The nucleotide messenger (p)ppGpp is an anti-inducer of the purine synthesis transcription regulator PurR in *Bacillus*

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ABSTRACT

The nucleotide messenger (p)ppGpp allows bacteria to adapt to fluctuating environments by reprogramming the transcriptome. Despite its well-recognized role in gene regulation, (p)ppGpp is only known to directly affect transcription in Proteobacteria by binding to the RNA polymerase. Here, we reveal a different mechanism of gene regulation by (p)ppGpp in Firmicutes: (p)ppGpp directly binds to the transcription factor PurR to downregulate purine biosynthesis gene expression upon amino acid starvation. We first identified PurR as a receptor of (p)ppGpp in *Bacillus anthracis*. A co-structure with *Bacillus subtilis* PurR reveals that (p)ppGpp binds to a PurR pocket reminiscent of the active site of phosphoribosyltransferase enzymes that has been repurposed to serve a purely regulatory role, where the effectors (p)ppGpp and PRPP compete to allosterically control transcription. PRPP inhibits PurR DNA binding to induce transcription of purine synthesis genes, whereas (p)ppGpp antagonizes PRPP to enhance PurR DNA binding and repress transcription. A (p)ppGpp-refractory purR mutant in *B. subtilis* fails to downregulate purine synthesis genes upon amino acid starvation. Our work establishes the precedent of (p)ppGpp as an effector of a classical transcription repressor and reveals the key function of (p)ppGpp in regulating nucleotide synthesis through gene regulation, from soil bacteria to pathogens.

INTRODUCTION

Nucleotide second messengers allow organisms to rewire transcription to reprogram physiology and lifestyle for survival in changing environments. In bacteria, nucleotide second messengers, such as the cyclic nucleotides cAMP, c-di-AMP and c-di-GMP, regulate transcription by binding to transcription factors as effectors and allosterically affecting the transcription factor’s binding to DNA regulatory elements (1–8). For example, cAMP, the key regulator of catabolite repression, was one of the first identified allosteric effectors of transcription, establishing the paradigm of gene regulation (9).

In contrast to cyclic nucleotides, another group of signaling nucleotides is the linear ‘alarmone’ nucleotides, including the ubiquitous nucleotide messengers ppGpp and pppGpp (collectively (p)ppGpp). (p)ppGpp is necessary for bacteria to adapt to environmental changes from nutrient availability to antibiotic assault (10–12). Since its discovery a half century ago, (p)ppGpp was best known for its profound role in gene regulation: downregulating the transcription of rRNA and tRNA operons and modulating global transcription in bacteria. (p)ppGpp binds RNA polymerase as well as the interface between RNA polymerase and the transcription factor DksA in Proteobacteria, and it binds the virulence regulator complex MglA-SspA in *Francisella tularensis* (13,14). However, unlike cyclic nucleotide second messengers, (p)ppGpp has not been known to act as an allosteric effector of a canonical promoter-binding transcription factor in bacteria.

In addition, (p)ppGpp has only been shown to regulate transcription directly in Proteobacteria (13–15). In other bacterial phyla, no transcription proteins have been shown to be directly regulated by (p)ppGpp. In Firmicutes, including the soil bacterium *Bacillus subtilis* and the pathogen *Bacillus anthracis*, (p)ppGpp affects the transcription of

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hundreds of genes, but the only known mechanism is indirect: via control of levels of GTP, which is the initiating nucleotide for ribosomal RNA genes and is an effector for the global regulator CodY (16–18). Therefore, the pervasive-ness of (pppGpp)’s role in direct gene expression remains unclear.

Here, we report the first example of (pppGpp) as an allosteric effector of a canonical transcription regulator, and the first example of transcription under direct control of (pppGpp) in bacterial species beyond the Proteobacteria phylum. We found that (pppGpp) directly regulates transcription in Bacillus by binding to the transcription regulator PurR and serving as its effector. PurR controls expression of de novo and salvage purine nucleotide biosynthesis genes in response to availability of external nucleobases (19,20). Here, we show that PurR also responds to amino acid starvation via (pppGpp) induction, which strongly increases PurR repression of purine nucleotide biosynthesis genes. PurR proteins across Firmicutes comprise a DNA binding domain and a regulatory domain resembling phosphoribosyltransferase (PRT) enzymes. PRT enzymes, including HPRT and XPRT, are purine salvage enzymes that use the substrate 5-phosphoribosyl-1-pyrophosphate (PRPP) to produce purine nucleotides such as GMP, XMP and IMP. However, the PRT domain on PurR lacks enzymatic activity and serves a purely regulatory role by binding PRPP to de-repress transcription (20,21). Our structural and biochemical analyses reveal that (pppGpp) also binds to the PRT regulatory domain and competes with PRPP to promote PurR binding to the promoter DNA, thus serving as a PurR anti-inducer. Our study highlights the conservation of the role of signaling nucleotides as allosteric effectors of gene regulation, not only by cyclic but also linear nucleotides.

MATERIALS AND METHODS

Cloning and strain construction

Primers, plasmids, and strains used in this study are listed in Supplementary Tables S3–S5. All primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). For selection in B. subtilis, media was supplemented with antibiotics when required: MLS (erythromycin at 0.5 μg/ml and lincomycin at 12.5 μg/ml) and spectinomycin (80 μg/ml). For E. coli, carbenicillin was used at 100 μg/ml.

B. subtilis purR was cloned into pLIC-trPC-HMA. PurR variants were made by megaprimer site-directed mutagene-sis of the plasmid (22). Inserts and mutants were confirmed by PCR amplification and Sanger sequencing.

B. subtilis NCIB 3610 purR:ermH1 was constructed by amplifying the purR:ermH1 genomic region from B. subtilis 168 purR:ermH1 (BKE00470, Bacillus Genetic Stock Center). The PCR product was transformed into JDW2809, and transformants were selected for MLS resistance. Positive transformants were confirmed by PCR.

To construct purR(D203A) and purR(Y102A) mutants in B. subtilis, CRISPR-Cas9 plasmids were cloned with Golden Gate assembly of pJW557 (amplified with primers oJW2775/2821), the appropriate repair template (gene fragment from GeneWiz, South Plainfield, NJ, USA), and the appropriate guide RNA. Plasmids were transformed into JDW159 (recA+ E. coli) with selection on carbenicillin. Purified plasmid was transformed into JDW2809 and transformants were selected for spectinomycin resistance. Transformants were re-streaked twice on LB at 45°C to cure the plasmid. Transformants were patched on spectinomycin, and the purR region of spectinomycin-sensitive colonies was sequenced to confirm the mutation. To construct the purR(Y102A/F205A) and purR(Y102A/K207A) double mutants, CRISPR-Cas9 plasmids were constructed with repair templates for purR(F205A) and purR(K207A) with the same guide RNA as purR(D203A). These plasmids were transformed into purR(Y102A).

To construct P_pure-sfGFP reporters, 500 bp DNA upstream of the start codon of purE in B. subtilis was amplified with primers oJW3438/3439 and cloned into pJW669 with Golden Gate assembly according to New England Biolabs (Ipswich, MA, USA) recommended protocol. The plasmid was transformed into JDW159 (recA+ E. coli) with selection on carbenicillin. Insert was confirmed by sequencing. Purified plasmid was transformed into JDW2809, JDW3970, JDW3975 and B. subtilis ΔyjbM ΔywaC. Transformants were selected on spectinomycin and streaked twice on LB at 45°C to cure the plasmid. Spectinomycin-sensitive colonies were confirmed by PCR. (pppGpp) with P_pure-sfGFP was constructed by transforming a relA:mls PCR fragment amplified with primers oJW902/903 into B. subtilis ΔyjbM ΔywaC amyE::P_pure-sfGFP (11). Transformants were selected for MLS resistance and confirmed by PCR.

Growth conditions

B. subtilis and E. coli strains were grown at 37°C unless otherwise noted. E. coli was grown in lysogeny broth (LB) or Terrific broth media. Media used to grow B. subtilis included LB and S7 defined minimal medium with 1% glucose (23). S7 was supplemented with 20 amino acids (18) or seven amino acids VILMTHE as needed. For experiments, a single colony of each strain was resuspended in 1X Spizizen salts (24) and spread on modified Spizizen minimal agar plates (1X Spizizen salts, 1% glucose, 2 mM MgCl2, 0.7 mM CaCl2, 50 mM MnCl2, 5 mM FeCl3, 1 mM ZnCl2, 2 mM thiamine, 1.5% agar and 0.1% glutamate). The minimal agar plates were supplemented with specific amino acids or 0.5% casamino acids where needed, especially in the case of amino acid auxotrophic (pppGpp)7. The plates were incubated ~16 h at 25–30°C and diluted to an OD600 = 0.005 in indicated liquid media for liquid culture experiments.

Growth curves were performed with a Synergy 2 plate reader (BioTek, Winooski, VT, USA) to measure OD600 and GFP fluorescence (Ex485/Em528). To measure growth rate, cells were washed from overnight plates, resuspended in S7 medium with 20aa at OD600 = 0.005, grown to OD600 ∼0.2, and diluted to OD600 0.005 in a clear 96-well plate (Corning, Corning, NY, USA) for measuring OD600 in the plate reader. Doubling times were calculated using a custom Python script that measured the fastest growth rate in a one hour window. To measure GFP fluorescence from the P_pure-sfGFP reporter, S7 medium with 20aa in a clear bottom black 96-well plate (Corning) was inoculated with part of a single colony of bacteria. Curves of OD600 and fluorescence were translated to a starting OD600 0.075 for
comparison across strains. To perform nutrient downshifts, cells were washed from overnight plates, resuspended in S7 medium with 20aa at OD$_{600}$ 0.01, and grown to OD$_{600}$ ~0.2. The medium was then filtered out with Costar Spin-X centrifuge filters (Corning, 0.45 μm) by centrifuging at 5000 × g for one min. The cells were washed three times with S7 medium without any amino acids (except for glutamate), resuspended in the same medium, and diluted in a 96-well plate at OD$_{600}$ 0.005 in both S7 medium with and without 20aa.

**ORFeome DRaCALA screen**

*Bacillus anthracis* Gateway® Clone Set containing plasmids bearing *B. anthracis* open reading frames was acquired from BEI Resources and used for Gateway cloning with the manufacturer’s protocol (Invitrogen, Waltham, MA, USA) into overexpression vectors pVL791 (10xHis tag, ampicillin-resistant) and pVL847 (10xHis-MBP tag, gentamycin-resistant) and transformed into *Escherichia coli* BL21 lacIq to produce two open reading frame proteome over-expression libraries (ORFeome library).

Cell lysate with overexpressed protein and purified protein were used for DRaCALA as described (25). 10 μl cell lysate or diluted, purified protein was mixed with 10 μl diluted [5-α-32P]-pppGpp (~0.2 nM) in a buffer containing 10 mM Tris pH 7.5, 100 mM NaCl and 5 mM MgCl$_2$, incubated at room temperature for 10 min. ~2 μl mixture was blotted onto nitrocellulose membrane (Amersham; GE Healthcare, Chicago, IL, USA) and allowed for diffusion and drying. The nitrocellulose membrane loaded with mixture was exposed on phosphor screen, which was scanned by a Typhoon FLA9000 scanner (GE Healthcare). Fraction of (p)pppGpp binding was analyzed as described (25).

**Protein purification**

PurR proteins were expressed in *E. coli* BL21 T7 Express lacIq (New England Biolabs) from the pLIC-trPC-HMA plasmid with an N-terminal hexahistidine-maltose binding protein (HisMBP) tag. Seed cultures at OD$_{600}$ ~0.5 were diluted 1:50 into Terrific broth and grown to OD$_{600}$ ~1.5 at 37°C prior to induction with 1 mM IPTG for four hours. Cultures were centrifuged at 10 000 × g for 30 min, the pellet was washed with 25 ml l × phosphate-buffered saline (PBS), and the pellet was stored at ~80°C.

Pellets were resuspended in Lysis Buffer (25 mM Tris–HCl pH 7.5, 300 mM NaCl, 10 mM imidazole; ~20 ml Lysis Buffer per l cell pellet) with Pierce protease inhibitor tablets (Thermo Fisher Scientific, Waltham, MA, USA) and DNase I (MilliporeSigma, Burlington, MA, USA; 500 U/l cell pellet). Cells were lysed via French press and centrifuged at 30 000 × g for 30 min. The supernatant was filtered through a 0.45 μm filter. HisMBP-tagged PurR was loaded onto a HisTrap FF column. Flowthrough was collected as untagged PurR. Untagged PurR was further purified via HiPrep 26/10 Sephacryl S-200 gel filtration (GE Healthcare) equilibrated in 20 mM HEPES pH 8, 300 mM NaCl and 50 mM (NH$_4$)$_2$SO$_4$. The peak corresponding to PurR was collected, concentrated, and concentration was measured with the Bradford assay. Typical concentrations were 10–20 mg/ml and yields were ~3–4 mg PurR/l culture. Protein was flash-frozen in liquid nitrogen and stored at ~80°C.

For small scale purification of HisMBP-tagged PurR, protein was expressed the same as above in a 5 ml volume. The cell pellet was stored at ~20°C until used. Cells were resuspended in one ml lysis buffer (20 mM sodium phosphate pH 8, 500 mM NaCl, and 10 mM imidazole). Cells were incubated with 1.6 mg lysozyme (MilliporeSigma) and 360 U Benzonase endonuclease (MilliporeSigma) for about one hour on ice for lysis. Lysate was centrifuged at 20 000 × g for 15 min. Supernatant was incubated with His Mag Sepharose beads (equilibrated with lysis buffer according to manufacturer’s instructions; GE Healthcare) for 30 min at 4°C rotating end-over-end. Beads were washed three times with 500 μl wash buffer (same as lysis buffer but with 40 mM imidazole). Protein was eluted three times with 250 μl elution buffer (same as lysis buffer but with 500 mM imidazole). Eluted protein was dialyzed into 20 mM HEPES pH 8, 300 mM NaCl, 20 mM (NH$_4$)$_2$SO$_4$, concentration was measured with $A_{280}$ and extinction coefficient, and flash frozen with liquid nitrogen for storage at ~80°C.

**DRaCALA**

[5-α-32P] pppGpp and ppGpp were purified as previously described (26,27). For PurR DRaCALA, protein in storage buffer was diluted to the appropriate concentration in 20 mM HEPES pH 8, 100 mM NaCl. 32P-labeled pppGpp or ppGpp was added in a final dilution of ~1:50 from its stock. The protein and ligand were incubated for 10 min and spotted onto Protran BA85 nitrocellulose (0.45 μm, GE Healthcare) with a replicator tool (VP 408FP652; V&P Scientific, Inc., San Diego, CA, USA). After the spots dried, the nitrocellulose was exposed to a phosphor screen for at least three hours and scanned with a Typhoon FLA9000 phosphorimager. Images were analyzed with ImageJ and fraction bound 32P-labeled (pppGpp was calculated as described (25). For ppGpp-PRPP competition assays, non-radiolabeled PRPP was first incubated with PurR (diluted as described above) for about two minutes before radiolabeled ppGpp was added. The reaction was then incubated for 10 min before being processed as described above. Data were analyzed with Prism v7 (GraphPad Software, San Diego, CA, USA), and binding curves were obtained by fitting the data to the equation: $Y = (B_{\text{max}} \times X^h) / (K_h^d + X^h)$, where $h$ represents the Hill cooperativity coefficient.

ppGpp-PRPP competition data were fit to a four-

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parameter logistic regression model in Prism. All DR-CALA reactions were performed in technical triplicate (separate reactions from the same protein preparation) and variance was determined as standard error of the mean.

**X-ray crystallography**

To obtain PurR-ppGpp crystals, the purified PurR protein was concentrated to 20 mg/ml and ppGpp was added to a final concentration of 1 mM. Crystal screens were performed using the hanging drop vapor diffusion method. The best crystals were obtained by mixing this complex 1:1 with a crystallization reservoir consisting of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.5 and 18% PEG 1500. The crystals grew at room temperature and took from 5 days to 1 week to reach optimal size. The crystals take the triclinic space group P1 and diffracted beyond 2.5 Å on synchrotron sources. The crystals were cryo-protected by dipping the crystals in a 1 μl drop consisting of the crystallization reagent supplemented with 15% glycerol for 2 s. The crystal was then placed directly in the cryo-stream. X-ray intensity data were collected at the Advanced Light Source (ALS) beamline 8.3.1. The data were integrated in MOSFLM and scaled using SCALA (28). Phaser in CCP4 was used to solve the structure by Molecular replacement using a dimer of the 1P4A B. subtilis PurR structure with the ligand and removed. Phaser readily located the three PurR dimers in the crystallographic asymmetric unit. Phenix (29) was then employed to perform refinement. After several rounds of refinement and refitting and optimizing of the model, density was evident for ppGpp molecules in four of the six PurR subunits; there was only weak density for the ppGpp of refinement and refitting and optimizing of the model, then employed to perform refinement. After several rounds in the crystallographic asymmetric unit. Phenix (29) was used to solve the structure by Molecular replacement using a dimer of the 1P4A B. subtilis PurR structure with the ligand and removed. Phaser readily located the three PurR dimers in the crystallographic asymmetric unit. Phenix (29) was then employed to perform refinement. After several rounds of refinement and refitting and optimizing of the model, density was evident for ppGpp molecules in four of the six PurR subunits; there was only weak density for the ppGpp molecules in the other two subunits. After fitting the ppGpp molecules, refinement in Phenix commenced and in the final stages of refinement ordered solvent molecules were added. The final model has work/free values of 19.4%/23.9% to 2.45 Å resolution and validation in Molprobity placed the structure in the top 98% of structures solved to similar resolutions.

**Electrophoretic mobility shift assay (EMSA)**

Gel shift assays were performed with untagged B. subtilis PurR, ppGpp, PRPP, and a 221 bp FAM-labeled DNA probe. The 221 bp probe was amplified from upstream of the pur operon with oJW4028/4029, and the 202 bp probe was amplified with oJW1285/1286. DNA concentration was measured using Quant-IT (Thermo Fisher Scientific). Protein was diluted from frozen stock to appropriate concentrations in 10 mM HEPES pH 8.0, 100 mM KCl and 10% glycerol. For reactions with the FAM-labeled DNA probe, the reaction contained 5 nM FAM-labeled DNA probe, 100 mM salmon sperm non-specific DNA (Invitrogen), 10 mM HEPES pH 7.5, 200 mM KCl, 10% glycerol, 0.2 mM EDTA, 5 mM MgCl2, 1 mM DTT and 0.5 mg/ml bovine serum albumin (BSA; protein standard, Bio-Rad, Hercules, CA, USA). For reactions with the unlabeled DNA probe, the conditions were the same except 50 mM KCl, no nonspecific DNA and 1 nM DNA probe. Twenty microliter reactions were prepared from a 5× buffer, 10× protein stock, 10× DNA probe stock and 10× ppGpp or PRPP stock. Reactions were mixed by pipetting and incubated at room temperature (~23°C) for 30 min. A 6% TBE polyacrylamide gel (Novex, Thermo Fisher Scientific) was pre-run at 10 V for 10 min at 4°C. Ten microliters of the sample were loaded and the gel was run at 100 V for ~100 min at 4°C. For the unlabeled 202 bp probe, gels were stained with 1X SYBR Gold (Thermo Fisher Scientific) at room temperature for 30 min and imaged on a Typhoon FLA9000. For the FAM-labeled probe, gels were imaged on a Typhoon FLA9000 with FAM detection settings.

**LC–MS quantification of PRPP and other nucleotides**

JDW2809 was grown in S7 defined media with 1% glucose and 0.1% potassium glutamate to OD₆₀₀ ~0.3. 5 ml culture was sampled before and after 0.5 mg/ml arginine hydroxamate treatment at defined time points. Samples were extracted and analyzed by LC–MS as described in previous publication (30). Concentration of PRPP was estimated as follows: PRPP concentration is 2.50 nmol/(mg dry weight) during exponential growth in minimal media (31). Given the dry weight/wet weight ratio of B. subtilis of 51.4% (32), the PRPP concentration is 2.50 × 51.4% = 1.29 nmol/mg wet weight. Finally, we calculated PRPP concentration after arginine hydroxamate treatment for 30 min to be ~0.7 mM by comparing the relative normalized ion counts to the untreated samples.

**Production of radiolabeled PurBox DNA probe**

To construct the DNA probe for DNase I footprinting, the fragment containing PurBoxes was amplified by PCR using oligos oJW4280/oJW4281. This fragment was cloned into pUC18 vector to construct pJW747 by restriction digestion and ligation with HindIII, BamHI and T4 DNA ligase (New England Biolabs). pJW747 was processed as follows to produce radiolabeled PurBox DNA probe: 7.5 μg pJW747 was firstly digested by 20 U BamHI at 37°C for 30 min. The product was phenol extracted and treated by 10 U Quick CIP (New England Biolabs) at 37°C for 30 min. The product was heated at 80°C for 10 min to deactivate Quick CIP and incubated with 10 U T4 polynucleotide kinase (New England Biolabs) and 120 μCi [³²P-α]-ATP (PerkinElmer, Waltham, MA, USA) to label the DNA. The product was heated at 65°C for 10 min to deactivate T4 polynucleotide kinase and treated by 20 U HindIII at 37°C for 30 min. The product was purified by phenol extraction and purified as described before (33).
treated with 3.2 μg/ml DNase I (Roche, Basel, Switzerland) for 40 s, and the reaction was stopped by adding 300 mM sodium acetate and 10 mM EDTA. The mix was purified by phenol extraction and dissolved in 5 μl gel loading buffer (8 M urea, 0.5× Tris-borate–EDTA buffer (TBE), 0.05% (w/v) bromophenol blue). The A + G ladder for footprinting was prepared as described before (33). DNA footprint gel electrophoresis was performed as described before (33). After electrophoresis, the dried gel was exposed to a phosphor screen for 48 h, and the screen was scanned on a Typhoon FLA9000 phosphorimager. The figure was analyzed by ImageJ for quantification of band intensities.

Western blot analysis

To test for α-PurR antibody activity, cultures were obtained by inoculating LB with single colonies of wild-type (JDW2809) and purR:ermHI B. subtilis (JDW3359). Ten mL of culture were pelleted at 4500 × g for 20 min and washed with one mL of 50 mM Tris pH 7.5 and 300 mM NaCl. The pellet was resuspended in 100 μl of lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 0.1 mg/ml lysozyme, 0.5 mM PMSF). Cells were lysed by incubation at 37°C for 30 min. An equal volume of 2× Laemmli buffer (Bio-Rad) was added and cells were incubated at 95°C for 10 min. The solution was centrifuged at maximum speed and the supernatant was transferred to a fresh tube. Ten micro liters of the cell lysate were loaded onto a 12.5% polyacrylamide gel and 20 μl secondary antibody (goat α-B. subtilis PurR rabbit serum (polyclonal, 1:500 dilution, Covance-Labcorp, Princeton, NJ, USA). The membrane was washed three times with PBST and three times with PBS − rabbit Alexa Fluor 647, Invitrogen). The membrane was washed three times with 10 mM Tris pH 7.5. The PurR ChIP protocol was adapted from previously published protocols (34,35). Cultures for ChIP-seq were grown from a starting OD600∼0.01. Cells were grown in minimal medium supplemented with the amino acids VILMTHE. For ChIP-seq with nucleobases, the medium was supplemented with 1× ACGU (Teknova, Hollister, CA, USA). For sample collection, cells were grown from a starting OD600∼0.01. Cells were grown to an OD600∼0.5 at 37°C and 250 RPM. For RHX treatment, an untreated sample was collected by adding ~50 ml culture into 1.4 ml 37% formaldehyde in a 50-ml conical. The conical was shaken at 37°C for 7–8 min prior to placing on ice. The remaining culture was treated with 0.5 mg/ml RHX and another ~50 ml was crosslinked 10 min after RHX addition. Crosslinked samples were treated with 3.2 μg/ml RNase A (Thermo Fisher Scientific) and incubating at 37°C for at least 1 h. Then, 48 μl elution buffer and 1.6 U Pro-
teinase K were added, incubated at 55°C for one hour, and then at 65°C overnight to reverse crosslinks. The DNA was isolated with a 1.8:1 SPRi cleanup and the concentration was quantified with Quant-iT (Thermo Fisher Scientific). Moving forward, 100 ng input DNA was processed with reactions similar to the ChiP samples, except the reaction volumes were 50 μl. Following blunting/5’ phosphorylation and A-tailing, the DNA was purified with a 1.8:1 SPRi cleanup. Following ligation, the DNA was purified with two successive 1:1 or 1:2.1 SPRi cleanups to eliminate adapter carryover. The final library was eluted with 22 μl ddH2O.

Quantitative PCR was used to determine the number of cycles for library amplification. In a 20 μl reaction, 2 μl of each sample DNA was combined with 0.2 μM each of oJW3536 and oJW3537, 1X EvaGreen dye (Biotium, Fremont, CA, USA), and KAPA high-fidelity low-bias polymerase (Roche). Samples were denatured in a Bio-Rad CFX qPCR thermocycler at 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s. The cycle quantification (Cq) value provided by the program was used as the number of cycles for full library amplification. The remaining 20 μl of library was amplified with KAPA polymerase and the primers and concentrations listed above. The reactions were denatured at 98°C for 30 s, cycled the Cq value through 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s before a final extension of 72°C for 5 min. Final amplified libraries were purified with a 1.6:1 SPRi cleanup and eluted with ddH2O.

ChIP-seq data analysis

Libraries were sequenced on the Illumina NextSeq platform at the University of Michigan Advanced Genomics Core. The analysis pipeline and scripts are available at https://github.com/bwanderson3/2021_PurR_ChIP_analysis.git. Briefly, quality control was determined with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters were removed with CutAdapt with arguments -quality-base = 33 -a AGATCGGAAGAGC -a CGGAAGAGCACAC -a GGGGGGGGGGGGGGGGGGGGG -n 3 -m 20 -match-read-wildcards -o. Using bowtie2 and arguments -U -q –end-to-end –very-sensitive-p 6 -phred33, wild-type B. subtilis reads were aligned to the B. subtilis NCIB 3610 genome (CP020102) from NCBI Reference Sequence Database. B. subtilis (p)ppGpp0 reads were aligned to the NCIB 3610 genome with the pBS32 megaplasmid (CP020102 concatenated with CP020103). Using a custom python script, coverage at a 10 bp resolution was bootstrapped and the median coverage of 100 bootstraps was used for downstream analysis. Coverage was smoothed with a custom python script. Enrichment was calculated as the median counts per million in a ChiP library divided by the median counts per million in its corresponding input sample for each 10 bp window. The input sequencing dataset for the first replicate was low quality, so the input datasets from the same strain background and treatment conditions of the third replicate were used to calculate enrichment for the first replicate. For plotting enrichment, coverage was smoothed to 50 bp windows and enrichment was calculated again as above.

To calculate the effect of (p)ppGpp on PurR occupancy genome-wide, bootstrapped coverage at a 10 bp resolution that was determined in the previous paragraph was used. The effect of (p)ppGpp was determined by calculating: (enrichment_post_RHX_wildtype/enrichment_pre_RHX_wildtype)/(enrichment_post_RHX_ppGpp/enrichment_pre_RHX_ppGpp0). This yielded 100 bootstraps for each biological replicate (replicate 1 for wild type was paired with replicate 1 for (p)ppGpp0 in the calculation). The median bootstrap value was determined from the 100 bootstraps for each biological replicate. Means and standard deviations were calculated from the medians of each biological replicate.

**PurR phylogenetic analysis**

GenBank files and genome sequences were downloaded from NCBI Assembly for 106 complete reference bacterial genomes. To build the phylogenetic tree, 16S rRNA sequences were extracted from the GenBank files with a custom python script, and three eukaryotic 18S rRNA sequences were included as an outgroup. The rRNA sequences were aligned in MEGA X with ClustalW (36). The best model for tree construction was chosen as General Time Reversible with gamma distributed categories and invariant sites, which had the lowest AIC with MEGA X’s model test software (37). The tree was constructed according to this model in MEGA X with extensive grafting and subtree pruning and 750 bootstrap replicates. The tree was modified and annotated in R using the ggtree package (38).

Standalone BLAST+ from NCBI was used to determine which reference genomes contained a B. subtilis or B. anthracis PurR homolog. A BLAST database was created from the FASTA files of the same reference genome sequences used to generate the phylogenetic tree. The 73 N-terminal residues of B. subtilis PurR were BLASTed against this database with blastn and a default E value of 10. This domain is unique to PurR according to Pfam (PF09182). The complete PurR sequence was not used due to homology of the PRT domain with PRT enzymes.

**Phylogenetic analysis of the PurR (p)ppGpp binding pocket**

Conservation analysis of the protein and binding pocket was conducted on 938 PurR homologs in the Pfam family PF09182. Amino acid sequences were downloaded from UniProt, and proteins were aligned with MUSCLE in MEGA X. A frequency logo was created from aligned (p)ppGpp binding residues using WebLogo (UC Berkeley; https://weblogo.berkeley.edu/logo.cgi). ConSurf analysis was conducted using the ConSurf server (http://consurf.tau.ac.il/) with conservation score determined by Bayesian inference (39,40). Conservation scores were mapped onto PDB ID 1O57.

**RESULTS**

Screening a B. anthracis ORF library identifies the purine transcription regulator PurR as a (p)ppGpp target

(p)ppGpp has been shown to bind to the RNA polymerase core enzyme in Proteobacteria (14,41,42). In contrast, in Firmicutes (p)ppGpp has not been shown to regulate transcription directly (43), despite regulating
many targets, including nucleotide synthesis enzymes, ribosome associated GTPases, and DNA replication enzymes (11,26,27,30,44,45). To uncover potential (p)ppGpp regulated transcriptional targets, we conducted a proteome based screen for proteins that directly bind (p)ppGpp in the pathogenic Firmicute Bacillus anthracis (30). We screened the 5341 open reading frame (ORF) library from B. anthracis, after placing each ORF into two expression constructs, one expressing the ORF with an N-terminal histidine (His) tag and the other with an N-terminal histidine maltose binding protein (HisMBP) tag. Each ORF in the His-tagged and HisMBP-tagged library was overexpressed and binding to $^{32}$P-pppGpp was assayed using differential radial capillary action of ligand assay (DRaCALA). The fraction of $^{32}$P-pppGpp bound to protein in each lysate was normalized as a $Z$-score for each plate (the number of standard deviations from the mean fraction $^{32}$P-pppGpp bound for each plate) to reduce the influence of plate-to-plate variation. We used a strict $Z$-score cutoff of 2.5 to identify proteins very likely to be (p)ppGpp targets (Figure 1A and B). This yielded known and new (p)ppGpp targets, as listed in Supplementary Table S1 and reported in (30).

Since (p)ppGpp binds many proteins in cell lysates, we sought to reduce nonspecific background binding by performing the same screen with a small amount (100 $\mu$M GTP) of non-radiolabeled GTP as a competitor (Figure 1C and D). Many (p)ppGpp binding proteins also bind GTP, so the non-radiolabeled GTP competitor reduces the $Z$-score of GTP binding proteins among others, leading to many hits losing significance in the presence of GTP (these are colored yellow in Figure 1A and B; Supplementary Table S1). This approach enriches for hits that specifically bind (p)ppGpp without competing with GTP. Strikingly, the purine synthesis transcription regulator PurR (BA0044) which had a $Z$-score slightly below the cutoff of 2.5 in the previous screens (Figure 1A and B), emerged as one of the strongest hits in the screens with GTP, only behind the known targets HPRT, XPRT, and YjbM (SasB) (Figure 1C and D, and Supplementary Table S1).

PurR is a transcription regulator in bacteria with the major role of repressing the transcription of genes responsible for de novo synthesis of IMP, the common precursor to the purine nucleotides ATP and GTP (Figure 1E) (20,46–48). PurR is known to be regulated by the availability of purine bases for salvage, thus is a critical regulator in ensuring balanced and efficient synthesis of purine nucleotides (20,49,50).

Homologs of the B. anthracis PurR are found almost exclusively in the bacterial phylum Firmicutes (Figure 1F). PurR proteins from other bacterial phyla, such as Proteobacteria, are structurally unrelated (51–53). To perform an in-depth study of the PurR-(p)ppGpp interaction, we turned to PurR from B. subtilis, a close relative of B. anthracis, since its PurR has been extensively characterized and the PurR homologs are similar (64% identical) (20). We purified untagged B. subtilis PurR and tested for binding to $^{32}$P-pGpp and $^{32}$P-pppGpp with DRaCALA (Figure 1G). Both ppGpp and pppGpp interacted with B. subtilis PurR similarly, with binding curves best fit with a $K_{d}$ of 4.4 $\mu$M and a Hill cooperativity coefficient of 1.5 (Figure 1G and H). Given that the physiological concentration of (p)ppGpp in Bacillus can reach up to mM during amino acid starvation (11), this interaction is likely to be relevant in vivo and represents a potential new regulation of gene expression of the PurR regulon by amino acid starvation.

Next, we tested whether (p)ppGpp interaction extends to PurR homologs in other Firmicutes beyond B. anthracis and B. subtilis, using purified PurR from the soil bacterium Clostridium sporogenes, the opportunistic pathogen Enterococcus faecalis, and the oral pathogen Streptococcus mutans. In all cases, we detected significant binding to $^{32}$P-ppGpp (Figure 1I). Altogether, our screen has identified a transcription factor in Firmicutes that (p)ppGpp binds tightly and specifically. (p)ppGpp binding to PurR suggests that it can act as an effector for the transcription factor.

(p)ppGpp binds to a conserved pocket on the effector binding domain of PurR

We next solved a co-structure of ppGpp bound to B. subtilis PurR to 2.45 Å resolution (Figure 2A and Supplementary Table S2). B. subtilis PurR comprises two domains, an N-terminal winged helix-turn-helix domain for DNA binding and a C-terminal effector binding domain. In contrast to E. coli PurR whose effector binding domain binds purine bases and is structurally similar to a ribose binding protein, the Firmicute PurR effector binding domain is similar to the phosphoribosyltransferase (PRT) class of enzymes (Figure 2A) (21,53). In the structure of the B. subtilis PurR-ppGpp complex, ppGpp binds to the PRT domain in a pocket defined by three loops found in all PRT proteins (II, III, IV; loop numbering from PRT enzymes) (Figure 2A and B) (54). The guanine ring of ppGpp is engaged in $\pi$-stacking interactions, sandwiched between the aromatic side chains of residues Phe205 and Tyr102 (Figure 2B). A Y102A variant interacts with $^{32}$P-ppGpp more weakly than wild type, supporting a role for Tyr102 in ppGpp binding (Supplementary Figure S1A). The 5'-phosphates of ppGpp nestle into a pocket formed by loop III and an $\alpha$-helix, where the backbone amides of loop III and the side chain of Thr211 coordinate the 5'-phosphates (Figure 2B). The 3'-phosphates extend upward and out of the binding pocket, where they interact with backbone amides of loop II and the side chains of Tyr102 and Lys207 (Figure 2B). The omit electron density for the 3'-phosphates is weaker than that of the 5'-phosphates (Supplementary Figure S1B) but can be unambiguously assigned to ppGpp when contoured down to lower sigma values. While (p)ppGpp is commonly co-crystallized with divalent cations (e.g. Mg$^{2+}$ or Mn$^{2+}$), we did not find evidence for metals in our structure. Accordingly, DRaCALA confirmed that Mg$^{2+}$ is not required for nor interferes with the interaction between $^{32}$P-ppGpp and PurR (Supplementary Figure S1C).

The (p)ppGpp binding pocket among Firmicutes PurR homologs is highly conserved. We aligned the (p)ppGpp binding residues from 938 B. subtilis PurR homologs in the Pfam database (Figure 2C). Of the 15 residues involved in the interaction, 11 are nearly perfectly conserved, and variants in other sites are highly similar (Figure 2C). For example, residue 102 is a tyrosine in most PurR homologs, including B. subtilis PurR, while phenylalanine is at the
Figure 1. DRaCALA screen identifies the transcription factor PurR as a (p)ppGpp target in Firmicutes. (A, B) Z-scores of 32P-pppGpp binding to an open reading frame (ORF) library from *B. anthracis*. His indicates hexahistidine-tagged ORFs, and HisMBP indicates hexahistidine maltose binding protein tagged ORFs. ORFs with Z-scores greater than 2.5σ are filled. The dashed horizontal line is at 2.5σ. The red circle is the PurR ORF. Yellow symbols indicate ORFs that were hits without GTP competition but were not hits with GTP (see C, D). These data were previously reported in Yang *et al.* (2020). (C, D) DRaCALA of ORF libraries from (A) and (B) with 100 μM non-radioactive GTP as a competitor to reveal (p)ppGpp-specific targets. 32P-pppGpp is at ~0.2 nM. (E) Schematic of PurR regulation of *de novo* ATP and GTP synthesis. (F) Cladogram constructed from 106 bacterial 16S rRNA and three eukaryotic 18S rRNA sequences. Tree branches colored according to the phylum. A red dot on the branch tip indicates that the species contains a *B. subtilis* PurR homolog. (G) Binding curve between purified untagged *B. subtilis* PurR and 32P-ppGpp (gray) and 32P-pppGpp (purple) obtained with DRaCALA. Error bars represent SEM of technical triplicate. Error bars are not visible when they are smaller than the height of the symbols. (H) Binding parameters from (G). mean ± standard error. *h* = Hill coefficient. (I) DRaCALA of 32P-pppGpp binding to 30 μM purified HisMBP-tagged PurR proteins from *Clostridium sporogenes* (Csp), *Enterococcus faecalis* (Efa), and *Streptococcus mutans* (Smu). Control is protein storage buffer.
Figure 2. ppGpp binds to the effector binding domain of PurR. (A) *B. subtilis* PurR dimer crystallized with ppGpp. The N-terminal DNA binding domain is blue and the C-terminal effector binding phosphoribosyltransferase (PRT) domain is salmon. (Right) Structure from left rotated 90° to show the ppGpp binding pockets on the effector domains. (B) ppGpp binding pocket with select interacting residues indicated in silver. Loops I–IV of the PRT binding pocket are labeled. See Supplementary Figure S1B for an omit electron density map. (C) Frequency logo of the (p)ppGpp binding residues from 938 PurR proteins homologous to *B. subtilis* PurR. The logo was made with WebLogo (UC Berkeley; https://weblogo.berkeley.edu/logo.cgi). (D) Overlay of apo PurR (green; PDB ID 1O57) and ppGpp-bound PurR (salmon). Bridging loop that is unresolved in apo but not with ppGpp is indicated. (E, F) Poisson-Boltzmann continuum electrostatics of the effector binding domain of apo (E) and ppGpp-bound (F) PurR. The scale of electrostatic potential is –5 (red) to + 5 (blue).

homologous position in the others. Also, residue 207 is a lysine in most homologs, including *B. subtilis* PurR, and arginine is at the homologous position in the remainder. Loop III residues Phe205 and Gly209/Gly210/Thr211 are nearly universally conserved. ConSurf analysis across the whole protein for the 938 PurR homologs shows that the (p)ppGpp binding pocket is more highly conserved than the remainder of the protein, even more than the DNA binding domain (Supplementary Figure S1D).

Structural comparison between apo PurR and ppGpp-bound PurR does not reveal drastic conformational or electrostatic differences in the DNA binding domain (Supplementary Figure S2A-C). ppGpp binding does affect the effector binding domain, which is important for oligomeric interaction between PurR subunits. A loop that bridges the monomer-monomer interface of a PurR dimer is unresolved in apo PurR but is resolved in ppGpp-bound PurR, suggesting that ppGpp binding stabilizes the conformation of this loop (Figure 2D). The effect of this change is that ppGpp significantly alters the electrostatics of the effector binding domain by creating a positively charged channel different from the apo protein (Figure 2E and F). Since it has been hypothesized that DNA wraps around PurR to interact with the effector binding domain (55), it is possible that the altered electrostatic surfaces affect the interactions with the electronegative DNA backbone.

(p)ppGpp competes with PRPP for binding PurR’s effector binding domain to alter PurR binding to promoter DNA

The effector binding domain of PurR is homologous to PRT enzymes, which catalyze phosphoribosyl transfer reactions with PRPP as a substrate. The PRT domain of PurR also binds PRPP but does not have enzymatic activity. PRPP binding instead de-represses the PurR regulon (20). The ppGpp binding site partially overlaps the PRPP binding site, as seen in an overlay of ppGpp and the PRPP analog, cPRPP, bound to PurR (Figure 3A) (55). Particularly, the 5′-phosphates of ppGpp overlap with the 5′-phosphate of PRPP in their interaction with loop III (Figure 3A). The overlap in this binding site suggests that they would compete for binding to PurR. Indeed, we found PRPP competes with 32P-ppGpp for binding PurR (Figure 3B). The binding pocket also contains interactions specific for each effector. PurR Tyr102 is flipped away from the pocket with PRPP bound and covers the guanine ring with ppGpp bound (Figure 3A). Asp203 sits below the ribose of PRPP but does not interact with ppGpp (Figure 3A). An alanine variant at
Figure 3. ppGpp competes with PRPP to allosterically regulate PurR-DNA interaction. (A) Overlay of PurR-ppGpp (salmon) with PurR-cPRPP (yellow; PDB ID 1P4A). The side chains of Y102 and D203 are shown. Loop II is hidden for clarity. (B) Competition between ³²P-ppGpp (~0.2 nM) and PRPP for binding to PurR and PurR D203A. Binding of ³²P-ppGpp was measured with DRAcALAL. Reactions were performed in technical triplicate, and error bars represent SEM. Error bars are not visible when their height is smaller than the height of the symbol. (C) EMSA showing PurR interaction with FAM-labeled 221 bp DNA with increasing PRPP concentrations and without ppGpp. (D) EMSA showing PurR interaction with DNA with increasing ppGpp concentration and with PRPP. The PurR concentration is 100 nM in (C) and (D). Similar EMSA results were observed with a non-labeled 202 bp probe from the same control region with a lower KCl concentration and no nonspecific DNA in the EMSA reaction (Supplementary Figure S3). (E) DNase I footprinting of PurR-PurBox interaction. ³²P-labeled PurBox fragment was incubated with or without PurR (50 nM), ppGpp (1 mM), and PRPP (16 μM), and then briefly digested by DNase I (3.2 μg/ml). The digestion product was analyzed by electrophoresis. The uncropped gel is in Supplementary Figure S4. (F) Relative intensity of PurBox 2 in PurR-PurBox DNA footprint. Relative intensity is the intensity of the PurBox 2 area normalized to the intensity of a reference area (raw data in Supplementary Figures S5 and S6). Error bars represent standard error of the mean for 4 replicates. A two-tailed two-sample equal-variance Student’s t test was performed between samples for statistical significance (*P ≤ 0.05; n.s. P > 0.05).

this position reduces the ability of PRPP to compete with ppGpp by 100-fold (Figure 3B), but ³²P-ppGpp binding is unaffected (Supplementary Figure S1A).

We next examined the role of (p)ppGpp/PRPP on PurR binding to a cognate binding sequence with electrophoretic mobility shift assays (EMSA). We used two DNA probes amplified from the PurR-binding promoter of the pur operon in B. subtilis, which encodes the genes necessary for de novo IMP synthesis. One probe was a FAM-labeled 221 bp probe that we detected with FAM-specific fluorescence, and the other probe was an unlabeled 202 bp probe that we detected with a nonspecific SYBR Gold dye. We added PurR to the FAM-labeled probe at high salt concentrations in the presence of unlabeled nonspecific DNA competitors to ensure the specificity of the interaction (Figure 3C), or we added PurR to the unlabeled probe in the absence of nonspecific DNA at lower salt concentrations (Supplementary Figure S3). In both cases, we observed a complete probe shift and the formation of larger molecular weight complexes, indicative of the PurR repressor-promoter complex. The presence of increasing concentrations of PRPP results in loss of the larger molecular weight PurR-promoter complex and an increase in unbound DNA (Figure 3C, left). This agrees with previous observations that PRPP is an inducer (anti-repressor) of PurR (20). However, in the presence of ppGpp (500 μM), PRPP no longer effectively prevents PurR-DNA interaction, and only a small portion of unbound probe was observed even at the highest PRPP concentration (Figure 3C, right). We also performed EMSA by titrating an increasing amount of ppGpp (Fig-
ure 3D, left). ppGpp on its own does not increase PurR-promoter association. However, in the presence of PRPP (62.5 μM), ppGpp effectively antagonizes PRPP, increasing PurR-promoter association (Figure 3D, right). These results suggest that ppGpp maintains PurR-DNA interaction and prevents PRPP from de-repressing PurR regulation.

To clearly address the effect of (p)ppGpp on PurR-DNA binding, we performed a DNA footprinting assay using the pur promoter DNA probe (Figures 3E, S4, S5, and S6 and quantification in Figure 3F). This promoter DNA probe included the two 14-bp inverted repeats, termed PurBoxes (Figure 3E), that each bind a PurR dimer (56). The unbound probe is susceptible to DNase I digestion, including the PurBox regions (Figure 3E, lane 2). Addition of PurR (Figure 3E, lanes 3 and 4) led to significant protection of the PurBoxes against DNase I cleavage, indicating PurR binding to DNA. Addition of PRPP, even at low concentration (Figure 3E, lanes 7 and 8), led to an increase of PurBox intensities, particularly in the PurBox 2 region. This indicates deprotection of this region due to PRPP-mediated PurR repression. Addition of ppGpp itself had little effect on PurBox 2 intensity (Figure 3E, lanes 5 and 6), but addition of ppGpp in the presence of PRPP alleviated PRPP’s effect on increasing the PurBox 2 intensity (Figure 3E, lanes 9 and 10). These results indicate that ppGpp works as an anti-inducer against PRPP to maintain PurR-PurBox binding.

Overall, we conclude that (p)ppGpp binds PurR at an effector binding pocket distal to the DNA binding domain and competes with the inducer ligand PRPP to enhance the PurR-DNA interaction. Because PRPP is a key intermediate for nucleotide biosynthesis, it is always present in vivo. Based on our LC-MS analysis and metabolite quantification from the literature (31,32), we estimate PRPP to be ~0.7 mM during amino acid starvation and ~1.29 mM during nutrient replete growth (Supplementary Figure S7). These concentrations of PRPP are more than sufficient to saturate PurR in vitro (Figure 3C), suggesting that if PurR is relying on PRPP alone for its regulation, then its regulon should always be induced in vivo. This apparent contradiction between high concentrations of PRPP and its regulatory role has been characterized as an ‘enigma’ (47). (p)ppGpp may help to resolve this enigma for PurR by antagonizing PRPP, which enhances PurR DNA binding and allows PurR to function as an effective repressor during conditions that lead to (p)ppGpp induction, such as upon amino acid starvation.

### Defining the PurR binding sites in the *B. subtilis* genome

(p)ppGpp binding to PurR and enhancing PurR–DNA interaction raised the possibility that in vivo (p)ppGpp accumulation during amino acid starvation directly regulates the PurR regulon. Since the PurR regulon in *B. subtilis* had not been systematically characterized, we turned to ChIP-seq to fully document PurR binding sites. We raised polyclonal antibodies against untagged *B. subtilis* PurR (Supplementary Figure S8) and collected ChIP samples from *B. subtilis* cells grown under a variety of conditions: with or without exogenous nucleobases and with or without amino acid starvation (Table 1). We first assessed ChIP-seq data with nucleobases to identify PurR binding sites genome-wide (Figure 4A). ChIP-seq identified 15 PurR binding sites (Figure 4A and 4B, Supplementary Figure S9, and Table 1), 9 of which had been previously identified (19).

### Table 1. PurR binding sites in *B. subtilis* identified with ChIP-seq

| Gene/operon | Function | Wild type (PurR enrichment\(^a\)) | (p)ppGpp (PurR enrichment) |
|-------------|----------|----------------------------------|---------------------------|
| +ACGU\(^b\) | −RHX\(^c\) | +RHX | −RHX | +RHX | PurBox |
| puxS-puxT | pyridoxal 5-phosphate (PLP) synthetase | 3.83 ± 0.72 | 1.63 ± 0.19 | 3.11 ± 0.52 | 1.36 ± 0.16 | 1.32 ± 0.10 | Y |
| purR-pux3 | purine repressor PurR | 11.30 ± 0.72 | 2.72 ± 0.43 | 7.19 ± 0.85 | 2.31 ± 0.43 | 1.79 ± 0.31 | Y |
| puxG | hypoxanthine/guanine permease | 21.92 ± 1.54 | 4.93 ± 0.46 | 8.19 ± 1.44 | 4.10 ± 0.98 | 2.81 ± 0.37 | Y |
| purEKBCSQLFMNHD | de novo IMP synthesis | 19.28 ± 1.15 | 5.55 ± 1.03 | 12.51 ± 1.95 | 3.65 ± 0.59 | 2.35 ± 0.44 | Y |
| steT | serine/threonine exchanger transporter | 42.10 ± 7.78 | 10.19 ± 1.46 | 26.37 ± 8.74 | 12.52 ± 5.95 | 5.28 ± 0.21 | Y |
| pycA | pyruvate carboxylase | 20.49 ± 3.54 | 4.60 ± 0.76 | 14.24 ± 3.66 | 2.68 ± 1.10 | 1.99 ± 0.32 | Y |
| bbdB | thiol-disulfide oxidoreductase | 3.31 ± 1.17 | 1.59 ± 0.07 | 3.40 ± 1.48 | 1.57 ± 0.48 | 2.03 ± 0.45 | N |
| opu-puxX | XPRT and xanthine permease | 13.35 ± 1.66 | 4.13 ± 0.53 | 9.97 ± 0.86 | 3.25 ± 0.88 | 2.45 ± 0.42 | Y |
| mscBfosD | transcription antirterminator NusB and methylene tetrahydrofolate dehydrogenase (FolD) | 25.81 ± 4.31 | 5.73 ± 0.73 | 15.40 ± 2.56 | 3.66 ± 0.67 | 3.67 ± 0.55 | Y |
| puxO | hypoxanthine/guanine permease | 34.14 ± 4.04 | 5.62 ± 0.60 | 14.18 ± 0.24 | 5.94 ± 2.06 | 2.74 ± 0.46 | Y |
| guaC | GMP reductase | 56.08 ± 4.55 | 10.85 ± 2.23 | 17.11 ± 3.38 | 16.46 ± 5.37 | 7.80 ± 1.40 | Y |
| opuA-BB-BC-BD | choline/arsenocholine ABC transporter | 9.64 ± 2.00 | 4.59 ± 0.39 | 9.71 ± 0.25 | 2.89 ± 0.68 | 5.91 ± 0.11 | N |
| yoaY \(^4\) | ABC transporter (glycine for betaine/carnitine/choline/arsenocholine) | 6.09 ± 1.85 | 3.38 ± 0.30 | 6.82 ± 0.87 | 2.30 ± 0.45 | 2.72 ± 0.33 | N |
| glyA | serine hydroxymethyltransferase | 25.55 ± 6.91 | 5.77 ± 0.84 | 9.12 ± 1.46 | 6.10 ± 1.45 | 4.06 ± 0.18 | Y |
| purA | adenylosuccinate synthetase | 50.75 ± 11.74 | 20.13 ± 4.09 | 34.25 ± 1.18 | 15.34 ± 4.20 | 7.90 ± 1.80 | Y |

\(^a\)Enrichment of reads in immunoprecipitated sample relative to input sample (see Materials and Methods). Data are the mean and standard deviation of biological triplicate of the maximum points of each of the ChIP peaks.

\(^b\)ACGU refers to the nucleobases adenine, cytosine, guanine, and uracil.

\(^c\)RHX is arginine hydroxamate, which mimics amino acid starvation.

\(^d\)The slash indicates a gene being transcribed in the opposite direction immediately upstream of the operon names in bold indicate new PurR regulatory sites identified in this study.
Figure 4. (p)ppGpp enhances PurR-DNA interaction during stress response. (A) Genome-wide view of mean PurR ChIP reads per million reads from B. subtilis grown with or without the nucleobases adenine, cytosine, guanine, and uracil. (B) Schematic of the GTP and ATP synthesis pathway in B. subtilis. Red arrows indicate PurR-controlled steps, as deduced from the respective genes being downstream of a PurR binding site. 10-fTHF = 10-formyl tetrahydrofolate; ser = serine; gly = glycine; thr = threonine; pyr = pyruvate; OAA = oxaloacetate; asp = aspartate; hpx = hypoxanthine; xan = xanthine; gua = guanine. Placements of pycA and steT in this pathway are inferred based on enzyme function and have not been verified. (C) PurR represses the 12-gene pur operon in B. subtilis, and PRPP is an inducing ligand. (D) Sequence logos of PurBox1 (top) and PurBox2 (bottom) sequences from 12 of the 15 PurR ChIP peaks. Logos were created using WebLogo (https://weblogo.berkeley.edu/logo.cgi). See Supplementary Figure S10 for complete sequences. (E) A plot showing the distance of PurBoxes from the ChIP enrichment peaks. The peak is represented by the dotted horizontal line at Y = 0. Peak location was determined at 10 bp resolution from the PurR ChIP sample obtained from (p)ppGpp-induced WT B. subtilis. The distance was calculated from the center of each PurBox (7th nt out of 15 nt PurBox) to the peak location. (F) Genome-wide view of mean PurR ChIP reads per million reads in WT and (p)ppGpp before and after arginine hydroxamate (RHX) treatment. Before RHX are the same data as without nucleobases in A. (G) PurR ChIP enrichment at the pur operon in WT and (p)ppGpp. The solid trace is the mean enrichment of biological triplicate, and SD is shown as the shaded region. The inset shows the PurBox sites (shaded box), −10 and −35 promoter sequences (blue box), and the transcription start site (arrow) upstream of the pur operon. The sequence for this site is shown in Supplementary Figure S12.
As expected, PurR enrichment was increased at all binding sites with nucleobases (Figure 4A). These PurR binding sites control expression of more than 30 genes, the majority of which are involved in purine nucleotide synthesis (Figure 4A). For example, PurR binds upstream of the 12-gene pur operon (purEKBCSQFLMNHD), which encodes all proteins necessary for de novo synthesis of the GTP/ATP precursor IMP from PRPP (Figure 4A and C). PurR also binds upstream of pbuG and pbuO (guanine/hypoxanthine permeases) and xpt-pxuX (xanthine phosphoribosyltransferase and xanthine permease), which altogether encode most proteins in the purine salvage pathway, and purA (the first dedicated step towards ATP synthesis) and guaC (GMP reductase that converts GMP to IMP). PurR controls expression of nusB-folD and glyA, which contribute to 10-formyltetrahydrofolate production necessary for de novo IMP synthesis (Figure 4B). Lastly, PurR binds upstream of its own promoter at the purR-yabJ operon.

Our ChIP-seq peaks include six regions not previously known to be PurR binding sites in B. subtilis. The promoters at three of these sites, pdsS-pdxT, steT, and pycA, contain the PurBoxes that were identified at the nine previously known PurR binding sites (Supplementary Figure S10 and Table 1) (19). A sequence logo shows that the PurBoxes at all 12 sites are characterized by a central CGAA motif (or TTCG for the inverted repeat) surrounded by A–T rich sequences, and the 14-bp PurBoxes are separated by a 16- or 17-bp spacer (Figure 4D and Supplementary Figure S10). Two of these new PurR binding sites can be linked to purine nucleotide metabolism. First, the gene steT encodes a serine/threonine exchanger that we hypothesize may be involved in 10-formyltetrahydrofolate regeneration (Figure 4B). This binding site is consistent with steT previously being predicted to be regulated by PurR in a global network analysis (57). Second, the gene pycA encodes pyruvate carboxylase, which can be linked to aspartate synthesis, a necessary metabolite for de novo IMP synthesis (Figure 4B). Unexpected binding sites include upstream of the pdsS-pdxT operon, which encodes the enzyme complex required for de novo PLP (vitamin B6) synthesis (58). There are at least 60 PLP-dependent proteins in B. subtilis, a majority of them involved in amino acid biosynthesis (59). Seven transcription factors bind PLP, including GabR which regulates γ-aminobutyrate utilization (60,61).

We also identified ChIP peaks at the opuB and opuC operons, which encode transporters involved in osmotic protection in B. subtilis (62,63). The final PurR binding site with the weakest ChIP enrichment is intragenic upstream of bdbB, encoding a thiol-disulfide reductase. However, canonical PurBoxes were not found at the opuC, opuB, or bdbB sites. Further investigation will be required to determine whether these are bona fide PurR binding sites or false positive ChIP-seq results.

Interestingly, for all sites with PurBoxes, the peak of PurR ChIP occupancy was offset from the PurBoxes (Figure 4E), suggesting that PurR protects DNA distal from the PurBox binding sequence, possibly through extended PurR oligomerization. Since PurR copy numbers range from 1000 to 3000 per cell in B. subtilis (64,65), there are ample PurR copies to extend beyond the PurBoxes at each binding site.

(pppGpp induction following amino acid starvation enhances promoter occupancy of PurR in B. subtilis)

Next, we examined PurR ChIP signals 10 min after amino acid starvation, which was mimicked using the drug arginine hydroxamate (RHX). RHX induces (pppGpp to millimolar levels in wild-type cells to turn on the stringent response (11). After RHX treatment, PurR ChIP signal increased at each PurR binding site in wild-type cells by an average of 2.2-fold (Figure 4F, Supplementary Figure S11, and Table 1). This included a 2.3-fold increase at the pur operon (Figure 4G and Table 1; see Supplementary Figure S12 for the pur operon promoter sequence with the PurR binding sites). On the other hand, the PurR ChIP signal remained unchanged or decreased slightly in a (pppGppnull ((pppGppβ) mutant (Figure 4G, Supplementary Figure S11, and Table 1), indicating that the increased enrichment in wild-type cells is dependent on (pppGpp.

To test how (pppGpp affects PurR occupancy independently of RHX treatment side-effects, we accounted for PurR occupancy changes due to RHX itself. We first calculated the effect of RHX treatment on PurR occupancy for both wild type and (pppGppβ separately by dividing the PurR enrichment after RHX by the enrichment before RHX treatment. Using these datasets with the effect of RHX accounted for, we were then able to determine the effect of (pppGpp induction on PurR enrichment by taking the ratio of wild type over (pppGppβ. We found a (pppGpp-dependent increase in PurR occupancy at all 12 sites with canonical PurBoxes (Supplementary Figure S13). These PurR binding sites in Table 1 in total control expression of 28 genes, suggesting that these genes’ expression is likely also controlled by (pppGpp.

(pppGpp binding to PurR down-regulates the expression of purine biosynthesis genes during nutrient downshift)

Next, we directly tested the hypothesis that (pppGpp increases repression of the PurR-regulated genes during nutrient adaptation in Bacillus cells. We analyzed our microarray-based transcriptomic profiles of B. subtilis cells under amino acid starvation (18). Strikingly, we observed that almost all the PurR regulated genes, including the pur operon, are downregulated up to 30-fold following amino acid starvation (Figure 5A and Supplementary Figure S14, Table 2). This downregulation is dependent on (pppGpp because little to no downregulation is observed in (pppGppβ cells. These data suggest that (pppGpp accumulation not only enhances PurR binding, but strongly downregulates the expression of genes involved in purine nucleotide synthesis through both de novo and salvage pathways.

To evaluate the role of (pppGpp on PurR regulation specifically, we engineered two PurR variants, PurRY102A/F203A and PurRK102A/K207A. Based on our structural data, these two variants have alterations in residues that interact specifically with ppGpp but not PRPP. We verified that both variants no longer bind to (pppGpp (Figure 5B), yet still bind to DNA probes and their binding to DNA is still inhibited by PRPP (Supplementary Figure S15). We introduced the corresponding mutations into the B. subtilis
Figure 5. (p)pGpp regulation of PurR is important for nutrient stress adaptation. (A) Change in transcript level of PurR-regulated genes after RHX treatment. In wild-type *B. subtilis*, genes are highly downregulated, with some genes in the *pur* operon being downregulated over 30-fold. In comparison, there is only a minor effect of RHX on expression of PurR-regulated genes in (p)pGpp0. These data are from Kriel et al. (2014). Mean of triplicate ± SD is shown. (B) DRacALA binding curves showing 32P-ppGpp interaction with PurR Y102A/F205A and Y102A/K207A. Experiments were performed in technical triplicate. Error bars representing SEM may be shorter than the height of the symbols. (C) Expression of a PurE-GFP reporter in wild-type, (p)pGpp0, *purR*Y102A/F205A, and *purR*Y102A/K207A *B. subtilis*. (D) Doubling times of *B. subtilis* wild type, ΔpurR, *purR*D203A, *purR*Y102A/F205A and *purR*Y102A/K207A in minimal medium (Min) and Min supplemented with 20 amino acids (Min + 20aa). An unpaired parametric two-tailed t-test with Welch’s correction was used to compare wild type and mutants’ doubling times (** P ≤ 0.01; all other pairings are not significant, P > 0.05). (E) Growth of wild type, *purR*Y102A/F205A and *purR*Y102A/K207A in minimal medium following a nutrient downshift from Min + 20aa to Min media. All growth experiments were performed in biological triplicate, and error bars are SEM of triplicate. For OD and fluorescence curves, error bars are represented by dotted lines.

Next, we engineered a GFP reporter controlled by the *pur* operon promoter and evaluated its expression. Using this reporter, we found that wild-type cells, but not the (p)pGpp0 cells, downregulate the expression of the *pur* reporter during entry into stationary phase (Figure 5C and Supplementary Figure S16A). The (p)pGpp-refractory *purR* mutants both fail to downregulate the *pur* reporter, strongly supporting our hypothesis that (p)pGpp interaction with PurR is necessary for repression of the PurR regulon during nutrient downshift.

Next, we examined the consequence of PurR-mediated transcriptional regulation of purine nucleotide synthesis on cell growth and nutrient transitions. We compared a strain with uninducible PurR repression (*purR*D203A, which lacks the ability to bind PRPP) (66,67), a ΔpurR strain, and the (p)pGpp-refractory *purR* mutant strains. All strains grew slowly in minimal medium (Figure 5D). However, in a richer medium supplemented with amino acids, all but the uninducible *purR*D203A decreased their doubling times (Figure 5D, WT versus *purR*D203A P = 0.003). The doubling times of the ΔpurR and (p)pGpp-refractory *purR* mutants were not significantly different from that of wild type. This is expected because cells have only basal levels of (p)pGpp in amino acid replete medium, thus the PurR regulon is expected to be induced by PRPP. The uninducible *purR* mutant displays comparative loss of fitness, suggesting that PurR regulon induction is important for growth under amino acid replete conditions.

Finally, we tested the importance of this interaction for cellular fitness on nutrient stress adaptation. We grew the genome at the endogenous *purR* locus to create (p)pGpp-refractory, separation of function mutants of *purR*. Next, we engineered a GFP reporter controlled by the *pur* operon promoter and evaluated its expression. Using this reporter, we found that wild-type cells, but not the (p)pGpp0 cells, downregulate the expression of the *pur* reporter during entry into stationary phase (Figure 5C and Supplementary Figure S16A). The (p)pGpp-refractory *purR* mutants both fail to downregulate the *pur* reporter, strongly supporting our hypothesis that (p)pGpp interaction with PurR is necessary for repression of the PurR regulon during nutrient downshift.
controls purine nucleotide biosynthesis by binding to multiple promoter sites in *B. subtilis*. (p)ppGpp competes with the PurR inducer PRPP for a binding pocket in the PurR effector binding domain, thus allosterically increasing PurR binding to the promoter DNA. This allows (p)ppGpp to serve as an anti-inducer to strongly repress both *de novo* and salvage purine nucleotide synthesis. Our discovery thus reveals a new regulatory pathway through which purine biosynthesis gene expression is inhibited during amino acid starvation to enhance bacterial adaptation to fluctuating environmental conditions.

### (p)ppGpp as a classical effector of a transcription regulator

Our study provides the first example of the well-characterized alarmone (p)ppGpp acting as an effector of a transcription repressor. Transcription factors often bind effectors that relay external signals to internal responses, allowing a cell to modulate its physiology to adapt to environmental changes. Well-known examples include LacI binding allolactose to derepress the *lac* operon when lactose is available (68), and TrpR binding tryptophan to enhance repression of the *trp* operon when tryptophan is available (69,70). Many nucleotide second messengers also bind transcription factors to relay signals. cAMP binds CRP as a co-activator to signal low carbon availability (5), c-di-GMP binds FleQ in *P. aeruginosa* to regulate flagella expression and exopolysaccharide synthesis (71), VpsT in *Vibrio cholerae* to regulate biofilm formation (72), and BldD in *Streptomyces* to regulate development (7), c-di-AMP binds DarR in *M. tuberculosis* to influence ion and membrane homeostasis (8). All these examples include cyclic nucleotides. However, our findings broaden regulation of transcription factors to include a linear nucleotide, the ‘magic spot’ alarmone (p)ppGpp. It conforms to the fundamental principle of the genetic switch in which cellular signals allosterically regulate a transcription factor, i.e. a small molecule effector binds to a regulatory domain of the transcription factor to affect its binding to cognate operator/promoter DNA. Our study provides the first example of (p)ppGpp as such a classical effector.

Our discovery that (p)ppGpp binds PurR makes it the first case of (p)ppGpp directly regulating transcription in Firmicutes and only the third structurally-characterized example of (p)ppGpp regulation of transcription machines. In *E. coli*, (p)ppGpp binds the RNA polymerase at two sites, the interface of ω and β’ and the interface of β’ and the transcription factor DksA (14,41,42). By binding these sites, (p)ppGpp is proposed to influence RNA polymerase interdomain interaction and stabilize DksA-RNA polymerase interactions (14). In *Francisella tularensis*, (p)ppGpp binds to the interface of the heterodimeric MglA-SspA complex, which interacts with the RNA polymerase to recruit the transcriptional activator PgrR (13). In Firmicutes, (p)ppGpp does not operate by either of these mechanisms. (p)ppGpp binds PurR to make the repressor inaccessible to the PRPP inducer, thus enhancing PurR binding to PurBox 2 (Figure 3E). The PurBoxes at the *pur* operon are just upstream of the -35 promoter element (Figure 4G and Supplementary Figure S12) (56). We have shown by ChIP-seq that PurR protects extensive promoter regions

### DISCUSSION

Transcription factors can relay environmental signals to transcriptional regulation through allosteric regulation by effector ligands. Here we discovered the first example of the ubiquitous alarmone (p)ppGpp acting as an effector for a transcription repressor. Our findings also demonstrate a direct regulation of transcription by (p)ppGpp in Firmicutes. (p)ppGpp binds the transcription repressor PurR, which

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**Table 2. Change in transcription from PurR-regulated genes after arginine hydroxamate treatment**

| Gene  | Wild type (log2 fold change) | (p)ppGpp6 (log2 fold change) |
|-------|-----------------------------|------------------------------|
| purE  | −4.15 ± 0.28               | −0.1 ± 0.18                  |
| purK  | −4.48 ± 0.22               | −0.04 ± 0.62                 |
| purB  | −6.01 ± 0.75               | −0.75 ± 0.59                 |
| purC  | −3.78 ± 0.22               | −0.79 ± 0.38                 |
| purS  | −5.86 ± 0.09               | −1.12 ± 0.31                 |
| purQ  | −6.15 ± 0.23               | −1.41 ± 0.63                 |
| purL  | −5.48 ± 0.4                | −1.08 ± 0.42                 |
| purF  | −5.49 ± 0.21               | −0.78 ± 0.96                 |
| purM  | −5.85 ± 0.17               | −1.35 ± 0.47                 |
| purN  | −4.21 ± 0.43               | −1.2 ± 0.71                  |
| purH  | −4.22 ± 0.15               | −0.99 ± 0.5                  |
| purD  | −2.38 ± 0.36               | −0.58 ± 0.31                 |
| purA  | −4.53 ± 0.42               | −0.36 ± 0.42                 |
| purR  | −1.2 ± 0.21                | 0.23 ± 0.11                  |
| nusB  | −1.77 ± 0.07               | 0.07 ± 0.15                  |
| gusC  | −2.32 ± 0.34               | −0.81 ± 0.08                 |
| pbuG  | −4.11 ± 0.29               | 0.23 ± 0.17                  |
| pbuO  | −3.68 ± 1.03               | −0.2 ± 0.28                  |
| xpt   | −1.5 ± 0.01                | −0.69 ± 0.06                 |
| pbuX  | −4.8 ± 0.67                | −0.96 ± 0.14                 |
| steT  | −4.12 ± 0.59               | −0.73 ± 0.29                 |
| pycA  | 1.65 ± 0.47                | −0.7 ± 0.08                  |
| pdsS  | −0.97 ± 0.25               | −0.88 ± 0.08                 |
| pdsT  | −0.87 ± 0.19               | −0.87 ± 0.17                 |

Log2 fold change in transcription from genes downstream of promoters with PurR-binding PurBoxes in *B. subtilis*. Note that pdsXS and pdsXT are downregulated in both wild type and (p)ppGpp6. This could be due to media differences between this study and Kriel et al. (2014) but requires further investigation. Data are mean ± SD. These data are adapted from Kriel et al. (2014).
and depletion of GTP (18). These include downregulation in Firmicutes, (p)ppGpp was previously only associated with (p)ppGpp indirectly activates the CodY regulon by decreasing CodY levels (75,76). Here, we have found that there is a (p)ppGpp-dependent increase in PurR binding upstream of 28 genes in B. subtilis after nutrient starvation (Supplementary Figure S13 and Table 1). Our work shows that nutrient deprivation signals (p)ppGpp synthesis, (p)ppGpp binds to PurR to outcompete the inducer PRPP and represses the synthesis of purine nucleotides to conserve energy. Thus we now expand our understanding of (p)ppGpp transcriptional regulon in Firmicutes beyond indirect regulation.

A conserved (p)ppGpp-binding motif shared among enzymes and a transcription factor

The nucleotide (p)ppGpp serves a highly pleiotropic role in protecting bacteria by interacting with a multitude of protein and riboswitch targets to remodel bacterial metabolomes, transcriptomes, and proteomes and to tune bacterial physiology for growth in varied environments (44,77–79). (p)ppGpp regulates its targets with a few relatively conserved direct binding motifs. In one case, (p)ppGpp binds to many GTPases through their GTP-binding motif (80–83). In another case, (p)ppGpp binds to a conserved PRPP-binding motif in the PRT fold in two enzymatic targets, the purine salvage enzymes HPRT and XPR (26,27). The (p)ppGpp binding site on PurR is the third PRT site also found to bind (p)ppGpp (Figure 6A). Here, the PRT enzyme is repurposed to become an effector binding regulatory domain of a transcription factor, the substrate PRPP is repurposed to become an inducer, and the enzyme inhibitor (p)ppGpp is repurposed to become an anti-inducer (Figure 6A). This striking evolutionary strategy highlights how nature utilizes limited motifs to evolve diverse regulatory targets of (p)ppGpp, allowing it to serve as a master regulator of nutrient stress.

Despite PurR, HPRT and XPR sharing the same (p)ppGpp binding motif, how ligand binding affects the function of each protein has diversified through distinct allosteric and oligomeric properties surrounding this binding pocket. For HPRT, robust (p)ppGpp inhibition is dependent on HPRT being a tetramer. Tetramerization allosterically opens the (p)ppGpp binding pocket by holding loop II at the dimer–dimer interface. Alteration of the dimer–dimer interface then allows diversification of (p)ppGpp regulation of HPRT across phylogenetically diverse organisms without changing the binding pocket (26). In XPR, two (p)ppGpp binding sites face one another across an XPR monomer–monomer interface in an XPR dimer. (p)ppGpp binding creates an electrostatic network with the bridging loop of the other monomer, inducing XPR dimerization, this enables a cooperativity of regulation with Hill coefficient of 2 (27). In this study, we have discovered a third allosteric property associated with this same motif. By binding its effector site on PurR, (p)ppGpp competes with an inducer of a transcription factor to enhance repression during stress.

Our work has also highlighted a broader (p)ppGpp binding motif shared among GTPases, PRTs, and other targets such as guanylate kinase (80,82,84,85). GTPases and guanylate kinase bind (p)ppGpp with a phosphate-binding loop (P-loop) motif, which is used to interact with nucleotides (86). While the PRT proteins lack the P-loop amino acid sequence, they are structurally reminiscent of the P-loop with...
Figure 6. Models of (p)ppGpp regulation of PurR and global (p)ppGpp
regulation of purine nucleotide synthesis in B. subtilis. (A) Schematic of
PurR evolution from PRT enzymes like XPRT. PurR repurposes the active
site of PRT enzymes to serve as an effector binding pocket, which PRPP
and ppGpp bind to allosterically regulate DNA binding. (B) GTP and
ATP synthesis in B. subtilis. (p)ppGpp regulates GTP synthesis at multiple
points, including inhibition of GTP synthesis enzymes (IMPDH, GMK,
HPRT, XPRT) and binding the transcription factor PurR. Red arrows rep-
resent steps in the PurR regulon.

Implication of (p)ppGpp-PRPP competition on nutritional
regulation of PurR regulon

During the evolutionary co-opting of the PRT enzyme as
the PurR effector binding regulatory domain, not only has
(p)ppGpp binding been conserved, so has the (p)ppGpp-
PRPP competition (Figure 6A). PRPP is the common sub-
strate for HPRT and XPRT, and (p)ppGpp inhibits HPRT
and XPRT by competing with PRPP (26,27). Here we have
shown that (p)ppGpp also competes with PRPP to bind
PurR. In all cases, PRPP is a signal to the cell for increased
nucleotide synthesis: it is directly converted to nucleotides
through purine salvage or it binds PurR to de-repress the de
novo IMP synthesis pathway (47). (p)ppGpp competes with
PRPP by binding the same binding site in each of these pro-
teins, shutting down nucleotide synthesis.

The competition between (p)ppGpp and PRPP for PurR
binding likely tunes transcription from the PurR regulon
during nutrient replete and nutrient starved growth. During
unstressed growth, PRPP concentrations range from hun-
dreds of micromolar to millimolar and (p)ppGpp is at con-
centrations < 50 μM (Supplementary Figure S7) (11,47).
Since both PRPP and (p)ppGpp bind PurR with $K_d \sim 5 \mu M$
(Figure 1H) (47), in nutrient replete conditions PRPP levels
dictate PurR binding and induce PurR-regulated genes for
rapid synthesis of GTP and ATP. PRPP-mediated induc-
tion can be overridden by salvageable nucleobases, which
repress the de novo purine operon by consuming PRPP upon
conversion to nucleotides and by binding riboswitches that
control select nucleotide synthesis genes (67,87).

During amino acid starvation, (p)ppGpp levels are ele-
vated up to >1 mM, allowing (p)ppGpp to compete with
PRPP and repress purine nucleotide synthesis. This not only
conserves energy and resources used for transcription and
translation of the PurR regulon, but enables the coordinated
use of PRPP, which is a common precursor of nu-
cleotides (GTP, ATP, UTP) and several amino acids (47).
In E. coli, a mutant for which (p)ppGpp fails to inhibit purine
nucleotide synthesis results in PRPP depletion due to over-
production of purine nucleotides (88). (p)ppGpp inhibition
of purine synthesis redirects PRPP from purine nucleotide
synthesis to UTP and amino acid synthesis during starva-
tion (88,89). We propose that, in Firmicutes, the direct com-
petition between (p)ppGpp and PRPP for PurR, HPRT and
XPRT (see Figure 6B) would serve the same purpose, al-
lowing (p)ppGpp to coordinate the consumption of PRPP
between purine synthesis and amino acid synthesis during
starvation. Interestingly, the (p)ppGpp-PRPP competition in
B. subtilis would allow maintenance of PRPP levels: when
(p)ppGpp accumulates after amino acid starvation, it di-
rectly prevents PRPP depletion by inhibiting its consump-
tion in purine salvage and by preventing PRPP from activat-
ing de novo IMP synthesis.

(p)ppGpp controls de novo purine synthesis by targeting dif-
ferent protein factors between bacterial species

Our results reveal that (p)ppGpp regulates de novo purine
synthesis across bacteria. The 11 reactions that synthesize
IMP from PRPP are necessary for de novo synthesis of
ATP and GTP. Until recently, there was no evidence that
(p)ppGpp directly regulated this pathway in bacteria. In E.
coli, it was recently shown that (p)ppGpp inhibits the ac-
tivity of PurF, the first step in the pathway, and (p)ppGpp
binding the RNA polymerase decreases the transcription of
all the genes in the pathway (15,78). We have shown that
(p)ppGpp regulation of de novo purine synthesis extends to
Firmicutes, albeit by an entirely different mechanism. While

a loop leading into the positive dipole of an α-helix. The
5′-phosphates of (p)ppGpp and PRPP fit into the pocket
created by the loop and are coordinated by the loop’s back-
bone (Figures 2D and 3A). This structural motif associ-
ated with (p)ppGpp binding may become more prevalent
as more (p)ppGpp binding targets are characterized.

(p)ppGpp regulates de novo synthesis of IMP in B. subtilis.
(p)ppGpp binding to PRPP and PurR results in conser-
vation of IMP and ATP at the expense of PRPP.
(p)ppGpp-mediated inhibition of IMP synthesis in E.
coli, which prevents PRPP depletion during amino acid
starvation, is conserved in Firmicutes.
Proteobacteria and Firmicutes have evolved different mechanisms for this regulation, they share the common theme of decreasing de novo synthesis upon (p)ppGpp induction. This theme is likely conserved across other bacteria as well.

With our data, we can begin building a model of (p)ppGpp regulating purine nucleotide synthesis at multiple checkpoints in Firmicutes. As we have shown, it regulates de novo and salvage nucleotide synthesis genes via its interaction with PurR (Figure 6B). (p)ppGpp also inhibits the activities of nucleobase salvage enzymes HPRT and XPR (11,26,27), and it potently inhibits guanylate kinase activity, which catalyzes GMP to GDP (Figure 6B) (85). Altogether, purine nucleotide synthesis has evolved to be sensitive to (p)ppGpp regulation at a majority of the synthesis steps. Since purine nucleotides are often a signal to increase growth, (p)ppGpp counteracts this during stressful conditions to control growth and enable survival.

DATA AVAILABILITY

ChIP-seq analysis pipeline and scripts are available at https://github.com/bwanderson3/2021_PurR_ChIP_analysis.git.

Atomic coordinates and structure factors for the reported crystal structure have been deposited with the Protein Data Bank under accession number 7RMW. ChIP-seq datasets have been deposited with the NCBI Gene Expression Omnibus under GSE185164.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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