Negative allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-stranded RNA

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The alarmone nucleotides guanosine pentaphosphate (pppGpp) and tetraphosphate (ppGpp), collectively referred to as (p)ppGpp, are key regulators of bacterial growth, stress adaptation, pathogenicity, and antibiotic tolerance. We show that the tetrameric small alarmone synthetase (SAS) RelQ from the Gram-positive pathogen *Enterococcus faecalis* is a sequence-specific RNA-binding protein. RelQ's enzymatic and RNA binding activities are subject to intricate allosteric regulation. (p)ppGpp synthesis is potently inhibited by the binding of single-stranded RNA. Conversely, RelQ's enzymatic activity destabilizes the RelQ:RNA complex. pppGpp, an allosteric activator of the enzyme, counteracts the effect of RNA. Tetramerization of RelQ is essential for this regulatory mechanism, because both RNA binding and enzymatic activity are abolished by deletion of the SAS-specific C-terminal helix 5α. The interplay of pppGpp binding, (p)ppGpp synthesis, and RNA binding unites two archetypal regulatory paradigms within a single protein. The mechanism is likely a prevalent but previously unappreciated regulatory switch used by the widely distributed bacterial SAS enzymes.

stringent response | (p)ppGpp | RNA–protein interaction | allosteric regulation | nucleotide signaling

The alarmone nucleotides guanosine pentaphosphate and tetraphosphate, collectively referred to as (p)ppGpp, are key regulators of bacterial growth, stress adaptation, pathogenicity, and antibiotic tolerance (reviewed in refs. 1–3). In *Escherichia coli*, (p)ppGpp signaling is orchestrated by two large multidomain proteins, RelA and SpoT, the namesakes of the RelA/SpoT homolog (RSH) protein family (4). Both RelA (5) and SpoT (6) synthesize (p)ppGpp using either GDP or GTP as substrates and ATP as a donor of the pyrophosphate moiety. SpoT, but not RelA, also can hydrolyze pppGpp and ppGpp, yielding GTP and GDP, respectively (7). The enzymatic activities of the two *E. coli* RSH enzymes are regulated allosterically. Synthesis of (p)ppGpp by RelA is strongly induced on amino acid limitation by so-called "starved" ribosomal complexes loaded with cognate deacylated tRNA in the A-site (8), and RelA activation is further potentiated by the product of the reaction, ppGpp (9). SpoT has both (p)ppGpp synthesis and hydrolysis activities and is regulated by numerous stress signals, including fatty acid (10), iron (11), and carbon source (6) limitations.

In the last decade, the repertoire of RSH enzymes has been expanded by the discovery of small, single-domain, monofunctional enzymes that either synthesize [small alarmone synthetases (SASs)] (12–14) or hydrolyze [small alarmone hydrolases (SAHs)] (15, 16) (p)ppGpp. Bacterial SAHs are largely uncharted territory, with our knowledge of these enzymes limited to mapping their phylogenetic distribution across the tree of life (16). The biological role and regulation of SAS enzymes are better understood. In contrast to allosterically regulated RelA and SpoT, induction of (p)ppGpp production by SASs in response to cell wall stress stimuli, such as alkaline shock or treatment with cell wall-active antibiotics, is believed to be effectuated chiefly via transcriptional up-regulation, leading to an increase in the enzyme's abundance (13, 17). The consequent increase in the (p)ppGpp level in turn renders bacteria more resilient to the signal that is inducing stress, e.g., tolerance to antibiotics targeting the cell wall (14, 17). Crystallographic analysis of the *Bacillus subtilis* RelQ (SAS1) revealed that it forms a tetramer that binds two pppGpp molecules at the interface between subunits, leading to an allosteric activation of the enzyme's catalytic activity (18). Activation by both ppGpp and pppGpp has been reported for RelQ from *Enterococcus faecalis* (19).

Using biochemical assays with *E. faecalis* SAS RelQ, we have discovered an unexpected regulatory interplay among (p)ppGpp binding, (p)ppGpp synthesis, and inhibition of the enzymatic activity by single-stranded RNA. This constitutes an example of two archetypical regulatory paradigms combined within a single protein—namely, an RNA-binding activity and a switch in catalytic activity in response to a second messenger. This provides insight into a previously unknown function of RelQ that is likely to be relevant for many other bacterial SAS enzymes.

Significance

Bacteria regulate their intracellular environment through two ubiquitous posttranscriptional regulatory mechanisms: enzymatic synthesis of small signaling molecules that allosterically regulate protein targets, and complex formation of RNA-binding proteins with target RNAs. We show that these two mechanisms can be combined within a single protein. The small alarmone synthetase RelQ from the Gram-positive pathogen *Enterococcus faecalis* enzymatically regulates the levels of alarmone nucleotide (p)ppGpp, a key regulator of stress adaptation, pathogenicity, and antibiotic tolerance. In its other role as an RNA-binding protein, RelQ interacts with single-stranded RNA in a sequence-specific manner. Because (p)ppGpp synthesis and pppGpp binding are mutually incompatible with RelQ:RNA complex formation, the RelQ:RNA interaction acts as a regulatory switch between inactive and active forms of the enzyme.

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Results

Enzymatic Activity of *E. faecalis* RelQ Is Inhibited by mRNA, and ppGpp Counteracts the Inhibition. As we have shown previously (19), the enzymatic activity of *E. faecalis* RelQ is insensitive to the addition of *E. coli* 70S ribosomes (Fig. 1*A*). This is unsurprising given that, unlike *E. coli* RelA (9), SAS RSH enzymes are not expected to interact with—or to be regulated by—ribosomes, because they lack the C-terminal domains mediating this interaction in long RSHs (20–22).

Unexpectedly, when we added the ultimate activator of *E. coli* RelA—namely "starved" ribosomal complexes assembled from 70S ribosomes, model mRNA(MF) coding for MF dipeptide, deacylated $tRNA^{Met}$ and $tRNA^{Phe}$—to RelQ, its synthetic activity was almost abolished (Fig. 1*A*). Because long ribosome-dependent RSHs interact directly with deacylated tRNA (21, 22), we tested whether the deacylated $tRNA^{Phe}$ is responsible for this inhibition. We found that $tRNA^{Phe}$ had little effect on RelQ in either the presence or absence of ppGpp. Because neither 70S ribosomes nor deacylated tRNA inhibit RelQ, via a process of elimination we concluded that the mRNA(MF) is responsible for RelQ inhibition by starved ribosomal complexes. Further experiments supported this conclusion. In the absence of externally added ppGpp, addition of 1 μM model mRNA(MF) abolished ppGpp synthesis by RelQ, whereas in the presence of 100 μM ppGpp, mRNA(MF) decreased the turnover rate of RelQ by only approximately sixfold, from 12.4 ± 0.6 to 1.5 ± 0.2 ppGpp molecules per RelQ per minute.

One possible explanation for this finding is that the mRNA nonspecifically binds and inhibits RSH enzymes. However, the addition of up to 10 μM mRNA(MF) had no effect on *E. coli* RelA activated by ribosomal complexes (Fig. S1), demonstrating that the inhibitory effect of mRNA(MF) is specific to SAS RelQ to the exclusion of the ribosome-associated RSH RelA.

Single-Stranded RNA Potently Inhibits RelQ in a Sequence-Specific Manner. We next investigated the specificity of RelQ inhibition by nucleic acids (Fig. 1 *B* and *C*). We characterized the effects of single-stranded (empty circles) and double-stranded (filled circles) RNA as well as the corresponding DNA in both the presence and absence of externally added 100 μM ppGpp (red and black traces, respectively). The oligonucleotides had a sequence identical to that of either model mRNA(MF) or its antisense. Single-stranded mRNA(MF) was found to be a very potent inhibitor of RelQ in the absence of externally added ppGpp; 150 nM mRNA virtually abolished ppGpp synthesis by 250 nM RelQ (i.e., 62.5 nM tetrameric RelQ) (Fig. 1*B*, empty black circles). The addition of 100 μM ppGpp mitigated this inhibition, but did not relieve it completely; at 1 μM, mRNA(MF) still inhibited RelQ activity by approximately fivefold (Fig. 1*B*, empty red circles). The inhibitory effect exhibited a pronounced sequence-specificity; single-stranded RNA with a complementary sequence—the antisense mRNA—was a considerably weaker inhibitor, despite having the same GC content (Fig. 1*C*). Double-stranded RNA was virtually inactive in both the presence and absence of ppGpp (Fig. 1*B*, filled red and black circles, respectively). Likewise, single- and double-stranded DNA were poor inhibitors; in the presence of 100 μM ppGpp, 2 μM single-stranded DNA had virtually no inhibitory effect on RelQ, and in the absence of ppGpp, it inhibited RelQ activity by approximately fivefold (Fig. S2*A*). Long-chain polyphosphate demonstrated no inhibitory effect when added in concentrations of up to 2 mM (Fig. S3).

Using the 24-nt-long inhibitory mRNA(MF) and its ineffective complementary antisense RNA as a starting point, we set out to define the sequence specificity for RelQ inhibition. By swapping the 5′ and 3′ halves of the two RNA molecules, we identified the 5′ half of the mRNA(MF) spanning the Shine–Dalgarno sequence AGGAGG as an essential element for the inhibitory activity (Fig. 2*A*). We then tested a series of 5′ and 3′ truncations of mRNA(MF) (Fig. 2*B*). The absence of six or nine 3′ terminal nucleotides (RNA 5 and 6) did not affect the inhibitory activity of the RNA, but the lack of an additional three nucleotides—which shortens the mRNA to its 5′ half (RNA 8)—significantly reduced the inhibitory effect. Because replacement of the 3′ AAA by UUU did not abrogate inhibition (RNA 7), we conclude that the loss of activity of RNA 8 is not due to the loss of a specific sequence element, but rather indicates the existence of a minimum length requirement between 12 and 15 nucleotides. Similar to the 5′ half (RNA 8), the 3′ half was also inactive (RNA 11); however, the addition of an extra six nucleotides at the 5′ (GAGGUA) nearly restored the activity (RNA 10).

Given our results suggesting that the activity is localized to the 5′ half of the mRNA(MF), we performed mutational studies (Fig. 2*C*) on a fully active RNA lacking the six 3′ nucleotides of mRNA(MF) (RNA 5). This RNA retains the three GG motifs, which are reminiscent of the GGA motifs that are essential for RNA binding by the bacterial global regulator Csr/Rsm (23). Although substitution of any one of the three GG repeats by CC did not affect the activity (RNAs 12–14), simultaneous mutation of two or three GG motifs significantly decreased the potency of RNAs 15 and 16 as a RelQ inhibitor, suggesting a possible consensus sequence.

To test this hypothesis, we introduced either one or two GG motifs in a poly(A) RNA (Fig. 2*D*). Although none of the four homopolymeric RNAs [poly(G), poly(C), poly(U), or poly(A)] significantly inhibited RelQ, the addition of two GG motifs, resulting in a GGAGG cluster (RNA 22), turned poly(A) into a potent inhibitor that completely abolished RelQ activity.

We conclude that RelQ inhibition by nucleic acids displays the following traits: (*i*) RNA is more efficient than DNA; (*ii*) single-stranded nucleic acids are more efficient than double-stranded nucleic acids; (*iii*) inhibition by single-stranded RNA is sequence-specific, with a tentative consensus of GGAGG; and (*iv*) ppGpp has a universal protective effect.

mRNA and pppGpp Reciprocally Destabilize Each Other's Binding to RelQ. Inhibition of RelQ's enzymatic activity by mRNA(MF) is indicative of complex formation between the two. We documented this interaction using an electrophoretic mobility shift assay (EMSA) (Fig. 3*A*). Unlike *E. faecalis* RelQ, *E. coli* RelA did not form a complex with mRNA (Fig. S4*A*), as was expected given the absence of RelA inhibition by mRNA(MF) (Fig. 1*B*). Similarly, neither double-stranded RNA nor DNA formed complexes with RelQ (Fig. S2*B*).

Because the addition of 100 μM ppGpp has a dramatic effect in enzymatic assays (Fig. 1), we tested whether ppGpp destabilizes the RelQ:mRNA(MF) complex. The addition of 100 μM ppGpp had a very mild destabilizing effect (Fig. 3*A*), which increased somewhat as ppGpp was titrated up to 1 mM into the system (Fig. S4*B*). Given that experiments with *B. subtilis* RelQ suggest that guanosine pentaphosphate pppGpp is a dramatically more potent effector of SASs (18), we titrated pppGpp in the EMSA assay (Fig. 3*B*). The binding of mRNA(MF) to RelQ was potently abrogated by pppGpp with an IC_{50} of 35 ± 6 μM.

According to the detailed balance argument (24), destabilization of the RelQ:mRNA(MF) complex by (p)ppGpp should be reciprocated by destabilization of (p)ppGpp binding in the presence of mRNA. We tested this prediction with a differential radial capillary action of ligand assay (DRaCALA) (25). In good agreement with results reported for *B. subtilis* RelQ (18), *E. faecalis* RelQ efficiently bound pppGpp (EC_{50}^{pppGpp} of 2.1 ± 0.1 μM), whereas ppGpp was a significantly poorer binder. Even in the presence of 20 μM RelQ, only 10% of ppGpp was associated with the protein, precluding quantitative analysis of the complex formation (Fig. 3*C*). ^{32}P-labeled pppGpp was displaced by mRNA(MF) with an IC_{50} of 2.8 ± 0.1 μM

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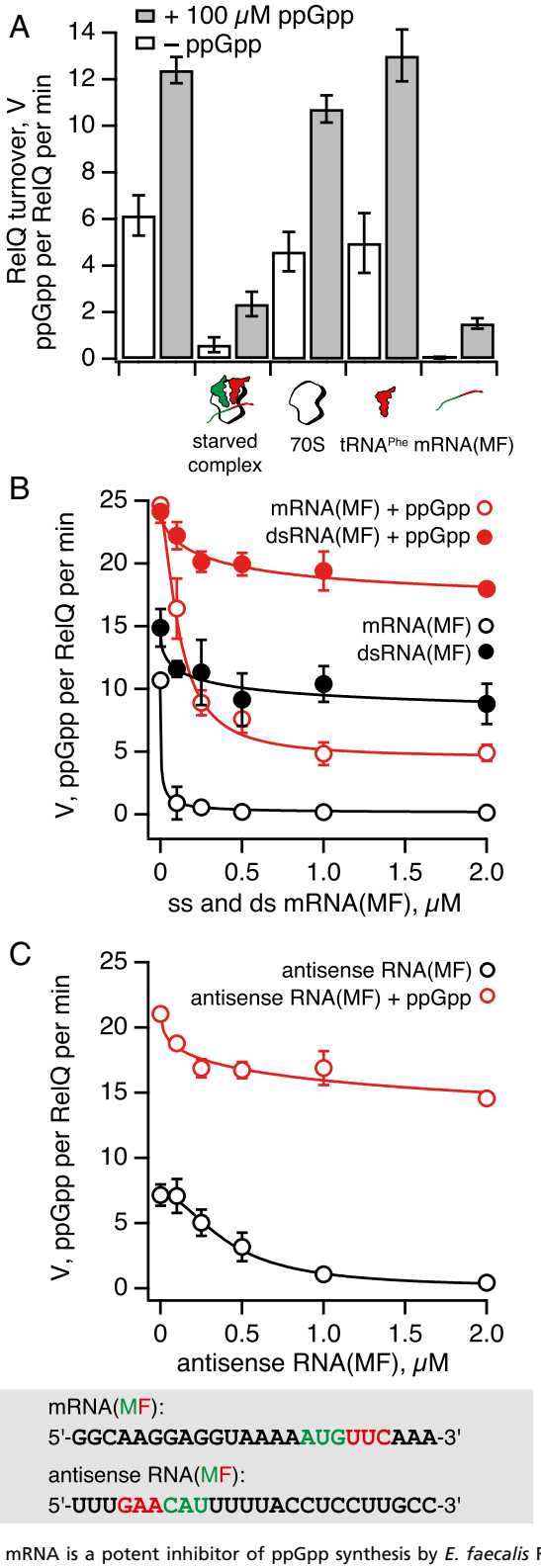

Fig. 1. mRNA is a potent inhibitor of ppGpp synthesis by *E. faecalis* RelQ. (*A*) ^{3}H ppGpp synthesis activity of 250 nM *E. faecalis* RelQ (62.5 nM tetrameric RelQ) in the presence (gray bars) and absence (empty bars) of 100 μM ppGpp, as well as starved ribosomal complexes or individual components thereof. Note that ppGpp is a strong activator of RelQ's enzymatic activity and mitigates the inhibition by starved ribosomal complexes or mRNA(MF). (*B* and *C*) Single-stranded mRNA inhibits RelQ's activity in a sequence-specific manner, and this inhibition is countered by ppGpp. Titrations were performed with increasing concentrations of either single-stranded (empty circles) or double-stranded (filled circles) RNA, in the absence (black circles) or presence (red circles) of 100 μM ppGpp. All reaction mixtures contained 250 nM (62.5 nM tetramer) *E. faecalis* RelQ, 300 μM ^{3}H GDP, and 1 mM ATP. Titration data were fitted with the 4PL model. Error bars represent SDs of the turnover estimates determined by linear regression. Each experiment was performed at least three times.

(Fig. 3*D*). A similar effect was observed with ^{32}P-labeled ppGpp (IC_{50} of 5.2 ± 1.9 μM), but not with ^{32}P-labeled ATP (Fig. S5*A*).

RelQ's Association with RNA Is Mutually Exclusive with ppGpp Synthesis. The moderate effects of ppGpp on the RelQ:mRNA(MF) interaction are in stark contrast to the nucleotide's dramatic effect on RelQ's enzymatic activity in the presence of mRNA. However, in enzymatic assays, ppGpp is always tested in the presence of RelQ substrates ATP and GDP. Therefore, we tested the effects of the simultaneous addition of both RelQ enzymatic substrates in an EMSA assay.

The simultaneous addition of ATP and GDP significantly destabilized the RelQ:mRNA(MF) complex, resulting in a supershift of the RelQ:mRNA(MF), suggesting a structural rearrangement (Fig. 4*A*). The addition of nucleotide combinations that are not accepted by the enzyme, such as CTP combined with GDP or substitution of ATP for nonhydrolyzable analog AMPCPP, did not destabilize the RelQ:mRNA(MF) complex (Fig. S4*C*). The effect of substrates on the RelQ:mRNA(MF) complex was indistinguishable in the presence or absence of ppGpp (Fig. 4*B*). This finding seemingly contradicts the very pronounced effect of externally added ppGpp observed in enzymatic assays (Fig. 1); however, the negated effect of ppGpp in the EMSA assays is due to efficient formation of the alarmone nucleotide in situ because of the excess of RelQ over mRNA [2 μM RelQ vs. 150 nM mRNA(MF)] (Fig. S6).

The addition of increasing concentrations of RelQ substrate GDP in the presence of ATP at a constant 1 mM concentration led to gradual destabilization of the RelQ:mRNA complex (Fig. 4*B*). Fitting the EMSA data to the 4-parameter logistic (4PL) model (Hill equation) (26) yielded a Hill coefficient, n_H, of 1.3 ± 0.4, which is in good agreement with the Michaelis–Menten-like behavior that we observed in previous enzymatic assays (19) but seemingly contradicts the strongly cooperative sigmoidal responses documented by Steinchen et al. (18). The likely cause of this difference is the absence (18) or presence (19) of externally added 100 μM ppGpp in enzymatic assays. The addition of ppGpp increased the enzyme's efficiency (V_{max}^{-ppGpp} of 19 ± 1 vs. V_{max}^{+ppGpp} of 51 ± 4 ppGpp per RelQ per minute) and rendered the response curve more Michaelis–Menten-like (n_H^{-ppGpp} of 3.6 ± 0.8 vs. n_H^{+ppGpp} of 1.8 ± 06) (Fig. 4*C*).

The absence of RelQ:RNA(MF) destabilization in the presence of AMPCPP and GDP (Fig. S4*C*) suggests that it is the very act of ppGpp synthesis, rather than binding of the substrates per se, that dislodges the mRNA from RelQ. To test this hypothesis, we used an enzymatically inactivated RelQ mutant in which a conserved aspartic acid residue in position 82 (EF2671 locus numbering) is substituted with glycine; a similar mutant of *B. subtilis* RelQ has been described previously (18, 27). The D82G RelQ protein exhibited no detectable ppGpp synthesis activity, and although it formed a complex with mRNA as efficiently as the wild type (WT), this complex was insensitive to the addition of ATP, GDP, and ppGpp (Fig. 4*D*).

Allosteric Regulator pppGpp and Substrate GDP Synergize in Protecting RelQ from RNA. Because pppGpp binds to RelQ considerably better than ppGpp and is more efficient in dislodging the mRNA(MF) (Fig. 3 and Fig. S4*A*), we tested pppGpp's protective effect against mRNA(MF) in enzymatic assays. Using either GDP or GTP as a substrate, we titrated ppGpp or pppGpp in the presence of mRNA(MF) (Fig. 5*A*). The RNA was added at a constant concentration of 1 μM to ensure complete inhibition of RelQ's enzymatic activity in the absence of allosteric nucleotide regulators. When GDP was used as a substrate, pppGpp had a dramatic protective effect, completely rescuing the inhibition by mRNA(MF) with

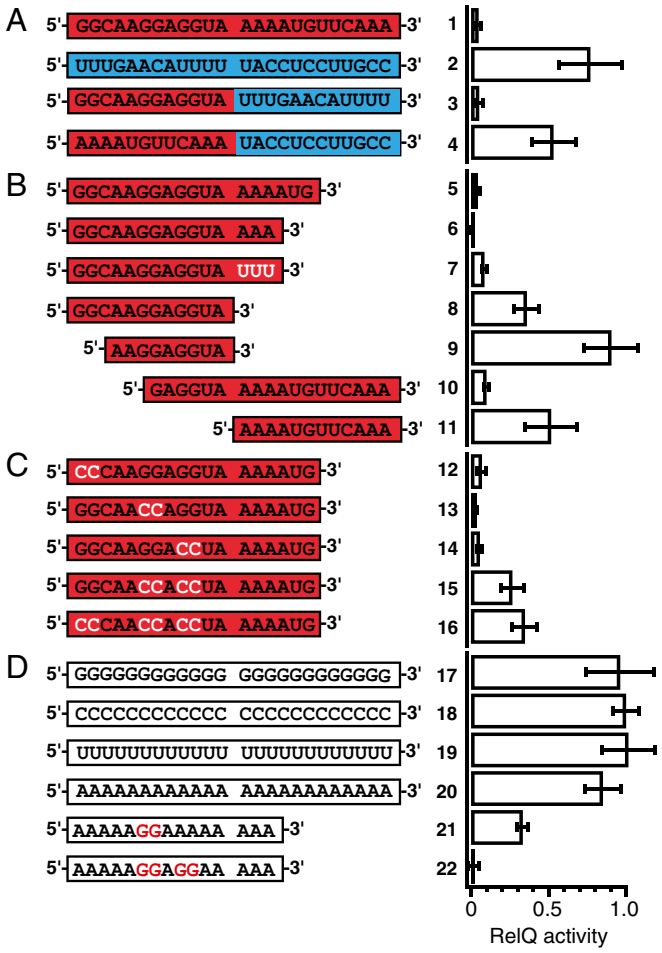

Fig. 2. Sequence specificity of RelQ inhibition by RNA. Here 24-nt-long mRNA(MF) (red) and its complementary antisense RNA (blue) were used as a positive and negative controls, respectively. Based on the two RNAs, we generated chimeras (*A*), cut-backs (*B*), and point mutants (*C*). We also reconstituted the inhibitory activity by adding two GG elements to otherwise inactive poly(A) RNA (*D*). To calculate the RelQ activity, the turnover rate (^{3}H ppGpp synthesized per RelQ per minute) in the presence of RNA was divided by that in the absence of RNA. All reaction mixtures contained 100 nM RNA, 250 nM (62.5 nM tetramer) *E. faecalis* RelQ, 300 μM ^{3}H GDP, and 1 mM ATP. Error bars represent SDs of the turnover estimates determined by linear regression. Each experiment was performed at least three times.

an EC_{50} of 21.4 ± 15 μM. ppGpp had a considerably weaker effect; at 100 μM, it restored RelQ's enzymatic activity to only 35%. In the case of GTP, the protective effect of pppGpp was significantly less pronounced (EC_{50} of 274 ± 5 μM), reflecting the lower catalytic efficiency of GTP utilization as a substrate (Fig. S7). Finally, when GTP was used as a substrate, ppGpp failed to rescue any enzymatic activity.

EMSA assays performed with WT and D82G RelQ variants in the presence of GTP and ATP as substrates and pppGpp as an allosteric activator showed that, similarly to the case of GDP, the catalytic activity of RelQ led to destabilization of the RelQ:mRNA(MF) complex (Fig. 5*B*). However, because pppGpp by itself has a dramatic destabilizing effect, it is impossible to discriminate between the effects of RelQ-mediated catalysis per se and that of pppGpp generated in situ in the EMSA reaction mixture.

Deletion of SAS-Specific C-Terminal Helix 5α, Which Is Essential for RelQ Tetramerization, Abrogates Both ppGpp Synthesis and RNA Binding. We next set out to test the connection between RelQ tetramerization and allosteric regulation by mRNA and ppGpp. Tetramerization has been proposed to play an important role in RelQ's enzymatic activity; the allosteric regulator (p)ppGpp binds at the interfaces between subunits, and the catalytic sites of monomers have been suggested to operate in a cooperative mode (18). We tested the role of tetramerization and subunit cross-talk in the regulation of RelQ's enzymatic activity using two types of perturbations: (*i*) formation of heterotetramers containing both WT and enzymatically inactive D82G subunits and (*ii*) complete abrogation of tetramerization via disruption of RelQ:RelQ contacts within the tetramer via deletion of the C-terminal helix 5α (amino acids 174–234; EF2671 locus numbering) (18) (Fig. 6 *A* and *B*).

Monomeric Δ5α RelQ was found to be enzymatically inactive (Fig. 6*C*), in good agreement with an earlier report of inactivation of *Mycobacterium smegmatis* MS_RHII-RSD on destabilization of oligomerization by 0.2% SDS (28). The truncated RelQ protein did not bind mRNA(MF) (Fig. 6*D*), suggesting that native complex formation is essential for protein function, and that disrupting it could serve as an off-switch exploited by an allosteric regulator. At the same time, formation of a heterotetramer of WT and D82G RelQ mutant did not affect the protein activity, even when D82G RelQ was added at fourfold excess over WT protein (Fig. S8), indicating that active sites of individual subunits are not strictly cooperative. Both WT RelQ and its D82G derivative formed stable tetramers that were not dissociated on the addition of substrates, mRNA, or a combination thereof (Fig. S9). We suggest that inhibition of RelQ by mRNA is mediated by rearrangement of the tetrameric structure, which is responsible for the observed supershift of the RelQ:mRNA complex migration in EMSA assays in the presence of GDP substrate (Fig. 4).

Discussion

Here we report that tetrameric RelQ is an example of an oligomeric bacterial RNA-binding protein. In contrast to well-studied

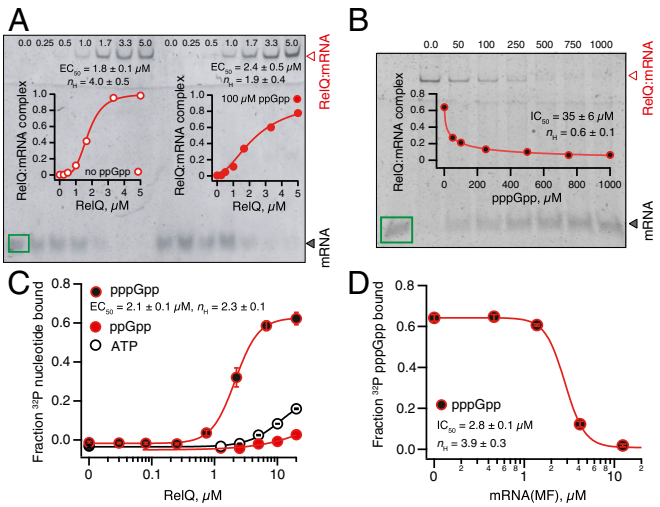

Fig. 3. mRNA and pppGpp have a destabilizing effect on each other's binding to RelQ. (*A*) Complex formation between 0.15 μM mRNA(MF) and increasing concentrations of *E. faecalis* RelQ was monitored by EMSA in the absence (empty circles) and presence (filled circles) of 100 μM ppGpp. (*B*) EMSA analysis of complex between 0.15 μM mRNA(MF) and 2 μM RelQ in the presence of increasing pppGpp concentrations. (*C*) Complex formation of increasing concentrations of RelQ with 50 nM ^{32}P-labeled ATP, ppGpp, or pppGpp monitored by DRaCALA. (*D*) ^{32}P pppGpp is displaced from 20 μM RelQ by increasing concentrations of mRNA(MF), as monitored by DRaCALA. Error bars represent SDs of the mean. Each experiment was performed at least three times.

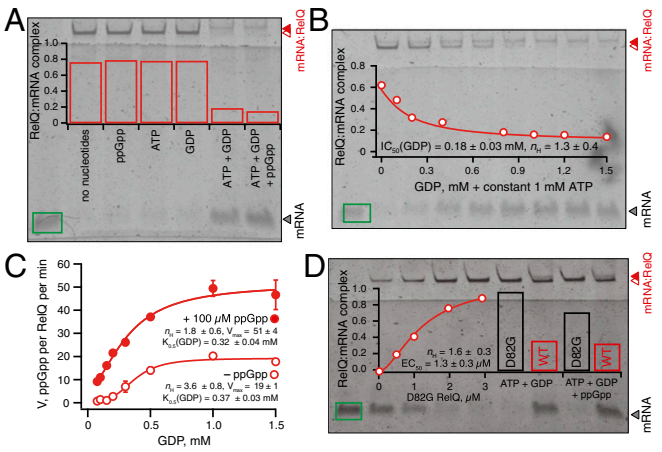

Fig. 4. RelQ binding to mRNA and ppGpp synthesis are mutually exclusive. (*A*) Although 1 mM ppGpp, ATP, or GDP alone does not affect the stability of the RelQ:mRNA(MF) complex, a combination of ATP and GDP has a strong destabilizing effect in both the presence and absence of 100 μM ppGpp. The positions of RelQ:mRNA (open red triangles) and supershifted complex in the presence of substrates (filled red triangles) are indicated to the left. (*B*) Increasing GDP substrate concentration in the presence of 1 mM ATP progressively destabilizes the RelQ:mRNA(MF) complex. (*C*) Addition of 100 μM ppGpp to RelQ both increases its catalytic efficiency (V_{max}) and relaxes the positive substrate cooperativity, as shown by a decrease in the Hill constant, n_H. (*D*) Enzymatically inactive RelQ mutant D82G (EF2671 locus numbering) forms the complex with mRNA(MF) as efficiently as the WT protein, whereas the addition of 1 mM ATP and GDP does not destabilize the complex in either the presence or absence of 100 μM ppGpp. Error bars represent SDs of the mean. Each experiment was performed at least three times.

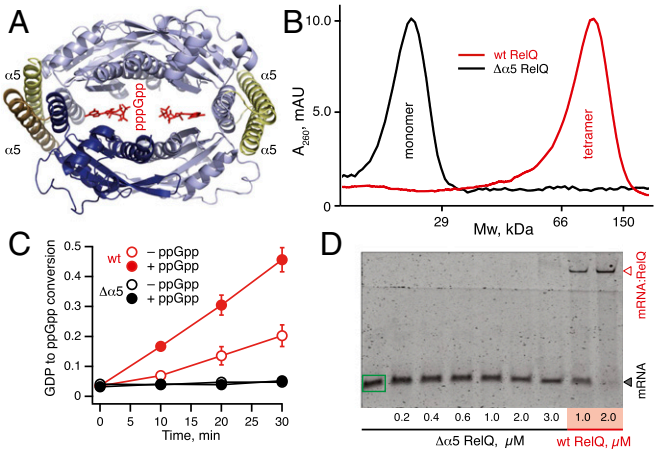

Fig. 6. An intact tetrameric structure is essential for ppGpp synthesis by RelQ. (*A*) C-terminal helix 5α (amino acids 174–234 in *E. faecalis* RelQ; EF2671 locus numbering) is highlighted in yellow in this homology model of *E. faecalis* RelQ based on the SAS1 tetramer of *B. subtilis* (18). Helix 5α, which forms contacts in tetrameric RelQ, is SAS-specific, i.e., absent in ribosome-associated RSHs such as RelA. Sequence alignment is shown in Fig. S10. Two allosteric pppGpp molecules are intercalated in the central cleft. (*B–D*) Deletion of the C-terminal α5 helix results in Δα5 RelQ, which is monomeric as shown by analytical gel filtration (*B*), enzymatically inactive in the presence or absence of 100 μM ppGpp (*C*), and unable to bind mRNA (*D*). Error bars represent SDs of the mean. Each experiment was performed at least three times.

RNA-binding proteins, such as hexameric Hfq (29) and dimeric Csr/Rsm (23), RelQ has the additional ability to synthesize and allosterically respond to the second messenger (p)ppGpp, thus combining two regulatory paradigms within a single protein. Through biochemical experimentation, we have demonstrated the mutual exclusivity of RelQ's activities as an RNA-binding protein and a signaling enzyme synthesizing and responding to the alarmone nucleotide messenger. RelQ's enzymatic activity is potently inhibited by association with single-stranded RNA, and we have identified GGAGG as a putative consensus sequence for inhibition. Association of the primary allosteric regulator pppGpp or, to a lesser extent, the secondary allosteric regulator ppGpp strongly counteracts the inhibition by RNA and destabilizes the RelQ:RNA complex. The protective effect is especially strong when the primary allosteric regulator pppGpp synergizes with the preferred substrate, GDP. Tetramerization of RelQ is apparently essential for this regulatory mechanism, given that both mRNA binding and enzymatic activity are abolished by deletion of the SAS-specific C-terminal helix 5α (Fig. S10).

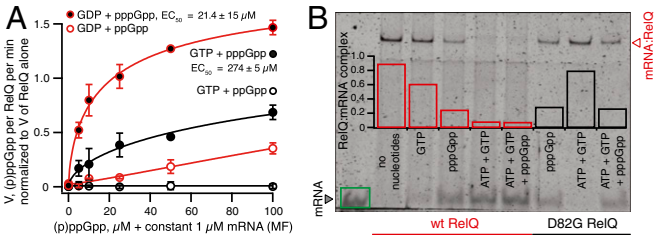

Fig. 5. The combination of GDP as a RelQ substrate and pppGpp as an allosteric regulator provides the best protective effect against RelQ inhibition by mRNA(MF). (*A*) The combination of the preferred substrate (GDP) and the best binding allosteric regulator (pppGpp) provides the strongest protective effect against mRNA(MF). All reaction mixtures contained 250 nM (62.5 nM tetramer) *E. faecalis* RelQ, 1 mM ATP, 1 μM mRNA(MF), 300 μM ^{3}H GDP/GTP, and increasing concentrations of ppGpp/pppGpp. Error bars represent SDs of the mean. Each experiment was performed at least three times. (*B*) EMSA analysis of complex formation between the WT and enzymatically inactive D82G mutant RelQ and 0.15 μM mRNA(MF) in the presence of 1 mM substrates GTP and ATP and 100 μM allosteric regulator pppGpp.

We propose a model for RelQ:RNA interaction as a regulatory switch between catalytically inactive and active forms of the enzyme (Fig. S11). Such a switch would mediate the cross-talk among cellular RSH enzymes by sensing the intracellular alarmone concentration. An increase in pppGpp (the primary nucleotide effector) and ppGpp (the secondary nucleotide effector) levels would allosterically stimulate RelQ's synthetase activity and drive the protein's dissociation from the RNA target. In principle, both the (p)ppGpp synthetic activity of RelQ and RNA binding can act as effectors in a cellular context; regulation of RelQ's enzymatic activity would result in modulation of intracellular alarmone levels, whereas regulation of RNA binding would directly affect a target RNA. The similarity of the putative consensus for efficient RelQ inhibition, GGAGG, identified by our mutational analysis and the Shine–Dalgarno sequence AGGAGG suggests the possibility that the RelQ:RNA interaction exerts its regulatory function via sequestration or occlusion of a ribosome-binding site of RelQ's mRNA target(s). Further investigations are needed to reveal the cellular RNA targets of RelQ and structural aspects of the interplay between mRNA binding and enzymatic activity. Given the broad evolutionary distribution of SAS enzymes, the allosteric regulatory interplay uncovered here for *E. faecalis* RelQ provides an example of a likely widespread regulatory mechanism.

Materials and Methods

Biochemical in vitro translation system from purified *E. coli* components and TLC measurements of ^{3}H ppGpp synthesis have been described previously (9). Enzymatic assays with *E. faecalis* RelQ were performed following the method of Gaca et al. (19). DRaCALA assays were performed as described by Roelofs et al. (25). Titration data were fitted with the 4PL model, or the Hill equation, $Y = (a - d)/(1 + (X/c)^b) + d$, following Sebaugh (26). In this equation, b is the slope factor or Hill coefficient, n_H; c is the half-response

concentration of the titrant (IC_{50}/EC_{50} for binding studies, $K_{0.5}$ for enzymatic assays); and *a* and *d* are the lower and higher plateaus, respectively ($d = V_{max}$ in enzymatic assays). The experimental procedures are described in detail in *SI Materials and Methods*.

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