Biochemical studies on *Francisella tularensis* RelA in (p)ppGpp Biosynthesis.

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Short Title: *Francisella tularensis* RelA is distinct from the model RelA

Summary statement: *Francisella tularensis* RelA shows significant sequence differences from other members of the RelA family of enzymes. Here we describe the functional similarities and differences between *Francisella tularensis* RelA and the model RelA from *Escherichia coli*.

ABSTRACT

The bacterial stringent response is induced by nutrient deprivation and is mediated by enzymes of the RSH superfamily that control concentrations of the "alarmones" (p)ppGpp. This regulatory pathway is present in the vast majority of pathogens and has been proposed as a potential antibacterial target. Current understanding of RelA mediated responses are based on biochemical studies using *Escherichia coli* as a model. In comparison, the *Francisella tularensis* RelA sequence contains a truncated regulatory C-terminal region and an unusual synthetase motif (EXSD). Biochemical analysis of *Francisella tularensis* RelA showed the similarities and differences of this enzyme compared to the model RelA from *Escherichia coli*. Purification of the enzyme yielded a stable dimer capable of reaching concentrations of 10 mg/mL. In contrast to other enzymes from the RelA/SpoT homologue superfamily, activity assays with *F. tularensis* RelA demonstrate a high degree of specificity for GTP as a pyrophosphate acceptor, with no measurable turnover for GDP. Steady state kinetic analysis of *F. tularensis* RelA gave saturation activity curves that best fitted a sigmoidal function. This kinetic profile can result from allosteric regulation and further measurements with potential allosteric regulators demonstrated activation by ppGpp with an EC_{50} of 60 ± 1.9 μM. Activation of *F. tularensis* RelA by stalled ribosomal complexes formed with ribosomes purified from *Escherichia coli* MRE600 was observed, but interestingly, significantly weaker activation with ribosomes isolated from *Francisella philomiragia*.

Key words: *Francisella*, RelA, ppGpp, synthetase, kinetics, stringent response

Abbreviations: *Ft*RelA, *Francisella tularensis* RelA; *Ec*RelA, *Escherichia coli* RelA; HPLC, High Performance Liquid Chromatography; IP RP HPLC Ion Pair Reverse Phase High Performance Liquid Chromatography; DMHA, *N*,*N*-Dimethylhexylamine; RSH, RelA/SpoT Homologue; (p)ppGpp, Guanosine penta- or tetra-phosphate; ppGpp, 5′.3′-dibisphosphate guanosine; pppGpp, 5′-triphosphate-3′-diphosphate guanosine; ACT domain, Aspartate kinase, Chorismate mutase, TyrA domain; CC domain, Conserved Cysteine domain; TGS domain, Threonyl-tRNA synthetase, GTPase and SpoT domain; IPTG, isopropyl β-D-1-thiogalactopyranoside; gDNA, genomic DNA; BSA, bovine serum albumin; DTT, DL-dithiothreitol; gDNA, genomic DNA; 2YT, 2 x YT medium.

INTRODUCTION

Bacteria rely on global metabolic regulation by the stringent response for survival as the environment becomes nutrient deficient [1, 2]. This response is orchestrated by the synthesis and subsequent downstream effects of the signalling molecules guanosine penta- and tetra-phosphate, collectively known as (p)ppGpp [3]. In β- and γ-proteobacteria, synthesis of these signalling molecules is catalysed by the enzymes RelA and SpoT, members of the long RelA/SpoT Homologue (RSH) superfamily [4]. Virtually ubiquitous across bacterial species, RSH proteins have been suggested as prospective novel antibacterial targets, with genetic knockouts showing attenuation in animal infection models [5-8].

In *E. coli*, and by inference other β- and γ- proteobacteria, the enzyme RelA is principally responsible for (p)ppGpp synthesis during amino acid starvation [9]. Since its discovery [10], the mechanism by which RelA activation leads to (p)ppGpp accumulation has been extensively studied [11-14]. A working hypothesis for RelA activation proposes that the enzyme is most active following its release from a stalled ribosomal complex [11, 15] (Fig. 1A). This hypothesis, termed the 'extended hopping model' [15], explains how the bacterium can respond to a reduced level of amino acids by sensing a hiatus in protein synthesis, and is supported by both *in vitro* [11-13] and *in vivo* [15] experimental evidence.

The protein sequence for long RSH proteins [4] can be divided into an N-terminal region (containing catalytic sites), and a C-terminal region (containing regulatory TGS, CC, Helical and ACT domains) connected by a linker region (Figure 1B) [4]. Long RSH proteins can be divided into bifunctional (Rel or SpoT) or monofunctional (RelA) enzymes. Both classes are capable of synthesising (p)ppGpp but only bifunctional enzymes are capable of (p)ppGpp hydrolysis [16]. Studies with RelA fragments indicate the C-terminal region functions to regulate the catalytic synthetase domain [17] and contains the recognition features that permit homodimerisation [18].

F. tularensis RelA (*Ft*RelA) contains a truncated C-terminal region, around 100 amino acids shorter than most RelAs, and is one of only three RelA sequences known to not contain an ACT domain [4] (Fig. 1B). Proteins containing ACT domains can be found throughout nature, displaying a conserved βαββαβ fold of the domain, and are involved in small molecule ligand recognition [19, 20]. This domain originally described as a 'conserved, evolutionary mobile module' is proposed to have evolutionarily fused with proteins to facilitate the regulation of their catalytic activity by the allosteric binding of small molecules [21].

Besides its truncated C-terminal region *Ft*RelA differs from the majority of other RelA enzymes in its synthetase active site motif. The synthetase domain from RSH proteins are reported to contain either an RXKD or EXDD motif, which are involved in the preferential binding of the pyrophosphate acceptor (i.e. GDP or GTP) [22]. In contrast, the synthetase domain in *Ft*RelA contains the alternative EXSD motif (Fig. 1C), which interestingly can be found in a wide spectrum of *Francisella* species (Supplementary Fig. S1). This alternative motif shows the replacement of the initial aspartate with a serine. The primary aspartate has been proposed to allow the co-ordination of a second magnesium ion [23]. The difference in this active site motif in *Ft*RelA raised the question of whether the (p)ppGpp synthetase activity in the presence of an EXSD motif is more closely aligned to the RSH enzymes featuring RXKD or EXDD motifs.

The functional analysis of *Ft*RelA described herein offers insights into the importance of the distinctive sequence of the synthetase domain and the absence of the ACT domain, compared to other RelA enzymes.

MATERIALS AND METHODS

DTT, BSA and antibiotics were purchased from Melford Laboratories (Suffolk, UK); polyacrylamide-bis polyacrylamide (30% w/v, 37:5:1), Bacto tryptone and yeast extract for culture media were purchased from Oxoid (Hampshire, UK). Chelating fast flow resin and Superdex 200 resin were purchased from GE Healthcare (Buckinghamshire, UK); primers were purchased from Eurofins (London, UK); restriction enzymes and *E. coli* strain K12 JM109 were purchased from New England Biolabs (Hertfordshire, UK); pET16b plasmid was purchased from Merck Chemicals (Middlesex, UK); *E. coli* RelA was expressed using a strain from the ASKA Clone library purchased from Shigen (Japan); *E. coli* MRE600 (C6) strain was purchased from NCTC (Hertfordshire, UK). *Francisella philomiragia* (ATCC 25015) was obtained from ATCC (Middlesex, UK); mRNA was purchased from ATDBio (Hampshire, UK). Unless stated otherwise all other reagents were purchased from Sigma Aldrich (Dorset, UK) or Fisher Scientific (Leicestershire, UK). Graphpad Prism version 6 for Windows was obtained from Graphpad Software, San Diego, California, USA.

Cloning of *F. tularensis relA*. The gene encoding *Ft*RelA, FTT_1508c, was amplified from *F. tularensis* subspecies *tularensis* SCHU S4 genomic DNA using a forward primer (5′-ccgccatgggtcatcatcatcatcatcaagttattgactctaaacttctagatagt) paired with a reverse primer (5′-cgcctcgagttagctgacctcttcattatcatc). The PCR product was digested with NcoI and XhoI and the resultant fragment was ligated into a backbone derived from NcoI/XhoI restricted pET16b. The sequence of the resultant plasmid pET16b::relA was verified by sequencing.

Expression of *Ft*RelA. The plasmid pET16b::relA was chemically transformed into *E. coli* BL21 (DE3) pLysS competent cells (Sigma Aldrich). Single colonies were used to inoculate 2YT media [24] (10 mL, containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol) and cultured overnight at 37 °C. The overnight culture was used as a 1% inoculum for flasks of 2YT (4 x 1.25 L) which were induced with IPTG (final concentration of 0.4 mM) when the absorbance at 600 nm (A_{600}) reached 0.6 and then cultured overnight at 16 °C. The cell pellet was then collected by centrifugation (average yield of 5 g/ 1 L of culture) and stored at -80 °C.

Purification of *Ft*RelA. Frozen cell pellet (typically 15 g) was resuspended (3 x v/w cell pellet) in Buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 3 mM β-mercaptoethanol, 15% v/v glycerol and 20 mM imidazole). Lysozyme (5-15 mg) and a Roche protease inhibitor tablet (1 per 50 mL) were added to the cell suspension and left to stir (4 °C, 30 min). Cells were lysed by sonication (4 °C, 20 x 30 s with 30 s rest), and cellular debris removed by centrifugation (Sorvall evolution, SS-34 rotor, 14000 rpm, 4 °C, 30 min). The resultant cleared lysate was applied (4 mL min^{-1}) to a Ni-IDA Sepharose Fast Flow Column (50 mL bed volume). The column was then washed (4 mL min^{-1}) with Buffer A until the absorption of the eluate at 280 nm (A_{280}) returned to baseline. Elution (4 mL min^{-1}) of *Ft*RelA was achieved using a gradient of imidazole from 20 to 500 mM (Buffer B, as Buffer A but with 500 mM imidazole) over 5 column volumes. Fractions containing *Ft*RelA were pooled and dialysed against Buffer C (2 x 1 L, 50 mM Tris pH 8.0, 300 mM NaCl, 1 mM DTT, 15% v/v glycerol) and concentrated to 10-15 mg/mL in an Amicon Pressure Cell (30 kDa PES filter, Sartorius) before overnight storage at 4 °C. The concentrated *Ft*RelA (3 mL) was then applied (2 mL min^{-1}) to a gel filtration column (HiLoad 26/60, Superdex 200, prep grade) pre-equilibrated in Buffer C. The purest fractions of *Ft*RelA, as judged by SDS-PAGE, were pooled and concentrated (as described previously) to ~4 mg/mL (~50 µM), then aliquoted

(typically 0.2 mL) and stored at -80 °C. For biochemical experiments, *Ft*RelA aliquots were defrosted and used only once. Each batch of *Ft*RelA was used within 8 weeks of freezing.

Characterization of *Ft*RelA Multimeric State by Gel Filtration. A Superdex 200 column (10 mm x 300 mm) was used to estimate the apparent molecular weight of purified *Ft*RelA (~1 mg/mL, ~13.5 μM).. Protein samples were applied (1 mL min^{-1}) to a pre-equilibrated column in Buffer D (50 mM Tris – HCl pH 7.5, 100 mM KCl). Cytochrome C, carbonic anhydrase, BSA, alcohol dehydrogenase and β-amylase (Sigma Aldrich) were used as protein standards for calibration of the column. Elution of the protein samples were monitored by absorbance at 280 nm.

HPLC Analysis of *Ft*RelA Activity. *Ft*RelA activity assays, substrate specificity assays and assays to identify and quantify activating factors were all analysed by IP RP HPLC, using methods adapted from Cordell *et al*. [25]. Injected samples (40 μL) were chromatographed on a reverse phase column [Gemini C18, 150 x 4.6 mm 5 micron (Phenomenex)] at a flow rate of 0.8 mL min^{-1} with UV detection at 260 nm. The mobile aqueous phase was 95% water with 5% methanol and organic phase was 20% water with 80% methanol. Both phases contained DMHA (15 mM) and were adjusted to pH 7.0 with acetic acid. Nucleotides were eluted with the following gradient: 0-5 min, isocratic, 25% organic buffer; 5-27 min, gradient 25-60%; 27-28 min, gradient 60-100%; 28-33 min, isocratic, 100%; 33-34 min, gradient 100-25%; 34-44 min, isocratic 25%.

Substrate specificity of *Ft*RelA. Reaction mixtures (100 μL) were prepared with components at the following final concentrations: ribonucleotide di- or triphosphates (2 mM, either G, T or C), ATP (2 mM), and *Ft*RelA (10 μM) in assay buffer (20 mM Tris pH 8.0, 15 mM KCl, 15 mM $MgCl_2$, 1 mM β-mercaptoethanol). Reactions were incubated at 30 °C for 60 min, prior to quenching by heating. All reactions quenched by heat were maintained at 80 °C for 2 min. The reaction mixtures were then kept on ice to ensure full protein precipitation (typically 10 min), before clearing by centrifugation (Biofuge Fresco microfuge, 12000 rpm, 4 °C, 5 min). An aliquot (40 μL) of the supernatant was then analysed by IP RP HPLC. Assays to determine substrate selectivity under modified conditions are described in Supplementary Methods 1 and 2.

Steady State Kinetics for *Ft*RelA and substrates. Reaction mixtures for time course experiments (1 mL) contained *Ft*RelA (10 μM) and were made up in assay buffer. When saturating, nucleotides (GTP or ATP) were included at 2 mM. Reactions were incubated at 30 °C and at selected time points, aliquots (100 μL) were withdrawn and reaction quenched by heating. Precipitated protein was removed as described previously and a sample (40 μL) was analysed by IP RP HPLC. The concentrations of nucleotides were quantified by comparison with a standard calibration curve. The formation of product nucleotides over time (never more than 15% substrate turnover) was fitted to a linear function to determine initial rates (Supplementary Table S1). Initial rates, *v*, were fitted to an allosteric sigmoidal function (Equation 1) using Graphpad Prism software where V_{max} is the rate of reaction at substrate saturation, $K_{1/2}$ is the concentration of substrate giving a rate of half of V_{max} and h is the apparent Hill constant.

Equation 1.

$$v = \frac{V_{max} S^h}{\left(K_{½}^h + S^h\right)}$$

^{31}P NMR Analysis of *Ft*RelA Activity. *Ft*RelA catalysed reaction time courses were monitored with ^{31}P NMR. All data were recorded on a Bruker AVII400 FT-NMR Spectrometer using a 10 mm auto-tune and match broadband probe tuned to the sample prior

to data collection (TD = 64k points; sweep width = 395 ppm; 512 scans using 90° pulse with a total acquisition time of 2.5 s per scan; chemical shifts referenced to H_3PO_4). Reaction mixtures (3 mL) were prepared in assay buffer and contained GTP (2 mM), ATP (2 mM), and 10% D_2O. The reaction was initiated through the addition of *Ft*RelA (10 μM), thoroughly mixed and then data collected at 25 °C for 22 min followed by 22 min bins for the duration of the experiment (typically 110 min). Peak integrals were calibrated against a spectrum recorded prior to initiation. At each substrate concentration the formation of product nucleotides over time was fitted to a linear function to determine the rate of product formation. These rates were then fitted to an allosteric sigmoidal curve (Equation 1).

Expression and Purification of Ribosomes. Ribosomes were isolated from both *F. philomiragia* and *E. coli* MRE600. For the purification of ribosomes from *E. coli*, overnight cultures of *E. coli* MRE600 (LB media, 10 mL) were used to inoculate flasks of LB media (1 L) supplemented with $MgSO_4$ (10 mM). Cultures were grown at 37 °C to an A_{600} of 0.4, cells were then pelleted by centrifugation (Sorvall evolution, SLC 6000 rotor, 4000 rpm, 4 °C, 30 min). For the purification of ribosomes from *F. philomiragia*, overnight cultures of *F. philomiragia* (Trypticase Soy Broth (TSB), 10 mL) were used to inoculate flasks of TSB (1 L) supplemented with L-cysteine (1 g/L). Culture was grown at 37 °C to an A_{600} of 1.0, and the cells collected by centrifugation as described above. Cell pellets were washed and stored as described by Maguire *et al.* [26]. SulfoLink-cysteine resin was prepared, and ribosomes purified as described for *E. coli* ribosomes by Maguire *et al.* [26]. Fractions (10 mL) containing ribosomes, as assessed by absorption at 260 nm, SDS-PAGE and measurement of protein content by the method of Bradford [27], were pooled and concentrated to 4.96 and 8.93 μM for *F. philomiragia* and *E. coli* respectively in an Amicon Pressure Cell (30 kDa PES filter, Sartorius). Concentrated ribosomes were aliquoted (200 μL) and stored at -80 °C. RNA was isolated from ribosomal samples using the GeneJet RNA purification kit (Thermo Scientific) and analysed by bleach RNA gel electrophoresis as described by Aranda *et al*.[28].

Ribosome Activated *Ft*RelA Activity Assays. Reaction mixtures containing stalled ribosomal complexes were prepared in assay buffer with purified ribosomes (0.2 μM), purified RelA (0.5 μM), ATP (2 mM) and each RNA species (0.3 μM). RNA species include: mRNA (5′-caaggagguaaaaauggucgucgcacgu) [12], $tRNA^{fmet}$ (from *E. coli* MRE600), and $tRNA^{val}$ (from *E. coli* MRE600). Reaction mixtures were incubated at 30 °C for 5 min prior to initiation by the addition of GTP (2 mM final concentration). Reaction mixtures were incubated for 60 min at 30 °C prior to quenching by heating. Precipitated protein was removed as described previously and a sample (40 μL) was analysed by IP RP HPLC.

Product activation of *Ft*RelA. The nucleotide ppGpp was synthesized and purified as described by Carmona *et al.* [29] with minor modifications (Supplementary Method 3). Reaction mixtures (100 μL) were prepared in assay buffer with components at the following final concentrations: ribonucleotide triphosphates (2 mM, both A and G), *Ft*RelA (5 μM) and purified ppGpp, AMP or KH_2PO_4 (at indicated concentrations). Reactions were incubated at 30 °C for 60 min prior to quenching by heating. Protein was precipitated and removed from samples as detailed above, prior to analysis of the sample by IP RP HPLC. Assays were prepared alongside a negative control containing no additional small molecular component. The EC_{50} value was calculated with a sigmoidal dose-response (variable slope) curve (Equation 2), using Graphpad Prism software where Y is the rate, Y_{max} is the maximum rate, Y_{min} is the basal rate and EC_{50} is the concentration of ligand required to give 50% of full activation and h is the apparent Hill slope.

Equation 2.

$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + 10^{(LogEC_{50} - X) - h}}$$

RESULTS

Expression and Purification of *Ft*RelA. The RelA encoding gene, FTT_1508c, from *Francisella tularensis* subspecies *tularensis* SCHU S4 was cloned with primers designed to encode an N-terminal hexahistidine (His_6) tag to facilitate subsequent protein purification. Optimized expression of *Ft*RelA in *E. coli* BL21 (DE3) pLysS was achieved by maintaining the cultures at 16 °C overnight after induction. The *Ft*RelA purification required two chromatographic steps with the initial purification using Ni-IDA affinity chromatography (Fig. 2A). SDS-PAGE analysis showed a distinct band corresponding to the expected molecular weight (74 kDa) of *Ft*RelA alongside several minor impurities (Fig. 2B). *Ft*RelA was further purified in a polishing step, using size exclusion chromatography (Superdex 200, Fig. 2C and 2D). The resultant highly purified *Ft*RelA was isolated in a yield of 12 mg/ g cell pellet and could be concentrated up to 10 mg/mL (~100 µM).

Multimeric State of Purified *Ft*RelA. The multimeric state of purified *Ft*RelA was determined using size exclusion chromatography and comparison with protein standards. *Ft*RelA was shown to form one distinct peak which had an apparent mass of 128 ± 1.57 kDa that approximately corresponds to a dimer state (calculated molecular mass of 148 kDa) (Fig. 3).

Substrate Specificity of *Ft*RelA. As other RSH enzymes have been shown to utilize GDP and GTP as pyrophosphate acceptors [30-33], the specificity of *Ft*RelA for these nucleotides was investigated. End-point *Ft*RelA activity assays were prepared with *Ft*RelA, ATP and either GDP, GTP or, as a negative control CTP (Supplementary Fig. S2). Efficient formation of AMP and the 3′-pyrophosphorylated product were only observed in the presence of GTP as a co-substrate (Fig. 4, Supplementary Fig. S2); GDP and CTP were not accepted as substrates. Very low concentrations of AMP are still measurable for activity assays with GDP as a pyrophosphate acceptor (Fig. 4), but the formation of ppGpp is not observed. *Ft*RelA remained selective for GTP as a pyrophosphate acceptor in the presence of stalled ribosomal complexes (Supplementary Fig. 3A) but accepted GDP as a substrate in the presence of methanol (30% v/v, Supplementary Fig. 3B)

Kinetic Characterization of *Ft*RelA Basal Activity Analysed by IP RP HPLC. To obtain steady state kinetic parameters for *Ft*RelA, a series of reaction time course experiments over a range of substrate concentrations were prepared. For time course experiments, initial rates resulting in less than 15% turnover of substrate were determined from HPLC quantification of products in aliquots withdrawn at 0, 10, 20 and 40 min (Fig. 5A and Supplementary Table S1). The HPLC analysis provided measurements of the products concentrations: the AMP peak was clearly resolved, however slow degradation of pppGpp to ppGpp resulted in two partially overlapping peaks, which were combined to give an overall (p)ppGpp concentration (Supplementary Fig. S4). A better fit of the velocity curves (plotting rate against concentration of substrate) was achieved for a sigmoidal (R^2 = 0.947) rather than a hyperbolic function (R^2 = 0.899) for AMP production (Fig. 5B and 5C and Table 1). The Lineweaver-Burk plot for *Ft*RelA steady state data was observed to curve in a manner consistent with positive cooperativity [34] (Fig. 5D). Fitting to Equation 1 yielded the kinetic parameters in Table 1 including $K_{1/2}$ which denotes the concentration of substrate at which half the maximal activity of the enzyme is achieved. The $K_{1/2}$ values for ATP as a substrate are within error when calculated from rate of formation of AMP or (p)ppGpp, with values of 259 ± 37.2 µM

and 332 ± 47.8 μM respectively. The $K_{1/2}$ values for GTP as a substrate when calculated from AMP or pppGpp formation are 800.7 ± 115.6 μM and 1095 ± 183.8 μM respectively, which are also within error.

Kinetic Characterization of *Ft*RelA Basal Activity by ^{31}P NMR. As the sigmoidal saturation kinetic profile of *Ft*RelA observed by HPLC analysis is somewhat unusual, we sought to verify it using ^{31}P NMR as an alternative measurement technique. Despite NMR not often being suitable for monitoring enzymatic reactions, due to its intrinsic low sensitivity and resultant long acquisition times, the relatively slow turnover of *Ft*RelA in the absence of activators (as judged by HPLC analysis) suggested ^{31}P NMR might also provide a useful approach for this enzyme. Using nucleotide standards dissolved in enzyme assay buffer, the signals for each phosphorus atom in the substrates and products were identified (Supplementary Table S2). In preliminary NMR experiments, some of these signals were observed to overlap including those for the 5′ α and 3′ α of ppGpp (~6 ppm). The signal for the α-phosphate of AMP (3.32 ppm) (Supplementary Fig. S5) however was well resolved. Despite the formation of an individual peak for the 5′-β phosphate of pppGpp (-19.88 ppm) (Supplementary Fig. S5), the observed overlap with the neighbouring peak (5′-β phosphate of GTP/ATP) and slow hydrolytic conversion of pppGpp to ppGpp resulted in an increased error in measuring pppGpp formation. This resulted in the higher derived rates and apparent V_{max} determined from calculated initial rates of pppGpp formation (Fig. 5).

The conversion of GTP to pppGpp was shown to occur in a highly specific manner, with only one minor by-product being observed, inorganic phosphate (1.94 ppm) (Supplementary Fig. S5), which accumulates during the activity assays. This observation is consistent with either the instability of pppGpp under assay conditions, or the presence of a very low level of contaminating phosphatase activity [35].

The intrinsic insensitivity of ^{31}P NMR results in a relatively weak signal, but 22 min spaced time points partly compensated for this. However, the resultant data comes with a caveat that a significant proportion of the substrates (~30%) had been turned over by the third time point (66 min). Somewhat surprisingly, these ^{31}P NMR time courses (Supplementary Fig. S6) showed approximate linearity in product formation to at least 1 hour and reaction rates were calculated within this linear range (Supplementary Fig. S7 and Table S3). The rates determined at a selected range of substrate concentrations were plotted to yield velocity curves (Fig. 5E and 5F). Once again, these fitted to a sigmoidal function comparable to the results observed with IP RP HPLC analysis of *Ft*RelA activity assays, including the $K_{1/2}$ and V_{max} for GTP and ATP (Supplementary Table S4). Data obtained by ^{31}P NMR should however be considered as an estimate only, due to the higher than normal proportion of substrate turnover measured. Kinetic data sets measured by ^{31}P NMR and HPLC were fitted to Equation 1 with V_{max} and $K_{1/2}$ as global (shared) constants to derive overall kinetic parameters (Table 2, Supplementary Fig. S8). For the reasons previously discussed the initial rates calculated by ^{31}P NMR analysis of pppGpp formation were omitted from this global fit.

Activation of *Ft*RelA by Small Molecules. Shyp *et al.* have described the positive regulation of *Ec*RelA by the product of GDP pyrophosphorylation, ppGpp [14]. Kinetic analysis using both HPLC and ^{31}P NMR methods showed positive cooperativity of *Ft*RelA activity and it was of interest to determine if small molecules such as ppGpp or other small ligands were regulating activity. The effect of adding putative activating factors, AMP, phosphate or ppGpp prior to initiating the activity assay at low (10 μM), medium (100 μM), or high (1000 μM) concentrations on the rate of product formation was measured using IP RP HPLC analysis. The background concentration of additional ppGpp or AMP was subtracted

to give the concentration of each product newly formed during the experiment. No significant activation was observed in the presence of additional inorganic phosphate (KH_2PO_4) or AMP (Fig. 6A); however, in the presence of medium to high concentrations of ppGpp, activation was observed. Repeating this activation measurement over a wider range of ppGpp concentrations (0-1000 μM) allowed determination of an EC_{50} for ppGpp of 60 ± 1.9 μM (Fig. 6B) and a maximal 1.5 fold activation, similar to the approximate 2 fold activation observed for *Ec*RelA [14].

Activation of *Ft*RelA by Stalled Ribosomal Complexes. Bio-safety considerations encouraged us to explore alternatives to *F. tularensis* as sources for ribosome isolation. Two alternative ribosome sources were investigated. Firstly the well characterized *E. coli* MRE600 strain which lacks ribonuclease I [36] and has consequently been widely used for ribosome purification [26, 37, 38]. Secondly, another member of the *Francisella* genus, *F. philomiragia*, which is of low virulence [39] and also encodes within its genome a RelA enzyme lacking the ACT domain in its C-terminus [4]. Maguire *et al*. developed an affinity chromatography method for ribosome purification using cysteine coupled SulfoLink resin (Pierce) [26]. Previous purification of ribosomes from clinical isolates of pathogenic bacteria using this method [26], suggested its potential use for the isolation of ribosomes from *F. philomiragia*. Ribosomes were isolated from bacterial cells collected from early-mid logarithmic phase cultures, as established by growth curves (Supplementary Fig. S9), to ensure the optimal recovery of ribosomes. The protein and RNA content of purified ribosomes were analysed primarily by SDS-PAGE and absorbance traces at 260 and 280 nm (Fig. 7 and Supplementary Fig. S10). Purification of ribosomes by this method can contain tRNA, which is thought to also interact with the resin [40]. Contaminating tRNA was removed from ribosomal preparations by ultrafiltration. Purified *E. coli* MRE600 ribosomes were shown to contain all ribosomal RNA species by bleach agarose gel electrophoresis (Supplementary Fig. S10B), however purified RNA from *F. philomiragia* proved too unstable for analysis by this method.

End-point (1 h) activity assays for both *E. coli* RelA (*Ec*RelA) and *Ft*RelA were prepared containing one of the following: *F. philomiragia* ribosomes, *E. coli* ribosomes or in the absence of any ribosomes. Rates are shown in units of pmol AMP per pmol RelA per min (Fig. 8) to allow comparison with previously published data for activated *Ec*RelA [41]. Both *Ec*RelA and *Ft*RelA demonstrated basal levels of activity in the absence of stalled ribosomes, as has been noted previously for *Ec*RelA and Rel_{Mtb} [42, 43]. *Ft*RelA displayed strong activation by *E. coli* ribosomes, with an 11 fold increase in activity compared to the basal level (Fig. 8). We were unable however to show strong activation of *Ft*RelA in the presence of *F. philomiragia* ribosomes, with only a modest 1.39 fold increase observed (Fig. 8). Conversely *Ec*RelA showed strong activation in the presence of either *F. philomiragia*, with an 11 fold increase, or *E. coli* ribosomes, with a 16 fold increase, respectively (Fig. 8). Maximal *Ft*RelA activity (701.5 ± 30.5 pmol AMP per pmol RelA per min) did not reach that of *Ec*RelA (2952 ± 99.14 pmol AMP per pmol RelA per min) under any conditions tested.

DISCUSSION

Virtually ubiquitous across bacterial species, the stringent response is coordinated by signalling molecules (p)ppGpp and is important in bacterial survival under nutrient deficient conditions. In β- and γ- proteobacteria the principle enzyme responsible for (p)ppGpp synthesis is RelA [4], and yet research on this enzyme has focused almost exclusively on *E. coli* RelA as a model enzyme [11-13, 30]. Here we discuss the relevance of the distinctive

synthetase active site motif and C-terminal truncation within *Ft*RelA in the functional similarities and differences when compared to other defined RelA enzymes.

Purification of this enzyme by nickel affinity chromatography followed by size exclusion chromatography yielded highly purified RelA, which could be concentrated to a tenfold higher concentration than that reported for *E. coli* RelA [13]. The higher solubility of *Ft*RelA may be of practical value in future crystallisation studies.

Previous work with *E. coli* RelA has demonstrated the enzyme's ability to dimerise via its C-terminal region [17, 18]. The ability of *Ft*RelA to therefore dimerise was of interest given its truncated C-terminus. Here however we demonstrate that *Ft*RelA forms a stable dimer upon purification. We postulate that the presence of the identified key residues for *Ec*RelA dimerization Cys-612, Asp-637 and Cys-638 [17] in *Ft*RelA facilitate the formation the dimer without the requirement of downstream residues. We note that this may not however be *Ft*RelAs natural multimeric state and might relate here to the high protein concentration. Further analysis of *Ft*RelA dimeric state over a range of concentrations may elucidate the enzymes multimeric state at concentrations closer to those found *in vivo*...

Previous work on synthetase activity in long RSH enzymes from *E. coli* [30], *M. tuberculosis* [44] and *B. subtilis* [31] has demonstrated a preference for either GDP or GTP as a substrate but invariably both are accepted. Data presented in this paper details the first example of a RelA enzyme which has an explicit specificity for one of these two main pyrophosphate acceptors. This specificity was also observed under activating conditions with stalled ribosomal complexes but interestingly not when *Ft*RelA was activated with the primary alcohol methanol (Supplementary Fig. S3). The effect of methanol on protein conformation has been previously demonstrated to strengthen hydrogen bonds and weaken hydrophobic interactions [45]. Structural alterations to the synthetase active site by methanol could therefore account for the acceptance of GDP as a pyrophosphate acceptor under these conditions and suggests the EXSD motif contributes to substrate specificity along with other structural elements of the synthetase domain. The potential importance of the structural transition in a putative catalytic loop within the synthetase domain of bifunctional enzymes with an RXKD motif has previously been reported [22]. It is therefore tempting to speculate that the EXSD motif contributes to the observed substrate specificity, although mutational analysis would be required to provide evidence for this. With equal Mg^{2+} concentrations across all activity assays, the acceptance of GDP in the presence of methanol leads us to suggest that the magnesium concentration is unlikely to be the dominant factor in determining this specificity. Further work will be required to elucidate the functional significance of this catalytic loop in RelA enzymes with the unusual EXSD motif.

Kinetic analysis of the *Ec*RelA in the absence of full activation has shown typical Michaelis-Menten kinetics [22, 30]. Here we demonstrate the kinetic profile for the *Ft*RelA in the absence of activating factors fits a sigmoidal curve, and yields the derived kinetic parameters (termed V_{max} and $K_{1/2}$). Measurement of both the nucleotide products AMP and (p)ppGpp by HPLC analysis gave comparable sigmoidal fits and calculated kinetic parameters. The observed sigmoidal curve was verified by a second technique, ^{31}P NMR spectroscopy, which yielded comparable kinetic parameters when calculated using AMP production for rate determination. The global fit of data gave a sigmoidal curve with an R^2 value of 0.93 and 0.88 for ATP and GTP respectively (with a worse fit resulting from a hyperbolic curve, $R^2 = 0.85$). A sigmoidal curve has been observed for the bifunctional long RSH enzyme, Rel_{Mtb}, synthetase activity in the absence of activating factors [22]. This kinetic profile was linked to the RXKD motif found in the synthetase active site [22].

The substrate specificity and cooperative kinetic effects during (p)ppGpp synthesis by *Ft*RelA more closely resembles that of bifunctional RSH enzymes rather than that of other RelA enzymes. Collectively these data demonstrate a divergence from the current classification system used for RelA enzymes [4, 22], and may indicate that *Ft*RelA is instead an example of a new distinct subclass of RelA enzymes within the protein superfamily.

Several mechanisms can account for a sigmoidal velocity curve, including the interesting possibility of allosteric regulation [46]. In 2012, Shyp *et al*. suggested ppGpp was responsible for positive feedback regulation of *E. coli* RelA by allosteric activation [14]. ACT domains have been shown to regulate enzyme catalytic activity by the downstream effects of binding small molecules, namely amino acids [19]. The regulatory small molecule involved in binding the ACT domain of RelA enzymes has yet to be identified. *Ft*RelA is one of only three RelA enzymes to not contain this domain [4], therefore the observation of *Ft*RelA activation by ppGpp (Fig. 6) is highly indicative that ppGpp is not the regulatory ligand for RelA enzymes ACT domains.

Experiments with *Ft*RelA demonstrated that the (p)ppGpp synthetase activity could be stimulated *in vitro* by the presence of stalled ribosomal complexes formed with ribosomes from alternative species (Fig. 8), as has been observed previously for RSH enzymes [47]. This weak activation of *Ft*RelA however pales in comparison to that achieved by *Ec*RelA, with maximal activities of 701.5 ± 30.5 pmol AMP per pmol RelA per min and 2952 ± 99.14 pmol AMP per pmol RelA per min respectively (Fig. 8). Conversely strong activation of *Ec*RelA (in the range of levels previously reported [11, 13]), was observed in the presence of stalled ribosomal complexes formed with either *F. philomiragia* or *E. coli* MRE600 ribosomes (Fig. 8). This strongly suggests that the observed lower sensitivity of *Ft*RelA to ribosomal activation is genuine and not related to the quality of purified ribosomes or the use of heterologous systems for ribosomal stalling. Key residues identified in *Ec*RelA involved in ribosomal binding were amino acids 550-682 [18]. At only 647 amino acids in length, *Ft*RelA is missing ~22 of these identified residues involved in *Ec*RelA ribosomal binding [18] and this could account for the weaker activation observed for *Ft*RelA by stalled ribosomal complexes. Future studies may identify the mechanistic relationship between this truncation and the observed reduced activation of *Ft*RelA.

CONCLUSIONS

*Ft*RelA contains a variety of amino acid sequence differences when compared to a wide range of other RelA enzymes, including a truncated C-terminus and an alternative EXSD active site motif. The current model for all RelA enzymes has been based on that from *E. coli*. Here we describe the similarities and differences of *Ft*RelA compared to the accepted model. Observed differences include the specificity of *Ft*RelA for the pyrophosphate acceptor GTP (except in the presence of methanol). Furthermore, the sigmoidal steady state kinetics observed for *Ft*RelA are unlike those reported for *Ec*RelA, but similar to that observed for the bifunctional RSH enzyme Rel_{Mtb}. Conversely *Ft*RelA behaves similarly to *Ec*RelA in its apparent ability to dimerise and its activation by both stalled ribosomal complexes and the nucleotide ppGpp. Comparison of the degree of activation by stalled ribosomal complexes for *Ft*RelA and *Ec*RelAs suggest the *Francisella* enzyme is more weakly activated. A deeper understanding of the underlying reasons behind the observed lower activation and its value to *Francisella* species forms an interesting objective for future research.

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Author Contribution Statement:
Rachael Wilkinson performed the biochemical experiments. Laura Batten developed the expression system for *Ft*RelA. Neil Wells and Rachael Wilkinson carried out the ^{31}P NMR experiments. Peter Roach and Petra Oyston devised and managed the project.

Declaration of Interest: The authors declare that they have no conflict of interest.

REFERENCES

1 Jain, V., Kumar, M. and Chatterji, D. (2006) ppGpp: stringent response and survival. The Journal of Microbiology. **44**, 1-10
2 Magnusson, L. U., Farewell, A. and Nyström, T. (2005) ppGpp: a global regulator in Escherichia coli. Trends Microbiol. **13**, 236-242
3 Wu, J. and Xie, J. (2009) Magic spot: (p) ppGpp. J. Cell. Physiol. **220**, 297-302
4 Atkinson, G. C., Tenson, T. and Hauryliuk, V. (2011) The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PLoS One. **6**, e23479
5 Dean, R. E., Ireland, P. M., Jordan, J. E., Titball, R. W. and Oyston, P. C. (2009) RelA regulates virulence and intracellular survival of Francisella novicida. Microbiology. **155**, 4104-4113
6 Haralalka, S., Nandi, S. and Bhadra, R. K. (2003) Mutation in the relA gene of Vibrio cholerae affects in vitro and in vivo expression of virulence factors. J. Bacteriol. **185**, 4672-4682
7 Gao, W., Chua, K., Davies, J. K., Newton, H. J., Seemann, T., Harrison, P. F., Holmes, N. E., Rhee, H.-W., Hong, J.-I., Hartland, E. L., Stinear, T. P. and Howden, B. P. (2010) Two Novel Point Mutations in Clinical Staphylococcus aureus Reduce Linezolid Susceptibility and Switch on the Stringent Response to Promote Persistent Infection. PLoS pathogens. **6**, e1000944
8 Muller, C. M., Conejero, L., Spink, N., Wand, M. E., Bancroft, G. J. and Titball, R. W. (2012) Role of RelA and SpoT in Burkholderia pseudomallei virulence and immunity. Infect. Immun. **80**, 3247-3255
9 Alföldi, L., Stent, G. S. and Clowes, R. C. (1962) The chromosomal site of the RNA control (RC) locus in Escherichia coli. J. Mol. Biol. **5**, 348-355
10 Cashel, M. and Gallant, J. (1969) Two compounds implicated in the function of the RC gene of Escherichia coli. Nature. **221**, 838-841

11 Wendrich, T. M., Blaha, G., Wilson, D. N., Marahiel, M. A. and Nierhaus, K. H. (2002) Dissection of the Mechanism for the Stringent Factor RelA. Mol. Cell. **10**, 779-788
12 Payoe, R. and Fahlman, R. P. (2011) Dependence of RelA-mediated (p)ppGpp formation on tRNA identity. Biochemistry. **50**, 3075-3083
13 Knutsson Jenvert, R. M. and Holmberg Schiavone, L. (2005) Characterization of the tRNA and ribosome-dependent pppGpp-synthesis by recombinant stringent factor from Escherichia coli. The FEBS journal. **272**, 685-695
14 Shyp, V., Tankov, S., Ermakov, A., Kudrin, P., English, B. P., Ehrenberg, M., Tenson, T., Elf, J. and Hauryliuk, V. (2012) Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. Embo Reports. **13**, 835-839
15 English, B. P., Hauryliuk, V., Sanamrad, A., Tankov, S., Dekker, N. H. and Elf, J. (2011) Single-molecule investigations of the stringent response machinery in living bacterial cells. Proc. Natl. Acad. Sci. U. S. A. **108**, E365-373
16 Heinemeyer, E. A. and Richter, D. (1978) Mechanism of the in vitro breakdown of guanosine 5'-diphosphate 3'-diphosphate in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **75**, 4180-4183
17 Gropp, M., Strausz, Y., Gross, M. and Glaser, G. (2001) Regulation of Escherichia coli RelA requires oligomerization of the C-terminal domain. J. Bacteriol. **183**, 570-579
18 Yang, X. and Ishiguro, E. E. (2001) Dimerization of the RelA protein of Escherichia coli. Biochem. Cell Biol. **79**, 729-736
19 Grant, G. A. (2006) The ACT Domain: A Small Molecule Binding Domain and Its Role as a Common Regulatory Element. J. Biol. Chem. **281**, 33825-33829
20 Lang, E. J., Cross, P. J., Mittelstadt, G., Jameson, G. B. and Parker, E. J. (2014) Allosteric ACTion: the varied ACT domains regulating enzymes of amino-acid metabolism. Curr. Opin. Struct. Biol. **29**, 102-111
21 Aravind, L. and Koonin, E. V. (1999) Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches1. J. Mol. Biol. **287**, 1023-1040
22 Sajish, M., Kalayil, S., Verma, S. K., Nandicoori, V. K. and Prakash, B. (2009) The Significance of EXDD and RXKD Motif Conservation in Rel Proteins. The Journal of biological chemistry. **284**, 9115-9123
23 Sajish, M., Tiwari, D., Rananaware, D., Nandicoori, V. K. and Prakash, B. (2007) A Charge Reversal Differentiates (p)ppGpp Synthesis by Monofunctional and Bifunctional Rel Proteins. Journal of Biological Chemistry. **282**, 34977-34983
24 Sambrook, J. and Russell, D. (2000) Molecular Cloning: A Labroatory Manual, Cold Spring Harbour. New York
25 Cordell, R. L., Hill, S. J., Ortori, C. A. and Barrett, D. A. (2008) Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry. Journal of Chromatography B. **871**, 115-124
26 Maguire, B. A., Wondrack, L. M., Contillo, L. G. and Xu, Z. (2008) A novel chromatography system to isolate active ribosomes from pathogenic bacteria. RNA. **14**, 188-195
27 Kruger, N. J. The Bradford Method For Protein Quantitation. In The Protein Protocols Handbook. pp. 17-24
28 Aranda, P. S., LaJoie, D. M. and Jorcyk, C. L. (2012) Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis. **33**, 366-369

29 Carmona, M., Rodriguez, M. J., Martinez-Costa, O. and De Lorenzo, V. (2000) In vivo and in vitro effects of (p)ppGpp on the sigma(54) promoter Pu of the TOL plasmid of Pseudomonas putida. J Bacteriol. **182**, 4711-4718
30 Cochran, J. W. and Byrne, R. W. (1974) Isolation and Properties of a Ribosome-bound Factor Required for ppGpp and pppGpp Synthesis in Escherichia coli. J. Biol. Chem. **249**, 353-360
31 Richter, D., Fehr, S. and Harder, R. (1979) The Guanosine 3′,5′-Bis(diphosphate) (ppGpp) Cycle. Eur. J. Biochem. **99**, 57-64
32 Avarbock, D., Avarbock, A. and Rubin, H. (2000) Differential Regulation of Opposing RelMtb Activities by the Aminoacylation State of a tRNA·Ribosome·mRNA·RelMtb Complex†. Biochemistry. **39**, 11640-11648
33 Sajish, M., Kalayil, S., Verma, S. K., Nandicoori, V. K. and Prakash, B. (2009) The significance of EXDD and RXKD motif conservation in Rel proteins. J. Biol. Chem. **284**, 9115-9123
34 Segel, I. H. (1975) Enzyme kinetics : behavior and analysis of rapid equilibrium and steady state enzyme systems. pp. 353-385, Wiley, New York
35 Mechold, U., Potrykus, K., Murphy, H., Murakami, K. S. and Cashel, M. (2013) Differential regulation by ppGpp versus pppGpp in Escherichia coli. Nucleic Acids Res. **41**, 6175-6189
36 Wade, H. E. and Robinson, H. K. (1966) Magnesium ion-independent ribonucleic acid depolymerases in bacteria. Biochem. J. **101**, 467-479
37 Lancaster, L., Kiel, M. C., Kaji, A. and Noller, H. F. (2002) Orientation of Ribosome Recycling Factor in the Ribosome from Directed Hydroxyl Radical Probing. Cell. **111**, 129-140
38 Dunkle, J. A., Xiong, L., Mankin, A. S. and Cate, J. H. D. (2010) Structures of the Escherichia coli ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. Proc. Natl. Acad. Sci. U. S. A. **107**, 17152-17157
39 Tarnvik, A. (2007) WHO Guidelines on Tularaemia. Geneva: World Health Organisation
40 Meskauskas, A., Leshin, J. A. and Dinman, J. D. (2011) Chromatographic Purification of Highly Active Yeast Ribosomes. Journal of Visualized Experiments : JoVE, 3214
41 Jenvert, R. M. and Schiavone, L. H. (2007) The flexible N-terminal domain of ribosomal protein L11 from Escherichia coli is necessary for the activation of stringent factor. J. Mol. Biol. **365**, 764-772
42 Knutsson Jenvert, R. M. and Holmberg Schiavone, L. (2005) Characterization of the tRNA and ribosome-dependent pppGpp-synthesis by recombinant stringent factor from Escherichia coli. The FEBS journal. **272**, 685-695
43 Avarbock, A., Avarbock, D., Teh, J.-S., Buckstein, M., Wang, Z.-m. and Rubin, H. (2005) Functional Regulation of the Opposing (p)ppGpp Synthetase/Hydrolase Activities of RelMtb from Mycobacterium tuberculosis†. Biochemistry. **44**, 9913-9923
44 Avarbock, D., Salem, J., Li, L.-s., Wang, Z.-m. and Rubin, H. (1999) Cloning and characterization of a bifunctional RelA/SpoT homologue from Mycobacterium tuberculosis. Gene. **233**, 261-269
45 Hwang, S., Shao, Q., Williams, H., Hilty, C. and Gao, Y. Q. (2011) Methanol Strengthens Hydrogen Bonds and Weakens Hydrophobic Interactions in Proteins – A Combined Molecular Dynamics and NMR study. The Journal of Physical Chemistry B. **115**, 6653-6660

46 Hammes, G. G. and Wu, C. W. (1974) Kinetics of Allosteric Enzymes. Annu. Rev. Biophys. Bioeng. **3**, 1-33
47 Lienert, I. and Richter, D. (1981) Sringent Control in an Extreme Thermophilic Bacterium. Preliminary Characterization of a Guanosine 3′,5′-Polyphosphate [(p)ppGpp] Synthesizing Enzyme from Thermus thermophilus. Journal of General Microbiology. **123**, 383-386

FIGURE LEGENDS

Figure 1. The extended hopping model for RelA activation, derived from the proposal of English *et al*. [15]. Under conditions of amino acid starvation, unacylated-tRNA accumulates and binds to the acceptor (A) site of the ribosome, leading to stalling. This results in the release of RelA from the ribosome in an activated conformation, which catalyzes the formation of (p)ppGpp. (B) Domain structure and corresponding amino acids for *Ec*RelA and *Ft*RelA. (C) Alignment of RelA enzymes synthetase domain active site motif from pathogenic bacteria.

Figure 2. The purification of *Ft*RelA by sequential Ni-IDA and size exclusion chromatography steps. (A) Absorption trace (280 nm) for the Ni-IDA purification of *Ft*RelA. (B) SDS-PAGE analysis of *Ft*RelA Ni-IDA purification. Lanes: 1, cleared lysate; 2, flow through; 3-7, eluate fractions from Ni-IDA chromatography. (C) Absorption trace (280 nm) for the size exclusion (Superdex 200) chromatography purification of *Ft*RelA. (D) SDS-PAGE analysis of *Ft*RelA purification. Lanes: 1-8, eluate fractions from size exclusion chromatography; those marked with * correspond to the peak similarly marked in panel C.

Figure 3. Analyzing the multimeric state of purified *Ft*RelA. (A) Absorption trace (280 nm) for size exclusion chromatography (Superdex 200) yielding a single peak with an apparent molecular weight of 128 ± 1.57 kDa corresponding to the dimeric state of the enzyme (calculated molecular mass of dimer 148 kDa). (B) Calibration curve for apparent molecular weight determination.

Figure 4. Substrate specificity of *Ft*RelA. *Ft*RelA activity assays, with either GTP (solid black line) or GDP (dashed green line) as the pyrophosphate acceptor, were stopped after 1 hour and analysed by IP RP-HPLC.

Figure 5. Kinetic analysis of *Ft*RelA Activity. (A) Time courses of AMP formation at varying GTP concentrations in *Ft*RelA activity assays determined by HPLC analysis; 0.25 mM (red), 0.4 mM (blue), 0.75 mM (green), 1 mM (lilac), 1.25 mM (orange), 1.75 mM (black), 2.75 mM (violet). (B) Saturation activity curves for ATP substrate, measured using HPLC analysis. (C) Saturation activity curves for GTP substrate, measured by HPLC analysis. (D) Lineweaver-Burk plot for *Ft*RelA calculated for ATP (black line) and GTP (green line) when analysed by IP RP HPLC. (E) Saturation activity curves for ATP substrate, measured by ^{31}P NMR analysis. (B) Saturation activity curves for GTP substrate, measured by ^{31}P NMR analysis. All saturations curves using rates derived from AMP formation are denoted with a black line and those derived from (p)ppGpp formation are denoted with a green line.

Figure 6. Activation of *Ft*RelA by potential small molecule activators. (A) Graph shows enzyme activity when incubated with low (10 μM), medium (100 μM) and high concentrations (1000 μM) of AMP (black), inorganic phosphate (white), or ppGpp (grey) in addition to substrates. (B) Dose-response (variable slope) curve for activation of *Ft*RelA by ppGpp.

Figure 7. Purification of *F. philomiragia* ribosomes by SulfoLink-cysteine chromatography. (A) Absorption traces at 260 nm (blue trace) and 280 nm (red trace) for fractions collected during purification. (B) SDS-PAGE analysis of *F. philomiragia* ribosome purification. Lanes:

1, cleared lysate; 2, flow through; 3-12, eluate fractions from SulfoLink-cysteine chromatography; those marked with * relate to the peak similarly marked in (A).

Figure 8. Activation of RelA by stalled ribosomal complexes. Activation of *Ft*RelA and *Ec*RelA by stalled ribosomal complexes comprised of either *F. philomiragia* ribosomes or *E. coli* MRE600 ribosomes, compared to basal RelA activity.

TABLES

Table 1. Kinetic parameters derived from HPLC analysis of *Ft*RelA activity assays for GTP and ATP substrates. R^2 is a measure of goodness of fit.

Substrate	ATP		GTP	
Product Measured	AMP	(p)ppGpp	AMP	(p)ppGpp
V_{max}/ x10^{-3} s^{-1}	9.46 ± 0.77	9.83 ± 0.82	10.59 ± 0.89	11.54 ± 1.5
$K_{1/2}$/ μM	259 ± 37.2	332 ± 47.8	800.7 ± 115.6	1095 ± 183.8
h	2.17 ± 0.7	2.71 ± 0.96	2.18 ± 0.53	3.07 ± 1.29
R^2	0.95	0.94	0.97	0.93

Table 2. Calculated Kinetic Parameters for *Ft*RelA when a global fit is applied to both ^{31}P NMR (AMP) and IP RP HPLC (AMP and pppGpp) data sets. R^2 is a measure of goodness of fit.

Substrate	ATP	GTP
V_{max}/ x10^{-3} s^{-1}	10.79 ± 0.59	12.6 ± 1.19
$K_{1/2}$/ μM	344.3 ± 33.95	1026 ± 134.5
h	2.26 ± 0.44	2.58 ± 0.70
R^2	0.932	0.881