The nucleotide pGpp acts as a third alarmone in *Bacillus*, with functions distinct from those of (p)ppGpp

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The alarmone nucleotides guanosine tetraphosphate and pentaphosphate, commonly referred to as (p)ppGpp, regulate bacterial responses to nutritional and other stresses. There is evidence for potential existence of a third alarmone, guanosine-5′-monophosphate-3′-diphosphate (pGpp), with less-clear functions. Here, we demonstrate the presence of pGpp in bacterial cells, and perform a comprehensive screening to identify proteins that interact respectively with pGpp, ppGpp and pppGpp in *Bacillus* species. Both ppGpp and pppGpp interact with proteins involved in inhibition of purine nucleotide biosynthesis and with GTPases that control ribosome assembly or activity. By contrast, pGpp interacts with purine biosynthesis proteins but not with the GTPases. In addition, we show that hydrolase NahA (also known as YvcI) efficiently produces pGpp by hydrolyzing (p)ppGpp, thus modulating alarmone composition and function. Deletion of nahA leads to reduction of pGpp levels, increased (p)ppGpp levels, slower growth recovery from nutrient downshift, and loss of competitive fitness. Our results support the existence and physiological relevance of pGpp as a third alarmone, with functions that can be distinct from those of (p)ppGpp.
Organisms from bacteria to humans rely on timely and appropriate responses to survive various environmental challenges. The stress signaling nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (ppppGpp) are conserved across bacterial species. When induced upon starvation and other stresses, they mediate multiple regulations and pathogeneses by dynamically remodeling the transcriptome, proteome, and metabolome of bacteria in a rapid and consistent manner. ppGpp interacts with diverse targets including RNA polymerases in *Escherichia coli*, replication enzyme primase in *Bacillus subtilis*, purine nucleotide biosynthesis enzymes, and GTPases involved in ribosome assembly. Identification of (p)pppGpp-binding targets on a proteome-wide scale is one way to unravel a more extensive regulatory network. However, because binding targets differ between different species and most interactomes have not been characterized, the conserved and diversifying features of these interactomes remain incompletely understood.

Another understudied aspect of (p)pppGpp regulation is whether ppGpp and pppGpp, while commonly referred to and characterized as a single species, target the same or different cellular pathways. In addition, there is evidence for potential existence of a third alarmone, guanosine-5'-monophosphate-3'-diphosphate (pGpp), since several small alarmone synthetases can synthesize pGpp in vitro. However, the clear demonstration of pGpp in bacterial cells has been challenging. More importantly, the regulation specificities and physiological importance of having multiple closely related alarmones in bacteria have not been systematically investigated.

Here we demonstrate pGpp as a third alarmone in Gram-positive bacteria by establishing its presence in cells, systematically identifying its interacting targets, and revealing a key enzyme for pGpp production through hydrolyzing (p)pppGpp. We also compare the targets of pGpp, ppGpp, and pppGpp through proteomic screens in *Bacillus anthracis*. We found that both ppppGpp and ppGpp regulate two major cellular pathways: purine synthesis and ribosome biogenesis. In contrast, pGpp strongly regulates purine synthesis targets but does not regulate ribosome biogenesis targets, indicating a separation of regulatory function for these alarmones. In *B. subtilis* and *B. anthracis*, ppGpp and pppGpp are efficiently produced from pppGpp and ppGpp by the Nucleoside Diphosphate linked to any moiety “X” (NuDX) NuDX alarmonc hydrolase A (hydrolyse NahA), both in vitro and in vivo. A ΔnahA mutant has significantly stronger accumulation of pppGpp and decreased accumulation of pGpp, as well as slower recovery from stationary phase and reduced competitive fitness against wild-type cells. Our work suggests a mechanism for the conversion and fine tuning of alarmonc regulation and the physiological production of the alarmonc pGpp.

**Results**

Proteome-wide screen for binding targets of pppGpp and ppGpp from *Bacillus anthracis*. To systematically characterize the binding targets of (p)pppGpp and identify novel (p)pppGpp-binding proteins in *Bacillus* species, we screened an open reading frame (ORF) library of 5341 ORFs from the pathogen *Bacillus anthracis* (Fig. 1a). Using Gateway cloning, we placed 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.

We first characterized the binding targets of ppGpp using the *B. anthracis* library. To this end, each ORF in the HisMBP-tagged library was overexpressed and binding to [5′-α-32P]-ppGpp was assayed using differential radial capillary action of ligand assay (DRA CALA) (Fig. 1a). The fraction of ligand bound to protein in each lysate was normalized as a Z-score of each plate to reduce the influence of plate-to-plate variation (Supplementary Data 1). We found that the strongest ppGpp-binding targets in *B. anthracis* can be categorized to three groups: (1) purine nucleotide synthesis proteins (Hpt1, Xpt, Gmk, GutC, PurA, and PurR); (2) ribosome and translation regulatory GTPases (HfX, Der, Obg, RbgA, TrmE, and Era); and (3) nucleotide hydrolytic enzymes, including NuDX hydrolases and nucleotide-dases (Fig. 1b). We compared these targets to those obtained from previous screens for ppGpp targets in *E. coli* and for an unseparated mix of pppGpp and ppGpp in *S. aureus*. Comparison of our results with these previous screens yielded conserved themes (Fig. 1b). Among the most conserved themes are the purine nucleotide synthesis proteins (Fig. 1c) and ribosome and translation regulation GTPases (Fig. 1d).

Next, we performed a separate screen to characterize the binding of the *B. anthracis* proteome to pppGpp (Fig. 1a). pppGpp is the predominant alarmonc induced upon amino acid starvation in *Bacillus* species, rising to a higher level than ppGpp. However, despite potential differences in specificity between ppGpp and pppGpp, the pppGpp interactome has not been systematically characterized in bacteria. We used both His-tagged and HisMBP-tagged libraries to test pppGpp binding. Using two differentially tagged libraries allows us to identify more potential hits and minimize false negatives. We found that pppGpp shares almost identical targets with ppGpp, with similar or reduced binding efficacy for most of its targets compared to ppGpp (Supplementary Data 1). By sharing targets with ppGpp, pppGpp also comprehensively regulates purine synthesis and ribosome assembly. We also found that several proteins bind to ppGpp but not pppGpp, including the small alarmone synthetase YjbM (SA51). This is expected for YjbM, since it is allosterically activated by ppGpp but not ppGpp.

NahA, a NuDX hydrolase among the (p)pppGpp interactome in *Bacillus*, hydrolyzes (p)pppGpp to produce ppGpp in vitro. The putative NuDX hydrolase, BA5385, was identified as a novel binding target of (p)pppGpp. Protein sequence alignment showed that BA5385 has homologs in different *Bacillus* species with extensive homology and a highly conserved NuDX box (Fig. S1). We cloned its homolog, YvcI, from the related species *Bacillus subtilis* and showed that overexpressed B. subtilis YvcI in cell lysate also binds ppGpp and pppGpp (Fig. 2a). The binding is highly specific, as non-radiolabeled ppGpp effectively competes with radiolabeled (p)pppGpp binding, whereas non-radiolabeled GTP failed to compete. EDTA eradicated (p)pppGpp binding to His-MBP-YvcI cell lysate, which implies that the divalent cation present in the reaction (Mg2+) is essential for (p)pppGpp binding to YvcI (Fig. 2a).

We noticed that while YvcI-overexpression cell lysate showed strong and specific binding to (p)pppGpp, the purified protein does not appear to bind (p)pppGpp in DRACALA (Fig. S2). This suggests that either YvcI requires a co-factor present in the lysate to bind to (p)pppGpp, or YvcI may rapidly hydrolyze (p)pppGpp and release the product. Therefore, we incubated purified YvcI with [5′-α-32P]- (p)pppGpp and ran the reaction product using TLC (Fig. S3). We found that YvcI can hydrolyze both ppGpp and pppGpp. We also tested the ability of YvcI to hydrolyze GTP and 8-oxo-GTP to sanitize guanosine nucleotide pool. YvcI failed to hydrolyze either GTP (Fig. S3a) or 8-oxo-GTP (Fig. S3c). The inability of YvcI to hydrolyze GTP despite the structural similarity between GTP and (p)pppGpp suggests that NahA is a specific (p)pppGpp hydrolase which requires its substrate to have pyrophosphate group on the 3′ end.
pppGpp and ppGpp can be hydrolyzed by the Rel enzyme, traditionally referred as RelA in *B. subtilis* 27–29, to produce GTP and GDP respectively. However, unlike Rel, YvcI hydrolyzed pppGpp and ppGpp to yield a single-nucleotide species that migrated differently than GTP (Fig. S3a) or GDP (Fig. S3b). To determine the identity of YvcI’s (p)ppGpp hydrolysis product, we analyzed the sample by liquid chromatography coupled with mass spectrometry (LC-MS), and compared to a pGpp standard produced by *E. faecalis* SAS (RelQ) in vitro 22. The LC-MS profile revealed a peak of the same mass over charge ratio (m/z) as GTP but with a different retention time (11.75 min versus 11.15 min for GTP). This result showed that the product has an intact 3′-β-32P]-pppGpp. If NahA cleaves between 5′-α and 5′-β phosphates, the reaction would yield 3′-β-32P]-pppGpp. In contrast, if NahA cleaves between 3′-α and 3′-β-phosphates, the reaction would yield free 32P-phosphate. TLC analysis revealed that the radioactive 32P after NahA hydrolysis of [3′-β-32P]-pppGpp co-migrates with the pGpp nucleotide rather than the free phosphate that migrates to the very end of TLC plate (Fig. 2c). This result showed that the product has an intact 3′-pyrophosphate group, confirming the product to be pGpp rather than the radioactive 32P-phosphate.

The production of pGpp from (p)ppGpp agrees with the NuDiX hydrolase function, inferring that pppGpp and ppGpp are hydrolyzed between the 5′-α and 5′-β phosphate groups to produce guanosine-5′-monophosphate-3′-diphosphate (pGpp). Therefore, we renamed the enzyme NuDiX alarmone hydrolase A (NahA).

### References

1. **Fig. 1** Proteome-wide DRaCALA screen identifies both conserved categories of binding targets and novel targets. a The *Bacillus anthracis* ORF donor vector library was recombined by Gateway cloning into overexpression vectors to generate ORFs with an N-terminal His-tag or HisMBP tag. The plasmids were transformed into *E. coli* for co-translation of recombinant proteins. Lysates of each ORF overexpressed in *E. coli* were assayed for binding to pppGpp, ppGpp, and pGpp using DRaCALA. b List of identified (p)ppGpp-binding targets in *E. coli*, *S. aureus*, and *B. anthracis* and ppGpp-binding targets in *B. anthracis*. ppGpp results were obtained using both His-tagged and His-MBP-tagged proteins. ppGpp and pGpp results were obtained using His-MBP-tagged proteins. NT not tested, NA not available due to the lack of homologous gene. c Enzymes in purine nucleotide synthesis, including HprT, Xpt, Gmk, and GuaC, bound both pGpp and (p)ppGpp; d GTPases, involved in ribosome biogenesis and translational control (Obg, HfIX, Der, RbgA, and Era), bound (p)ppGpp, but not pGpp.
than ppGp. Finally, quantification of \([3'\beta-32P]\)-pppGpp hydrolysis by NahA showed that the decrease of substrate radioactivity mirrored the increase of the single product radioactivity (Fig. 2d), demonstrating the product is exclusively pGpp.

As a NuDiX hydrolase, NahA has been reported to have a modest activity in removing the 5′-phosphate of mRNA30. We found that NahA is far more efficient at hydrolyzing (p)ppGpp than at decapping mRNA. Enzymatic assays revealed that NahA hydrolyzes ppGpp following Michaelis–Menten kinetics, with a \(k_{\text{cat}}\) of \(1.22 \pm 0.17 \text{s}^{-1}\) and a \(K_m\) of \(7.5 \pm 2.3 \mu\text{M}\) (Fig. 2e and Table 1).

![Fig. 2 NahA (Yvcl) produces pGpp via (p)ppGpp hydrolysis.](image)

**Table 1.** Kinetic parameters of (p)ppGpp hydrolysis by NahA.

|        | ppGpp | pppGpp |
|--------|-------|--------|
| \(k_{\text{cat}}\) (s\(^{-1}\)) | 1.22 \(\pm\) 0.17 | 10.0 \(\pm\) 0.5 |
| \(K_m\) (\(\mu\text{M}\)) | 7.5 \(\pm\) 2.3 | 177.4 \(\pm\) 0.4 |
| Hill coefficient \(n\) | 1 | 2.78 \(\pm\) 0.47 |
**Fig. 3** pGpp is produced by NahA in vivo and has a distinct binding spectrum compared to (p)pppGpp. **a** LC-MS analyses of pGpp, ppGpp, and pppGpp of wild type and ΔnahA in log phase and stationary phase. Normalized ion count is ion count per OD600 norm per unit volume of the culture. Error bars represent standard errors of the mean of three biological replicates. A two-tailed two-sample equal-variance Student’s t test was performed between samples indicated by asterisks. Asterisks indicate statistical significance of differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). **b** In vitro hydrolysis of pGpp, ppGpp, and pppGpp by B. subtilis Rel. For each nucleotide, 200 nM Rel was incubated with a mix of 100 μM unlabeled alarmone and a small amount of 5′-α-32P-labeled version. The degradation of the alarmone was analyzed by TLC to measure the decreased radioactivity. Error bars represent standard errors of the mean of three replicates. **c** DRaCALA of purified His-MBP-tagged B. anthracis proteins (1 μM) with <0.1 nM of identical amount of 5′-α-32P-labeled pGpp, ppGpp, and pppGpp. **d** Quantification of DRaCALA in c. Error bars represent standard errors of the mean of three replicates except for control group (six replicates). **e** HprT enzymatic activities in the presence of indicated concentrations of pGpp, ppGpp, and pppGpp. The reaction was performed with 20 nM HPRT, 1 mM PRPP, and 50 μM guanine14. Error bars represent standard errors of the mean from three replicates. **f**–**h** Titrations of B. anthracis GTPases and quantification of their binding to pGpp, ppGpp and pppGpp using DRaCALA: GTPases HIX (**f**), Obg (**g**), and translation elongation factor G (EF-G) (**h**). Error bars represent standard errors of the mean of three replicates. **i** Schematic showing the relationship between pGpp-binding targets and (p)pppGpp-binding targets.

Table 1). NahA also effectively and cooperatively hydrolyzes pppGpp with a Hill coefficient of 2.78 ± 0.47, kcat of 10.0 ± 0.5 s⁻¹ and Km of 177.4 ± 0.4 μM (Fig. 2e and Table 1). In contrast, its kcat to decap RNA is ~0.0003 s⁻¹ (estimation based on published figure)30. The difference in kcat in vitro suggests that NahA’s major function is to regulate (p)pppGpp rather than to decap the 5′-cap of mRNA.

NahA hydrolyzes (p)pppGpp to produce pGpp in vivo. NahA was previously identified as a constitutively expressed protein with ~600 copies per cell31. To examine its impact on (p)pppGpp in vivo, we engineered a nahA deletion strain, and developed an LC-MS quantification for pppGpp, ppGpp, and pGpp in B. subtilis cells (see Methods section). LC-MS measurement of cell extracts showed that ΔnahA cells accumulate more pppGpp and pppGpp than wild-type cells during both log phase and stationary phase, in agreement with NahA’s ability to hydrolyze (p)pppGpp (Fig. 3a). In contrast, ΔnahA mutant has much less pGpp than wild-type cells (Fig. 3a). Specifically, during log phase, pGpp can hardly be detected in ΔnahA (Fig. S4a). When we complement ΔnahA with an overexpressed copy of nahA, the pGpp levels increased to more than wild-type levels (Figure S4). These results support the function of NahA in producing pGpp.

We also used the drug arginine hydroxamate (RHX) which mimics amino acid starvation to induce accumulation of (p)pppGpp12. Using both LC-MS and TLC, we observed rapid accumulation of (p)pppGpp after RHX treatment, with ΔnahA cells showing stronger (p)pppGpp accumulation than wild-type cells (Fig. S5a–d). We still observed the accumulation of pGpp in ΔnahA cells, although to a much less extent than wild-type cells.
Protein binding spectrum of pGpp is distinct from (p)ppGpp hydrolysis or is a bona fide alarmone with its own regulatory targets, we used DRaCALA to systematically screen the B. anthracis library for pGpp-binding targets (Fig. 1a, b and Supplementary Data 1). Our screen showed that pGpp binds strongly to multiple purine nucleotide synthesis enzymes (Fig. 1c), but to none of the (p)ppGpp-binding ribosome and translation regulation GTPases (Fig. 1d). We then purified selected pGpp and (p)ppGpp-binding targets and tested with [5′-α-32P]-labeled pGpp, ppGpp, and pppGpp using DRaCALA (Fig. 3c, d). These results confirmed strong pGpp binding to guanosine nucleotide synthesis proteins (Hpt1, Gmk, and Xpt). Enzyme kinetic assay of Xpt32 and HprT (Fig. 3e) confirmed that pGpp inhibited their activities more potently than ppGpp and pppGpp. In contrast, GTPases involved in ribosome biogenesis (Hix, Obg, Der) bind ppGpp but not pGpp (Fig. 3d). Titration analysis of these GTPases showed their strong affinity to ppGpp, modest affinity to pppGpp and lack of affinity to pGpp (Fig. 3f–h and Supplementary Table 1). We conclude that among the two main groups of conserved interaction targets of (p)ppGpp, pGpp exclusively regulates the purine pathway, but not the GTPases, thus can serve as a specialized signal (Fig. 3i).

Protein binding spectrum of pGpp is distinct from (p)ppGpp.

To understand whether pGpp is just an intermediate of (p)ppGpp hydrolysis or is a bona fide alarmone with its own regulatory targets, we used DRaCALA to systematically screen the B. anthracis library for pGpp-binding targets (Fig. 1a, b and Supplementary Data 1). Our screen showed that pGpp binds strongly to multiple purine nucleotide synthesis enzymes (Fig. 1c), but to none of the (p)ppGpp-binding ribosome and translation regulation GTPases (Fig. 1d). We then purified selected pGpp and (p)ppGpp-binding targets and tested with [5′-α-32P]-labeled pGpp, ppGpp, and pppGpp using DRaCALA (Fig. 3c, d). These results confirmed strong pGpp binding to guanosine nucleotide synthesis proteins (Hpt1, Gmk, and Xpt). Enzyme kinetic assay of Xpt32 and HprT (Fig. 3e) confirmed that pGpp inhibited their activities more potently than ppGpp and pppGpp. In contrast, GTPases involved in ribosome biogenesis (Hix, Obg, Der) bind ppGpp but not pGpp (Fig. 3d). Titration analysis of these GTPases showed their strong affinity to ppGpp, modest affinity to pppGpp and lack of affinity to pGpp (Fig. 3f–h and Supplementary Table 1). We conclude that among the two main groups of conserved interaction targets of (p)ppGpp, pGpp exclusively regulates the purine pathway, but not the GTPases, thus can serve as a specialized signal (Fig. 3i).

naha mutant exhibits stronger inhibition of translation upon amino acid starvation, delayed outgrowth, and loss of competitive fitness. The fact that pGpp does not directly regulate ribosome biogenesis and translation implicates an in vivo function of NahA: reducing (p)ppGpp levels to alleviate translation inhibition upon stress, while still keeping purine biosynthesis in check. To test this hypothesis, we analyzed the effects of naha on cellular growth and metabolism. We first compared the key metabolites (NTPs, NDPs, NMPs, nucleosides, and nucleobases)

**Fig. 4** The effect of NahA on purine nucleotides, translation, fitness, and growth recovery of B. subtilis cells. a Hierarchical clustering of selected metabolites in exponential growth and stationary phase wild type and ΔnahA cells. Metabolites were measured by LC-MS. Normalized ion count is ion count per OD_{600nm} per unit volume of the culture. b Protein translation rate of wild type and ΔnahA cells 40 min after amino acid starvation. Translation rate was measured by a 5-min pulse of [35S]-methionine incorporation into TCA-precipitable fraction. Error bars represent standard errors of the mean from three replicates. c Relative fitness of ΔnahA and wild type strains obtained from a 7-day serial dilution competition assay. Error bar represents standard deviation from three repeats. d Doubling times of ΔnahA and wild-type cells in log phase. A two-tailed two-sample equal-variance Student’s t test was performed between samples indicated by asterisks. Asterisk indicates statistical significance of difference (*P ≤ 0.05, **P ≤ 0.001, n.s. P > 0.05). e Growth recovery from transient nutrient downshift. Log phase cultures of wild type, ΔnahA and naha complementation (Δnaha IPTG-naha) strains in rich media with 20 amino acids were firstly downshifted in minimum media without amino acids for 10 min, and then diluted in rich media for outgrowth. Result of growth recovery from similar downshift with the presence of guanosine is shown in Figure S6c. Error bars in d, e represent standard errors from six replicates.
between wild-type cells and the nahA mutant, using LC-MS analyses of exponential growth and stationary phase cells (Fig. 4a and Supplementary Data 2). Despite much higher (p)ppGpp levels and much lower pGpp levels in the nahA mutant, most purine nucleotides exhibit very little difference, corroborating with the observation that pGpp regulates purine metabolism similarly to (p)ppGpp.

Next, we examined the effect of nahA on protein translation. We measured total protein translation rate of wild type and ΔnahA B. subtilis by pulsed incorporation of 35S-methionine (Fig. 4b). We induced amino acid starvation either by resuspending cells from amino acid replete medium to minimal medium, or by adding the nonfunctional amino acid analog arginine hydroxamate. In both cases, the rate of 35S-methionine incorporation is significantly lower in ΔnahA than in wild type, indicating a stronger inhibition of translation (Figs. 4b and S6a).

Finally, we examined the impact of nahA on fitness of B. subtilis. We performed a growth competition assay in which a mixture of ΔnahA and wild-type cells were grown to saturation and then diluted repeatedly for days. The proportion of ΔnahA rapidly decreased (Fig. S6b), indicating significant loss of fitness compared to wild-type cells (Fig. 4c). We found that ΔnahA has a similar doubling time as wild-type cells (Fig. 4d), but a longer lag phase in adjusting to starvation (Fig. 4e). Together, these results suggest that NahA tunes alarmone composition and alarmone levels to promote B. subtilis adjustment to nutrient fluctuation and optimizes growth and fitness.

Discussion

Alarmones are universal stress signaling nucleotides in bacteria, however, the repertoire of alarmones and the target spectrums for each alarmone are incompletely understood. In this work, we have comprehensively characterized the interactomes of the related alarmones ppGpp and pGpp and established the in vivo presence of pGpp as a closely related alarmone in Gram-positive Bacillus species. We characterized the direct targets of (p)ppGpp by screening an open reading frame expression library from B. anthracis. From this screen, we identified an enzyme NahA that converts (p)ppGpp to pGpp as efficient means to produce pGpp and to reduce (p)ppGpp concentrations, thus regulating the composition of the alarmones. We demonstrated that pGpp binds a distinct subset of protein receptors of (p)ppGpp. We also identified a key role of NahA in nutrient adaptation, suggesting that regulating alarmone composition may serve as a separation-of-function strategy for optimal adaptation.

Conservation of pppGpp and pGpp regulation of purine biosynthesis and ribosome biogenesis pathways across different species of bacteria. (p)ppGpp regulates diverse cellular targets that differ between different bacteria. For example, (p)ppGpp directly binds to RNA polymerase in E. coli to control the transcription of ribosomal and tRNA operons yet the (p)ppGpp-binding sites on RNA polymerase are not conserved beyond proteobacteria4-8. Instead, (p)ppGpp accumulation in firmicutes strongly down-regulates synthesis of GTP, the exclusive transcription initiating nucleotides of RNA and tRNA operons in firmicutes, to achieve a similar transcription control with different direct targets12,33,34. Therefore, identifying whether certain aspects of (p)ppGpp regulation are conserved among bacterial species is important for understanding the principles of bacterial survival and adaptation.

Our DRaCALA screen with a B. anthracis library revealed many ppGpp and pppGpp binding targets in this pathogenic Gram-positive bacterium. Comparing ppGpp-binding targets in B. anthracis to S. aureus18 and E. coli15,20 identified novel targets but more importantly, revealed a clear theme of conservation. Most notably, (p)ppGpp in all three species binds to multiple proteins in two key pathways: (1) purine nucleotide synthesis, (2) translation-related GTPases including ribosome biogenesis factors.

The regulation of purine nucleotide synthesis in firmicutes includes well characterized targets Gmk and HprT whose regulation by (p)ppGpp protects Bacillus subtilis against nutrient changes like amino acid starvation and purine fluctuation, preventing cells from accumulating toxic high levels of intracellular GTP12,14,35. These also include new-found likely targets such as enzymes GuaC and PurA and the de novo pathway transcription factor PurR. Intriguingly, in the evolutionarily distant E. coli, the ppGpp targets in the purine biosynthesis pathway are different. For example, Gmk is not regulated by (p)ppGpp in E. coli25. On the other hand, (p)ppGpp directly targets the E. coli de novo enzyme PurE25, a target that is not conserved in firmicutes. Therefore, despite differences in precise targets, (p)ppGpp extensively regulates the purine biosynthesis pathway in evolutionarily diverse bacteria (Fig. 1c), highlighting this critical physiological role of (p)ppGpp.

We also found that (p)ppGpp interacts with essential GTPases that are implicated in ribosome biogenesis and translation in B. subtilis and B. anthracis (Fig. 1d). (p)ppGpp’s targets in GTPases from E. coli to firmicutes are conserved16-19, HIlX, Obg, and Era, are also (p)ppGpp-binding proteins in E. coli and S. aureus15,18,20. Der and TrmE were identified in E. coli although not in S. aureus as (p)ppGpp-binding proteins15,20, RbgA does not exist in E. coli and it is identified as a (p)ppGpp-binding protein in S. aureus18. HIlX is a ribosome-splitting factor which may rescue stalled ribosomes under stressed conditions36, and mediates the dissociation of hibernating 100S ribosome to resume normal translation37. Obg16, Der38, and RbgA39,40 participate in the maturation of the 50 S subunit of ribosome. Era functions in the assembly of the functional 70 S ribosome complex41. TrmE functions in the maturation of tRNA to facilitate translation42. (p) ppGpp can be produced by amino acid starvation-induced translational stress or by defects in tRNA maturation43. Thus the conservation of (p)ppGpp regulation of GTPase targets highlights the key function of (p)ppGpp in quality control of ribosome biogenesis and regulation of translation.

The third major alarmone pGpp in Gram-positive species and its specific regulatory effect. In addition to pppGpp and ppGpp, it was long suspected that in B. subtilis, there are additional alarmones such as pppApp, pGpp, and ppGpp accumulating during the stringent response44,45. Here we detected pGpp in B. subtilis cells, characterized an enzyme for its production in vivo and in vitro, and identified its targets. In contrast to our DRaCALA screens for pppGpp and ppGpp which identify similar targets and functionalities between these two alarmones, our DRaCALA screen for pGpp displays a strong difference from (p)ppGpp with regarding to two conserved pathways. The affinity and inhibitory potency of pGpp for purine nucleotide synthesis enzymes are equivalent or higher than that of (p)ppGpp. In contrast, pGpp’s affinity to GTPases involved in translational regulation is much lower, or completely absent, compared to (p)ppGpp. The distinct profile of target receptors establishes pGpp as a different alarmone from (p)ppGpp, allowing fine tuning of bacterial stress response. We propose a model for the function of NahA in growth recovery and competitive fitness by its role in transforming the alarmones. In wild-type cells, (p)ppGpp produced in response to amino acid starvation will be hydrolyzed in part by NahA to pGpp. (p)ppGpp concentration in wild-type cells...
during amino acid starvation can reach >1 mM. Given the copy number of NahA as ~600 copies per cell and its maximum velocities for (p)ppGpp hydrolysis, (p)ppGpp concentrations observed in wild-type cells is a balance of synthesis by enzymes Rel and SAS1/2, and hydrolysis by Rel and NahA. Thus, in ΔnahA cells, (p)ppGpp accumulates higher than in wild-type cells during amino acid starvation. Stronger inhibition of translation due to overly high level of (p)ppGpp in ΔnahA leads to slower growth recovery after nutrient stress and thus leads to fitness loss when co-cultured with wild-type strain. In addition, because pGpp appears to be hydrolyzed more efficiently by RelA than ppGpp and pppGpp (Fig. 3b), another role of NahA is perhaps to suppress (p)ppGpp removal to promote growth recovery.

In E. coli, (p)ppGpp-binding proteins also include NuDX hydrolases NudG and MutT [20]. Like NahA, NudG and MutT also hydrolyze (p)ppGpp. Unlike NahA, NudG and MutT produce guanosine 5'-monophosphate 3'-monophosphate (pGp) rather than pGpp. Similarly, in the bacterium Thermus thermophilus, the NuDX hydrolase Ndx8 is also found to hydrolyze (p)ppGpp to produce pGp [46]. Sequence alignment shows that the homology with NahA is mostly restricted in the NuDX box that is shared by Bacillus subtilis ΔnahA. ΔnahA leads to slower growth compared to wild type [45]. Expression of ΔnahA in ΔrelA also leads to slower growth compared to ΔrelA [47]. Therefore, because of the strong inhibition of translation due to overly high level of (p)ppGpp, the role of NahA is to hydrolyze (p)ppGpp to protect E. coli against toxicity caused by overly high level of (p)ppGpp. This suggests that NahA is essential for cell fitness survival during amino acid starvation. This finding appears to contradict with the finding by Kozomara et al. [48] that NahA is dispensable in E. coli under nitrogen starvation and reverses the growth defect of ΔrelAΔmutT. A possible explanation is that in ΔrelAΔmutT cells, the toxicity of overly high level of (p)ppGpp is relieved by strong inhibition of translation due to overly high level of pGpp produced by MutT. In this case, the role of NahA is to protect E. coli against toxicity caused by overly high level of pGpp during amino acid starvation.

Methods

Bacillus anthracis ORFeome Library Construction. Bacillus anthracis Genotype—Clone Set containing plasmids bearing B. anthracis open reading frames was acquired from BEI Resources and used for Gateway cloning (Invitrogen protocol) into overexpression vectors pVL7916 [10] (His-tag ampicillin-resistant) and pVL8474 (10×His-MBP tag, gentamycin-resistant) and transformed into Escherichia coli strain BL21 lacIq to produce two open reading frame proteome over-expression libraries (ORFeome library). The resulting ORFeome library contains 5139 ORFs from the genome of B. anthracis str. Ames (91.2% of 5632 ORFs in the genome). The corresponding proteins were expressed in E. coli and the cells were lysed to prepare the overexpression lysates for the downstream analysis. E. coli strain containing overexpression vectors were grown in LB-M9 media (7 g/L NaHPO4, 2 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl, 2 g/L glucose, 1 g/L sodium succinate dibasic hexahydrate; 10 g/L tryptophan, 5 g/L yeast extract, and 3 mM MgSO4) at 30 °C and the overexpression was induced by 0.5 mM IPTG. Cells were collected by centrifugation at 5000 g 4 °C for 10 min. Cells were resuspended in 10% original volume of resuspension/lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.25 mg/mL lysozyme, 0.01 mg/mL DNase I, and 1 mM PMSF). Cells were then lysed by three rounds of ~80 °C freezing and room temperature thawing.

Plasmid and strain construction. Plasmid for NahA purification was constructed as follows. To generate a His-SUMO-NahA plasmid, the nahA sequence was PCR amplified using primers pJW301/pJW302 and incorporated into pET-SUMO vector backbone amplified using primers pOL3194/pOL3195 by Golden Gate assembly method (New England Biolabs) to generate pJW739. To generate a His-MBP-NahA plasmid, the nahA sequence was PCR amplified using primers pW3274/pW3275 and incorporated into pVL8474 (His-MBP tag overexpression vector) by Gateway cloning method (Invitrogen) to generate pJW742.

Nucleotide preparation. (p)ppGpp was synthesized in vitro from 8 mM ATP and 6 mM GTP using RelSeq1 [35]. For production of 5'-β-32P-(p)ppGpp, 750 μCi/μL 32P-γ-ATP (3000 Ci/mmol; PerkinElmer) was used instead of radioactive-labeled GTP. For production of 5'-β-32P-(p)ppGpp, 750 μCi/μL 32P-γ-ATP (3000 Ci/mmol; PerkinElmer) was used instead of non-radioabeled ATP. ppGpp was synthesized from (p)ppGpp using GppA [37]. For purification of nucleotides, the reaction mix was diluted in Buffer A (0.1 mM LiCl, 0.3 mM EDTA, 25 mM Tris pH 7.5). The mixture was loaded onto 1 ml Buffer A with 155 mM LiCl with 1 ml fractions, followed by 5 column volumes of Buffer A with 500 mM LiCl with 1 ml fractions. The radioactivity and purity of radioactive nucleotides were analyzed by thin-layer chromatography and phosphorimaging.

Overexpression and purification of NahA. His-tagged NahA was purified by Ni-NTA affinity column, followed by SUMO protease cleavage to remove the tag. To express His-tagged protein, His-SUMO-NahA vector (pJW739) was transformed into E. coli BL21(DE3) lacI7 by chemical transformation. A single colony of the corresponding strain was grown in LB with 30 μg/mL kanamycin overnight.

### Table 2 Strains used in this work.

| ID       | Construct          | Source |
|----------|--------------------|--------|
| pJW736   | pDR111 amyE::Phyperspank::nahA (amp, spec) | This work |
| pJW737   | pBP41-gRNA(nahA)ΔnahA (amp, spec) | This work |
| pJW739   | pE-SUMO-Bacillus subtilis nahA (kan) | This work |
| pJW742   | pVL8474-Bacillus subtilis nahA (gentamyacin) | This work |

### Table 3 Plasmids used in this work.

| ID       | Construct          | Source |
|----------|--------------------|--------|
| pVL8474  | 10×His-MBP tag, gentamycin-resistant | This work |
| pJW301   | His-His tag ampicillin-resistant | This work |

### Table 4 Strains used in this work.

| ID       | Construct          | Source |
|----------|--------------------|--------|
| pJW736   | pDR111 amyE::Phyperspank::nahA (amp, spec) | This work |
| pJW737   | pBP41-gRNA(nahA)ΔnahA (amp, spec) | This work |
| pJW739   | pE-SUMO-Bacillus subtilis nahA (kan) | This work |
Table 4 Primers used in this work.

| ID   | Sequence                                                                 |
|------|--------------------------------------------------------------------------|
| aJW2775 | GACGGTCTGTCAGTGGTCTAAGGTACGATTGCGATGTCGATCGATTTGAGG                     |
| aJW2821 | GTAGTTGCTCTAAGGATTTGCGGATGTCGATCGATCGATTTGAGG                     |
| aJW3194 | GCGGTTGCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3195 | GCGGTTGCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3274 | GGAGGAACAGTTGTGACAAAGAGGAGTGGTACGATCTGGAAGATGAAAGATGAAATGGGTAGAGAG   |
| aJW3275 | GGAGGAACAGTTGTGACAAAGAGGAGTGGTACGATCTGGAAGATGAAAGATGAAATGGGTAGAGAG   |
| aJW3382 | GCTGAGATTATCTTACACGGAGAG                                              |
| aJW3383 | CATTCCACTCTCATGACGAGGGG                                              |
| aJW3400 | GCGGTTGCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3401 | GCGGTTGCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3498 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3499 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3500 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3501 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3502 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3503 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3504 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3505 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3519 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |
| aJW3520 | GACGGTCTCCTCACTTGACGACCAACACACCAACCA                                |

Overnight culture was 1:100 diluted in LB-M9 media with 30 µg/mL kanamycin and grown at 30 °C with shaking for 4 h. After adding 1 mM IPTG for induction, the culture was further grown at 30 °C for 4 h. Then the culture was centrifuged at 4000×g for 30 min at 4 °C and the supernatant was discarded. The pellet was stored at −80 °C before cell lysis. All the following steps were performed at 4 °C. Pellet was resuspended in Lysis Buffer (50 mM Tris-HCl pH 8, 10% sucrose w/v, and 300 mM NaCl) and lysed by French press. Cell lysate was centrifuged at 15,000 rpm for 30 min, and the supernatant was collected, filtered through 0.45-µm pore-size cellulose syringe filter. His-SUMO-NahA filtered supernatant was purified using a 5-mL HisTrap FF column (GE Healthcare) equipped on an ÄKTA FPLC apparatus (GE Healthcare). SUMO Buffer A (50 mM Tris-HCl, 25 mM imidazole, 500 mM NaCl, and 5% glycerol v/v) was used as washing buffer, and SUMO Buffer C (50 mM Tris-HCl, 500 mM NaCl, 5% glycerol v/v, and 1 mM MgCl2, indicated concentrations of non-radioactive and 32P-radiolabeled (p)ppGpp, or pGpp). For (p)ppGpp and pGpp hydrolysis by B. subtilis RelA, 100 nM purified NahA was added to a reaction mix containing 40 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, indicated concentrations of non-radioactive and 32P-radiolabeled (p)ppGpp. For (p)ppGpp and pGpp hydrolysis by B. subtilis RelA, 100 nM purified NahA was added to a reaction mix containing 25 mM Tris-HCl pH 7.5, 1 mM MnCl2, 100 µM non-radioactive, and ~0.2 nM 32P-radiolabeled (p)ppGpp, pGpp, or pGpp. At each indicated time points after the reaction started, 10 µL reaction mix was aliquoted into 10 µL ice-chilled 0.8 M formic acid. Samples at each time point were resolved by thin-layer chromatography on PEI-cellulose plates with 1.5 M KH2PO4 (pH 3.4). Nucleotide levels were quantified as mentioned above and the phosphorimager counts of substrate and product were used to calculate the concentration of product by a formula: 

\[
\text{cp} = \text{c0} \times \frac{V_f}{V_i + \text{cp}}
\]  

(3)

Here \(\text{cp}\) was the concentration of product, \(\text{c0}\) was the concentration of substrate before the reaction starts, \(V_f\) was the phosphorimager count of product, and \(V_i\) was the phosphorimager count of substrate. Initial rates of hydrolysis (\(v_0\)) were calculated using the slope of the initial linear part of \(c_p\) over time curve at different initial substrate concentrations. Michaelis–Menten constant (\(K_m\)) and catalytic rate constant (\(V_{max}\)) were obtained by fitting the data of \(V_f/V_i\) to the model

\[
V_f = \frac{V_{max} \cdot c_p}{K_m + c_p}
\]  

(4)

by MATLAB (R2016b), where \(c_p\) was the concentration of NahA, \(c_i\) was the initial concentration of substrate, and \(V_f\) was the Hill's coefficient (for (p)ppGpp hydrolysis, fix \(n = 1\)).

LC-MS quantification of metabolites. Cells were grown to designated OD_{600nm}. In all, 5 mL of cultures were sampled and filtered through PTFE membrane (Sartorius) at time points before and after 0.5 mg/mL ariginine hydroxamate treatment. Membranes with cell pellet were submerged in 3 mL extraction solvent mix (on ice 50:50 (v/v) chloroform/water) to quench metabolism, lyse the cells and extract metabolites. Mixture of cell extracts were centrifuged at 5000×g for 10 min to remove organic phase, then centrifuged at 20,000×g for 10 min to remove cell debris. Samples were frozen at −80 °C if not analyzed immediately. Samples were analyzed using an HPLC-tandem MS (HPLC-MS/MS) system consisting of a Vanquish UHPLC system linked to heated electrospray ionization (ESI, negative mode) on a hybrid quadrupole high resolution mass spectrometer (Q-Exactive Orbitrap, Thermo Scientific) operated in full-scan selected ion monitoring (MS-SIM) mode to detect targeted metabolites based on their accurate masses. MS parameters were set to a resolution of 70,000, an automatic gain control (AGC) of 1e6, a maximum injection time of 40 ms, and a scan range of 90–1000 m/z. LC was performed on an Aquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters). Total run time was 30 min with a flow rate of 0.2 mL/min, using Solvent A (97.3 (v/v) water/methanol, 10 mM tributylamine pH 8–8.5 adjusted with ~9 mM acetic acid) as the mobile phase.
acid) and 100% acetonitrile as Solvent B. The gradient was as follows: 0 min, 5% B; 2.5 min, 5% B; 19 min, 100% B; 23.5 min 100% B; 24 min, 5% B; and 30 min, 5% B. Raw output data from the MS was converted to mZXML format using inhouse-developed software, and quantification of metabolites were performed by using the Metabolomics Analysis and Visualization Engine (MAVEN 2011.6.17, http://genomics-pubs.princeton.edu/mzroll/index.php) software suite. Normalized ion count was defined and calculated as the ion count per OD_{600} per unit volume (5 mL) of the culture.

Growth competition assay. nahA::ermB mutant and wild-type cells were mixed in LB broth to an OD_{600} of 0.03 and grown at 37°C with vigorous shaking. After every 24-h period, the stationary phase culture was back-diluted in fresh LB broth to an OD_{600} of 0.02, for a total period of 7 days. Each day, the culture was sampled, serially diluted, and spread over LB agar and LB agar containing 0.5 μg/mL erythromycin/12.5 μg/mL lincomycin to obtain the CFU of total bacteria and erythromycin-resistant strain, respectively. Relative fitness was calculated by the formula:

$$w = \frac{\log_{10}(\text{CFU}_{\text{ermR}}/\text{CFU}_{\text{ermS}})}{\log_{10}(\text{CFU}_{\text{ermS}}/\text{CFU}_{\text{ermR}})}$$

10. References

Data availability

Source data are provided with this paper.

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