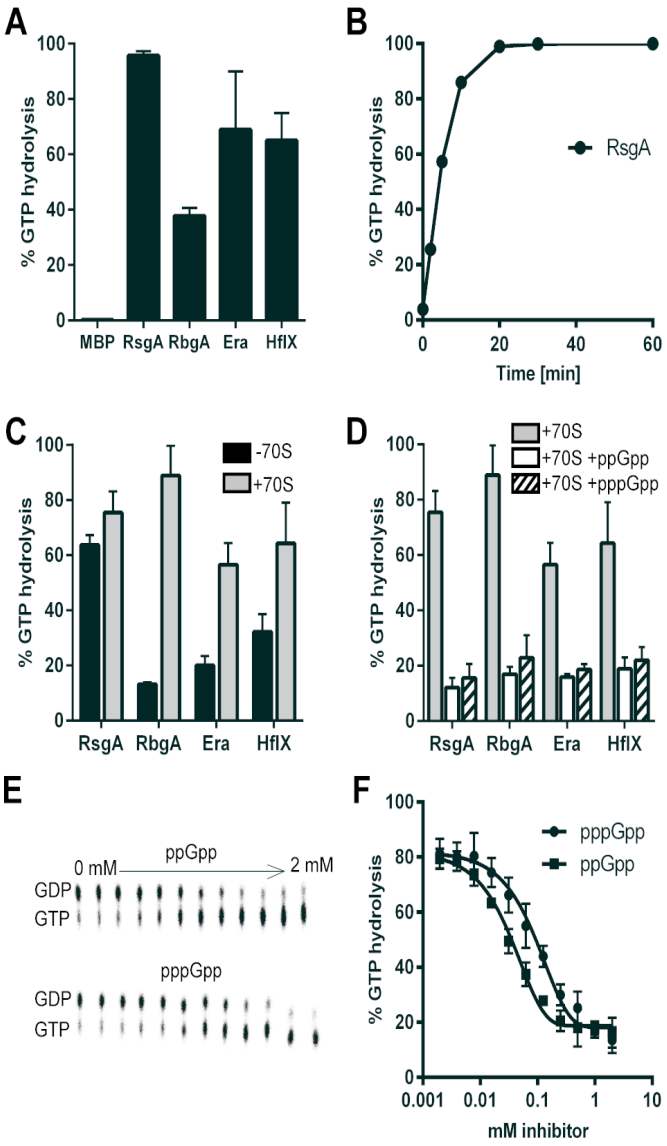

Fig. 2. GTPase activity assays in the presence or absence of ribosomes and (p)ppGpp. (*A*) The GTPase activity of recombinant RsgA, RbgA, Era and HflX were determined by incubating 10 μM protein with α-^{32}P-GTP overnight at 37°C. Hydrolysis was monitored by TLC and the % GDP formed was quantified using ImageJ and values plotted using GraphPad Prism. (*B*) The enzymatic activity of RsgA was monitored as above with samples withdrawn over a 1 h period. (*C*) Quantification of GTPase activity in the presence of 70S ribosomes. GTPase assays were set up as above in the absence or presence of 70S ribosomes. Reactions with RsgA were stopped after 10 min, while reactions with RbgA, Era and HflX were incubated for 60 min. (*D*) Analysis of GTPase activity of all four target proteins in the presence of (p)ppGpp. Hydrolysis in the presence of 70S ribosomes was monitored in the presence of either 1 mM ppGpp or pppGpp. Reactions with RsgA were stopped after 10 min, while reactions with RbgA, Era and HflX were incubated for 60 min. (*E*) The activity of RsgA was monitored in the presence of increasing concentrations of ppGpp and pppGpp. Reactions were stopped after 10 min and analyzed by TLC. (*F*) Quantification of the GTPase activity of RsgA in the presence of (p)ppGpp. The enzyme reactions were set up as in (*E*) and the % GDP formed quantified using ImageJ. The data were fitted using a dose response inhibition algorithm in GraphPad Prism with the corresponding IC50 value given. All experiments were performed in triplicate and averages and standard deviations were plotted using GraphPad Prism.

and few direct binding proteins have been identified. It has been established that the depletion of cellular GTP, the substrate for (p)ppGpp synthesizing enzymes, plays a significant role in initiating the stringent response in these organisms (27). Decreased GTP levels lead to a decrease in the transcription of mRNAs with a GTP initiating nucleotide, which in Gram-positive bacteria includes most rRNA promoters (26). Aside from substrate depletion, (p)ppGpp also actively inhibit GTP synthesis in *Bacillus subtilis* and *Enterococcus faecalis* by blocking the functions of HprT and Gmk, two enzymes involved in the GTP synthesis pathway (28-30). GTP levels are also important in some species for the activation of CodY, a global transcriptional regulator. In a GTP-bound state CodY binds to DNA and represses the transcription of a number of genes involved in the adaptation to nutrient limitation. However, upon entry of cells in stationary phase, GTP levels decrease leading to the release of CodY from DNA, derepression and transcription of target genes (31). Intracellular GTP levels do therefore play a significant role in modulating the stringent response. However, given the identification of multiple (p)ppGpp-binding proteins in *E. coli* it seems unlikely that GTP homeostasis is the sole regulatory function for (p)ppGpp in Gram-positive species.

In this study we used a genome-wide nucleotide-protein interaction screen to identify novel targets for the stringent response nucleotides (p)ppGpp in *S. aureus.* In addition to confirming that both HprT and Gmk from *S. aureus* can interact with these nucleotides, we demonstrate that (p)ppGpp bind with high affinity and specificity to five putative GTPases - RsgA, RbgA, Era, HflX and ObgE implicated in ribosome assembly. Characterization of RsgA, RbgA, Era, HflX revealed that their GTPase activity is increased in the presence of ribosomes but inhibited by the stringent response nucleotides. With this we identify a new mech-

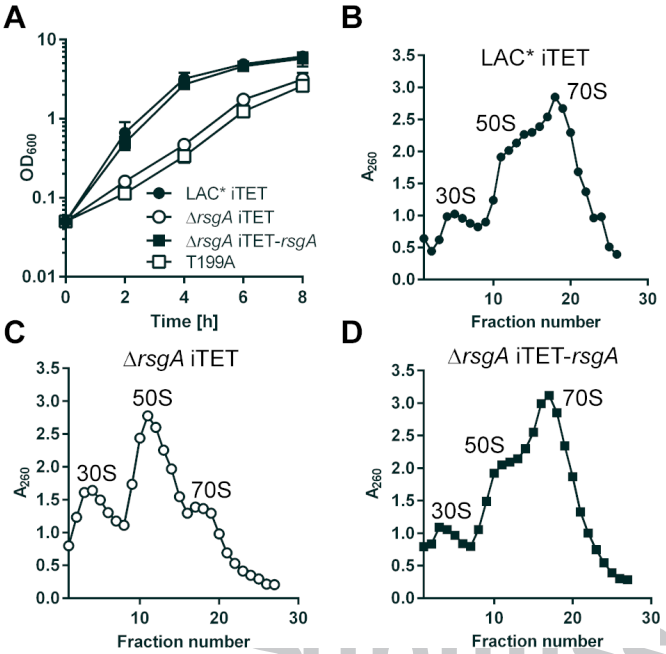

Fig. 3. Deletion of *rsgA* negatively affects the growth and ribosomal composition of *S. aureus*. (*A*) Growth of *S. aureus* strains LAC* iTET, LAC*Δ*rsgA* iTET, LAC*Δ*rsgA* iTET-*rsgA* and LAC*Δ*rsgA* iTET-*rsgA* T199A. Overnight cultures grown in the presence of 100 ng/ml Atet were diluted to an OD_{600} of 0.01 (time = 0 h) and grown in the presence of Atet for 8 h. Growth curves were performed 3 times and average OD_{600} readings and standard deviations plotted. (*B-D*) Effect of *rsgA* deletion on ribosomal profiles. Extracts from wild-type LAC* iTET (*B*), LAC*Δ*rsgA* iTET (*C*) and LAC*Δ*rsgA* iTET-*rsgA* (*D*) grown to exponential phase were fractionated by sucrose density gradient centrifugation. Gradients were fractionated by upwards displacement and analyzed for RNA content by measuring the absorbance at 260 nm. Experiments were performed in triplicate with one representative graph shown.

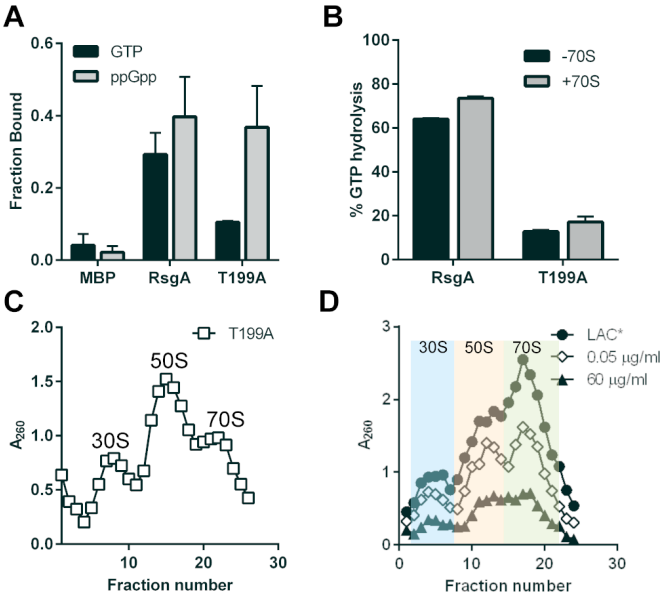

Fig. 4. GTPase activity of RsgA is crucial for its function. (*A*) DRaCALA with purified recombinant MBP, MBP-RsgA (RsgA) or MPB-RsgA-T199A (T199A) protein and ^{32}P-labeled GTP and ppGpp. All experiments were carried out in triplicate. The data were plotted using the GraphPad Prism software. (*B*) The GTPase activity of recombinant RsgA and the T199A variant were analyzed in the absence or presence of 70S ribosomes. Hydrolysis was monitored by TLC, the % GDP formed was quantified using ImageJ and values plotted using GraphPad Prism. (*C*) Effect of inactivation of GTPase activity on ribosomal profiles. Strain LAC*Δ*rsgA* iTET-*rsgA* T199A was fractionated by sucrose density gradient centrifugation and analyzed for RNA content by measuring the absorbance at 260 nm. (*D*) Effect of (p)ppGpp production on ribosomal profiles. The production of (p)ppGpp was induced by the addition of 0.05 μg/ml or 60 μg/ml mupirocin to exponentially grown cultures. 30 min post induction cells were harvested and extracts analyzed by sucrose density gradient centrifugation. Peaks corresponding to 70S, 50S and 30S are highlighted in green, orange and blue, respectively. Experiments were performed in triplicate with one representative graph shown.

anism by which the stringent response alarmones can control cell proliferation in Gram-positive bacteria at a post-transcriptional level by actively interfering with ribosome assembly to inhibit cell growth and promote antimicrobial tolerance.

Results

Identification of (p)ppGpp-binding proteins using a genome-wide nucleotide-protein interaction screen

Our previous work demonstrated the use of a differential radial capillary action of ligand assay (DRaCALA)-based ORFeome screen as a high-throughput platform for identifying interaction partners for c-di-AMP (32). In order to adapt this screen to identify (p)ppGpp binding proteins, radiolabeled (p)ppGpp was synthesized (Figs. S1A, S1B and S1C) and used in combination with an *S. aureus* protein expression library that contains 2,343 ORFs from the genome of the *S. aureus* strain COL (85.5% of the total number of ORFs in the genome) fused to a His-MBP-tag and expressed in *E. coli*. To perform the genome-wide screen, the *S. aureus* His-MBP protein expression library strains were grown up, protein expression induced and crude whole cell extracts prepared. These lysates were arrayed in a 96 well format and used in DRaCALA binding assays with a 1:1 mix of radiolabeled pppGpp:ppGpp (Fig. S1D). An average fraction bound for each plate was calculated as described by Roelofs *et al.* (33) and positive interactions were deemed as being 2.4-times greater than the background. This led to the identification of seven putative (p)ppGpp target proteins.

To interrogate the binding further, the plasmid from each of the seven strains was sequenced to confirm the identity of each gene and retransformed into *E. coli* cells. Protein expression was once again induced, whole cell lysates prepared and binding to both pppGpp and ppGpp examined (Fig. 1A). Fraction bound values higher than the empty vector negative control were observed for all seven strains, indicating positive binding interactions. Following this, all seven His-MBP-fused proteins were purified by Ni^{2+}-affinity and size exclusion chromatography (Fig. S1E) and the recombinant proteins used in DRaCALA binding assays with radiolabeled (p)ppGpp (Fig. 1B). Binding assays confirmed positive interactions for six of the target proteins, namely HprT, Gmk, RsgA, RbgA, Era and HflX. The binding to YqeH however, was very weak preventing a determination of binding affinity. For this reason, this protein was not investigated further.

(p)ppGpp bind specifically to HprT and Gmk from *S. aureus* to inhibit their function

Of the six identified (p)ppGpp-binding proteins, two have previously been shown to interact with these nucleotides, namely Gmk and HprT, two proteins involved in GTP synthesis. Gmk is the enzyme responsible for the conversion of GMP to GDP during *de novo* synthesis of GTP, whilst HprT is involved in the salvage pathway, converting both hypoxanthine to IMP and guanine to GMP. The activities of these enzymes from both *B. subtilis* and *E. faecalis,* as well as Gmk from *S. aureus* (Gmk_{SA}), have been shown to be inhibited in the presence of (p)ppGpp, thus lowering intracellular GTP levels to a range that supports survival during starvation (28-30).

Using the purified *S. aureus* Gmk and HprT proteins in DRaCALAs, we show here that both proteins have stronger affinities for ppGpp and pppGpp over GTP (Figs. S2A and S2B, Table S1).

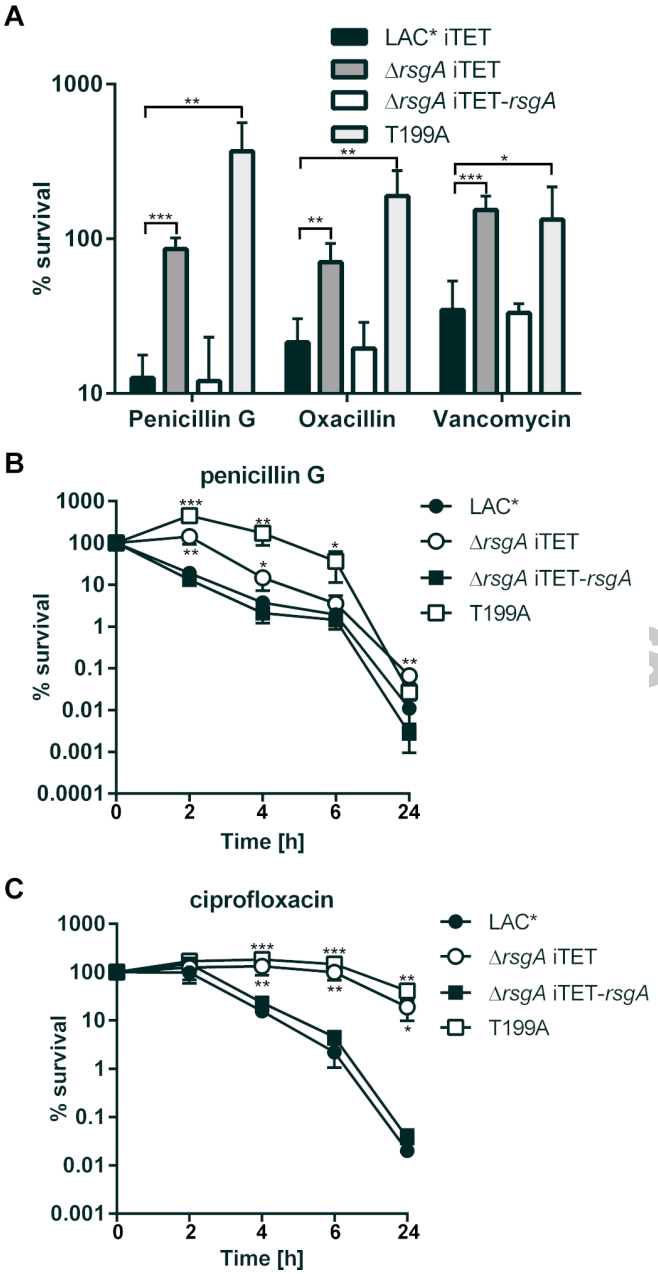

Fig. 5. Strains lacking RsgA, or producing an inactive GTPase variant, exhibit increased survival upon exposure to antimicrobials. (*A*) Exponentially growing LAC* iTET, LAC*Δ*rsgA* iTET, LAC*Δ*rsgA* iTET-*rsgA* and LAC*Δ*rsgA* iTET-*rsgA* T199A cells were exposed to 20 times the MIC of penicillin G, oxacillin and vancomycin. Percentage survival of the mutants and complemented strains after 3 h exposure was compared to that of the wild-type. % survival was calculated by dividing the number of cfu/ml after antibiotic treatment by the number of cfu/ml prior to addition of the antibiotics. Five independent experiments were performed with the averages and standard deviations shown. (*B and C*) Exponentially growing strains were exposed to 20 times the MIC of penicillin G (*B*) or ciprofloxacin (*C*). Percentage survival at the indicated time-points was calculated as for (*A*). Four independent experiments were performed with the averages and standard deviations shown. For statistical analysis, a two-tailed two sample equal variance Student's *t*-test was performed between LAC* iTET and LAC*Δ*rsgA* iTET or LAC*Δ*rsgA* iTET-*rsgA* T199A. Asterisks indicate statistically significant differences (*, P 0.05; **, P 0.01; ***, P 0.001).

Additionally, it was noted that these interactions are specific as only an excess of cold unlabeled ppGpp but not any of the other nucleotides tested could compete for binding with labeled ppGpp

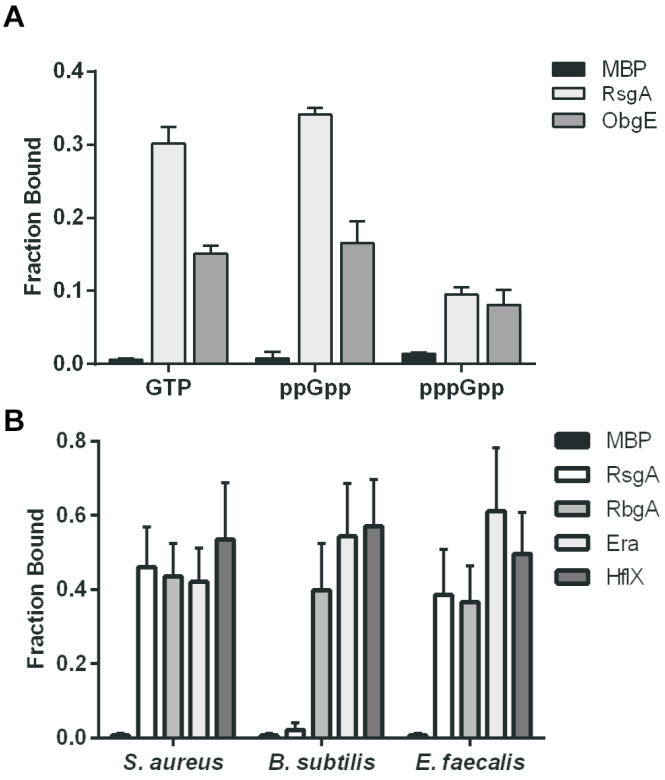

Fig. 6. (p)ppGpp bind GTPases from multiple Gram-positive species. DRaCALAs were performed with purified recombinant (*A*) MBP-tagged ObgE from *S. aureus* and the indicated ^{32}P-labeled nucleotides or (*B*) His-tagged RsgA, RbgA, Era and HflX proteins from *S. aureus*, *B. subtilis* and *E. faecalis* and ^{32}P-labeled ppGpp. All experiment were carried out in triplicate. The data were plotted using the GraphPad Prism software.

or pppGpp (Figs. S2C and S2D). To examine whether (p)ppGpp can directly inhibit the function of the staphylococcal HprT enzyme, and to confirm that Gmk_{SA} can be inhibited, the enzymatic activities of both proteins were monitored in the presence of both ppGpp and pppGpp. Enzymatic assays monitoring the conversion of guanine to GMP by HprT or GMP to GDP by Gmk were set up as previously described (28). This analysis revealed that the HprT and Gmk enzymes from *S. aureus* are inhibited by both ppGpp and pppGpp (Figs. S2E and S2F).

RsgA, RbgA, Era and HflX are putative GTPases involved in ribosomal biogenesis

Of the four remaining putative (p)ppGpp-binding proteins identified from the *S. aureus* strain COL Gateway Clone Set, RsgA is annotated as a hypothetical protein that has 33% identity over 89% of the protein to the *E. coli* ribosome small-subunit-dependent GTPase A. RbgA and HflX are described as putative GTP-binding proteins. RbgA, whilst not present in *E. coli* and other γ-proteobacteria, shows 55% identity over 94% of the protein to the ribosome biogenesis GTPase A from *B. subtilis* and HflX has 43% identity over 81% of the protein to the high frequency lysogenization locus X GTPase from *E. coli*. Finally, Era is annotated as a GTP-binding protein that has 40% identity over 97% of its length to the *E. coli* Ras-like protein from *E. coli*.

Little is known about the functions of these proteins in *S. aureus*. RsgA from *S. aureus*, *E. coli* and *B. subtilis* is a non-essential protein that is nonetheless important for normal growth (34-36), while both RbgA and Era are essential (37-42). Unlike eukaryotic GTPases that have roles in membrane signaling, members of this family of prokaryotic GTPases appear to have functions linked to ribosome assembly. In *E. coli* it has been demonstrated that Era and RsgA bind to the 30S subunit of the ribosome and are

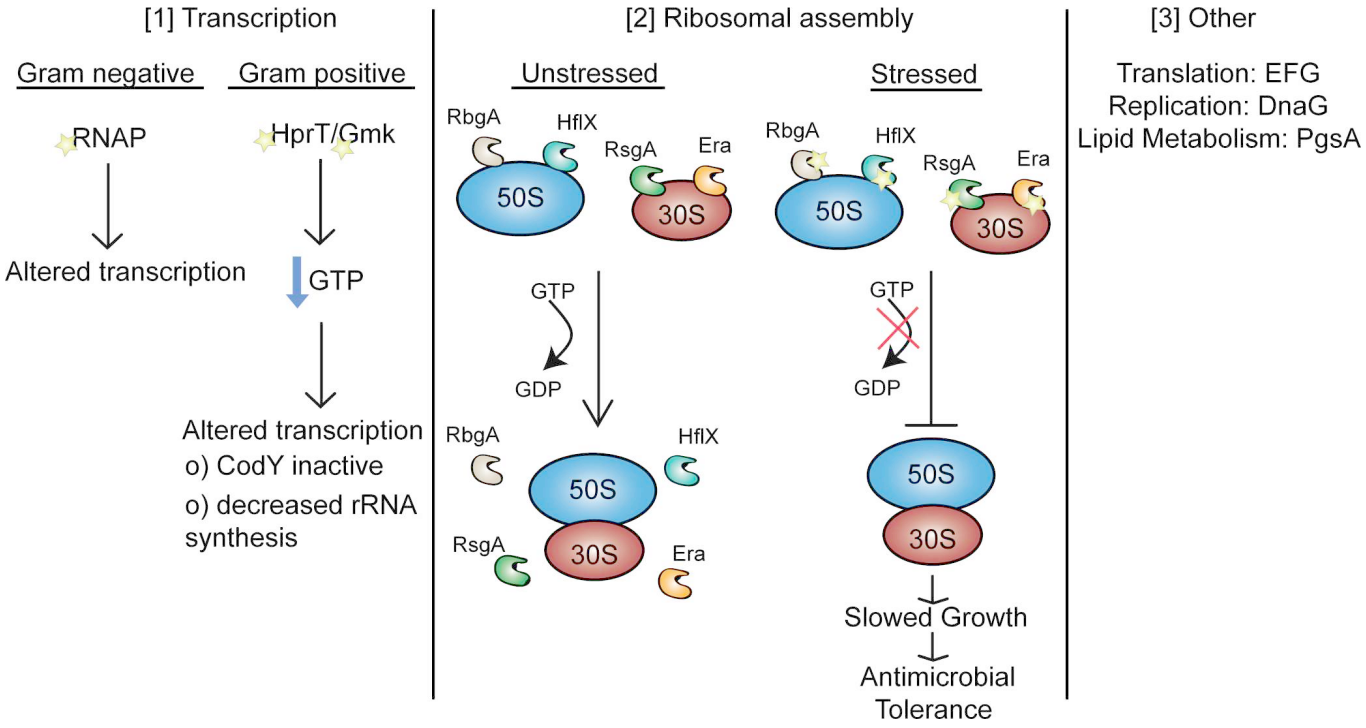

Fig. 7. Model depicting the functions of (p)ppGpp. Upon exposure to nutrient deprivation the bacteria respond by activating the stringent response. This response is controlled by two nucleotide messengers – ppGpp and pppGpp, which function to shut down active growth and promote survival. [1] Once synthesized these nucleotides can bind to the RNAP in Gram-negative bacteria leading to altered transcription and decreased growth. In Gram-positive bacteria these nucleotides instead bind to HprT and Gmk, two enzymes involved in the GTP synthesis pathway. Here they inactivate the functions of these enzymes, resulting in decreased intracellular levels of GTP. This in turn results in altered transcription of a number of genes, mediated in part by the GTP-regulated control of the transcriptional repressor CodY and also by a decrease in the availability of GTP as an initiating nucleotide for transcription. [2] (p)ppGpp can bind to bacterial GTPases. In unstressed cells these proteins associate with the ribosome and are thought to control the ribosome maturation processes leading to the formation of 70S ribosomes. In stressed cells the synthesis and binding of (p)ppGpp to these enzymes inhibits their GTPase activity, resulting in decreased 50S and 30S association and a reduction in the number of mature 70S ribosomes. This in turn slows growth, a consequence of which is an increase in the tolerance of bacterial cells to antimicrobials. [3] In addition to factors controlling transcription and ribosomal assembly, (p)ppGpp can also bind to proteins involved in translation, such as the elongation factors EFG and EF-Tu, replication, such as DnaG or lipid metabolism. Binding of (p)ppGpp to these proteins inhibits their function, again promoting a slower growth state.

critical for 30S ribosomal subunit biogenesis (36, 43, 44). Cryo-electron micrograph images of both proteins in complex with the 30S subunit suggest a chaperoning role, where they may prevent premature association of the 30S with the 50S subunit presumably until the 30S subunit has fully matured (44, 45). Indeed depletion of these proteins in bacterial cells leads to a decrease in 70S ribosomes with a buildup of 50S and 30S subunits (36, 43, 46). In contrast, both RbgA and HflX have been shown to bind to the 50S subunit and are required for its biogenesis, as cells depleted for RbgA show a reduction in 70S ribosomes, while free 50S subunits are completely missing (40, 42, 47, 48, 49). HflX has also been implicated as a ribosome-splitting factor, involved in rescuing stalled ribosomes during stress (50).

(p)ppGpp binds specifically to the four novel target proteins, RsgA, RbgA, Era and HflX

In order to determine binding kinetics and interaction specificities between (p)ppGpp and the four putative GTPases, DRaCALAs were performed with the purified proteins. Binding affinities in the low μM range were established for all the proteins and ppGpp (Fig. S3 and Table S1). With the exception of RsgA, the affinities of all four proteins to pppGpp and GTP were 4-16 times weaker, indicating that ppGpp may be a more potent effector in *S. aureus* than pppGpp (Fig. S3 and Table S1). RsgA, on the other hand, bound ppGpp and GTP with similar affinities, suggesting that the occupancy of the binding site with either ligand is going to depend heavily on the intracellular nucleotide concentration at any given time during the growth cycle. Additionally it was determined that the interactions between each of these proteins and ppGpp are specific as only an excess of cold unlabeled ppGpp, but not any of the other nucleotides tested, could completely compete for binding with labeled ppGpp (Fig. S4).

RsgA, RbgA, Era and HflX are GTPases, the activities of which are inhibited by (p)ppGpp

To examine whether these four proteins function as GTPases, the proteins were incubated with radiolabeled GTP and the hydrolysis to GDP monitored by thin layer chromatography (TLC). While the control protein MBP was unable to hydrolyze GTP even after overnight incubation, all four (p)ppGpp-binding proteins hydrolyzed GTP, however to varying degrees (Fig. 2A). As RsgA was able to fully hydrolyze GTP upon overnight incubation, a time-course was performed with the enzyme, revealing that full hydrolysis of GTP to GDP occurred within 20 min (Fig. 2B). Previous work on RsgA from *E. coli* reported that the activity of the protein is increased in the presence of ribosomes (36, 51). To determine the effect of ribosomes on the activity of all four GTPases, 70S ribosomes were purified from the community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strain LAC* and included in the GTP hydrolysis assays. While only a slight increase in the enzymatic activity was observed for RsgA, a dramatic increase in activity was noted for RbgA, Era and HflX (Fig. 2C), indicating that these proteins are indeed all intracellular GTPases, the activities of which are stimulated in the presence of the ribosome.

Next, to determine the effect of (p)ppGpp on the enzymatic function, hydrolysis assays were performed in the presence of

ribosomes and either 1 mM ppGpp or pppGpp. Interestingly, the hydrolysis activity of all four GTPases was significantly inhibited in the presence of either one of the stringent response nucleotides (Fig. 2D). To examine this in more detail the activity of RsgA in the presence of increasing amounts of ppGpp or pppGpp was monitored by TLC, revealing an IC50 of 56.8 ± 8.23 μM for ppGpp and 151 ± 18.9 μM for pppGpp (Figs. 2E and 2F). During stringent response activation, the levels of (p)ppGpp in the bacterial cell rise to 1-2 mM (28, 52), levels that are more than sufficient to inhibit the functions of these enzymes. Altogether, these data reveal that RsgA, RbgA, Era and HflX function as GTPases, the activities of which increase upon association with the ribosome and are inhibited upon interaction with (p)ppGpp. These data further suggest, that upon induction of the stringent response, where cellular levels of (p)ppGpp increase to 1-2 mM and levels of GTP fall, the activities of these enzymes are inhibited, which could affect the assembly of functional ribosomes.

The absence of RsgA, or the inhibition of its GTPase activity, reduces intracellular levels of 70S ribosomes and slows the growth of *S. aureus*

We next wished to examine the contribution of ribosomal GTPases to the growth and viability of *S. aureus.* Both *rbgA* and *era* are essential genes in this organism and so the construction of deletion mutants was not possible. RsgA and HflX, on the other hand, are encoded by non-essential genes and *S. aureus* strains with in-frame deletions in these genes were constructed in the CA-MRSA background strain LAC*. While no growth defect was observed under the conditions tested for the *hflX* mutant strain, the *rsgA* mutant strain grew significantly slower than the wild-type LAC* (Fig. 3A). This growth defect could be complemented fully by the introduction of a plasmid with the *rsgA* gene expressed under anhydrotetracycline-inducible control (Fig. 3A). As RsgA is reported to be involved in ensuring 30S subunit maturation prior to binding the 50S subunit (45), we sought to examine the effect of its absence on the ribosomal content in *S. aureus*. To this end the ribosomal profiles from extracts of the wild-type strain LAC*, the *rsgA* mutant and the complemented strain were evaluated by sucrose density gradient centrifugation (Figs. 3B, 3C and 3D). These profiles revealed that in the absence of RsgA cells contained reduced levels of intact 70S ribosomes, with a concomitant build-up of 50S and 30S subunits, which is in agreement with previous observations that RsgA has a role in ribosomal subunit association.

As reported above, binding of (p)ppGpp inhibits the GTPase activity of RsgA and as shown here a decrease in the number of mature ribosomes is observed in *S. aureus* in its absence. To determine whether inhibiting the GTPase activity of RsgA alone is sufficient to cause this phenotype the nucleotides encoding for a threonine residue at position 199 in the switch 1 region of the GTPase domain of RsgA were mutated to encode for an alanine in order to abolish GTPase activity. This protein variant should have reduced GTPase activity and hence mimic a protein in which the GTPase activity has been inhibited by (p)ppGpp. This variant was expressed and purified from *E. coli* cells. Nucleotide binding assays showed that ppGpp could still interact with this protein variant, indicating that it is not essential for ppGpp binding, however GTP binding was, as expected, severely diminished (Fig. 4A). In agreement with the decrease in GTP binding, the GTPase activity of the protein was drastically reduced, even in the presence of ribosomes (Fig. 4B). This *rsgA* T199A allele was then also introduced on a complementing plasmid into the *rsgA* mutant strain LAC*Δ*rsgA*, creating strain LAC*Δ*rsgA* iTET-*rsgA* T199A. Monitoring the growth of this strain revealed a significant defect, similar to that of the *rsgA* mutant, confirming that inactivation of the GTPase function of this protein results in a slower growth phenotype (Fig. 3A). Next the ribosomal profile for strain LAC*Δ*rsgA* iTET-*rsgA* T199A was determined by sucrose density gradient centrifugation, revealing that there is, similar to the *rsgA* mutant, a reduction in the amount of mature 70S ribosomes (Fig. 4C). Together these results suggest that in the absence of RsgA, or upon inactivation of its GTPase activity, the maturation of ribosomes is severely affected.

It is known that induction of the stringent response causes a reduction in the overall quantity of ribosomes being produced due to ppGpp-mediated decreases in rRNA transcripts (4, 53). Our previous observations led us to suspect that upon synthesis of (p)ppGpp this nucleotide would also bind to the four ribosomal GTPases and inhibit their activity resulting in a decrease in intact 70S ribosomes. To examine what effect (p)ppGpp synthesis has on the ribosomal profile of wild-type staphylococcal cells, the synthesis of (p)ppGpp was triggered by the addition of a low (0.05 μg/ml) or a high (60 μg/ml) dose of mupirocin for 30 min, conditions known to induce the stringent response, and extracts were analyzed by sucrose density gradient centrifugation. As expected the overall level of ribosomes in the cell was decreased compared to wild-type (Fig. 4D). In addition, the ratio of intact 70S to 50S and 30S subunits altered from 1/0.59/0.29 for the wild-type to 1/0.83/0.44 with the addition of 0.05 μg/ml mupirocin and to 1/0.92/0.36 in the presence of high levels of mupirocin, revealing that the levels of 70S ribosomes were indeed decreased after induction of the stringent response compared to untreated cells grown in the absence of mupirocin (Fig. 4D). Altogether these data lead us to propose a role for (p)ppGpp in binding to intracellular GTPases in order to inhibit ribosomal assembly and promote slow growth.

Inhibition of GTPase activity leads to increased tolerance to antimicrobials

It has been reported that bacterial cultures naturally contain subpopulations of slower growing cells that are associated with persistence and tolerance to antimicrobials (9, 54, 55). In Gram-negative bacteria such as *E. coli* this persistence phenotype has been linked to intracellular (p)ppGpp levels, where high levels of (p)ppGpp activate toxin-antitoxin systems leading to a reduced bacterial growth rate (9). To investigate whether the slower growth phenotype observed in this study as a result of the inactivation of GTPase activity also results in tolerance to antimicrobials, exponentially growing cells of LAC* iTET, LAC*Δ*rsgA* iTET, LAC*Δ*rsgA* iTET-*rsgA* and LAC*Δ*rsgA* iTET-*rsgA* T199A were firstly exposed to three bactericidal antimicrobials, namely the penicillins penicillin G and oxacillin, and the glycopeptide vancomycin (Fig. 5A). Both the Δ*rsgA* mutant and the Δ*rsgA* iTET-*rsgA* T199A strain expressing the inactive GTPase variant, showed increased survival against all three antimicrobials compared to the wild-type, with the introduction of a plasmid expressing the *rsgA* gene restoring susceptibility to wild-type levels (Fig. 5A). To examine this in more detail the strains were exposed to both penicillin G and the fluoroquinolone ciprofloxacin and cfu counts determined over a 24 h period (Fig. 5B and C). Exposure to penicillin G revealed statistically significant differences between the wild-type and mutant strains at the earlier time-points, which became less dramatic over time (Fig. 5B). Incubation of strains with ciprofloxacin showed a highly significant increase in survival for the mutant strains that was still clearly observable after 24 h (Fig. 5C). Together these data indicate that GTPase inactivation leads to an increase in bacterial survival upon exposure to a number of different types of antimicrobials.

GTPases from a number of Gram-positive species are also targets of the stringent response alarmones

RsgA, RbgA, HflX and Era are enzymes belonging to the Era/Obg subfamily of GTPases. ObgE from *E. coli* has been shown to interact with (p)ppGpp (56). To examine whether the homologue of this protein from *S. aureus* also interacts with these nucleotides, the *S. aureus* ObgE protein was purified and binding to (p)ppGpp determined by DRaCALA (Fig. 6A). This revealed

a positive interaction that was somewhat weaker than the binding of ppGpp to RsgA. This weaker affinity is potentially why the protein was not identified as an interacting partner using the whole cell lysate screen.

In order to determine whether the binding of (p)ppGpp to the GTPases identified in this work is specific for *Staphylococcus* or is a more general regulatory mechanism used by a number of Gram-positive species, the *rsgA, rbgA, era* and *hflX* genes from *B. subtilis* and *E. faecalis*, as well as *S. aureus* as a control, were amplified and cloned in a vector allowing the expression of the respective proteins as N-terminal His-tagged fusion proteins. All proteins were subsequently expressed in *E. coli* and purified by Ni^{2+}-affinity chromatography. DRaCALA binding assays with radiolabeled ppGpp revealed a positive interaction between the nucleotide and all *S. aureus* and *E. faecalis* His-tagged proteins (Fig. 6B). Of the *B. subtilis* homologues, all with the exception of $RsgA_{BS}$ showed strong binding (Fig. 6B). RsgA from *S. aureus* and *B. subtilis* share 45% identity but must have amino acid differences at the binding site for ppGpp that prevent binding. Altogether these data suggest that the binding and inhibition of bacterial GTPases upon induction of the stringent response is likely a ubiquitous process in Gram-positive bacteria.

Discussion

Upon detection of an environmental stress, bacteria utilize the nucleotides (p)ppGpp to mediate a complex and multipronged approach leading to cells rapidly shutting down growth and entering a persistent state that promotes drug tolerance. The work presented here demonstrates the use of a genome-wide nucleotide-protein interaction screen to systematically identify target proteins for (p)ppGpp in order to unravel the mechanisms behind this process. As expected this screen identified two previously known target proteins for these nucleotides, HprT and Gmk, providing validation for the screening technique. In addition we have identified four previously uncharacterized *S. aureus* GTPases as binding partners.

GTPases are a superfamily of ubiquitous enzymes with roles in signal transduction, cell division and protein translation. This superfamily consists of several subfamilies, grouped based on identity and function, which include the translation elongation factor subfamily, the FtsY/Ffh subfamily, the Era subfamily and the Obg subfamily. The translation elongation-factor group contains a number of proteins, the functions of which have been demonstrated to be inhibited by (p)ppGpp. For instance (p)ppGpp can inhibit the activities of the elongation factors EFG and EF-Tu (57, 58), as well as the initiation factor IF2 (59), which power the translocation of the ribosome during protein synthesis, the binding of new aminoacyl tRNAs to the ribosome and the formation of the initiation complex, respectively. Several bacterial GTPases of the Era/Obg subfamilies, into which RsgA, RbgA, HflX and Era group, are known to function in ribosomal assembly, more specifically in the maturation of the individual 50S and 30S ribosomal subunits prior to mature 70S formation. Only one of these proteins, ObgE from *E. coli*, is known to interact with (p)ppGpp (56). ObgE has been implicated in DNA replication (60) and has also been shown to bind to the Gram-negative (p)ppGpp synthetase/hydrolase enzyme SpoT from *E. coli* (61). Similar to RsgA, RbgA, HflX and Era, it has recently been shown that this protein also has a role in 50S and 30S ribosomal subunit association and that (p)ppGpp binding to this protein can enhance the association of ObgE with the 50S subunit of the ribosome (59).

In the present work we identify four putative GTPases in *S. aureus* that have the ability to bind specifically and with high affinity to both ppGpp and pppGpp. Enzymatic analysis reveals that these enzymes are all active GTPases, the activities of which are enhanced in the presence of ribosomes but are inhibited when they are bound to (p)ppGpp. The identification of these new target proteins allows us to propose an additional mechanism by which cells undergoing stress can utilize (p)ppGpp in order to rapidly shut down growth, namely by preventing the assembly of 70S ribosomes (Fig. 7).

The stringent response alarmones help bacteria to adjust their growth to stress conditions in a number of different ways (Fig. 7): [Method 1] In the α, β and γ-proteobacteria, (p)ppGpp bind to the RNAP and in conjunction with the transcription factor DksA, alter the transcription of approximately one third of the genome (3, 14, 62). Due to alterations in amino acid sequences that render (p)ppGpp unable to bind, the RNAP is not a target for (p)ppGpp in the Firmicutes, the Actinobacteria or Deinococcus-Thermus genera (26, 63). Instead (p)ppGpp regulate transcription by binding to HprT and Gmk, enzymes involved in the GTP synthesis pathway (28, 30). These nucleotides are able to bind with high affinity and specificity to both of these enzymes, resulting in an inhibition of enzymatic function (Fig. S2 and Table S1)(28). This inhibition results in a decrease in cellular GTP levels triggering a de-repression of the transcriptional regulator CodY, as well as inhibiting the transcription of many rRNA genes due to the lack of availability of GTP as an initiating nucleotide (27, 28). Of note is that while Gmk is present in Gram-negative species, this protein is not able to bind (p)ppGpp due to conformational changes in the nucleotide-binding pocket, suggesting that the regulation of GTP levels in this way may be unique to Gram-positive organisms (30); [Method 2] ppGpp can interact with GTPases involved in ribosomal assembly to inhibit the association of the 50S and 30S subunits, as now shown in this study. In normal unstressed cells RbgA and HflX bind to the 50S subunit (40, 42, 48, 49), while both RsgA and Era bind to the 30S subunit and interact with the 16S rRNA (35, 36, 43, 44). There is evidence to suggest that this occurs while the proteins are in the GTP-bound state, as for RsgA, RbgA and ObgE, the inhibition of GTPase activity by the binding of non-hydrolysable analogue of GTP causes increased association of the protein to ribosomal subunits (36, 45, 64). Here the proteins are thought to have a caretaking or checkpoint role where they could function to facilitate proper RNA folding or processing or could promote correct protein-protein or protein-RNA interactions. Support for this conclusion comes from the observation that a *B. subtilis* strain depleted for RbgA shows an increase in immature 50S subunits, caused by the incorrect incorporation of the ribosomal protein L6 prior to the binding of other late assembly proteins (42). Additionally it has been shown that deletions of both *era* and *rsgA* results in an accumulation of immature 17S RNA, a precursor of 16S RNA (36, 44), with cryo-EM images suggest a chaperoning role in processing the 3' end of rRNA (44, 45). Furthermore the position at which the RsgA and Era proteins bind to the 30S subunit, as revealed in cryo-EM studies, likely prevents the formation of a complex with the 50S subunit while they are bound (44, 45). Upon the sensing of an as yet unknown signal these proteins are then released from the 50S and 30S subunits by GTP hydrolysis, allowing the now mature subunits to interact and form 70S ribosomes. In this way these GTPases control ribosome assembly and so protein synthesis. In strains where these proteins are absent it is likely that the subunits fail to successfully mature, seriously affecting association and mature 70S formation (36, 43).

Once (p)ppGpp is present in the cell, we show that these nucleotides can interact with high affinity with RsgA, RbgA, Era and HflX (Fig. S3, S4 and Table S1) and efficiently inhibit their GTPase activity (Fig. 2). In an *rsgA* mutant strain, the lack of GTPase activity results in a decrease in ribosomal subunit association, resulting in fewer mature 70S ribosomes (Fig. 3 and 4) (36). The decrease in 70S ribosomes would lead to a stall in protein production, which could explain the observed slower growth phenotype seen for the *rsgA* mutants in the absence of

GTPase activity (Fig. 3A), as well as the increase in antimicrobial tolerance (Fig. 5). Further investigation is needed to conclusively say if *rbgA*, *era* or *hflX* mutant strains behave in a similar fashion; [Method 3] In addition to transcription and ribosomal assembly these nucleotides can also bind to a number of other targets such as the *E. coli* proteins PlsB and PgsA to shut down lipid metabolism (65), to DnaG from both Gram-negative and Gram-positive bacteria to inhibit DNA replication (66) or the elongation factor GTPases from *E. coli* to inhibit protein translation (57, 58).

Together these modes of growth inhibition combine to ensure a rapid shut down in bacterial growth. While the exact biochemical mechanism by which (p)ppGpp can inhibit GTPase activity has not yet been fully elucidated, the data presented here clearly point to the control of ribosomal assembly as a potent contributor to bacterial stress survival.

Methods

Bacterial strains and culture conditions. *E. coli* strains were grown in LB or LB-M9 (67) and *S. aureus* strains in TSB at 37°C with aeration. Strains and primers used are listed in Tables S2 and S3. The *S. aureus* (MRSA), Strain COL Gateway® Clone Set, Recombinant in *Escherichia coli*, Plates 1-25, NR-19277 was obtained through BEI Resources, NIAID, NIH. Information on strain construction is provided in SI Methods.

Protein purifications. Proteins were purified from 1-2 L *E. coli* cultures. Cultures were grown to an OD_{600} 0.5-0.7, protein expression induced with 1 mM IPTG and incubated overnight at 16°C. Protein purifications were performed by nickel affinity and size exclusion chromatography as previously described (68, 69). Protein concentrations were determined by A_{280} readings.

Construction of the *S. aureus* ORFeome expression library. 2,343 *E. coli* strains containing pDONR221 vectors with *S. aureus* strain COL ORFs (BEI Resources, NIAID, NIH) were grown in 1.5 ml LB-M9 in 2 ml 96-well deep dishes selecting for kanamycin resistance. The cultures were centrifuged and the plasmids extracted using 96-well MultiScreen$_{HTS}$ PLASMID plates (Millipore). The *S. aureus* gateway ORFeome library was shuttled from the pDONR221 entry plasmids into the protein overexpression destination vector pVL847-GW using LR clonase enzyme II as per manufacturer's guidelines (Invitrogen). Subsequently, the destination plasmid library was introduced into *E. coli* strain T7IQ selecting for gentamicin resistance.

Preparation of *E. coli* whole cell lysates. Protein expression strains were grown in LB-M9 medium overnight at 30°C and protein induction subsequently induced for 6 h with 1 mM IPTG. Bacteria were collected by centrifugation and suspended in 1/10th of their original volume in 40 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ binding buffer containing 2 mM PMSF, 20 µg/ml DNase and 0.5 mg/ml lysozyme. Cells were lysed by 3 freeze/thaw cycles. Lysates were directly used in binding assays or stored at -20°C.

Differential radial capillary action of ligand assay (DRaCALA). This assay was performed as described previously with slight modifications as outlined in SI Methods (32, 33).

Synthesis of (p)ppGpp. ^{32}P-labeled pppGpp was synthesized from α-^{32}P-GTP (Perkin Elmer) by incubating 55.5 nM α-^{32}P-GTP with 2 µM Rel*seq* protein in 25 mM Bis-Tris propane pH 9, 100 mM NaCl, 15 mM $MgCl_2$ binding buffer, using 8 mM ATP as the phosphate donor, at 37°C for 1 h. The Rel*seq* protein was separated from the radiolabeled pppGpp by filtration on 3 kDa cut-off spin column. To synthesize ^{32}P-ppGpp, the ^{32}P-pppGpp was incubated with 1 µM of the phosphatase GppA for 15 min at 37°C. The GppA protein was separated from the radiolabeled ppGpp by filtration on 3 kDa cut-off spin column. Reaction products were visualized by spotting 1 µl on PEI-cellulose F TLC plates (Merck Millipore) and separation in 1.5 M KH_2PO_4, pH 3.6. The radioactive spots were visualized using an LA 7000 Typhoon PhosphorImager. Unlabeled (p)ppGpp was synthesized in the same way but with the addition of 6 mM GTP instead of the 55.5 nM α-^{32}P-GTP. Spiking a duplicate reaction with radiolabeled GTP confirmed complete conversion of GTP to (p)ppGpp.

GTP hydrolysis assays. The ability of proteins to hydrolyze GTP to GDP was determined by incubating 10 µM recombinant protein with 2.78 nM α-^{32}P-GTP in 40 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ at 37°C for the indicated times. Ribosomes at a final concentration of 118 nM and increasing concentrations of ppGpp or pppGpp were added to the initial mixture where indicated. The reactions were inactivated with the addition of formic acid to a final concentration of 1.2 M. Precipitated proteins were pelleted by centrifugation at 17,000 x g for 10 min. Reaction products were then visualized by spotting 1 µl on PEI-cellulose F TLC plates (Merck Millipore) followed by separation in 1 M KH_2PO_4, pH 3.6 buffer. The radioactive spots were visualized using an LA 7000 Typhoon PhosphorImager and images quantified using ImageJ.

Enzymatic assays. Gmk and HprT activity assays were performed as previously described and are outlined in the SI Methods (28).

70S ribosome purification. 70S ribosomes were purified as detailed by Daigle and Brown with the following exceptions (51): Ribosomes were purified from 4 L of the *S. aureus* strain LAC* grown in TSB medium. The *S. aureus* culture was grown to an OD_{600} of 0.8 before the addition of 100 µg/ml chloramphenicol. Following a 3 min incubation at 37°C cultures were allowed to cool to 4°C before centrifugation. Cells were suspended in buffer A (20 mM Tris-HCl pH 7.5, 10.5 mM magnesium acetate, 100 mM NH_4Cl, 0.5 mM EDTA, 3 mM 2-mercaptoethanol) and lysed with 0.2 µg/ml lysostaphin and 75 ng/ml DNase for 30 min at 37°C. Lysates were centrifuged at 30,000 x g for 1 h and the protocol continued as per Daigle and Brown (51).

Ribosomal profiles from *S. aureus* cell extracts. Crude isolations of ribosomes from *S. aureus* cell extracts were achieved as described by Uicker *et al* with some modifications (42). Briefly, 150 ml cultures of the different *S. aureus* strains were grown to an OD_{600} of 0.6 in TSB 100 ng/ml Atet. For induction of the stringent response mupirocin was added to cultures 30 min prior to harvesting. Cultures were allowed to cool to 4°C before centrifugation. The cells were suspended in lysis buffer (80 mM Tris-HCl pH 7.8, 7 mM magnesium acetate, 150 mM NH_4Cl and 2.5 mM DTT), normalized to an OD_{600} of 25, lysed by the addition of 0.2 µg/ml lysostaphin and 75 ng/ml DNase and incubated for 30 min at 37°C. The extracts were centrifuged at 17,000 x g for 5 min and subsequently 500 µl were layered onto 10-25% sucrose density gradients in 10 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$ and 300 mM KCl. Gradients were centrifuged for 3.5 h at 210,000 x g. Gradients were fractionated by upwards displacement of 250 µl aliquots, which were analyzed for RNA content at an absorbance of 260 nm.

Antimicrobial tolerance assay. Overnight cultures of *S. aureus* strains in TSB containing 100 ng/ml Atet were diluted to an OD_{600} of 0.05 and grown until an OD_{600} of 0.4 was reached. 1.5 ml aliquots were then incubated with 20 times the MIC value for vancomycin (40 µg/ml), oxacillin (1.28 mg/ml), penicillin G (20 µg/ml) or ciprofloxacin (320 µg/ml) as previously determined by e-test strips. Aliquots were further incubated at 37°C for the times indicated. CFU counts were determined by removing 500 µl samples, centrifuging and suspending cells in fresh medium. The cells were subsequently serially diluted and plated. % survival was calculated by dividing the number of cfu/ml after antibiotic treatment by the number of cfu/ml prior to addition of the antibiotics.

Footnote. Author contributions: R.M.C. and A.G. designed research; R.M.C., L.E.B., and A.W. performed research; R.M.C., L.E.B., A.W., and A.G. analyzed data; and R.M.C. and A.G. wrote the paper.

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