The autoregulation of *Escherichia coli* RelA through intramolecular interactions

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Abstract

When subjected to amino acid starvation, *Escherichia coli* cells rapidly produce the alarmone nucleotide (p)ppGpp. Accumulation of (p)ppGpp potentiates transcriptional rewiring and a dormant cellular phenotype that promotes survival during stress conditions. RelA, the enzyme responsible for this stress response, has to be tightly regulated in order to avoid aberrant (p)ppGpp synthesis in non-stressed conditions. Conversely, it is well documented that in the absence of stress, the N-terminal domain (NTD) of RelA constitutively produces (p)ppGpp. The C-terminal domain (CTD) has thus been proposed to regulate the N-terminal synthase domain off the ribosome, but there are currently conflicting reports regarding the mechanistic interplay. Here, we present data that shows high ectopic expression of RelA-CTD inhibits translation in a (p)ppGpp-independent manner, suggesting that CTD directly interacts with the ribosome. Furthermore, we reveal that growth permitting low level expression of RelA-CTD does not sequester the activation of native RelA, indicating that the activation of RelA is not regulated by dimerization through the CTD as previously proposed. Our findings show that the (p)ppGpp synthetic activity of RelA is negatively regulated by the presence of the RRM subdomain of the CTD, suggesting existence of possible intramolecular interaction between RRM and NTD. Considering the detrimental effects of unchecked RelA-NTD (p)ppGpp synthesis, or untimely association of the CTD with the translation machinery, the autoinhibition of RelA is vital for maintaining high cellular fitness.

1 Introduction

Bacteria, including major pathogens, have evolved mechanisms enabling them to rapidly adapt to a plethora of nutritional or environmental changes. They do so by evoking the “stringent response” (SR). The SR is marked by accumulation of two unusual phosphorylated derivatives of GTP and GDP, known as guanosine pentaphosphate and guanosine tetraphosphate (collectively referred to as (p)ppGpp). The stringent response and (p)ppGpp have important roles in the regulation of bacterial virulence, survival during host invasion, and antibiotic resistance (Dalebroux et al., 2010;Dalebroux and Swanson, 2012;Hauryliuk et al., 2015).

RelA/SpoT homolog (RSH) are ubiquitous bacterial enzymes responsible for regulation of the intracellular levels of (p)ppGpp. In *E. coli*, and most beta and gamma-proteobacteria, the SR is orchestrated by two RSHs called RelA and SpoT. RSH can be separated into two distinct domains, the catalytic N-terminal domain (NTD) and the regulatory C-terminal domain (CTD, Figure 1A). The N-terminal half of these enzymes includes a (p)ppGpp hydrolase domain (HD) as well as a (p)ppGpp synthesis domain (SYNTH). The C-terminal domain (CTD) is comprised of four subdomains: Thr-tRNA synthetase, GTPase and SpoT domain (TGS domain); helical domain; zing finger domain (ZFD); and the RNA recognitions motif (RRM). RelA is the best studied of all of the RSH and its regulation is the focus of this study.

RelA has no hydrolytic activity, despite having a hydrolase domain, but possesses a strong ribosome-dependent (p)ppGpp synthetic activity that is activated in response to uncharged tRNA accumulation upon amino acid deprivation (reviewed in (Hauryliuk et al., 2015). *In vitro*, RelA poses a very low (p)ppGpp synthetic activity that is not stimulated by deacylated tRNA in the absence of ribosomes (Wendrich et al., 2002;Knutsson Jenvert and Holmberg Schiavone, 2005;Kudrin et al., 2018). Deletion of the CTD results in constitutive (p)ppGpp synthetic activity *in vivo* (Schreiber et al., 1991;Svitil et al., 1993;Gropp et al., 2001), suggesting that the CTD is essential for regulation of the synthetic activity of RelA.

Two distinct and opposing models have been presented for the regulatory role of the CTD. The first, was proposed by three independent groups (Gropp et al., 2001;Yang and Ishiguro, 2001;Jain et al., 2006). These reports suggested that under optimal growth conditions RelA exists as a synthetically inactive dimer and upon amino acid starvation the enzyme is activated in the monomeric form. The second model contradicts the dimerization model and was proposed by a study on Rel from *Streptococcus equisimilis* (Rel^{seq}), where it was suggested that the CTD of Rel negatively regulates (p)ppGpp synthesis by making intramolecular contacts with the NTD (Mechold et al., 2002). Taken together, the regulatory role of the CTD is an accepted premise, but the the manner in which the activity of RelA is regulated is open to question.

Here, we provide evidence that RelA is regulated through intramolecular (autoinhibition) rather than intermolecular (dimerization) interactions. We show that the CTD of RelA can readily impair translation and thus growth. Furthermore, we demonstrate that contacts between NTD and the CTD are essential for keeping in check the otherwise toxic effects of either domain.

2 Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

Tables listing the strains, plasmids and oligonucleotides used in this study are shown in the Supplementary Material. For growth assays in Luria–Bertani (LB) medium or on LB agar, the relevant strains were grown overnight in LB medium supplemented with antibiotic selection. For growth assays on SMG (Serine, Methionine and Glycine) plates, the strains were grown overnight in M9-glucose minimal medium (1 x M9 salts, 1 ug ul-1 thiamine, 1 mM MgCl2, 0.1 mM CaCl, pH 7.4; 10 x M9 Salts: 80 g Na2HPO4, 30 g KH2PO4, 5 g NaCl per 1 L) supplemented with antibiotics for plasmid selection. The SMG plates were made by supplementing M9-glucose minimal medium with 0.7% agarose, 100 μg/ml of Serine, Methionine and Glycine and appropriate antibiotic selection. Expression from LacI-regulated promoters in solid or liquid media was induced by the addition of 1 mM of IPTG. Bacterial cultures were routinely grown overnight at 37°C, and plates were incubated at 37°C for 18 h, 30 μg/ml ampicillin was used to select for low copy number plasmid pNDM220 and its derivatives, and 100 μg/ml ampicillin was used to select for high copy number plasmid pMG25

and its derivatives). All growth curves were performed in flasks, grown at 37°C, with shaking at 160 rpm, unless otherwise indicated.

2.2 (p)ppGpp measurement

To determine the (p)ppGpp levels in cells we modified the method from (Cashel, 1994;Tian et al., 2016). In brief, overnight cultures were diluted 100-fold in 5 ml of MOPS glucose minimal medium supplemented with all nucleobases (10 µg/ml of each) and incubated at 37°C with shaking. All of the strains had similar growth rates in this medium. At an optical density at 600nm (OD_{600}) of ~0.5, cells were diluted 10-fold to an OD_{600} of ~0.05 and were left to grow with shaking at 37°C with $H_3{}^{32}PO_4$ (100 µCi/ml). When looking at the effect of $RelA^{CTD}$ expressed from the low copy number plasmid (Figure 2C and Supplementary Figure 4), 1 mM IPTG was also added at this point. The cells were then grown for ~2 generations (OD_{600} of ~0.2) before amino acid starvation was induced by the addition of either valine (500 µg/ml – Sigma Aldrich) or serine hydroxamate (400 µg/ml – Sigma Aldrich). Whereas, due to its inhibitory effect on growth, $RelA^{CTD}$ was expressed from the high copy number plasmid for 30 min after the cells were grown for ~2 generations (OD_{600} of ~0.2). Subsequently, amino acid starvation was induced by the addition of valine (Supplementary Figure 1A). Fifty-microliter samples were withdrawn before and at various times (see figures) after the induction of amino acid starvation. With the induction of RelA and $RelA^{\Delta RRM}$ (Figure 3C), after labelling of cells for ~2 generations, 1 mM IPTG was added and 50 µl samples were taken at -2, 10, 30, and 60 min. The reactions were stopped by the addition of 10 µl of ice-cold 2 M formic acid. A 10 µl aliquot of each reaction mixture was loaded on polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plates (GE Healthcare) and separated by chromatography in 1.5M potassium phosphate at pH 3.4. The TLC plates were revealed by phosphorimaging (GE Healthcare) and analysed using ImageQuant software (GE Healthcare). The increase in the level of (p)ppGpp was normalized to the basal level (time zero) for each strain.

2.3 Western blotting

For the detection of overproduced RelA and $RelA^{\Delta RRM}$ the appropriate plasmid was induced as described in the text and samples taken at the indicated time points. At each time point, 1ml samples were taken and the cells were harvested by centrifugation at 4°C. Subsequently, the cell pellet was resuspended in an appropriate volume of 1x LDS loading buffer (Life Technologies) and boiled for 5 min. 20 µl of the samples was then resolved on a 4-12% NuPAGE gel (Life Technologies). Subsequently, the proteins were transferred using a semi-dry blotting apparatus (Hoefer Scientific Instruments) to a PVDF membrane (Amersham) and incubated with the primary antibody in PBS with 0.1% Tween-20 and 5% milk powder. The primary antibodies used in this study were raised in rabbit against a peptide sequence from the ZFD of RelA (Eurogentec). Next, membranes were incubated with HR conjugated anti-rabbit IgG antibody (Sigma) in PBS with 0.1% Tween-20 and 5% milk powder. Finally, the protein bands were visualised using Pierce ECL chemiluminescence substrate (Thermo scientific) according to manufacturer's instructions and the signal was detected in an Imagequant LAS4100.

2.4 Monitoring translation efficiency

Overnight *E. coli* MG1655 cells harbouring the CII-YFP plasmid (pSEM3034UR2 – kindly donated by Szabolcs Semsey) and pMG25 (-ve), pMG25::*relA*, pMG25::$relA^{G251E}$, pMG25::$relA^{CTD}$ were grown into balanced growth in MOPS glucose minimal medium, before back-dilution to 0.05 OD_{600} into flat-bottom 96-well microtiter plate (Sterilin, Thermo Scientific) and placed in Synergy H1 (BioTek) microplate reader. Subsequently, the cultured were grown at 37°C with continuous orbital

shaking. Every 15 minutes growth measured by taking the optical density (OD_{600}), and the fluorescence was read from the bottom at 485nm excitation and the 520nm emission wavelengths.

3 Results

3.1 High expression of Rel-CTD is inhibitory to growth

The oligomerization state of the carboxyl-terminal domain (CTD) was suggested to be essential for the regulation of (p)ppGpp synthetic activation of RelA (Gropp et al., 2001;Yang and Ishiguro, 2001;Jain et al., 2006). Previous studies proposed a model in which oligomeric RelA is enzymatically inactive and dissociation to the monomeric form is required for activation during the stringent response. The same groups reported that overexpression of RelA-CTD (designated $RelA^{CTD}$) was able to silence the stringent response via direct interaction and inhibition of native RelA.

Survival and growth of *E. coli* on minimal medium (starvation) plates supplemented with serine, methionine, and glycine (SMG), plates is dependent on a functional RelA-mediated stringent response (Uzan and Danchin, 1978). To investigate the effect of $RelA^{CTD}$ expression on RelA activity, we assayed the viability of wild-type *E. coli* MG1655 (wt) cells growing on SMG. In agreement with the previous studies, when expressing $RelA^{CTD}$ from a high copy number plasmid (pMG25::$RelA^{CTD}$, $P_{A1/O4/O3}$ promoter), growth was severely inhibited on SMG when compared to the empty vector control (Figure 1B). As seen from the thin layer chromatography assays (TLCs) in Supplementary Figure 1A (left side), when cells encounter amino acid starvation, levels of (p)ppGpp significantly increase. Whereas, expression of $RelA^{CTD}$ from the high copy vector inhibits the response of native RelA to amino acid starvation and the levels of (p)ppGpp in the cells do not increase beyond the basal levels (Supplementary Figure 1A, right side). Thus, in accordance with the previous reports, RelA is not activated in cells expressing high levels of $RelA^{CTD}$. Supporting the model that RelA is regulated by oligomerisation via the CTD.

The ZFD domain (previously referred to as CC, conserved cysteine as per (Atkinson et al., 2011) was suggested to be required for dimerization of RelA (Gropp et al., 2001;Yang and Ishiguro, 2001;Jain et al., 2006). Mutational analysis of the ZFD domain suggested three specific residues (C612G, D637R, and C638F) located in the conserved region of this subdomain, are essential for this process as expression of these mutants in $RelA^{CTD}$ were unable to suppress RelA activity (Gropp et al., 2001;Jain et al., 2006). To test this, we individually introduced these three mutations into the high copy plasmid encoding $RelA^{CTD}$ to see whether the negative dominance of the CTD is abolished. The mentioned amino acid changes do not affect the expression or the stability of C-terminal fragments (Gropp et al., 2001). To our surprise, overexpression of mutant variants of $RelA^{CTD}$ still inhibited growth of wt cells on SMG, contradicting the published work (Figure 1B). Therefore, high levels of $RelA^{CTD}$, with or without the mutated conserved residues, impaired the stringent response.

To ensure that the observed effect of $RelA^{CTD}$ overexpression on the stringent response is solely mediated by inactivation of RelA, we investigated the effect of $RelA^{CTD}$ expression in wt as well as Δ*relA* backgrounds in both rich liquid and solid medium under unstarved conditions where functional RelA is not required. Surprisingly, a prominent growth defect was seen in the wt background, either on solid or in liquid medium (Figure 1C). Identical inhibition in growth was also seen in a Δ*relA* background (Figure 1C). Additionally, high levels of $RelA^{CTD}$ bearing mutations in the above-mentioned conserved residues (C612G, D637R, and C638F) were also inhibitory to growth in unstarved conditions (Supplementary Figure 2). In contradiction with previous reports, the above

data suggests that the inhibition of growth achieved by high level expression of $RelA^{CTD}$, is independent of RelA or the stringent response.

3.2 Low expression of Rel-CTD does not affect activation of RelA

As evident from the above data, high expression of $RelA^{CTD}$ was inhibitory to growth and thus, no conclusions could be drawn with regard to its effect on the regulation of RelA. Ectopic expression of $RelA^{CTD}$ from a high copy number plasmid resulted in accumulation of a considerable amount of the recombinant protein in the cell (Supplementary Figure 1B). Given that there are only modest numbers of RelA molecules in the cell (Pedersen and Kjeldgaard, 1977;Justesen et al., 1986;Li et al., 2014;Schmidt et al., 2016), we switched to a very low copy number R1 plasmid (pNDM220, $P_{A1/O4/O3}$ promoter). First we checked to see if expression of the full-length RelA from this plasmid (pNDM220::*relA*) can restore the growth of a strain lacking *relA* (*E. coli* MG1655Δ*relA*) on SMG (starvation) plate. As seen from Supplementary Figure 3, expression of RelA in a Δ*relA* background from this low copy number plasmid was enough to result in similar growth as wt cells on the SMG minimal media plate. Notably, RelA bearing mutations in the conserved ZFD domain also showed full complementation (Supplementary Figure 3). As expected, the empty plasmid control and the synthetically null RelA (G251E, designated RelA* (Gropp et al., 2001)) failed to grow (Supplementary Figure 3). This data indicates that the expression from this plasmid resulted in more physiologically relevant numbers of RelA.

Subsequently, in order to investigate the effect of $RelA^{CTD}$ on the regulation of RelA, the above experiments were repeated with ectopic expression of $RelA^{CTD}$ from the mentioned low copy number R1 plasmid (pNDM220::$relA^{CTD}$, $P_{A1/O4/O3}$ promoter). As seen from Figure 2A, lower levels of $RelA^{CTD}$ were not inhibitory to growth under unstarved conditions on LB agar. Likewise, such concentrations of $RelA^{CTD}$ did not affect the growth of starved wt cells on SMG plate (Figure 2B) or the out growth in SMG media (Supplementary Figure 5B), suggesting that native RelA in these conditions is still active. We then tested the profile of the of RelA mediated response upon amino acid starvation by measuring (p)ppGpp produced *in vivo*. As the SMG plate assay suggested (Figure 2B), low levels of $RelA^{CTD}$ in the cell did not affect activation of endogenous RelA upon induction of the stringent response by addition of either valine (Figure 2C) or serine hydroxamate (Supplementary Figure 4). Furthermore, in case additional domains other than the CTD are required, we wanted to explore the possibility of dimerization by two full length RelA enzymes. We hypothesise that if RelA is capable of dimerization, that endogenous expression of a catalytically inactive RelA mutant would bind and titrate native RelA, shifting the equilibrium away from free and active RelA in the cell, thus impairing the response to amino acid starvation. In order to test this theory, we repeated the above experiments with the inactive RelA mutant, RelA* (pNDM220::*relA**). As seen from Supplementary Figure 5, low expression of RelA* did not affect growth on SMG plate or media, and did not affect the activation of endogenous RelA upon induction of amino acid starvation. Taken together, on the contrary to the previous reports, our data indicates that the activation of RelA is not regulated through intermolecular interactions.

3.3 RelA-CTD impairs translation

The above data with the low expression vector suggests that $RelA^{CTD}$ does not inhibit RelA activation by forming dimers. However, the question remained as to why high levels of $RelA^{CTD}$ is inhibitory to growth (Figure 1C). All recent studies have unanimously showed that the CTD is critical for RelA binding to the ribosome (Agirrezabala et al., 2013;Arenz et al., 2016;Brown et al., 2016;Loveland et al., 2016;Winther et al., 2018). Furthermore, biochemical studies have shown that

upon overexpression of RelA^{CTD}, the majority of the protein is ribosome bound (Yang and Ishiguro, 2001). Therefore, we hypothesised that the toxicity associated with high levels of RelA^{CTD} may be due to inhibition of translation. To monitor the efficiency of translation upon expression of RelA variants (RelA, RelA*, and RelA^{CTD}), we transformed wt cells with a low copy number plasmid encoding bacteriophage λ CII protein fused to Venus yellow fluorescence protein (CII-YFP) which allows the indirect monitoring of translation via measuring of fluorescence (Svenningsen et al., 2019).

As expected, wt cells with the empty vector exhibited increased levels of fluorescence correlated with growth, demonstrating active translation (Figures 3A and B). Wild-type cells harbouring CII-YFP were grown exponentially before RelA and its variants were induced. High expression of full-length RelA results in accumulation of (p)ppGpp (Schreiber et al., 1991;Svitil et al., 1993;Gropp et al., 2001) and the alarmone is a potent inhibitor of bacterial growth targeting both transcription (Sorensen et al., 1994;Molodtsov et al., 2018) and translation (Milon et al., 2006;Corrigan et al., 2016). Thus, as expected, upon strong expression of RelA, growth was severely inhibited (Figure 3A). Consequently, the levels of fluorescence failed to increase, indicating arrest of translation (Figure 3B). Ectopic expression of RelA^{CTD} in mid-exponentially growing cells resulted in slower rather than total arrest of growth (Figure 3A), but the levels of fluorescence, as with full-length RelA, did not increase suggesting translation is impaired (Figure 3B). Subsequently, to test whether the presence of the NTD could regulate the inhibitory effect of the CTD, we checked the effect of expression of full-length RelA* devoid of (p)ppGpp synthetic activity (Supplementary Figure 3). As seen from Figure 3A, high expression of RelA* was inhibitory to growth, but to a lesser extent than RelA^{CTD}. Moreover, the levels of fluorescence gradually increased with expression of RelA* indicating continuous lower rates of translation in these cells. Notably, the inhibitory effect of RelA^{CTD} and RelA* in the cell was more pronounced when overnight cultures were back diluted to a lower optical density and allowed more time to accumulate the expressed product, suggesting that toxicity is dose dependant (Supplementary Figure 2B). Overall, the above data suggests that RelA^{CTD} is inhibitory to growth as translation is impaired upon expression. RelA* is less toxic to growth and the presence of the NTD appears to moderate the toxic effect of the CTD.

3.4 Regulation of RelA by Autoinhibition

The above data suggests that activation of RelA is not regulated by dimerization, as RelA^{CTD} inhibits translation and growth independently of RelA and at closer to physiological levels, RelA^{CTD} has no effect on growth or viability during starvation conditions. Moreover, we observed that the presence of RelA NTD dampens the toxic effect of the CTD truncate, which is also suggestive of the presence of RelA intramolecular interactions. From this we infer that the CTD may in turn function to regulate the (p)ppGpp synthetic activity of the NTD in an autoinhibitory manner. In order to test this theory, we decided to systematically remove subdomains of the regulatory CTD (Figure 1A) to see whether this autoinhibition is lost and at what point the enzyme becomes more promiscuous in regulation to synthesis (p)ppGpp. Growth rate is inversely proportional to the (p)ppGpp concentrations (Ryals et al., 1982), and thus, increases in cellular levels of (p)ppGpp are inhibitory to growth. For this reason, expression of the full-length RelA in wt cells from the low copy number plasmid was slightly inhibitory to growth (Figure 4A). Whereas, as expected, the expression of the constitutively active RelA^{NTD} (designated RelA^{NTD}) (Schreiber et al., 1991;Svitil et al., 1993;Gropp et al., 2001) from the same plasmid resulted in a more pronounced inhibition of growth (Figure 4A). Interestingly, expression of RelA lacking the RRM subdomain (RelA$^{\Delta RRM}$, Figure 1A), renders the enzyme more toxic to the cells than RelA^{NTD} (Figure 4A). Western blot analysis showed that upon expression,

RelA$^{\Delta RRM}$ has similar abundance as RelA in the cells (Figure 4C). Thus, showing that the RRM subdomain is essential for the regulation of the (p)ppGpp synthetic activity of RelA.

To confirm that RelA variant toxicity was dependant on synthetic activity, we repeated the above plate assay with both RelA and RelA$^{\Delta RRM}$ harbouring the G251E mutation which nullifies the (p)ppGpp synthetic activity (Gropp et al., 2001). As seen in Figure 4B, the inhibition of growth was indeed dependant on the synthetic activity of RelA and RelA$^{\Delta RRM}$, suggesting growth inhibition to be (p)ppGpp dependent. Furthermore, we measured the levels of (p)ppGpp upon expression of RelA and RelA$^{\Delta RRM}$ in exponentially growing wt cells. Expression of RelA resulted in a gradual increase in the (p)ppGpp levels (Figure 4D). Whereas, the expression of RelA$^{\Delta RRM}$ resulted in around twice more (p)ppGpp in the cells at indicated timepoints (Figure 4), which can explain its inhibitory effect on growth (Figures 4A and B). Therefore, we experimentally show for the first time, that the synthetase activity of *E. coli* RelA is regulated by intramolecular interactions and that the deletion of the RRM subdomain is able to alleviate the auto-inhibitory effect of the CTD.

4 Discussion

RelA is a ribosome-dependent (p)ppGpp synthetase that is activated upon amino acid starvation. Here, we have explored the regulation of RelA. We show evidence that the catalytic activity of RelA is not regulated through oligomerization, as previously suggested (Gropp et al., 2001;Yang and Ishiguro, 2001;Jain et al., 2006), but rather by autoinhibition as was suggested for Rel^{seq} (Mechold et al., 2002). The autoinhibition is essential for both controlling the binding of RelA to the ribosomes, as well as regulating the (p)ppGpp synthetic activity.

We initially set out to investigate the negative effect of the expression of RelA^{CTD} on (p)ppGpp accumulation during amino acid deprivation and the possible regulation of RelA through dimerization. Our initial finding was that high levels of RelA^{CTD} is inhibitory to growth in a RelA-independent manner. This suggests that the previous studies (Gropp et al., 2001;Yang and Ishiguro, 2001) might have overlooked the direct perturbations of the translation machinery caused by RelA^{CTD}. It is the CTD of RelA that is responsible for anchoring the enzyme to the ribosome during amino acid starvation (Arenz et al., 2016;Brown et al., 2016;Loveland et al., 2016;Winther et al., 2018). Recent structural data revealed that RelA cannot interact with active translation due to steric clashes with the elongation factors EF-Tu and ET-G (Loveland et al., 2016). Moreover, earlier studies showed that RelA^{CTD} upon expression is ribosome bound (Yang and Ishiguro, 2001) and that starved ribosomal complexes could pull-down RelA through its CTD (Agirrezabala et al., 2013). Recently, the CTD of Rel from *Staphylococcus aureus* was shown to dock the enzyme to the translational machinery (Gratani et al., 2018). Taken together, we propose that high levels of RelA^{CTD} likely hinder translation by outcompeting the elongation factors from the ribosomal A-site and thus cause compromised growth. Similarly, upon amino acid starvation, activation of the modest molecules of endogenous RelA would be inhibited as it would be outcompeted from the stalled ribosomes by RelA^{CTD} resulting in a relaxed phenotype (Supplementary Figure 1). Furthermore, we ruled out the dimerization model as low levels of RelA^{CTD} or RelA* did not affect the response of wt cells (MG1655) to amino acid starvation induced by the addition of either valine or serine-hydroxamate (Figures 2C and Supplementary Figure 4).

The toxic effect of the RelA^{NTD} due to its constitutive (p)ppGpp synthetic activity is well documented (Schreiber et al., 1991;Svitil et al., 1993;Gropp et al., 2001). Here, we show that RelA^{CTD} also has toxic effects on the cell as it impairs translation. By taking advantage of a known single amino acid change in RelA that renders the enzyme synthetically null (G251E, RelA*), and RelA lacking the

RRM subdomain ($RelA^{\Delta RRM}$), we propose a model whereby contacts between the NTD and the CTD keep RelA in the folded conformation, which is unable to associate with the ribosome and therefore synthesise (p)ppGpp. These intramolecular interactions are essential for regulating the (p)ppGpp synthetic activity of the NTD (Mechold et al., 2002), and also to prevent the unnecessary binding of the CTD to the ribosomes. Therefore, the NTD and the CTD of RelA counteract each other's toxic effects on growth.

We truncated RelA from the C-terminus and found that the (p)ppGpp synthetic activity of the enzyme becomes unregulated in the absence of the RRM subdomain. Expression of $RelA^{\Delta RRM}$ was more inhibitory to growth than $RelA^{NTD}$ (Figure 4A). We recently showed that upon amino acid starvation $RelA^{\Delta RRM}$ has impaired binding to the ribosomal A-site finger of the 23S rRNA but retained interactions with the 16S rRNA and the Sarcin-Ricin loop (SRL) (Winther et al., 2018). $RelA^{NTD}$ is known to be highly unstable and decays with a half-life of only a few minutes (Schreiber et al., 1991), while, the stability of $RelA^{\Delta RRM}$ appears to be similar to the full-length RelA, which has half-life of more than 2 hours (Schreiber et al., 1991). Therefore, the data suggests that $RelA^{\Delta RRM}$ loses autoinhibition and its expression is more toxic than $RelA^{NTD}$ possibly due to its greater stability. We propose, the loss of the autoinhibition likely favours the enzyme to take up an unfolded conformation, which is more likely to be recruited to the ribosome whereby (p)ppGpp is able to be synthesised. Conversely, the loss of autoinhibition might increase the likelihood of $RelA^{\Delta RRM}$ activation independently of the ribosome. Further work is required to determine the regulatory role of the RRM domain and to also determine whether the other subdomains of the CTD are also involved in the autoinhibition. It also remains to be determined which subdomain(s) is required for the enzyme's stability.

Regulation through autoinhibition was previously suggested for a homolog of RelA, Rel from *Streptococcus equisimilis* (Rel^{seq}) (Mechold et al., 2002). In this study, it was shown that the $Rel^{seq-CTD}$ can bring order to the otherwise constitutively active and unregulated $Rel^{seq-NTD}$. Moreover, crosstalk between the two domains were shown in *E. coli*. A chimera of $RelA^{CTD}$ fused to $Rel^{seq-NTD}$ brought back a response to amino acid starvation to an otherwise none-responsive Rel^{seq}. These findings lead to the hypothesis that in optimal growth conditions, Rel^{seq} exists in the folded conformation where the hydrolase domain is active. When contacts between the Rel^{seq} NTD and CTD are altered, for example by binding to deacylated tRNA and the ribosome during the stringent response, the enzyme opens up resulting the activation of the synthetase domain. Moreover, the crystal structure of the NTD of Rel^{seq} revealed two distinct conformations; (p)ppGpp hydrolase OFF/(p)ppGpp synthetase ON and hydrolase ON/synthetase OFF (Hogg et al., 2004). Our results are consistent with these reports. During optimal growth, we propose that RelA exists in the folded conformation where the pseudo hydrolase domain is ON. Suggesting, that upon amino acid starvation, contacts between the NTD and CTD are lost and RelA is recruited to the ribosome in the open conformation where the synthetase domain becomes ON.

Based on the presented data, together with the available data from the literature, we present a model for the regulation and activation of RelA. During optimal growth conditions, RelA exists in a closed conformation or an autoinhibited state. This is where the contacts between the RRM subdomain of the CTD can negatively regulate the (p)ppGpp synthetic activity of the NTD. In the autoinhibited state RelA produces very low levels of (p)ppGpp (Hauryliuk et al., 2015;Kudrin et al., 2018), and the presence of the unacylated tRNA alone is not enough to alleviate the autoinhibition (Wendrich et al., 2002;Knutsson Jenvert and Holmberg Schiavone, 2005;Kudrin et al., 2018). Upon encountering amino acid starvation, as a preformed complex, RelA loads the cognate deacylated tRNA onto the ribosomal A-site (Winther et al., 2018). Binding to the ribosome leads to the loss of autoinhibition

resulting in the "open" conformation of RelA and thus activation (Wendrich et al., 2002;Arenz et al., 2016;Brown et al., 2016;Loveland et al., 2016;Kudrin et al., 2018;Winther et al., 2018).

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6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

MR conceived and designed the study, KJT and MR performed experiments, contributed to the data analysis, and drafted and revised the manuscript. Both authors have read and approved the final version of this manuscript.

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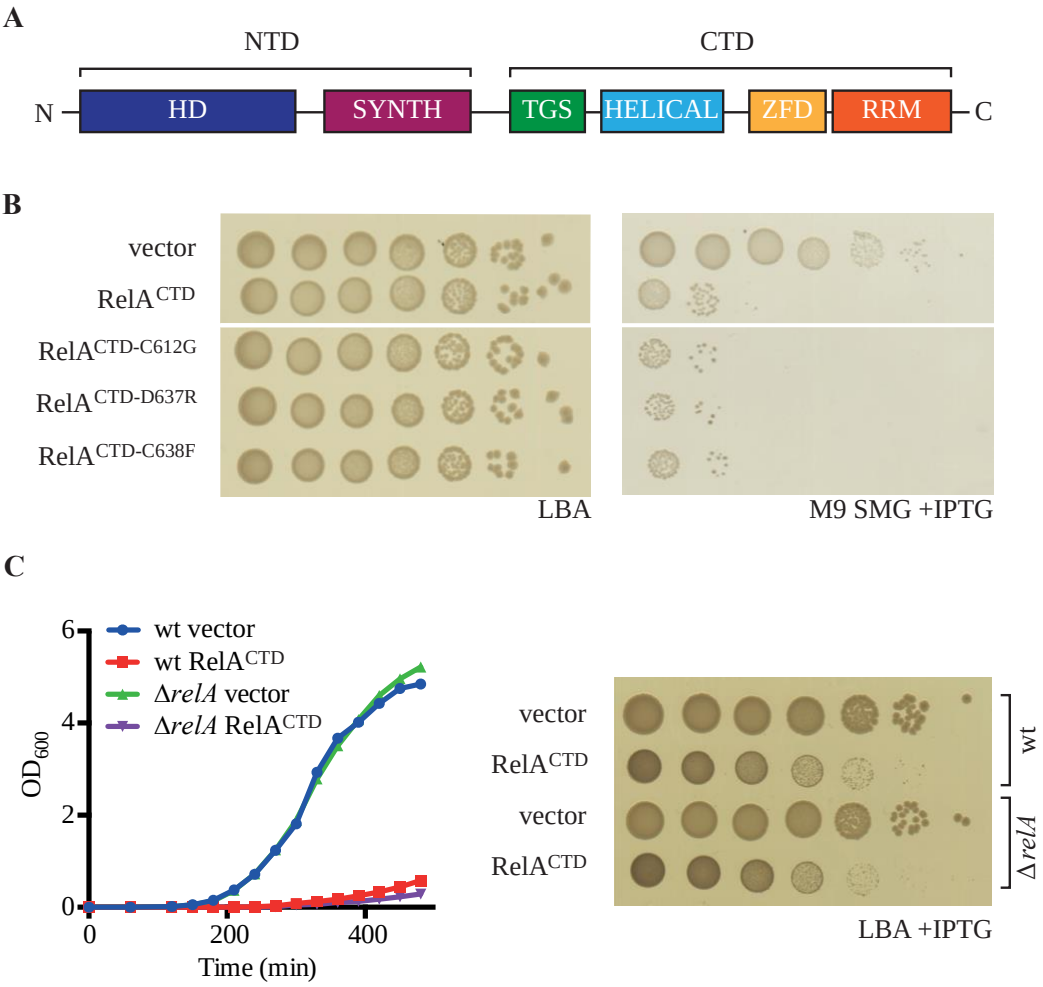

Figure 1. High expression of RelA-CTD is inhibitory to growth independently of native RelA activity. A. Domain structure of RelA, the enzymatically inactive (p)ppGpp hydrolysis (HD) and a functional (p)ppGpp synthesis (SYNTH) domains form the NTD. TGS (ThrRS, GTPase and SpoT), Helical, ZFD (Zinc Finger Domain) and RRM (RNA recognition motif) domains comprise the 'regulatory' CTD. **B.** *E. coli* MG1655 (wt) cells were transformed with high copy IPTG inducible vector, pMG25 (vector), pMG25::$relA^{CTD}$, pMG25::$RelA^{CTD\text{-}C612G}$, pMG25::$RelA^{CTD\text{-}D637R}$, and pMG25::$RelA^{CTD\text{-}C638F}$. The transformed cells were grown at 37°C overnight in M9 minimal medium with 100 µg/ml ampicillin. Ten-fold serial dilutions were made and spotted onto LB agar (LBA) supplemented with 100 µg/ml ampicillin, as a loading control, and onto SMG plates supplemented with 100 µg/ml ampicillin and 1 mM IPTG to induce *relA* variant expression. Growth was examined after incubation at 37°C for 18 h. Here and after, the growth assays originate from a single plate and were brought together. **C.** Overnight cultures of *E. coli* MG1655 (wt) and MG1655Δ*relA* (Δ*relA*) transformed with pMG25 (vector) or pMG25::$relA^{CTD}$, grown in LB with 100 µg/ml ampicillin, were diluted 1/1,000 into fresh LB supplemented with 100 µg/ml ampicillin and 1 mM IPTG, growth was then monitored by sampling at regular time points where OD_{600} measurements were taken. Ten-fold serial dilutions of the same overnights, were made and spotted onto LB agar supplemented with 1 mM IPTG. Growth on the plate was examined after incubation at 37°C for 18 h.

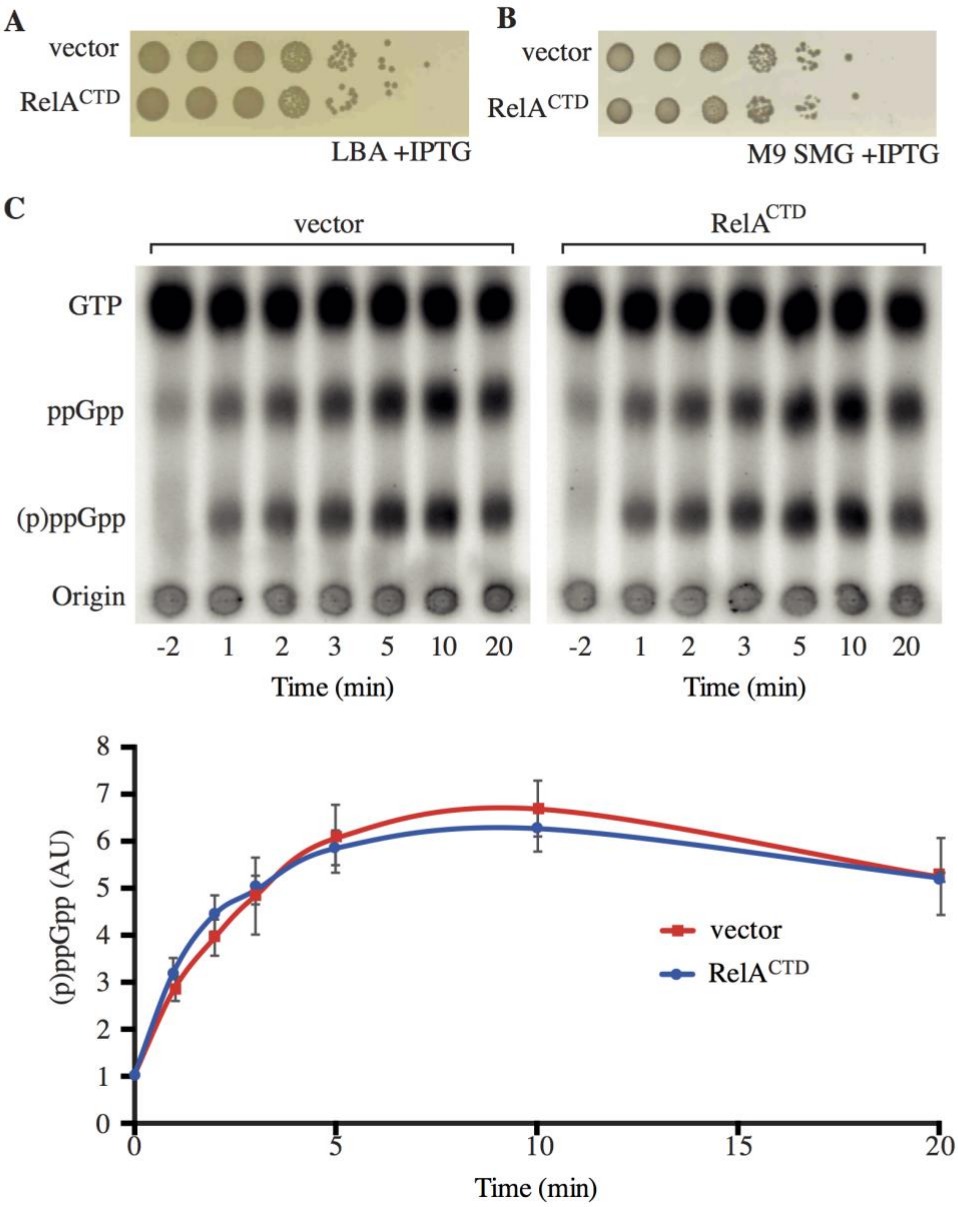

Figure 2. Expression of RelA-CTD does not affect RelA-mediated (p)ppGpp synthesis. A and **B.** Overnight cultures of *E. coli* MG1655, transformed with low copy IPTG inducible vector, pNDM220 (vector) or pNDM220::*relA*^{CTD}, were grown at 37°C overnight in M9 minimal medium with 30 µg/ml ampicillin. Ten-fold serial dilutions were made and spotted onto LB agar (LBA) and SMG plates, both supplemented with 30 µg/ml ampicillin and 1 mM IPTG. Growth was examined after incubation at 37°C for 18 h. **C.** Representative audioradiogram of a PEI Cellulose TLC plate showing (p)ppGpp accumulation of *E. coli* MG1655 carrying pNDM220 (vector) or pNDM220::*relA*^{CTD} upon valine-induced isoleucine starvation. The curves represent the average fold increase for three independent measurements, and the error bars represent standard errors. The levels of (p)ppGpp were normalized to the pre-starved level for each strain at -2 minutes. See experimental procedures for more details.

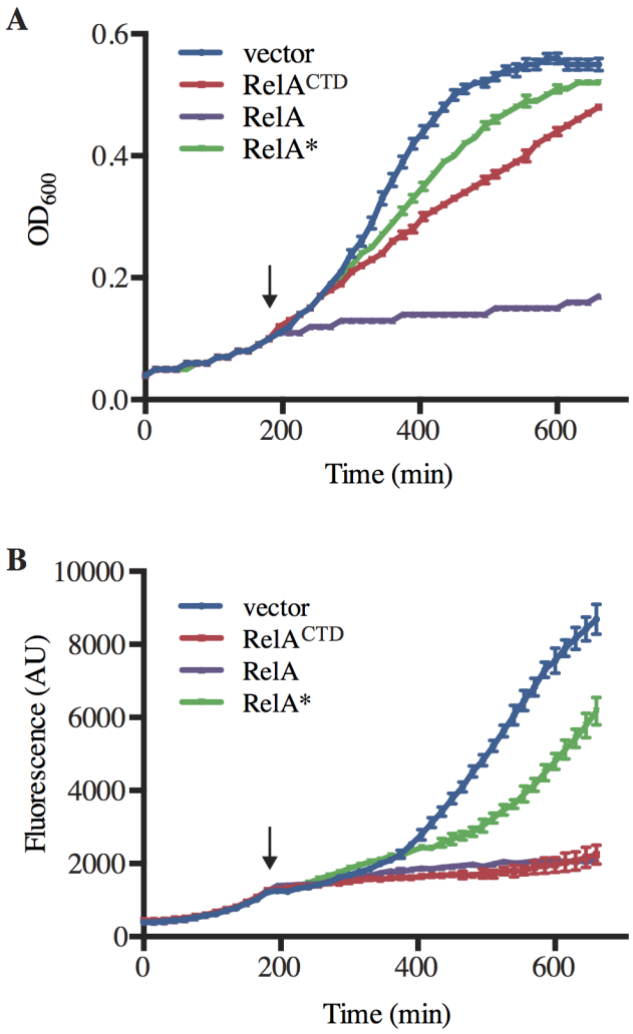

Figure 3. High expression of RelA-CTD impairs translation. Exponentially growing *E. coli* MG1655 cells harbouring the CII-YFP plasmid were back-diluted to OD_{600} 0.05, before the expression of *relA*, *relA**, or *relA*^{CTD} from high copy vector pMG25 was induced by addition of 1 mM IPTG (indicated by the arrow). Growth, measured at 600 nm (OD_{600}) (**A**) and the fluorescence of YFP, measured at 520 nm (**B**) was monitored every 15 minutes for 11 h. See materials and methods for more details.

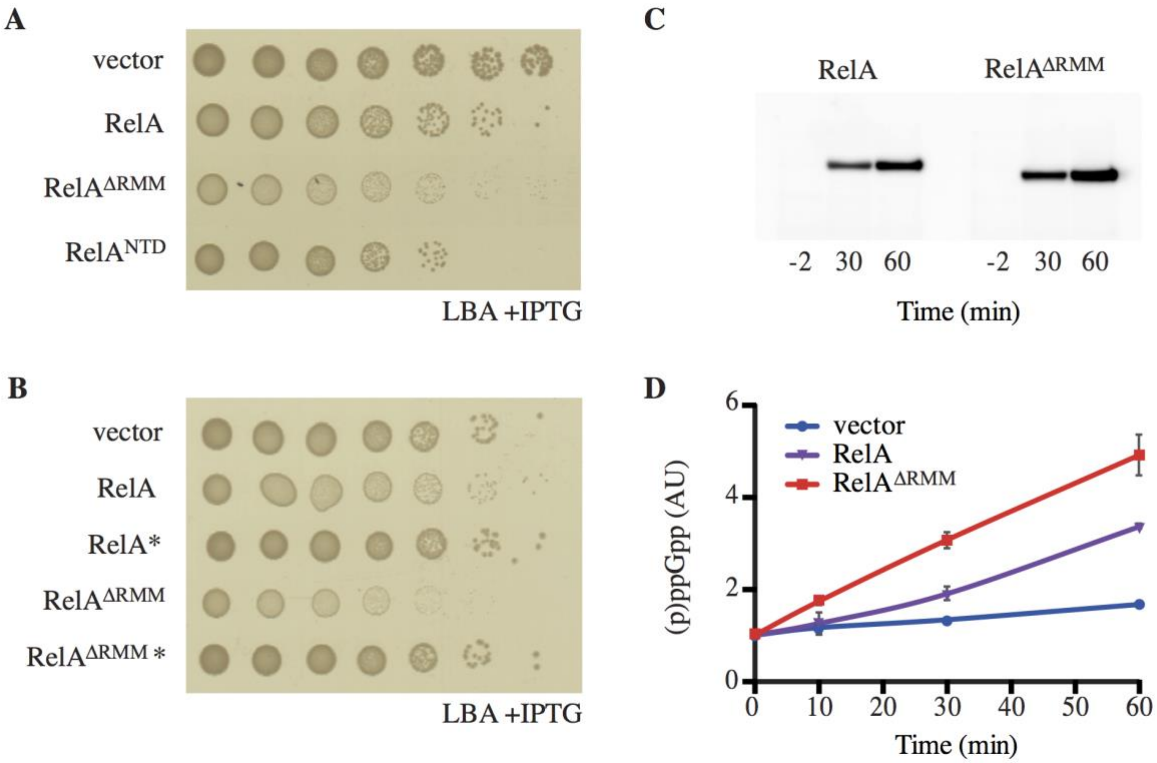

Figure 4. Truncation of RelA leads to overactivation of (p)ppGpp synthesis. A and **B.** *E. coli* MG1655 cells were transformed with low copy IPTG inducible vector, pNDM220 (vector), pNDM220::*relA*, pNDM220::*relA**, pNDM220::$relA^{\Delta RRM}$, pNDM220::$relA^{\Delta RRM}$*, or pNDM220::$relA^{NTD}$. Ten-fold serial dilutions of overnight LB cultures were made and spotted onto LB agar (LBA) supplemented with 30 µg/ml ampicillin and 1 mM IPTG. Growth was examined after incubation at 37°C for 18 h. **C.** *E. coli* MG1655Δ*relA* transformed with pNDM220::*relA* or pNDM220::$relA^{\Delta RRM}$ were grown at 37°C to OD_{600} 0.5, before 1 mM IPTG was added for plasmid induction. Samples were withdrawn at the mentioned times (minutes after induction) and western blotting was performed as described in the experimental procedures. **D.** *E. coli* MG1655 carrying pNDM220 (vector), pNDM220::*relA*, or pNDM220::$relA^{\Delta RRM}$ were grown exponentially in MOPS minimal medium with 30 µg/ml ampicillin before1 mM IPTG was added, for plasmid induction at time 0 minutes. The curves represent the average fold increase of (p)ppGpp for two independent measurements and the error bars represent standard errors. The levels of (p)ppGpp were normalized to the pre-starved level (-2 minutes) for each strain.