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Video Article

A Purification and *In Vitro* Activity Assay for a (p)ppGpp Synthetase from *Clostridium difficile*

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Abstract

Kinase and pyrophosphokinase enzymes transfer the gamma phosphate or the beta-gamma pyrophosphate moiety from nucleotide triphosphate precursors to substrates to create phosphorylated products. The use of γ -³²P labeled NTP precursors allows simultaneous monitoring of substrate utilization and product formation by radiography. Thin layer chromatography (TLC) on cellulose plates allows rapid separation and sensitive quantification of substrate and product. We present a method for utilizing the thin-layer chromatography to assay the pyrophosphokinase activity of a purified (p)ppGpp synthetase. This method has previously been used to characterize the activity of cyclic nucleotide and dinucleotide synthetases and is broadly suitable for characterizing the activity of any enzyme that hydrolyzes a nucleotide triphosphate bond or transfers a terminal phosphate from a phosphate donor to another molecule.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58547/>

Introduction

Kinase and pyrophosphokinase (or diphospho-kinase) enzymes transfer phosphates from nucleotide triphosphate (NTP) precursors to substrate molecules. The substrates can include other nucleotides, amino acids or proteins, carbohydrates, and lipids¹. Bioinformatic analyses can sometimes predict an enzyme's cognate substrate or substrates based on the similarity to characterized enzymes, but experimental validation is still necessary. Similarly, the affinity of an enzyme for its substrate(s) and the rate at which it catalyzes the phosphor-transfer reaction, and the effects of co-factors, inhibitors, or other enzyme effectors must be determined experimentally. To avoid depletion of the ATP precursor by other ATP-consuming enzymes present in bacterial cytoplasm, quantitative activity assays require purified protein.

Protein purification by metal affinity chromatography has been covered thoroughly in the literature^{2,3}. Histidine tags consisting of six consecutive histidine residues appended to the N- or C-terminus of a recombinant protein allow rapid purification by metal affinity chromatography^{4,5,6}. These sequences are small compared to the proteins they modify and typically have a minimal effect on protein function, although they can sometimes alter protein stability and/or enzyme kinetics^{7,8}. Histidine tags at the N- and C-termini of the same protein can have different effects, which are difficult to predict without knowing the structure of the protein in question. Histidine tags are typically incorporated during the cloning of a recombinant protein by designing primers that encode six histidine residues, either immediately 3' to the ATG start codon or immediately 5' to the stop codon of the open reading frame. After amplification, the hexahistidine-containing gene is ligated into a vector under the control of an inducible promoter and expressed, typically in a laboratory strain of *E. coli*. The recombinant protein can then be isolated on an affinity resin containing immobilized divalent cations (typically nickel or cobalt)⁹. Contaminating native metal-binding proteins can be removed by titration with imidazole, which competitively displaces bound protein². Finally, the target protein is eluted from the column with higher concentrations of imidazole. There are several commercial sources for immobilized metal cation resins, and the manufacturers provide recommendations for the buffer conditions and imidazole concentrations. After elution, protein may be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dialyzed, or used immediately in functional assays.

There are several methods to indirectly monitor kinase activity by coupling ATP phosphate bond hydrolysis to a second reaction that releases or excites a fluorophore or generates chemiluminescence, but these reactions have multiple moving parts and can be logistically challenging¹⁰. The most straightforward way to specifically measure phosphor-transfer activity is to directly monitor the transfer of a radiolabeled phosphate group from a commercially available γ -³²P NTP precursor to a non-radiolabeled substrate^{11,12,13}. Mixtures of radiolabeled substrates and products can be separated and quantified by thin layer chromatography (TLC). TLC utilizes the differential mobility of solutes in a given solvent by allowing the solvent (liquid phase) to migrate by capillary action across a surface (solid phase) upon which a mixture of solutes has been adsorbed¹⁴. Solute that are small and/or lack favorable interactions with the solid phase will migrate longer distances from their initial location than solutes with higher molecular weights or great affinities for the solid. For examination of phosphor-transfer, phosphate moieties increase the molecular weight of molecules they are added to, and add negative ionic charge at neutral or acidic pH^{11,12,14}. This decreases their mobility on a basic surface such as PEI-cellulose. When developed in acidic potassium phosphate buffer, mixtures of mono-, di-, tri-, tetra-, and pentaphosphate

species can be readily separated on PEI-cellulose, allowing quantification of each species (**Figure 2, Figure 3**). Such assays can be performed using cell lysates containing the enzyme of interest, but this includes the potential for the activity of other kinases, phosphatases, and general ATPases to deplete the substrate and/or product. For a quantitative *in vitro* assessment of enzyme activity, it is necessary to purify the enzyme of interest.

Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) are ribonucleotide signaling molecules formed by the transfer of a pyrophosphate group from an adenosine triphosphate (ATP) precursor to, respectively, a guanosine diphosphate (GDP) or guanosine tetraphosphate (GTP) substrate¹⁵. These single ribonucleotide signals, collectively known as (p)ppGpp, mediate a cell-wide response to environmental stress known as the stringent response in diverse bacterial species^{15,16}. Two conserved classes of enzymes catalyze the formation of (p)ppGpp^{15,17}. Rel/Spo homolog (RSH) enzymes are 'long' bifunctional (p)ppGpp synthetase/hydrolases named for their similarity to the RelA and SpoT (p)ppGpp metabolic enzymes from *Escherichia coli* which contain synthetase, hydrolase, and regulatory domains, while small alarmone synthetase (SAS) enzymes are short monofunctional synthetases found exclusively in Gram positive bacteria^{15,17,18}. The spore-forming Gram-positive bacterium *Clostridium difficile* encodes putative RSH and SAS genes¹⁹. Here, we present initial activity assays that confirm that the *C. difficile* RSH enzyme is a catalytically active (p)ppGpp synthetase.

Protocol

1. Inducible Overexpression of a Histidine-tagged Protein

- Amplify *rsh* from *C. difficile* R20291 genomic DNA.
 - Use a high-fidelity polymerase and follow the manufacturer's instructions.
 - Amplify *C. difficile rsh* using primers *rsh_F*(CAGGTACCGGTTATATGCATGATAAAGAATTACAAG) and *rsh_R*(CCCTGCAGCTAATGGTGATGGTGATGGTGATTTGTCATTCTATAAATAC), which introduces a C-terminal hexahistidine tag. NOTE: The KpnI and PstI cut sites in the primer sequences are bolded.
 - Digest the pMMBneo vector and the *rsh-his6* PCR product with PstI and KpnI restriction cut sites at 37 °C for 45 min.
 - Purify the linearized vector and the PCR fragments via agarose gel electrophoresis and subsequent purification with a DNA gel extraction kit.
 - Measure the 280 nm absorbance (A_{280}) of the vector and the amplified PCR product. Use the equation

$$[DNA] \frac{\mu g}{mL} = A_{260} \times dilution\ factor \times 50$$
 to determine the DNA concentration of each DNA fragment.
- Ligate *rsh* into pMMBneo for expression.
 - Combine 25 ng of digested PMMBneo vector, 125 ng of *rsh-his6* gene product, 2 μ L of 10x ligase buffer, and 1 μ L of DNA ligase. Use the nuclease free water to adjust the total volume to 20 μ L. Incubate at 16 °C for 16 h. NOTE: The efficacy of ligation depends on fragment size and must be adjusted based on manufacturer protocols.
 - Transform the ligated vector product into *E. coli* DH5- α or another *recA*- plasmid maintenance strain. Select for transformed cells on LB plates with 100 μ g/mL kanamycin and incubate for 16–24 h at 37 °C.
 - Pick four colonies from the transformation plate (s) and streak each on a fresh plate containing 100 μ g/mL kanamycin for DNA isolation. Incubate the new plates at 37 °C for 16–24 h and select one isolate for the subsequent protein expression.
 - To confirm the successful ligation of the intact *rsh* protein coding region, pick a colony of the chosen isolate into 20 μ L of water, heat at 95 °C for 10 min, and use as a template for a confirmatory PCR using the same primer used to amplify the gene.
- Transform the verified plasmid according to standard transformation protocols for *E. coli* bacterial plasmid transformation into *E. coli* BL21 system for high yield protein production.
- Express RSH-His6 in *E. coli*.
 - Select a single colony of *E. coli* BL21 transformed with pMMBneo::*rsh* and inoculate 2 mL of LB medium with 50 μ g/mL kanamycin. Incubate at 37 °C 12–16 h while shaking at 250 rpm.
 - Inoculate 500 mL of LB medium containing 50 μ g/mL kanamycin with 0.5 mL of the overnight culture for cell growth and protein expression.
 - Incubate the expression culture at 37 °C and 250 rpm in an incubator shaker until the cell density reaches an OD₆₀₀ 0.167 at 37 °C.
 - Reduce the incubator temperature to 30 °C and wait 30 min for the culture temperature to drop.
 - Induce RSH expression by adding isopropyl β -D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. Allow the induction to take place overnight (for 16–18 h) at 30 °C, shaking at 250 rpm.
 - Pellet by centrifugation at 3,080 x g for 30 min at 4 °C. NOTE: The pellet can be stored overnight at -20 °C for purifying target protein the following day without noticeable loss of protein yield or enzymatic activity.

2. Protein Purification by Nickel Affinity Chromatography

NOTE: Continue directly with protein purification steps provided below after clarifying the cell lysate. Storing clarified lysate at 4 °C overnight for subsequent protein purification reduces the protein yield.

- Purify protein using 1 mL of Nickel-Nitriloacetic Acid (Ni-NTA) resin on a gravity column.

1. The day before use, equilibrate the column overnight at 4 °C with 2 mL of equilibration buffer (10 mM Tris-HCl pH 7.79, 300 mM NaCl, 50 mM NaH₂PO₄, 0.5 mg/mL lysozyme, 5 mM MgCl₂, 10 mM imidazole, 0.25 mM DTT, 5 mM phenylmethane sulfonyl fluoride (PMSF), 10% glycerol).
 2. The following day bring the column from 4 °C to RT prior to loading the clarified lysate and let it stand for ~2–3 h.
NOTE: Bringing the column to thermal equilibrium is crucial to avoid air bubbles forming within the column.
 3. Resuspend the pellet obtained in step 1.3.6 in the lysis buffer (10 mM Tris-HCl pH 7.8, 300 mM NaCl, 5 mM MgCl₂, 50 mM NaH₂PO₄, 10% glycerol, 0.5 mg/mL lysozyme, 10 mM imidazole, 0.25 mM DTT and 5 mM PMSF).
 4. Sonicate cells on ice for 8 x 10 s intervals, pausing 30 s between pulses.
 5. Clarify the lysate by centrifugation at 3,080 x g for 30 min at 4 °C using a microcentrifuge.
 6. Prepare lysate with equal volumes of lysis buffer then apply the prepped clarified lysate to the column and collect the flow-through.
 7. Reapply clarified lysate flow-through to the column and collect the secondary flow-through.
 8. Wash the column with wash buffer 1 (10 mM Tris-HCl (pH 7.79), 300 mM NaCl, 5 mM MgCl₂, 50 mM NaH₂PO₄, 30 mM imidazole, 10% glycerol). Collect the flow-through.
NOTE: Inclusion of 5 mM MgCl₂ in the wash and elution buffers is important for enzymatic activity of the purified protein.
 9. Wash the column with wash buffer 2 (10 mM Tris-HCl (pH 7.79), 300 mM NaCl, 5 mM MgCl₂, 50 mM NaH₂PO₄ and 50 mM imidazole). Collect the flow-through.
 10. Apply 2 mL elution buffer (10 mM Tris-HCl (pH 7.79), 300 mM NaCl, 50 mM NaH₂PO₄, 10% glycerol and 75 mM imidazole). Collect flow-through in two fractions of 1 mL each.
NOTE: The column after this step can be stored in equilibration buffer at 4 °C if the same protein will be purified in the next purification assay. The column can be used up to 3 times if stored properly.
2. Assess column fractions for purity by SDS-PAGE.
 1. To qualitatively assess protein purification, run 20 µL aliquots of all column fractions on a 4%/10% polyacrylamide gel for 60 min at 170 V.
 2. Stain the gel with 0.1% Coomassie blue at room temperature for 5 h, rocking gently on a benchtop rocker.
 3. Destain the gel in 40% methanol, 10% glacial acetic acid overnight at room temperature, rocking on a benchtop rocker.
NOTE: A representative gel is pictured in **Figure 1**.
 3. Dialyze elute fraction 2 overnight at 4 °C.
 1. Dialyze against dialysis buffer (15.7 mM Tris-HCl (pH 7.6), 471.9 mM NaCl, 15.69 mM MgCl₂, 1.57 mM DTT, 1.5 mM PMSF, and 15.7% glycerol) at a 200:1 ratio using a 1 mL dialysis device with a 20 kDa molecular weight cut-off (MWCO).
 2. Determine the concentration of dialyzed protein sample by measuring absorbance at 280 nm and using the calculated molar extinction coefficient 82085 M⁻¹cm⁻¹²⁰.
 3. Store 5 µL aliquots of the dialyzed protein sample in aliquots at -80 °C until use.

3. Protein Activity Assay by Thin Layer Chromatography

1. Prepare the thin layer chromatography plate.
 1. Prior to performing the reaction, prepare polyethyleneimine (PEI)-cellulose plates by washing in deionized water. Place the plates in a glass chamber with double distilled water to a depth of ~0.5 cm.
 2. Allow water to migrate to the top of the plate.
NOTE: Washing the plates is not strictly necessary, as plates may be used without it, but washing does increase the clarity of resulting images. Washing the plates in two perpendicular directions further ensures that any contaminants present in the resin are isolated in one corner of the plate (**Figure 2**).
 3. Bring the plates out of the glass chamber and leave on a benchtop rack to dry overnight (12–18 h).
 4. Mark the dry plates 2.0 cm from one edge with a soft pencil to indicate where the samples will be applied for TLC. For 2 µL samples, apply samples no less than 1.0 cm apart (**Figure 2**).
NOTE: This allows clear separation between adjacent spot, which is critical for signal quantification. As long as the cellulose resin is not scratched, small pencil marks on the surface will not interfere with solvent migration.
 5. When planning experiments, always leave one spot on each plate unused.
NOTE: This will provide a blank lane for sample quantification (**Figure 2**). A 20 cm TLC plate will have room for 19 spots.
2. Enzyme activity assay
 1. Prepare a 5x buffer mix containing 50 mM Tris-HCl (pH 7.5), 25 mM ammonium acetate, 10 mM KCl, 1 mM DTT and 0.6 mM ATP.
NOTE: This mix may be prepared in large quantities and frozen in 10 µL aliquots for later use. Do not subject mix to multiple freeze-thaw cycles.
 2. Prepare individual reactions containing 3 µM RSH, 1x buffer mix, 0.6 mM GDP, 1.2 mM MgCl₂. Add 1.0 µCi of γ-³²P-ATP per 10 µL of reaction and use nuclease free water to bring the reaction up to a desired volume. Add the RSH after the other components have been mixed, as the addition of RSH to the nucleotide-containing mix initiates the enzymatic activity assay.
NOTE: Final reaction volume will depend on the number of timepoints sampled. To sample 2 µL/timepoint, assemble 10 µL of reaction mixture for each 4 timepoints.
 3. To control for ATP hydrolysis from contaminating nuclease activity, assemble a 10 µL reaction containing no protein and incubate it in parallel. Spot 2 µL samples at t = 0 and at the end of the experiment to ensure that ATP was not hydrolyzed in the absence of protein.
 4. Immediately upon addition of RSH, remove 2 µL and spot it onto the labeled PEI-cellulose plate as the t = 0 min sample.
 5. Incubate the reaction at 37 °C, removing 2 µL aliquots at desired timepoints.
NOTE: Enzymatic activity will cease when the sample is adsorbed onto the cellulose plate. Wait 10–30 min after the last spot is added to plate before development to ensure complete adsorption and sample drying.
3. Thin layer chromatography

1. Fill the chromatography chamber with 1.5 M 1.5 M KH_2PO_4 (pH 3.64) to a depth of 0.5 cm.
NOTE: The volume needed will depend on the dimensions of the chromatography tank. Any glass container with a level bottom that is wide enough to allow insertion of the TLC plate without bending may be used as a developing tank with the addition of a cover. The TLC plate can be cut into narrower strips with a clean razorblade to enable development in a glass beaker covered in plastic film.
2. Immerse the bottom edge of the plate in solvent. Allow the solvent to migrate to the top of the plate (~90 min).
NOTE: While solvent migration will halt at the top of the plate and samples will not be lost or run together during a longer immersion, plates should not be left in solvent overnight. Immersions longer than 4 h can cause the resin to detach from the plate backing and result in loss of signal.
3. Remove the plate from the chromatography tank and place it on a benchtop drying rack.
4. Allow the plate to air dry overnight.
NOTE: Drying may be accelerated by the use of a hair drier. Dryness can be assessed by the color of the resin, which will darken when wet and return to the color of an unused plate when completely dry.
5. After the plate is dry, wrap the plate in plastic film to avoid transfer of radioactive material to the imaging cassette and analyze by autoradiography (**Figure 3**).

4. Data analysis

1. Expose the PEI-cellulose plate containing separated reactions to a phosphorimager cassette for 4 h at room temperature.
NOTE: This is sufficient exposure to yield a very clear image using the indicated concentrations of fresh $\gamma\text{-}^{32}\text{P}\text{-ATP}$. If lower amounts of radiolabelled substrate are used, exposure time can be increased to 12-16 h.
2. Image the cassette on a phosphorimager.
3. Using imaging software with a graphical user interface, draw Regions of Interest (ROIs) by selecting **Draw Rectangle** and using the mouse to draw rectangular ROIs around one entire lane and the ATP and ppGpp spots contained within that lane (**Figure 3**).
4. Use the **Select, Copy, and Paste** commands (or corresponding commands based on the imaging software being used) to draw identical ROIs within the other lanes to ensure that the ROIs are measuring signal within identical areas in each lane. Include ROIs from an unused lane, to be used as blanks.
5. Using the **Analyze | Tools | ROI Manager | Add** commands of the imaging software, select all of the ROIs drawn on the PEI cellulose plate.
6. Using the **Analyze | Set Measurements | Measure** commands, quantify the signal intensity within each ROI and export the measurements as a spreadsheet (**Figure 3**). Subtract blank ROI values from experimental signals.
7. Calculate what percentage of the blanked signal within each lane is attributable to ATP and ppGpp using the formulas

$$\% \text{ ATP} = \frac{\text{blanked ATP}}{\text{blanked total signal}} \times 100 \text{ and } \% \text{ ppGpp} = \frac{\text{blanked ppGpp}}{\text{blanked total signal}} \times 100.$$

NOTE: ROIs may be drawn and quantitated using commercial software compatible with the phosphorimager or freely available ImageJ software (National Institutes of Health).

Representative Results

We present a method for the affinity purification of a (p)ppGpp synthetase from *Clostridium difficile* and the assessment of its enzymatic activity. **Figure 1** demonstrates the protein purification achieved by metal affinity chromatography. The second elution (E2) fraction from this purification was dialyzed and used for the enzymatic activity assay. **Figure 2** details the necessary steps to prepare for and carry out pyrophosphotransferase assays by thin layer chromatography. **Figure 3** illustrates how data from these experiments is quantitated with an emphasis on appropriate blanking and conversion of signal intensity to percentages. In **Figure 4A**, we present a representative TLC autoradiograph of a (p)ppGpp synthetase assay using purified RSH, a *C. difficile* (p)ppGpp synthetase. The initial spot location, ppGpp, and ATP spots are clearly visible on the autoradiograph, as is the solvent front that is visible because it contains trace amounts of radiolabeled inorganic phosphate. Molecules with more phosphoric acid moieties exhibit less mobility from the initial spot location, as they have greater molecular weights and more negative ionic charge; this impedes their mobility on the basic PEI-cellulose. Over the course of 120 min at 37 °C, ppGpp accumulation and ATP depletion are negligible in reactions lacking the synthetase enzyme, while both ATP depletion and ppGpp accumulation are readily apparent in the presence of RSH **Figure 4A**). **Figure 4B** shows the absolute ATP and ppGpp signals from four experiments. **Figure 4C** shows the same data as **Figure 4B** with the absolute signals converted to percentages of the total radioactive signal. This minimizes inaccuracies due to pipetting error and/or radioactive decay and allows data from experiments performed on different days to be pooled without introducing uncertainty to the data.

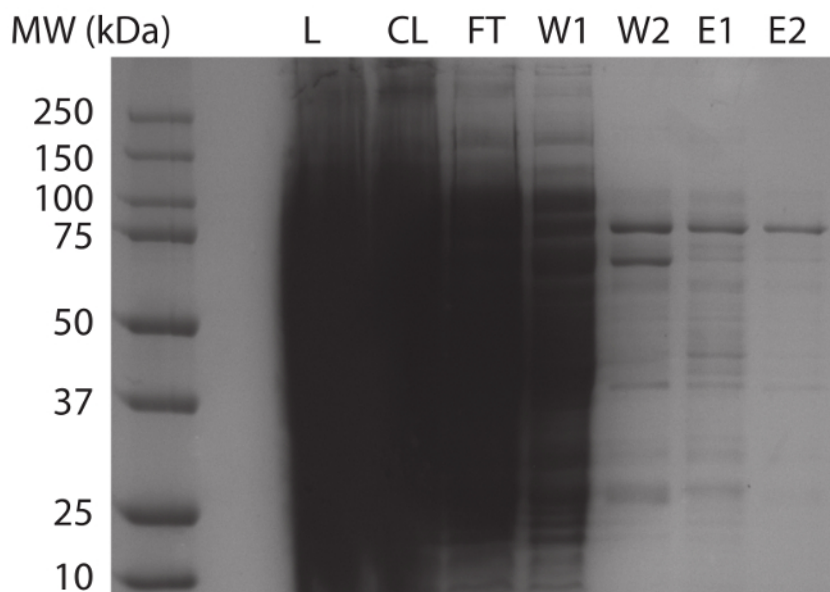


Figure 1: Nickel affinity purification of RSH. A Coomassie-stained SDS-PAGE gel showing lysate (L) and centrifuged lysate (CL) of induced BL21 pMMB::*rsh-his6* as well as the flow-through (FT), wash 1 (W1), wash 2, and elution (E1 and E2) fractions after nickel affinity purification. The E2 fraction was dialyzed and used for subsequent enzymatic assays. Molecular weights of protein size standards are shown on the right. [Please click here to view a larger version of this figure.](#)

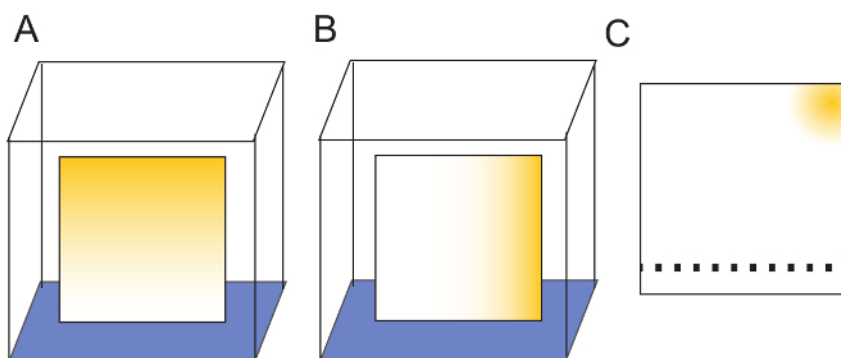


Figure 2: Preparation of TLC plates. (A) Plates are washed in one dimension by placing the bottom edge in water (blue). Contaminants (yellow) migrate to the top of the plate with the solvent. (B) After a plate is dried completely, it is washed in a second dimension by rotating it 90° relative to the first wash and again allowing water to migrate to the top of the plate. (C) After washing, any contaminants are isolated in one corner of the plate. The resin of a washed TLC plate may be marked gently with a soft pencil to indicate where samples should be spotted. For 2 μ L samples, a minimum of 1 cm between spots will ensure adequate sample separation. Samples are spotted 2 cm from the 'bottom' of the plate. After sample application, solvent is allowed to run to the 'top,' where any contaminants will have been isolated by the water washes. [Please click here to view a larger version of this figure.](#)

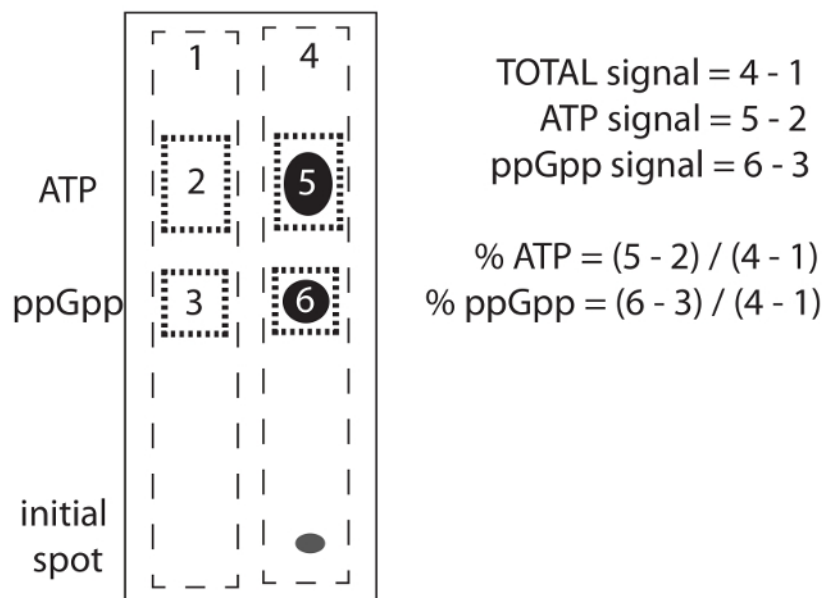


Figure 3: Signal quantification. Regions of interest (ROIs) defining the total, ATP, and ppGpp signal are shown for a blank lane and an experimental lane. Signal intensity within each blank ROI is subtracted from the experimental value, and the ATP and ppGpp signals are normalized to the total signal using the equations shown to present the percentage of the total radioactive signal attributable to ATP and ppGpp. [Please click here to view a larger version of this figure.](#)

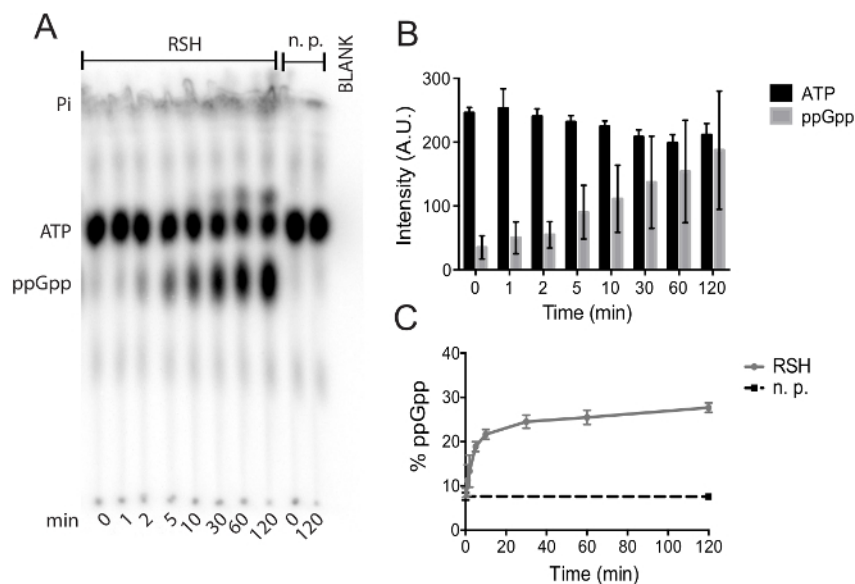


Figure 4. Autoradiograph of a representative TLC plate: (A) This image shows the solvent front, ATP, ppGpp, and initial spot locations of a reaction carried out using purified *C. difficile* RSH. A control reaction containing no protein (n.p.) allows quantification of uncatalyzed ATP hydrolysis while a blank lane allows accurate signal quantification. (B) Absolute signal intensities of ATP and ppGpp during 120 minutes of incubation with *C. difficile* RSH. Shown are the means and standard deviations of four independent experiments. (C) The same data from (B) with the ppGpp signal presented as a percentage of the total radioactive signal. ppGpp accumulation in the no protein (n.p.) control reaction is shown in black. Shown are the means and standard deviations of two independent experiments with two technical replicates each. [Please click here to view a larger version of this figure.](#)

Discussion

Here we report the purification of His-tagged RSH from *C. difficile* and present a method for activity quantification using radiolabeled thin layer chromatography. This method has previously been used to assess the activity of diguanylate cyclase enzymes from *C. difficile*, as well as

(p)ppGpp synthetase, nucleotide cyclase, kinase and phosphodiesterase enzymes from other organisms^{11,12,13,21}. While the method is not novel, it is broadly applicable to many types of assay, and we hope researchers will find its presentation in video format helpful.

The most critical steps within the protocol are the protein purification (steps 2.1.3–2.1.10), reaction preparation for thin layer chromatography (step 2.2–2.3), and data analysis (step 3.4). We have found the following modifications to be especially helpful: the addition of MgCl₂ to the buffers used for nickel column purification (steps 2.1.3–2.1.10) is crucial for the enzymatic activity of the purified protein, and the presentation of the enzyme activity data as % ppGpp produced rather than absolute ppGpp produced ensures that data collected on different days, with different of γ -³²P-ATP are consistent. This method is accessible to any research group with access to a phosphorimager, and the data analysis is straightforward. By quantitating phosphotransfer activity as a percentage of ³²P converted to ppGpp, we ensure data reproducibility. Because very small reaction volumes are spotted onto the PEI-cellulose plates, there is significant potential for slight pipetting inaccuracies to introduce significant error in the absolute quantity of γ -³²P-ATP or ³²P-ppGpp in a given lane on the TLC plate, but the distribution of the radioactive signal between the possible forms is independent of the total signal present. In addition, the total radioactive signal in a given lane can depend on the age of the γ -³²P-ATP used in the experiment. ³²P has a half-life of 14.3 days, so independent assays performed several days apart can show substantial differences in the total radioactive signal detected but the relative amounts of radiolabeled ATP and ppGpp depend only on enzyme activity. Presenting the data as 'percent ppGpp' rather than absolute ppGpp signal prevents the introduction of random noise from pipetting error or radioactive decay. This is illustrated by the differences between **Figure 4B** and **4C**. Both display the means and standard deviations of the data from the same four experiments, but the data in **Figure 4C** has been normalized to the total radioactive signal.

We have determined that the *C. difficile* RSH enzyme is a functional ppGpp synthetase *in vitro*, capable of rapid pyrophosphotransfer from an ATP phosphodonator and a GDP acceptor. This reaction is dependent on magnesium, which coordinates ATP and guanosine binding by RSH family enzymes²². We have modified the manufacturer protocols for nickel affinity chromatography to include 5 mM MgCl₂ in the lysis and wash buffers as well as the elution buffer because we have found that purification in the absence of magnesium is detrimental to the enzymatic activity of the purified protein. This suggests that magnesium ions may play a non-catalytic role in stabilizing protein structure in the absence of nucleotide binding, but further structural characterization will be necessary to confirm this.

To our knowledge, this work is the first published report of ppGpp synthesis in *C. difficile* and indicates that this organism is likely to utilize a (p)ppGpp-mediated stringent response to survive extracellular stress. (p)ppGpp metabolism has never before been reported in this important human pathogen. Given that the stringent response is implicated in persistence in many other pathogens, it is likely that (p)ppGpp-mediated signaling may play a role in the high stress tolerance of *C. difficile* cells and the high recurrence rate of *C. difficile* infection^{23,24,25}.

Disclosures

The authors declare no competing financial interests or other conflicts of interest.

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