Protein synthesis controls phosphate homeostasis

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Phosphorus is an essential element assimilated largely as orthophosphate (Pi). Cells respond to Pi starvation by importing Pi from their surroundings. We now report that impaired protein synthesis alone triggers a Pi starvation response even when Pi is plentiful in the extracellular milieu. In the bacterium *Salmonella enterica* serovar Typhimurium, this response entails phosphorylation of the regulatory protein PhoB and transcription of PhoB-dependent Pi transporter genes and is eliminated upon stimulation of adenosine triphosphate (ATP) hydrolysis. When protein synthesis is impaired due to low cytoplasmic magnesium (Mg^{2+}), *Salmonella* triggers the Pi starvation response because ribosomes are destabilized, which reduces ATP consumption and thus free cytoplasmic Pi. This response is transient because low cytoplasmic Mg^{2+} promotes an uptake in Mg^{2+} and a decrease in ATP levels, which stabilizes ribosomes, resulting in ATP consumption and Pi increase, thus ending the response. Notably, pharmacological inhibition of protein synthesis also elicited a Pi starvation response in the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. Our findings identify a regulatory connection between protein synthesis and Pi homeostasis that is widespread in nature.

[*Keywords*: ATP; Mg^{2+}; Pi; PhoB/PhoR; PhoP/PhoQ; translation]

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Phosphorus is an integral component of a large repertoire of biological molecules ranging from nucleotides and nucleic acids to lipids and sugar phosphates (Pis). Phosphorus is assimilated and used in the form of Pis (Wanner 1996; Kroger and Fuchs 1999) during the synthesis of adenosine triphosphate (ATP). Because Pi groups carry negative charges, they exist as salts with positively charged ionic species, prominently magnesium (Mg^{2+}), the most abundant divalent cation in living cells (Maguire and Cowan 2002; Wolf and Cittadini 2003; Pontes et al. 2015b). For example, Mg^{2+} neutralizes negative charges in ATP (Storer and Cornish-Bowden 1976; Maguire and Cowan 2002; Wolf and Cittadini 2003) and ribosomal RNAs (rRNAs) (Wolf and Cittadini 2003; Klein et al. 2004; Pontes et al. 2016). Given that protein synthesis is the activity that consumes the majority of cellular ATP (Stouthamer 1973) and that Mg^{2+} is required for the assembly of functional ribosomes (Wolf and Cittadini 2003; Klein et al. 2004; Pontes et al. 2016), we reasoned that cells coordinate protein synthesis with the metabolisms of Pi and Mg^{2+}.

Enteric bacteria harbor distinct regulatory systems that maintain the cytoplasmic levels of Pi and Mg^{2+} within the physiological range. On the one hand, the PhoB/PhoR system consists of the membrane-bound sensor PhoR, which responds to low Pi by activating PhoB (Horiuchi et al. 1959; Makino et al. 1988, 1989; Yamada et al. 1990; Gao and Stock 2013b), a transcriptional regulator that promotes its own expression as well as that of many genes, including those specifying Pi transporters (Rosenberg et al. 1977; Cox et al. 1981; Shinagawa et al. 1983; Makino et al. 1988, 1989; Gao and Stock 2013a, 2015) and enzymes that scavenge Pi from extracytoplasmic organic molecules (Fig. 1; Horiuchi et al. 1959; Makino et al. 1988, 1989; Hsieh and Wanner 2010).

On the other hand, the PhoP/PhoQ system consists of the sensor PhoQ, which responds to low extracytoplasmic Mg^{2+} by activating PhoP (Soncini et al. 1996; Véscovi et al. 1996), a regulatory protein that promotes its own transcription (Soncini et al. 1995) as well as that of dozens of genes (Zwir et al. 2012; Groisman et al. 2013). In *Salmonella enterica* serovar Typhimurium, where the PhoP/PhoQ system is best understood (Groisman et al. 2013), PhoP promotes transcription of genes that mediate the chemical modification of Pis in the bacterial outer membrane (Guo et al. 1997; Bishop et al. 2000; Shi et al. 2004),

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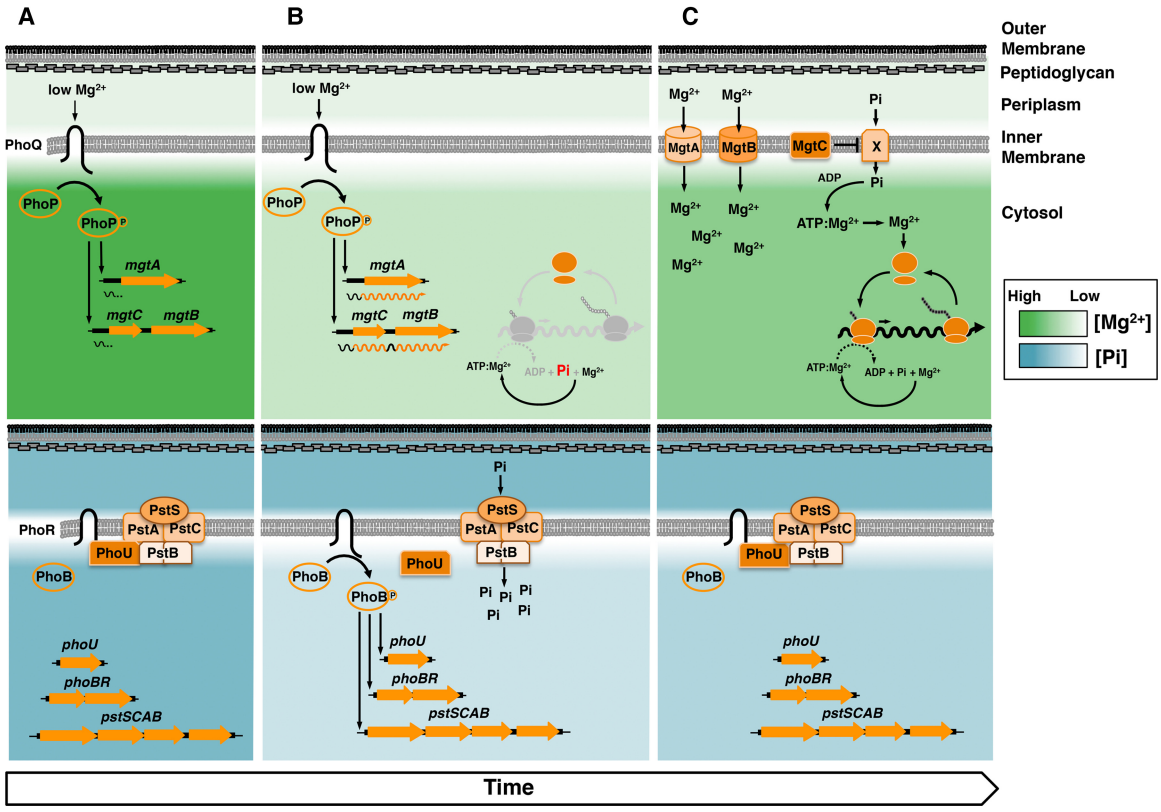

Figure 1. Cartoon representation of the PhoP/PhoQ and PhoB/PhoR systems, regulated targets, and cellular behavior during Pi starvation induced in low cytoplasmic Mg^{2+}. (*A*, *top*) Low extracytoplasmic Mg^{2+} activates PhoQ, which promotes the phosphorylated state of PhoP (PhoP-P), which in turn promotes transcription initiation from the *mgtA* and *mgtCB* promoters. Transcription stops before RNA polymerase (RNAP) reaches the *mgtA*- and *mgtCB*-coding regions. (*Bottom*) High extracellular and intracellular free Pi promotes an interaction between the sensor kinase PhoR and the PstB component of the high-affinity Pi transporter via the regulatory protein PhoU, which inhibits the activity of the PhoB/PhoR system. (*B*, *top*) As free cytoplasmic Mg^{2+} decreases, ribosome subunits are unable to associate efficiently, translation slows down, ATP consumption decreases, and transcription elongation into *mgtA*- and *mgtCB*-coding regions takes place. (*Bottom*) The decrease in ATP consumption prevents the liberation of Pi into the cytosol, leading to a decrease in free cytoplasmic Pi. The low concentration of free cytoplasmic Pi disrupts the inhibitory activity of PhoU, resulting in PhoB/PhoR activation even when extracytoplasmic Pi is high. (*C*, *top*) Restoration of free cytoplasmic Mg^{2+} by the MgtA and MgtB proteins importing Mg^{2+} into the cytoplasm and the MgtC protein reducing the importation of Pi (via an unknown mechanism/Pi transporter X), which feeds into the synthesis of the Mg^{2+}-chelating ATP. The increase in free cytoplasmic Mg^{2+} restores translation, which results in ATP hydrolysis and recycling of intracellular Pi. (*Bottom*) Elevated ATP hydrolysis increases free cytoplasmic Pi levels, restoring the PhoU-mediated inhibition of PhoB/PhoR activity.

Mg^{2+} uptake into the cytoplasm (Soncini et al. 1996; Véscovi et al. 1996), and inhibition of the ATP-generating F_1F_o ATPase (Fig. 1; Lee et al. 2013).

We now report that the metabolisms of Pi and Mg^{2+} are linked to each other and to the translation status of the cell. We establish that cytoplasmic Mg^{2+} limitation activates the *Salmonella* PhoB/PhoR system even when Pi levels in the bacterium's surroundings are high. We determine that protein synthesis inhibitors activate the PhoB/PhoR system in both *Salmonella* and *Escherichia coli* and trigger a Pi starvation response in *Saccharomyces cerevisiae*. Moreover, genomic analysis reveals that genes involved in Pi metabolism are often adjacent to those responsible for Mg^{2+} homeostasis in the chromosomes of divergent bacterial species. Our findings demonstrate that the coordination of Pi and Mg^{2+} metabolisms with ribosome activity exhibits a broad phylogenetic distribution. This is true even though the particular gene products responsible for this coordination vary across species. The identified coordination of Pi and Mg^{2+} metabolisms with ribosome activity likely reflects the universality of phosphorus as a cellular building block, Mg^{2+} as a biological Pi counter ion, and both ions as central players in protein synthesis.

Results

Cytoplasmic Mg^{2+} limitation induces a Pi starvation response

When bacteria are placed in low-Mg^{2+} medium, low extracellular Mg^{2+} activates the PhoP/PhoQ system (Fig. 1A; Véscovi et al. 1996). Initially, bacteria grow and consume the limited Mg^{2+} present in the medium via the housekeeping Mg^{2+} transporter CorA (Snavely et al. 1989b). Eventually, as CorA is unable to maintain an adequate

supply of Mg^{2+}, the concentration of cytoplasmic Mg^{2+} drops to levels that compromise ribosome assembly (Pontes et al. 2016). Impaired translation and/or direct detection of low cytoplasmic Mg^{2+} promotes transcription of the *mgtA*-, *mgtB*-, and *mgtC*-coding regions via the formation of secondary structures in the leader regions of their respective mRNAs that hinder transcription termination within the leader region (Cromie et al. 2006; Spinelli et al. 2008; Park et al. 2010; Zhao et al. 2011; Lee and Groisman 2012a,b). The *mgtA* and *mgtB* genes specify two distinct Mg^{2+} transporters (Snavely et al. 1989a,b), and the *mgtC* gene specifies an inhibitor of the F_1F_o ATPase (Lee et al. 2013). Production of the MgtA, MgtB, and MgtC proteins increases the concentration of free cytosolic Mg^{2+}, inhibits ribosome production, and stabilizes the remaining ribosomes (Pontes et al. 2016).

To identify additional genes differentially transcribed in response to a decrease in cytoplasmic Mg^{2+} concentration, we assessed RNA polymerase (RNAP) occupancy of the *Salmonella* genome by chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) following growth in defined liquid medium with two different Mg^{2+} concentrations (10 or 50 µM). Growth in either Mg^{2+} concentration activates the PhoP/PhoQ system (Véscovi et al. 1996; Cromie et al. 2006) and results in a similar optical density (OD_{600}) of the bacterial cultures by 4 h (Supplemental Fig. S1A). However, the threshold of cytoplasmic Mg^{2+} concentration that triggers transcription of the *mgtA*-, *mgtB*-, and *mgtC*-coding regions takes place following 4 h of growth in 10 µM Mg^{2+} but not in 50 µM Mg^{2+} (Cromie et al. 2006; Spinelli et al. 2008).

RNAP occupancy was similar under 10 and 50 µM Mg^{2+} for the vast majority of genes, including the PhoP-activated *phoPQ*, *slyA*, and *slyB* genes (Fig. 2A) and those not regulated by PhoP, such as the *ompA*, *ybdQ*, and *his* genes (Supplemental Fig. S1B). In contrast, RNAP occupancy of the *mgtA*-, *mgtB*-, and *mgtC*-coding regions was much higher following growth in 10 µM Mg^{2+} than in 50 µM Mg^{2+} (Fig. 2A), in agreement with previous mRNA and transcriptional reporter data (Cromie et al. 2006; Spinelli et al. 2008).

Surprisingly, RNAP occupancies of the PhoB-activated *phoBR, pstSCAB*, and *phoU* genes were higher during growth in 10 µM Mg^{2+} than in 50 µM Mg^{2+} (Fig. 2B). This result was unexpected for two reasons. First, Pi was abundant in the growth medium (~1.16 mM), implying that PhoR should be promoting the inactive state of the PhoB protein, resulting in low levels of PhoB-activated mRNAs (Gao and Stock 2013a,b). Second, the promoters of the *phoBR*, *pstSAB*, and *phoU* genes lack sequences resembling PhoP-binding sites (Kato et al. 1999; Zwir et al. 2012), suggesting that PhoP does not directly activate these promoters.

In agreement with the RNAP ChIP-seq results (Fig. 2B), quantitative PCR (qPCR) revealed that the mRNA levels of the *phoB*, *pstB*, *pstC*, and *pstS* genes were fivefold to 25-fold higher following growth in 10 µM Mg^{2+} than in 50 µM Mg^{2+} (Supplemental Fig. S1C). In contrast, no differences in mRNA amounts were observed for the *slyB*, *ompA*, and *ybdQ* genes (Supplemental Fig. S1C). In sum, low cytoplasmic Mg^{2+} promotes transcription of both PhoB-activated genes and PhoP-activated genes that maintain protein synthesis (Pontes et al. 2016).

Delayed activation of PhoB in bacteria experiencing low Mg^{2+} or low Pi

To examine the expression behavior of PhoB-dependent genes resulting from changes in cytoplasmic Mg^{2+}, we monitored the fluorescence of wild-type *Salmonella* harboring a medium copy number plasmid with a transcriptional fusion between the PhoB-activated *phoB* promoter and a promoterless *gfp* gene. This approach enabled a temporal resolution of the response to cytoplasmic Mg^{2+} concentration in an organism with an otherwise wild-type genome.

Fluorescence was low in the first 2 h of growth in 10 µM Mg^{2+}, increased dramatically by 3 h, and remained at the same high levels for the following 2 h (Fig. 2C). When grown in 50 µM Mg^{2+}, fluorescence increased only at 4 h and reached values less than one-fifth of those observed during growth in 10 µM Mg^{2+} (Fig. 2C). Organisms experiencing ≥100 µM Mg^{2+} displayed no change in fluorescence (Fig. 2C). Thus, activation of the *phoB* promoter requires growth in <100 µM Mg^{2+} for >2 h.

Transcription from the *phoB* promoter is in contrast to that from the PhoP-activated *phoP* promoter because the fluorescence of a strain harboring a transcriptional fusion between the *phoP* promoter and a promoterless *gfp* gene increased in medium with ≤500 µM Mg^{2+} in the first hour and continued to increase over the course of the experiment (Fig. 2D). As expected, fluorescence remained low in wild-type *Salmonella* carrying the vector control harboring a promoterless *gfp* gene (Fig. 2E, pVector) and was not impacted by the Mg^{2+} concentration in the medium in a strain in which the *lac* promoter transcribes a promoterless *gfp* gene (Fig. 2E, $pGFP_{ON}$).

In contrast, low Pi does not activate the PhoP/PhoQ system: Fluorescence of the strain harboring the *phoP-gfp* fusion was the same regardless of the Pi concentration in the medium (Fig. 2G). Control experiments revealed that fluorescence of wild-type *Salmonella* harboring the *phoB-gfp* fusion increased dramatically at 3 h in medium with ≤100 µM Pi and at 4 h in medium with 250 µM Pi (Fig. 2F). No increase in fluorescence was observed in organisms grown in ≥500 µM Pi (Fig. 2F) or in the strains harboring the vector control (Fig. 2H, pVector). Additionally, when *gfp* was transcribed from the *lac* promoter, fluorescence was only 50% lower in cells grown in low Pi relative to those grown in high-Pi medium (Fig. 2H), indicating that low Pi per se does not lead to increased GFP fluorescence. Cumulatively, the results presented above establish that both low Mg^{2+} and low Pi activate the PhoB/PhoR system after a delay of several hours.

The Mg^{2+} transporters MgtA and MgtB and the ATPase inhibitor MgtC regulate PhoB activity in opposite ways

To explore whether the PhoB activation (Fig. 2B) and the transcription of the *mgtA*-, *mgtB*-, and *mgtC*-coding

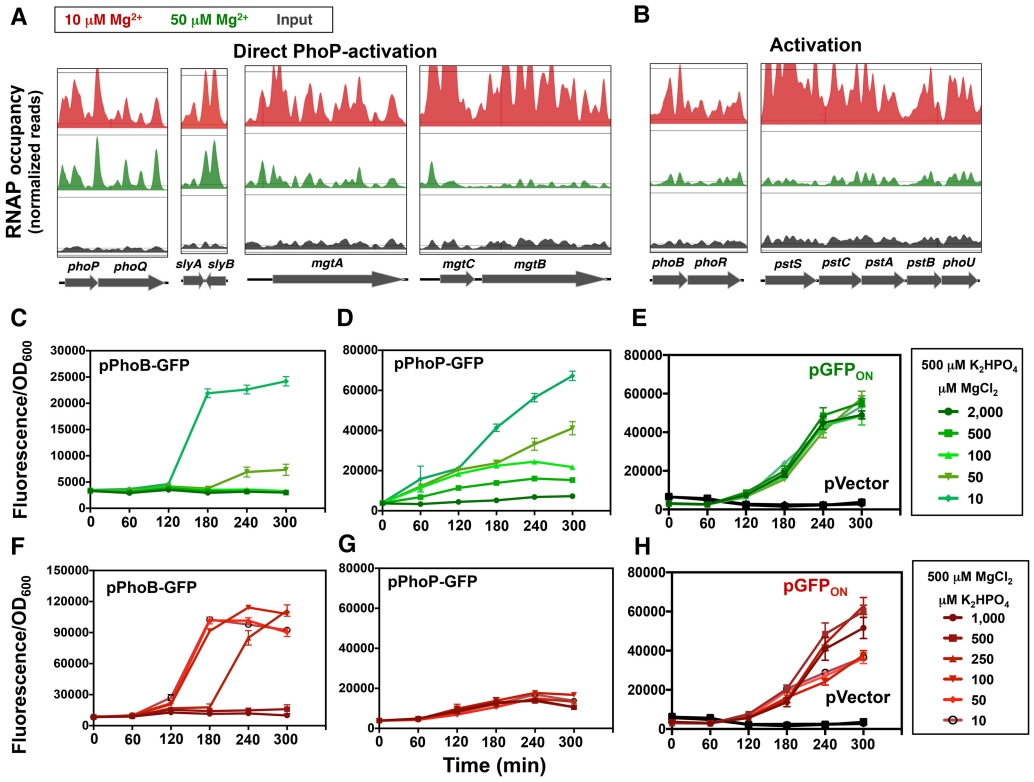

Figure 2. Low cytoplasmic Mg^{2+} induces a transcriptional signature of Pi starvation. (*A*,*B*) RNAP ChIP-seq results of selected genes in wild-type *Salmonella* (14028s) grown in N-minimal medium containing 10 μM Mg^{2+} (red) or 50 μM $MgCl_2$ (green) to an OD_{600} of ~0.3. Input (nonimmunoprecipitated DNA) is also shown (black). Peak heights were normalized to the total number of assembled nucleotides. (*C*–*E*) Fluorescence from wild-type *Salmonella* (14028s) harboring pPhoB-GFP (*C*), pPhoP-GFP (*D*), and the promoterless GFP vector pVector and inducible GFP vector $pGFP_{ON}$ (pUH-GFP; 325 μM IPTG) (*E*) grown in MOPS medium containing 500 μM K_2HPO_4 and the indicated concentrations of $MgCl_2$. (*F*–*H*) Fluorescence from wild-type *Salmonella* (14028s) harboring pPhoB-GFP (*F*), pPhoP-GFP (*G*), and the promoterless GFP vector pVector and constitutive GFP vector $pGFP_{ON}$ (pUH-GFP; 100 μM IPTG) (*H*) grown in MOPS medium containing 500 μM $MgCl_2$ and the indicated concentrations of K_2HPO_4. Error bars represent the standard deviations. Graphs are representative of at least three independent experiments with a total of at least six biological replicates. See also Supplemental Figure S1.

regions taking place in low cytoplasmic Mg^{2+} (Figs. 1B, 2A; Cromie et al. 2006; Spinelli et al. 2008) are physiologically linked, we examined fluorescence over time in isogenic strains harboring *gfp* transcriptional fusions to the *phoB* promoter, the *mgtA* promoter and leader region, and the *mgtC* promoter and leader region (*mgtB* follows *mgtC* in the *mgtCBR* operon) (Fig. 1).

Transcription from the *phoB-gfp* fusion preceded that of the *mgtA-gfp* and *mgtC-gfp* fusions following a shift from 10 mM to 10 μM Mg^{2+} (Fig. 3A); that is, fluorescence from the *phoB-gfp* fusion began at 120 min, remained stable for 80 min, and decreased during the following 16.6 h (Fig. 3A). In contrast, fluorescence from the *mgtA-gfp* and *mgtC-gfp* fusions began at 135 min and continued to increase throughout the course of the experiment (Fig. 3A). As expected, the strain with the vector control displayed no major changes in fluorescence (Fig. 3A). Thus, the *phoB-gfp* fusion differs from the *mgtA-gfp* and *mgtC-gfp* fusions in both the time of induction and the behavior in the subsequent 17 h.

We reasoned that PhoB activation results from disturbances in free cytoplasmic Mg^{2+} occurring prior to the production of the MgtA, MgtB, and MgtC proteins, which maintain physiological levels of free cytoplasmic Mg^{2+} by importing Mg^{2+} ions and reducing the levels of Mg^{2+}-chelating ATP molecules (Pontes et al. 2016). If so, the decrease in fluorescence of the PhoB-activated *phoB-gfp* fusion happening after transcription of the *mgtA*- and *mgtCB*-coding regions (Fig. 3A) may reflect changes brought about by the MgtA, MgtB, and/or MgtC proteins. This hypothesis predicts that inactivation of the *mgtA*, *mgtB*, and/or *mgtC* genes should abolish the decrease in fluorescence of the *phoB-gfp* fusion observed in wild-type cells at 200 min (Fig. 3A) but not alter the time at which fluorescence starts.

As hypothesized, fluorescence from the *phoB-gfp* fusion began at ~120 min in wild-type, *mgtC*, and *mgtA mgtB* *Salmonella* (Fig. 3B). However, whereas fluorescence decreased in the wild-type strain after 200 min (Fig. 3B), it rose throughout the course of the experiment in the *mgtA mgtB* double mutant (Fig. 3B). Unexpectedly, fluorescence was lower in the *mgtC* mutant than in wild-type *Salmonella* (Fig. 3B). The behavior of the strain harboring the *phoB-gfp* fusion reflects activation of the PhoB

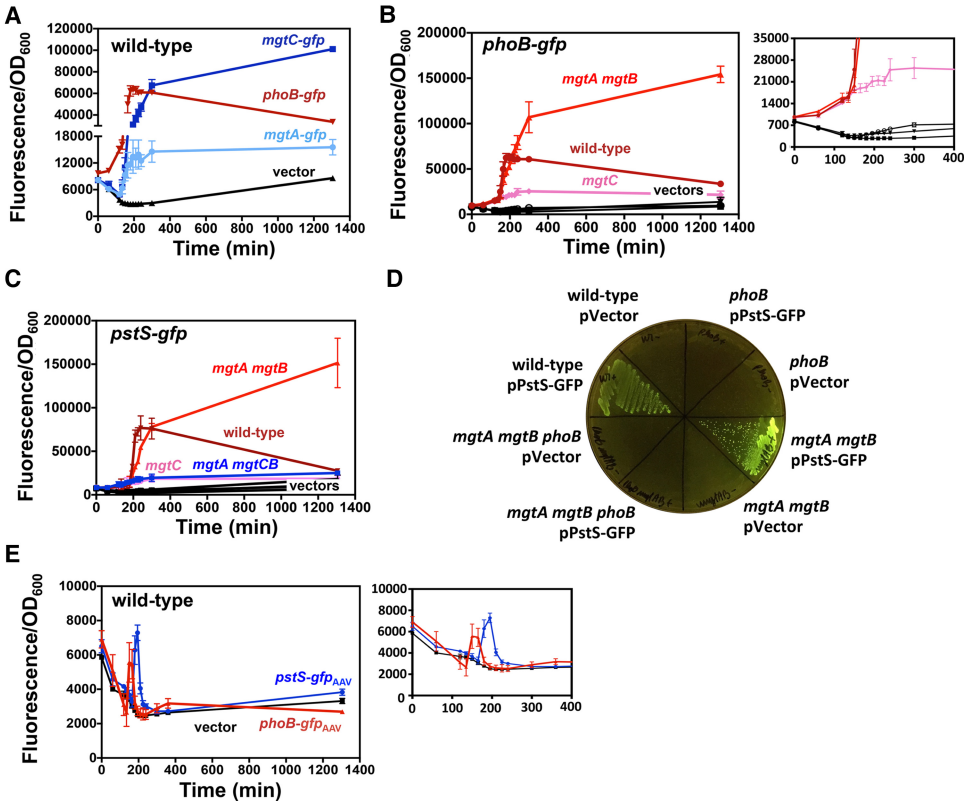

Figure 3. The Mg^{2+} transporters MgtA and MgtB and the ATPase inhibitor MgtC regulate PhoB activity in opposite ways. (*A*) Fluorescence from wild-type *Salmonella* (14028s) harboring pPhoB-GFP (*phoB-gfp*), pMgtA-leader-GFP (*mgtA-gfp*), pMgtC-leader-GFP (*mgtC-gfp*), or the promoterless GFP vector pVector plasmid. (*B*) Fluorescence from wild-type (14028s), *mgtA mgtB* (EG17048), and *mgtC* (EL4) *Salmonella* harboring pPhoB-GFP or pVector. (*C*) Fluorescence from wild-type (14028s), *mgtA mgtB* (EG17048), *mgtC* (EL4), and *mgtA mgtCB* (MP363) *Salmonella* harboring pPstS-GFP or pVector. (*D*) Fluorescence from wild-type (14028s), *phoB* (EG9054), *mgtA mgtB* (EG17048), and *mgtA mgtB* (EG17048) and *phoB mgtA mgtB* (MP1184) *Salmonella* following 24 h of growth on solid medium. (*E*) Fluorescence from wild-type *Salmonella* (14028s) harboring pPhoB-GFP_{AAV}, pPstS-GFP_{AAV}, or $pVector_{AAV}$. For all experiments, cells were grown in MOPS liquid or on solid (1% agarose) medium containing 10 µM $MgCl_2$ and 500 µM K_2HPO_4. Error bars represent the standard deviations. Graphs are representative of at least three independent experiments with a total of at least six biological replicates. See also Supplemental Figure S2.

protein (as opposed to being confined to the *phoB* promoter) because the wild-type, *mgtC*, and *mgtA mgtB* strains displayed analogous fluorescence patterns when harboring a *gfp* fusion to the *pstS* promoter (Fig. 3C), which is also activated by PhoB (Makino et al. 1988). The *mgtC* mutation is epistatic to the *mgtA* and *mgtB* mutations because fluorescence was similarly low in the *mgtC* single mutant and *mgtA mgtB mgtC* triple mutant (Fig. 3C). Control experiments showed unchanged low fluorescence in strains harboring the vector control (Fig. 3B,C).

The PhoB protein is responsible for the transcriptional induction of the *phoB-gfp* and *pstS-gfp* fusions taking place in low Mg^{2+} because deletion of the *phoB* gene eliminated the high fluorescence exhibited by wild-type and *mgtA mgtB* strains harboring the *pstS-gfp* fusion on solid medium containing 10 µM Mg^{2+} (Fig. 3D). In agreement with the results described above (Fig. 3B,C), the *mgtA mgtB* double mutant and the *mgtC* mutant displayed more and less fluorescence than the wild-type strain, respectively, when harboring either the *phoB-gfp* (Supplemental Fig. S2A) or *pstS-gfp* (Supplemental Fig. S2B) fusion. There was no fluorescence in strains harboring the vector control (Supplemental Fig. S2A,B).

Taken together, the results presented in this section indicate that low cytoplasmic Mg^{2+} activates the PhoB protein and that the Mg^{2+} transporters MgtA and MgtB and the ATPase inhibitor MgtC regulate PhoB activity in opposite ways.

MgtC activates PhoB by decreasing cytoplasmic Pi levels

To understand how MgtC activates the PhoB protein, we probed the effects of time and levels of *mgtC* induction and whether Mg^{2+} controls PhoB activity independently of its effect on MgtC expression (Spinelli et al. 2008). Fluorescence from the *pstS-gfp* fusion increased sharply in an *mgtC* mutant harboring a plasmid with a copy of the *mgtC*-coding region under the control of a derivative of the *lac* promoter (pMgtC) following addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), the gratuitous

inducer of the *lac* promoter (Fig. 4A). MgtC acts in a dose-dependent manner because fluorescence progressively rose as the IPTG concentration increased (Fig. 4A) and surpassed that of the wild-type strain carrying the plasmid vector at ≥250 µM IPTG (Fig. 4A). As expected, fluorescence from the *mgtC* mutant harboring the vector control remained low and unaffected by the presence of IPTG (Fig. 4A); the same was true when no IPTG was added to the *mgtC*/pMgtC strain (Fig. 4A).

The wild-type strain harboring the vector control instead of pMgtC fluoresced much later than the *mgtC*/pMgtC strain treated with IPTG (Fig. 4B). This result reflects normal transcriptional control of the *mgtC*-coding region by the wild-type promoter (Soncini et al. 1996) and leader (Cromie et al. 2006; Spinelli et al. 2008) regions.

MgtC activates PhoB independently of the Mg^{2+} concentration in the medium because IPTG induced fluorescence from *phoB-gfp* even when the *mgtC*/pMgtC strain was grown in 1 mM Mg^{2+} (Fig. 4C), a condition in which the *mgtC*-coding region is not transcribed in wild-type *Salmonella* (Spinelli et al. 2008). In contrast, wild-type *Salmonella* harboring the vector control fluoresced following growth in 10 µM Mg^{2+} but not in 1 mM Mg^{2+} (Fig. 4C). The fluorescence of the *mgtC* mutant harboring the vector control remained low in all tested conditions (Fig. 4C).

We reasoned that MgtC activates PhoB by decreasing cytoplasmic Pi levels because low Pi medium activates PhoB (Horiuchi et al. 1959; Makino et al. 1988, 1989) and because the sensor PhoR has a small periplasmic region. In agreement with this notion, Pi levels were 18-fold to 25-fold lower in wild-type *Salmonella* than in the *mgtC* mutant following 5 h of growth in 10 µM Mg^{2+} (Fig. 4D). (This time was chosen because wild-type *Salmonella* exhibited low steady-state levels of *phoB* and *pstS* transcription [Fig. 3A–C,E], suggesting that intracellular Pi levels are stable.) Moreover, *mgtC* expression from a heterologous promoter lowered intracellular Pi levels in the *mgtC* mutant, whereas the vector control had no effect (Fig. 4D). In sum, MgtC activates PhoB by reducing cytoplasmic Pi levels.

The MgtA and MgtB proteins prevent a sustained Pi starvation response when Pi is plentiful

The PhoB protein is hyperactivated in the *mgtA mgtB* double mutant following growth in 10 µM Mg^{2+} for 120 min (Figs. 3B,C, 5A,C,D; Supplemental Fig. S2). Thus, we anticipated the *mgtA mgtB* mutant to have low

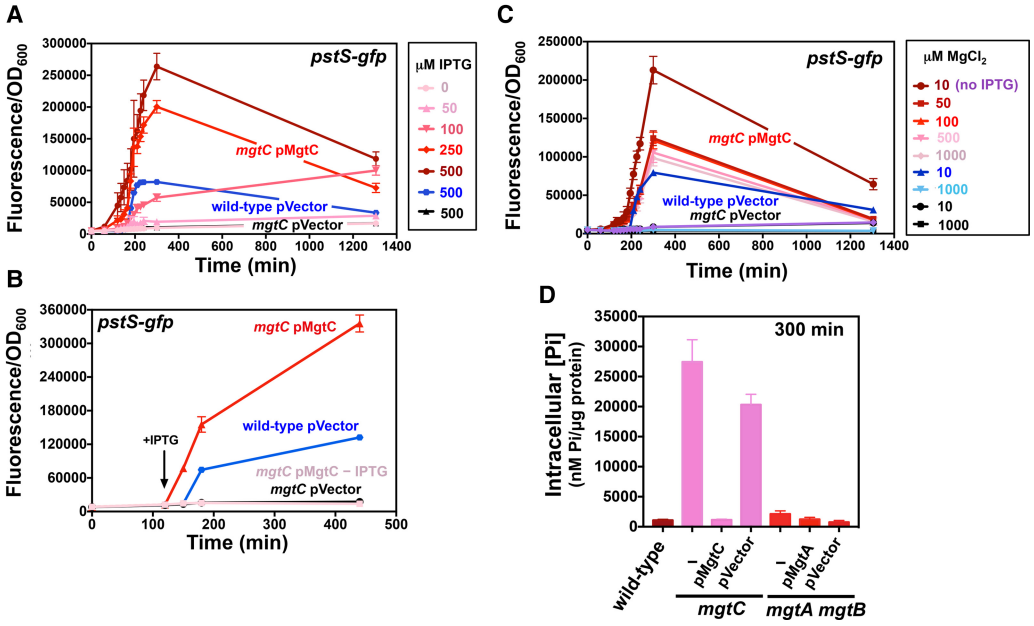

Figure 4. MgtC reduces cytoplasmic Pi during cytoplasmic Mg^{2+} starvation. (*A*) Fluorescence from wild-type (14028s) and *mgtC* (EL4) *Salmonella* carrying pPstS-GFPc and either pUHE-21 (pVector) or pUHE-MgtC (pMgtC) grown in the presence of different concentrations of IPTG. The fluorescence of *mgtC* (EL4)/pMgtC cultures lacking IPTG is also shown. (*B*) Fluorescence from wild-type (14028s) and *mgtC* (EL4) strains carrying pPstS-GFPc and pVector in the presence of 500 µM IPTG and *mgtC* (EL4)/pMgtC at the indicated IPTG concentrations. The arrow indicates the time when 250 µM IPTG was added to the cultures. (*C*) Fluorescence from wild-type (14028s) and *mgtC* (EL4) *Salmonella* carrying pPstS-GFPc and pVector during growth in 10 or 1000 µM $MgCl_2$ and of *mgtC* (EL4)/pMgtC *Salmonella* in the presence of the indicated $MgCl_2$ concentrations. Unless indicated in the figure, bacteria were grown in the presence of 250 µM IPTG. (*D*) Total intracellular Pi in wild type (14028s), *mgtC* (EL4), or *mgtC* (EL4)/pMgtC or pVector, *mgtA mgtB* (EG17048) *Salmonella*, and *mgtA mgtB* (EG17048)/pMgtA or pVector following 300 min of growth. All experiments were carried out in MOPS medium containing 500 µM K_2HPO_4 and either 10 µM $MgCl_2$ or the specified $MgCl_2$ concentration. Error bars represent the standard deviations. Graphs are representative of at least three independent experiments with a total of at least six biological replicates. See also Supplemental Figure S2.

cytoplasmic Pi. Surprisingly, the *mgtA mgtB* double mutant exhibited wild-type levels of cytoplasmic Pi (Fig. 4D). What, then, activates PhoB in the *mgtA mgtB* double mutant or in the wild-type strain before the MgtA and MgtB proteins are expressed?

We hypothesized that PhoB activation results from Pi being sequestered in ATP molecules that are not effectively recycled when translation is compromised. In agreement with this notion, translation consumes the majority of cellular ATP (Stouthamer 1973), and the *mgtA mgtB* double mutant, unable to efficiently assemble ribosomes and carry out translation (Pontes et al. 2016), displays higher ATP levels than the wild-type strain (Supplemental Fig. S3A; Pontes et al. 2016). If this hypothesis is correct, releasing Pi trapped in ATP molecules should decrease PhoB activity.

As hypothesized, fluorescence from the *pstS-gfp* fusion was inversely proportional to the IPTG concentration in a culture of the *mgtA mgtB* double mutant carrying plasmid pATPase (Fig. 5A), which promotes IPTG-inducible ATP hydrolysis (Supplemental Fig. S3B). In contrast, there was no change in fluorescence in the isogenic strain with the vector control (Fig. 5A). Fluorescence of an isogenic strain with the PhoP-activated *phoP-gfp* fusion was unaffected by the presence of pATPase (Fig. 5B), indicating that the pATPase plasmid does not cause a systemic decrease in transcription. Taken together, these results indicate that MgtA and MgtB inhibit PhoB activation by maintaining the integrity of ATP-hydrolyzing reactions.

The Mg^{2+} transporters MgtA and MgtB are ~50% identical at the amino acid level but display different specificities and modes of regulation (Snavely et al. 1991; Tao et al. 1995; Groisman et al. 2013). We determined that either Mg^{2+} transporter is sufficient for the decrease in PhoB activation after it reaches a peak; that is, fluorescence from the *pstS-gfp* fusion in the *mgtA mgtB* double mutant carrying the IPTG-inducible pMgtA decreased as the IPTG concentration increased (Fig. 5C), but no decrease in fluorescence was observed in the isogenic strain harboring the vector control (Fig. 5C). In contrast to the sustained high fluorescence displayed by the *mgtA mgtB* double mutant (Figs. 3B,C, 5A,C,D), fluorescence from the *phoB-gfp* fusion increased and subsequently decreased in the *mgtA* and *mgtB* single mutants (Fig. 5D), like in the wild-type strain (Fig. 5D). However, the *mgtA* and *mgtB* single mutants achieved higher fluorescence peak values than the wild-type strain (Fig. 5D). As expected, the fluorescence of strains carrying the vector control remained low throughout the course of the experiment (Fig. 5D). Thus, the MgtA and MgtB proteins prevent sustained activation of the PhoB protein.

The data presented above argue that PhoB activation is transient in wild-type *Salmonella* experiencing low cytoplasmic Mg^{2+}, lasting only until the MgtA and MgtB proteins normalize Mg^{2+} concentration to levels that support ribosome assembly (Pontes et al. 2016). To test this possibility, we examined the behavior of a set of isogenic strains harboring plasmid-borne transcriptional fusions

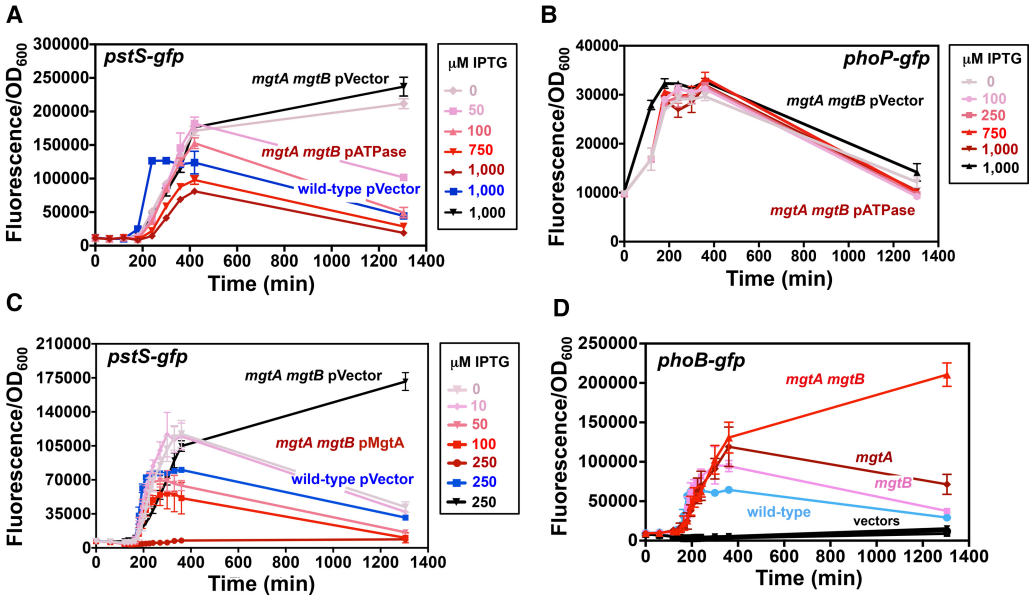

Figure 5. Free cytoplasmic Mg^{2+} inhibits Pi starvation response. (*A*) Fluorescence from wild-type (14028s), *mgtA* (EG16735), *mgtB* (EL5), and *mgtA mgtB* (EG17048) *Salmonella* harboring pPhoB-GFP. (*B*) Fluorescence from wild-type *Salmonella* (14028s) carrying pPstS-GFPc and pUHE-21 (pVector) and from *mgtA mgtB* mutant (EG17048) harboring pPstS-GFPc and either pUHE-21 (pVector) or pUHE-MgtA (pMgtA) in the presence of the indicated IPTG concentrations. (*C*) Fluorescence from wild-type *Salmonella* (14028s) carrying pPstS-GFPc and pVector and from *mgtA mgtB* mutant *Salmonella* (EG17048) harboring pPstS-GFPc and either pVector or pUHE-ATPase (pATPase) in the presence of the indicated IPTG concentrations. (*D*) Fluorescence from *mgtA mgtB* (EG17048) *Salmonella* harboring pPhoP-GFP and either pATPase or pVector in the presence of various IPTG concentrations. For all experiments, cells were grown in MOPS liquid medium containing 10 μM $MgCl_2$ and 500 μM K_2HPO_4. Error bars represent the standard deviations. Graphs are representative of at least three independent experiments with a total of at least six biological replicates. See also Supplemental Figures S2 and S3.

of the PhoB-activated *phoB* and *pstS* promoters to a *gfp* gene specifying an unstable GFP variant (gfp_{AAV}) (Andersen et al. 1998), which allowed examination of short-lived changes in transcriptional activity.

Wild-type *Salmonella* harboring the *phoB-gfp*$_{AAV}$ or *pstS-gfp*$_{AAV}$ fusion displayed a sharp increase in fluorescence that lasted 45 min when shifted to 10 µM Mg^{2+} (Fig. 3E). In contrast, no changes in fluorescence were observed in the isogenic strain carrying the vector control (Fig. 3E). In agreement with these results, wild-type *Salmonella* harboring the *phoB-gfp* or *pstS-gfp* fusion were fluorescent following overnight growth on 10 µM Mg^{2+} solid medium (Supplemental Fig. S2A–C) but not when harboring fusions of the same two promoters to the gfp_{AAV} variant (Supplemental Fig. S2D–F). In contrast, the *mgtA mgtB* double mutant with either the *phoB-gfp*$_{AAV}$ or *pstS-gfp*$_{AAV}$ fusion was fluorescent after overnight growth on 10 µM Mg^{2+} (Supplemental Fig. S2E,F). Thus, by promoting Pi recycling, the MgtA and MgtB proteins avoid a Pi starvation response.

A physiological connection between protein synthesis and PhoB activation

Bacteria link metabolic energy with translation potential (Gaal et al. 1997). For example, the translation inhibitor chloramphenicol promotes an increase in cytoplasmic ATP levels (Bagnara and Finch 1973). Under normal physiological conditions (i.e., in the absence of translation inhibitors), an increase in ATP levels stimulates rRNA transcription (Gaal et al. 1997; Schneider et al. 2002; Murray et al. 2003), which derepresses expression of ribosomal proteins (Nomura et al. 1984; Zengel and Lindahl 1994), resulting in a larger number of translating ribosomes and boosting translation rates (Bremer and Dennis 1996; Murray et al. 2003).

We reasoned that the translation inhibitor chloramphenicol activates the PhoB/PhoR system because Pi is largely incorporated through assimilation into ATP molecules (Wanner 1996; Kroger and Fuchs 1999) and because translation is the activity that uses most cellular ATP (Stouthamer 1973). Therefore, if translation is inhibited, Pi would remain trapped in ATP, which bacteria might experience as low cytoplasmic Pi even if the Pi concentration in their surroundings is high.

As proposed, addition of chloramphenicol to wild-type *Salmonella* increased both cytoplasmic ATP levels (Fig. 6A) and the amounts of the *phoB* (~16-fold) and *pstS* (~40-fold) mRNAs (Fig. 6B). Notably, these increases took place in the presence of 2 mM Pi, a concentration that normally hinders activation of the PhoB/PhoR system (Horiuchi et al. 1959; Makino et al. 1988, 1989). The large increases in *phoB* and *pstS* mRNA amounts appear specific to PhoB-activated genes because they were not observed in a *phoB* mutant (Fig. 6C) and because the mRNA level of the PhoB-independent *thrS* gene was not altered (Fig. 6B).

If excessive ATP resulting from the inhibition of translation promotes PhoB activation, then chloramphenicol should increase the fraction of phosphorylated relative to unphosphorylated PhoB protein. As predicted, we established that when *Salmonella* was grown in an intermediate Pi concentration to promote PhoB activation, the addition of chloramphenicol increased the ratio PhoB-P to PhoB (Fig. 6D). Liberating Pi from ATP by expressing

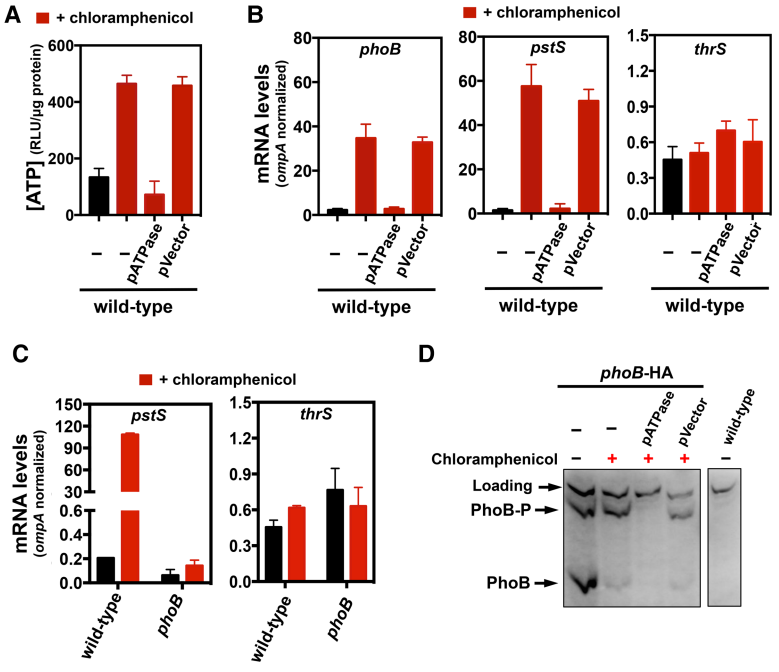

Figure 6. Translation recycles intracellular Pi from ATP, preventing a Pi starvation response. (*A*) Intracellular ATP levels of wild-type *Salmonella* (14028s) lacking or harboring either pUHE-ATPase (pATPase) or pUHE-21 (pVector). (*B*) mRNA amounts of the *phoB*, *pstS*, and *thrS* genes produced by wild-type *Salmonella* (14028s) lacking or harboring either pATPase or pVector. (*C*) mRNA amounts of the *pstS* and *thrS* genes produced by wild-type (14028s) and *phoB* (EG9054) *Salmonella*. For *A–C*, cells were grown in MOPS containing 10 mM $MgCl_2$ and 2 mM K_2HPO_4 for 2 h followed by 30 min of treatment with 1 mM IPTG (when harboring pATPase or pVector) followed by 30 min of treatment with 25 µg/mL chloramphenicol. mRNA amounts were normalized to those of the *ompA* gene. (*D*) Western blot analysis of extracts prepared from wild-type (14028s) and *phoB*-HA (MP1429) *Salmonella* and *phoB*-HA (MP1429) *Salmonella* harboring either pATPase or pVector following separation on Phos-tag SDS-PAGE to detect PhoB-HA and PhoB-HA-P. Cells were grown in MOPS containing 10 mM $MgCl_2$ and 200 µM K_2HPO_4 for 2 h and treated for 35 min with 1 mM IPTG followed by chloramphenicol as in *A–C*. Error bars represent the standard deviations. Graphs are representative of at least three independent experiments with a total of at least six biological replicates. See also Supplemental Figures S3 and S4.

pATPase prior to chloramphenicol addition prevented PhoB activation, whereas the vector control had no effect (Fig. 6D). Because PhoB positively regulates its own transcription, cells experiencing pATPase prior to chloramphenicol addition had undetectable levels of PhoB protein (Fig. 6D).

The increases in ATP levels and transcription of PhoB-dependent genes taking place upon chloramphenicol treatment result from inhibition of protein synthesis (rather than from a different action of chloramphenicol) because similar increases in ATP levels (Supplemental Fig. S4A) and *phoB* and *pstS* mRNA amounts (Supplemental Fig. S4B) were observed when translation was inhibited with spectinomycin. Furthermore, they result from Pi being trapped in ATP because the pATPase plasmid prevented rises in ATP (Fig. 6A), *pstS*, and *phoB* mRNA amounts (Fig. 6B) and PhoB-P levels (Fig. 6D) caused by chloramphenicol. This is in contrast to cultures harboring the vector control, which failed to overcome the effect of chloramphenicol (Fig. 6A,B,D). Taken together, these results indicate that ATP consumption by active ribosomes increases the intracellular pools of free Pi, thereby reducing PhoB activity.

Translation inhibitors promote Pi starvation responses in E. coli *and* S. cerevisiae

We hypothesized that inhibition of protein synthesis triggers a Pi starvation response in other organisms because all living cells use ATP to synthesize and power ribosomes and because Pi is a precursor of ATP. Chloramphenicol treatment increased the mRNA levels of the *phoB* and *pstS* genes in *E. coli* (Supplemental Fig. S4D), just as it did in *Salmonella* (Fig. 6B). The latter increase is caused by Pi being trapped in ATP because the pATPase plasmid normalized ATP levels (Supplemental Fig. S4C) and prevented the increase in the levels of *phoB* and *pstS* mRNAs (Supplemental Fig. S4D), whereas the vector control had no effect (Supplemental Fig. S4C,D).

The Pi starvation response triggered by protein synthesis inhibitors extends beyond the bacterial domain; that is, the inhibitor of eukaryotic translation cycloheximide increased the mRNA levels of the *PHO89* and *PHO84* genes in *S. cerevisiae* (Supplemental Fig. S4E), which specify high-affinity Pi transporters normally induced when Pi is limited (Willsky and Malamy 1980; Bun-Ya et al. 1991; Auesukaree et al. 2003). In contrast, the mRNA levels of MAG1, which is induced by DNA damage (Chen and Samson 1991), remained unaltered (Supplemental Fig. S4E). These results argue that ATP hydrolysis resulting from translation maintains Pi homeostasis by replenishing free Pi to the cytosol.

Discussion

We uncovered a fundamental relationship between protein synthesis and the cytoplasmic levels of Pi and Mg^{2+}. This relationship enables cells to coordinate Mg^{2+} availability with Pi acquisition. Moreover, it protects the integrity of critical processes, such as translation, that require the stabilization of negative charges of Pi-containing biomolecules by Mg^{2+} ions (Pontes et al. 2016). Specifically, a decrease in free cytoplasmic Mg^{2+} triggers a Pi starvation response that lasts until cells normalize the pools of free cytoplasmic Mg^{2+} by promoting Mg^{2+} transport into the cytosol and inhibiting the acquisition of Pi. The resulting physiological changes reduce growth rate (Pontes et al. 2016), enabling cells to maintain viability (Soncini et al. 1996) and achieve higher growth yields (Blanc-Potard and Groisman 1997; Pontes et al. 2015a).

We determined that conditions that compromise translation trigger a Pi starvation response even when Pi is plentiful in a cell's surroundings (Fig. 7). This is because

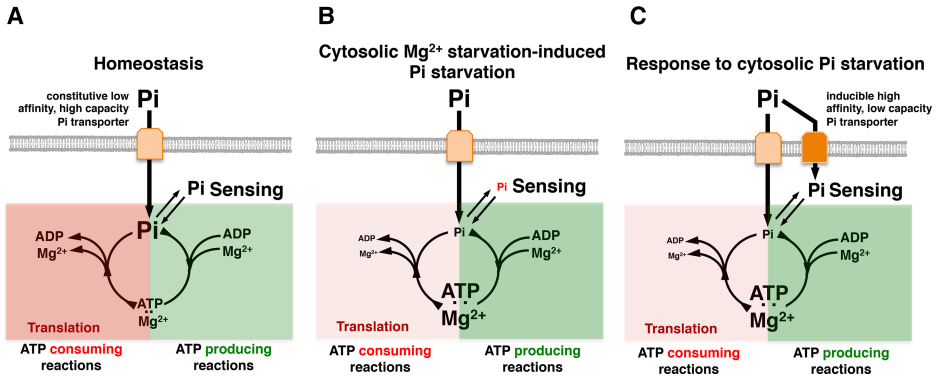

Figure 7. Free cytoplasmic Mg^{2+} inhibits Pi starvation response. (*A*) When neither Pi nor Mg^{2+} are limiting in the cytoplasm, extracellular Pi is imported via constitutive low-affinity high-capacity Pi transporters. Free intracellular Pi is sensed by components of the cellular machinery and incorporated into ATP. Pi from ATP is transferred to other biomolecules and assimilated into the cell or liberated back into the cytosol through ATP hydrolysis (e.g., the result of active ribosomes). (*B*) During Pi starvation, the intracellular concentration of free Pi decreases either because Pi is limited in the extracellular environment or because low free cytoplasmic Mg^{2+} levels compromise the function of cellular activities that recycle Pi from ATP (i.e., translation). (*C*) The cellular machinery senses a drop in free intracellular Pi, eliciting a Pi starvation response that is characterized, among other things, by the expression of high-affinity Pi transporters. See also Supplemental Figure S5.

translation is the cellular activity that consumes most cellular ATP (Stouthamer 1973), and Pi incorporation into ATP enables its assimilation into cells through cycles of ATP synthesis and hydrolysis (Wanner 1996; Kroger and Fuchs 1999). For instance, a decrease in free cytoplasmic Mg^{2+} reduces the number of active ribosomes by compromising ribosome assembly, thereby drastically lowering the fraction of ATP-consuming reactions (Fig. 7A; Pontes et al. 2016). As the available Pi transporters are unable to maintain a steady supply of Pi relative to the fraction being incorporated into ATP molecules, the levels of free cytoplasmic Pi decrease (Fig. 7B). This decrease activates the PhoB/PhoR system, promoting the phosphorylated state of PhoB (Fig. 6D) and transcription of a high-affinity Pi transporter (Figs. 6B, 7C). That translation inhibitors activate the PhoB/PhoR system (Fig. 6B–D; Supplemental Fig. S4B,D) by "trapping" cytoplasmic Pi in ATP molecules is supported by the prevention of PhoB/PhoR activation resulting from stimulation of ATP hydrolysis using the pATPase plasmid (Fig. 6A,B; Supplemental Fig. S4A–D).

Our results provide a physiological explanation for the finding that mutations conditionally expanding cytoplasmic pools of adenine nucleotides promote expression of PhoB-dependent genes even when Pi is abundant in the growth medium (Wilkins 1972); that is, when the adenine pool increases, cells experience low cytoplasmic Pi because Pi is trapped as ATP. Moreover, our results elucidate a reciprocal regulatory relationship between translation and Pi homeostasis: Pi acquisition allows the synthesis and operation of ribosomes, which in turn control Pi acquisition. Notably, the majority of Pi in living cells is present in ATP and rRNA (Bremer and Dennis 1996; Elser et al. 2003; Gillooly et al. 2005).

The intimate relationship between translation and the PhoB/PhoR system is further supported by the bacterial behavior elicited when protein synthesis is compromised due to an increase in the levels of the alarmone (p)ppGpp: Transcription of PhoB-dependent genes is inhibited even when environmental Pi is limited (Spira et al. 1995; Spira and Yagil 1998). Thus, just as an increase in ATP (Fig. 6A) drives expression of Pi acquisition genes (Fig. 6B), (p)ppGpp-mediated inhibition of rRNA transcription (Sarmientos et al. 1983) and adenine biosynthesis (Pao and Dyess 1981; Hou et al. 1999) alleviates the consumption of free cytoplasmic Pi pools, preventing PhoB/PhoR activation even when cells experience low environmental Pi.

Our findings indicate that the PhoB/PhoR system is activated by conditions promoting a decrease in free cytoplasmic Pi (Scholten and Tommassen 1993; Hoffer and Tommassen 2001) even in organisms experiencing high Pi in their surroundings (Fig. 1). Furthermore, they argue against the prevailing notion that the sensor PhoR responds to extracytoplasmic Pi (Hsieh and Wanner 2010). Moreover, they are in agreement with the proposal that induction of the *PHO* genes in *S. cerevisiae* is triggered by low cytoplasmic (as opposed to extracytoplasmic) Pi (Auesukaree et al. 2004).

The PhoU protein inhibits PhoB activation through an interaction with PhoR and the PstB high-affinity Pi transporter component (Fig. 1; Gardner et al. 2014). In *E. coli*, inactivation of *phoU* results in the hyperactivation of PhoB (Steed and Wanner 1993; Rice et al. 2009). Notably, mutations in *phoU* result in severe growth defects that can be suppressed by inactivation of either *phoB* or the PhoB-activated high-affinity Pi transporter encoded by the *pstSCBA* operon (Steed and Wanner 1993) or ameliorated by decreasing the Pi concentration in the growth medium (Rice et al. 2009). This indicates that excessive cytoplamic Pi inhibits *E. coli* growth. The cytosolic activation of the PhoB/PhoR system constitutes a simple mechanism to avoid the deleterious effects resulting from high cytoplasmic Pi that would inevitably arise from premature *pstSCBA* expression.

In prokaryotes, natural selection often favors close genomic linkage among genes participating in related or complementary cellular processes, presumably because acquisition of certain adjacent gene pairs by horizontal gene transfer is more beneficial than the acquisition of either gene alone (Lawrence 1997, 2002). In support of the physiological interplay between Pi and Mg^{2+}, genomic analysis reveals that genes involved in the acquisition and/or metabolism of Pi are often found in close proximity to those responsible for the maintenance of free cytoplasmic Mg^{2+} (Supplemental Fig. S5). For instance, the *mgtC* gene of the gram-negative bacterium *Polynucleobacter asymbioticus* is adjacent to *phoBR*, *phoU*, and *pstSCAB* homologs; the *corA* Mg^{2+} transporter gene of the gram-positive *Bacillus cereus* is located near one specifying a phosphatase (Supplemental Fig. S5). Notably, close linkage between these classes of genes is also observed in archaeal species such as *Halorhabdus utahensis*, *Natrialba magadii*, and *Haloarcula* spp. (Supplemental Fig. S5).

Finally, cells typically rely on Mg^{2+} to neutralize the negative charge of Pi-containing molecules. These include the rRNA Pi backbone, which stabilizes the ribosome structure, enabling translation (Gesteland 1966; Klein et al. 2004; Pontes et al. 2016); the DNA Pi backbone, which stabilizes base pairing and base stacking in chromosomes (Shaw and Wang 1993); and ATP and other (deoxy)nucleotide triphosphates [(d)NTPs], enabling catalysis of most (d)NTP-dependent reactions, such as those carried out by DNA polymerase and RNAP (Kornberg and Gefter 1972; Zaychikov et al. 1996; Cowan 2002). Given that these fundamental cellular processes require Mg^{2+}, we postulate that inhibition of Pi acquisition during cytoplasmic Mg^{2+} starvation is widespread in nature.

Materials and methods

Microbial strains, plasmids, and growth conditions

Microbial strains and plasmids used in this study are listed in Supplemental Table S1. All *S. enterica* serovar Typhimurium strains were derived from strain 14028s (Fields et al. 1986) and constructed by λ-red-mediated recombination (Datta et al. 2006) followed by phage P22-mediated transductions as described (Davis et al. 1980). *E. coli* MG1655 (Blattner et al. 1997) and *S. cerevisiae* DY1457 (Askwith et al. 1994) were used in physiological

experiments. *S. cerevisiae* was grown in YPD medium supplemented with 5 mM $MgSO_4$ at 250 rpm and 30°C; when necessary, cultures were supplemented with 250 µg/mL cycloheximide. Bacterial strains used in transformation or recombination were grown in LB medium at 30°C or 37°C (Datta et al. 2006; Khetrapal et al. 2015). When required, LB medium was supplemented with 100 µg/mL ampicillin, 20 µg/mL chloramphenicol, 50 µg/mL kanamycin, and/or 30 mM glucose or rhamnose.

In ChIP-seq experiments, *S. enterica* was grown in N-minimal medium (Véscovi et al. 1996) containing 10 or 50 µM $MgCl_2$. Unless stated otherwise, physiological experiments with bacteria were carried out at 37°C with shaking at 250 rpm in MOPS medium (Neidhardt et al. 1974) lacking $CaCl_2$ (to avoid repression of the PhoP/PhoQ system) (Véscovi et al. 1996) and supplemented with 0.1% (w/v) bacto casamino acids (BD Difco) and 30 mM glucose. Experiments were conducted as follows: After overnight (~16- to 20-h) growth in MOPS containing high Mg^{2+} (10 mM $MgCl_2$) and Pi (2 mM K_2HPO_4), cells washed twice in medium lacking Mg^{2+} and Pi and inoculated (1:100) in fresh medium containing 10 mM Mg^{2+} and 2 mM Pi, 10 mM Mg^{2+} and 250 µM Pi, or 10 µM Mg^{2+} and 500 µM Pi and propagated at 37°C with shaking at 250 rpm. (It should be noted that at a concentration of 0.1% [w/v] bacto casamino acids [BD Difco], the medium already contained ~163 µM Pi.) During physiological experiments, selection of plasmids was accomplished by the addition of ampicillin at 35 µg/mL (overnight growth) or 15 µg/mL (experimental condition) and/or chloramphenicol at 20 µg/mL (overnight growth) or 8 µg/mL (experimental condition). Unless specified otherwise, heterologous expression of proteins was achieved by treatment of cultures with 100 µM (pMgtA), 250 µM (pMgtC), or 1 mM (pATPase) IPTG. In experiments involving pharmacological inhibition of translation, bacteria were treated with approximately three times their minimal inhibitory concentration: either 25 µg/mL chloramphenicol or 500 µg/mL spectinomycin.

Recombineering and qPCR primers

Generation of chromosomal mutations was carried out using PCR products generated with primers listed in Supplemental Table S2, as described (Datta et al. 2006). The counterselection method developed by Khetrapal et al. (2015) was used to construct strain MP1429.

Construction of plasmids

Phusion high-fidelity DNA polymerase (New England BioLabs) was used in reactions with primers listed in Supplemental Table S2. PCR products were assembled into pFPV25 or pFPV25$_{AAV}$ (digested with restriction enzymes BamHI and EcoRI), pUHE-21-2-*lacI*^{q} (digested with BamHI and HinDIII), or pACYC184 (digested with SalI and HinDIII) using NEBuilder HiFi DNA assembly cloning kit (New England BioLabs). The integrity of constructs was verified by DNA sequencing and/or functional assays.

RNAP ChIP-seq

ChIP assays were performed as described (Shin and Groisman 2005) with the modifications described in the Supplemental Material.

RNA extraction, cDNA synthesis, and qPCR

For all experiments involving bacteria, RNA extraction was carried out using RNeasy (Qiagen) according to the manufacturer's instructions. For *S. cerevisiae*, RNA extraction was carried as described (Aiba et al. 1981) with the minor modifications described in the Supplemental Material. cDNA was synthesized from RNA samples using the SuperScript VILO cDNA synthesis lit (Thermo Fisher Scientific) according to the manufacturer's instructions. Relative amounts of cDNA were determined using standard curves obtained from qPCR with serially diluted wild-type *E. coli*, *Salmonella*, or *S. cerevisiae* genomic DNA. Quantification of cDNA was performed by real-time PCR using Fast SYBR Green master mix (Thermo Fisher Scientific) in an ABI 7500 sequence detection system (Thermo Fisher Scientific).

Estimation of intracellular ATP

Intracellular ATP was estimated as described (Pontes et al. 2015a) with the minor modifications described in the Supplemental Material.

Estimation of intracellular Pi

Total Pi in the samples was estimated from crude cell extracts using the molybdenum blue method as described (Kanno et al. 2016). Total Pi levels in samples were normalized by the amount of protein in the samples. Detailed sample extraction and Pi and protein measurements are described in the Supplemental Material.

Monitoring gene expression via fluorescence

Fluorescence derived from reporter constructs was measured into clear-bottomed 96-well black plates (Corning) using a Synergy H1 plate reader (BioTek). Green fluorescence was measured with excitation 485 nm and emission 535 nm, and the absorbance in each well was measured at 600 nm. Detailed sample extraction and Pi and protein measurements are described in the Supplemental Material.

Phos-tag SDS-PAGE and Western blotting

Phos-tag SDS-PAGE and Western blotting were carried out as described (Wayne et al. 2012) with the modifications described in the Supplemental Material.

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Author contributions: M.H.P. and E.A.G. conceived the study and designed the research, M.H.P. performed the research, and M.H.P. and E.A.G. analyzed the data and wrote the paper.

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