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Cellular Adaptations to Cytoplasmic Mg^{2+} Limitation

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Abstract

Mg^{2+} is the most abundant divalent cation in living cells. It is essential for charge neutralization, macromolecule stabilization, and the assembly and activity of ribosomes and as a cofactor for enzymatic reactions. When experiencing low cytoplasmic Mg^{2+}, bacteria adopt two main strategies: They increase the abundance and activity of Mg^{2+} importers and decrease the abundance of Mg^{2+}-chelating ATP and rRNA. These changes reduce regulated proteolysis by ATP-dependent proteases and protein synthesis in a systemic fashion. In many bacterial species, the transcriptional regulator PhoP controls expression of proteins mediating these changes. The 5′ leader region of some mRNAs responds to low cytoplasmic Mg^{2+} or to disruptions in translation of open reading frames in the leader regions by furthering expression of the associated coding regions, which specify proteins mediating survival when the cytoplasmic Mg^{2+} concentration is low. Microbial species often utilize similar adaptation strategies to cope with low cytoplasmic Mg^{2+} despite relying on different genes to do so.

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INTRODUCTION

All living organisms rely on ions to carry out biological processes. Mg^{2+} is the most abundant divalent cation in living cells (71). Mg^{2+} has essential roles as a counterion for negative charges, stabilizer of molecular and macromolecular structures, and cofactor for many enzymatic reactions across the three domains of life. Multiple chemical properties make Mg^{2+} the divalent cation most widely used in these processes. First, Mg^{2+} has a relatively small ionic radius and high affinity for oxygen, allowing it to form strong water-metal interactions (124). These water molecules can form hydrogen bonds with numerous other biomolecules. Second, the millimolar concentrations of Mg^{2+} inside the cell greatly outweigh the micromolar concentrations of other divalent cations (35, 71). The sheer abundance of Mg^{2+} renders it the most likely divalent cation to bind structures and macromolecules in living cells that exhibit higher affinities for other divalent cations (35).

The high cytoplasmic concentration of Mg^{2+} may stem from its ubiquitous nature in the environment. As the eighth-most abundant element in the Earth's crust, Mg^{2+} is available to organisms residing in nearly every habitat (56). In addition to its environmental bioavailability, Mg^{2+} is highly soluble in the presence of inorganic phosphate and bicarbonate relative to other metal ions, which

may be the reason for its role as the major cofactor in electrophilic catalysis in living cells (43). In other words, cations that can functionally replace Mg^{2+} in Mg^{2+}-dependent reactions in vitro appear to have been selected against in vivo because of their poor solubility or potential toxicity. For instance, the rate of incorrect nucleotide incorporation during in vitro transcription is five times lower when reactions are carried out with Fe^{2+} rather than with Mg^{2+} (43). Likewise, Fe^{2+} and Mn^{2+} can functionally replace ribosomal Mg^{2+} under anoxic conditions and support translation in vitro (14). However, despite being more abundant than Mg^{2+} in the Earth's crust, Fe^{2+} is readily oxidized to Fe^{3+}, which is poorly soluble at neutral pH (6). In addition, Fe^{2+} can cause widespread cellular damage by catalyzing the formation of reactive oxygen species (34).

Mg^{2+} plays critical roles in all bacterial subcellular compartments. The concentration of free Mg^{2+} is ~1 mM in the cytoplasm, and the total intracellular Mg^{2+} concentration is ~50 mM (48). Bacteria have different needs for Mg^{2+} inside and outside the cytoplasm, which results in different distributions of Mg^{2+}. Bacteria have thus developed distinct mechanisms for sensing and responding to extracytoplasmic (40) versus cytoplasmic (29, 32) Mg^{2+} limitation, allowing them to differentially regulate pathways and enact a coordinated response to the specific Mg^{2+} limitation condition experienced.

Outside of the cytoplasm, Mg^{2+} acts as a counterion to maintain the integrity of bacterial membranes and cell surfaces. Gram-negative bacteria possess two membranes with a layer of peptidoglycan in between, collectively termed the cell envelope. The outer leaflet of the asymmetric outer membrane is composed of lipopolysaccharide (LPS), which contains phosphate groups that confer upon the cell a negative charge (94). This charge must be neutralized to prevent electrostatic repulsion among adjacent LPS molecules (2). Multiple divalent ions, including Ca^{2+} and Mn^{2+}, contribute to charge neutralization, but Mg^{2+} carries the largest responsibility by far (64). In addition to stabilizing the LPS, Mg^{2+} binds membrane-embedded proteins, helping them maintain conformations critical for function (78, 120). Chelation of divalent cations leads to increased permeability of the outer membrane, resulting in increased susceptibility to antibiotics (116). The LPS appears to serve as a Mg^{2+} reservoir because it contains about one-third of the total Mg^{2+} in *Escherichia coli* (and likely in other species) (49). Certain species tap into this Mg^{2+} reservoir by chemically modifying some of the LPS phosphates that are normally neutralized by Mg^{2+}, thereby making Mg^{2+} available to be imported into the cytoplasm and used in activities that exhibit a strict dependence on Mg^{2+} (19, 49).

Gram-positive bacteria have a single membrane surrounding their cytoplasm. They have a cell wall with lipoteichoic acids (LTAs) and wall teichoic acids (WTAs) as the main components (112, 113). LTA is anchored to the cytoplasmic membrane, whereas WTA is covalently bound to the peptidoglycan. The polyphosphates of teichoic acids and the carboxylates of peptidoglycan confer a negative charge to the cell wall that is neutralized by divalent cations, primarily Mg^{2+}. The peptidoglycan has higher affinity for Mg^{2+} than WTA does but a smaller binding capacity (113), which together are hypothesized to facilitate Mg^{2+} movement toward the cytoplasmic membrane where the Mg^{2+} transporters are located. Mg^{2+} bound to teichoic acids constitutes a reservoir that gram-positive bacteria can exploit when there is an increased need for cytoplasmic Mg^{2+} (113). In *Bacillus subtilis*, growth in a Mg^{2+}-limited medium alters overall cell wall composition such that its teichoic acid content is increased (77). The upregulation of WTA biosynthesis increases the cell's capacity and affinity for Mg^{2+} adsorption (77), which may have the effect of helping with assimilation of Mg^{2+} into the intracellular environment (15). In *Staphylococcus aureus*, D-alanine modification of the ribitol and glycerol groups in WTA and LTA, respectively, reduces the surface negative charge conferred by the phosphates in teichoic acids (95). Transcription of the genes responsible for D-alanylation of teichoic acids is repressed when the Mg^{2+} concentration is plentiful (59), suggesting a physiological connection between teichoic acid modification and

Mg^{2+} homeostasis analogous to the connection between LPS modification and Mg^{2+} homeostasis discussed above for gram-negative bacteria.

The majority of cytoplasmic Mg^{2+} is bound to nucleotides, nucleic acids, enzymes, and other cellular components, while ~5% constitutes a free-ion pool (78, 90). The diverse functions of these components reflect similarly diverse needs for Mg^{2+} in the cytoplasm. For example, cells rely on Mg^{2+}-ATP for energy and Mg^{2+}-GTP for signaling. That is, nucleotide triphosphates (NTPs) are typically found in complex with Mg^{2+} when biologically active because Mg^{2+} is especially effective at stabilizing phosphate-containing species (124).

Mg^{2+} neutralizes the backbone phosphate groups in DNA and RNA while acting as a cofactor for enzymes during DNA and RNA synthesis (119). Protein synthesis is likewise dependent on Mg^{2+} due to its critical roles in rRNA folding that is necessary for ribosome assembly (92), and due to GTP neutralization, because GTP energizes multiple proteins required for translation (97). Given that the majority of cell processes are carried out by proteins, and that DNA replication, transcription, and translation all depend on Mg^{2+}, bacteria adjust a wide range of cellular activities when the cytoplasmic Mg^{2+} concentration drops below the threshold required for normal protein synthesis (90). Thus, the response to Mg^{2+} limitation allows bacteria to preserve critical cellular processes, the absence of which would be incompatible with life. That is, mounting and sustaining a response to low cytoplasmic Mg^{2+} relies on proteins that still need to be actively transcribed from genes and synthesized even while the cell is experiencing low Mg^{2+} stress.

In this review, we examine bacterial adaptation to low cytoplasmic Mg^{2+}. We first discuss the mechanisms by which bacteria detect Mg^{2+} limitation in the cytoplasm. We then describe the physiological adaptations that bacteria use to respond to this condition, as well as the effects that these adaptations have on other regulatory systems. Some of these adaptations are critical for bacterial pathogenesis because the mammalian protein Slc11a1 (also known as Nramp1) confers resistance to intracellular pathogens (123) and causes Mg^{2+} starvation in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) when this gram-negative bacterium is in host tissues (31). *S.* Typhimurium, in turn, relies on a Mg^{2+} transporter to survive in Slc11a1-competent macrophages (132). The contents of this article reflect that the majority of research on cellular adaptations to cytoplasmic Mg^{2+} limitation has been conducted with *S.* Typhimurium (48). Finally, we identify responses to low cytoplasmic Mg^{2+} that are conserved in prokaryotic and eukaryotic organisms in spite of differences in the genes mediating the responses.

DETECTING LOW CYTOPLASMIC Mg^{2+}

When a bacterium encounters low extracytoplasmic Mg^{2+}, it takes time for the cytoplasmic Mg^{2+} concentration to drop below the threshold that triggers the physiological adaptations enabling bacterial survival when the cytoplasmic Mg^{2+} concentration is suboptimal (83). Therefore, depending on when bacteria are examined after a switch to low-Mg^{2+} media, the observed behaviors may reflect the response to low extracytoplasmic Mg^{2+}, low cytoplasmic Mg^{2+}, or both (83, 130, 133).

The mRNAs specifying all three Mg^{2+} transporters in *S.* Typhimurium—MgtA, MgtB, and CorA—harbor unusually long leader regions that can adopt alternative secondary structures in response to specific cytoplasmic signals that, in turn, determine whether transcription proceeds into the associated coding regions or terminates within the leaders. These cytoplasmic signals include disruptions in the translation of open reading frames (ORFs) located within the leaders and a decrease in the cytoplasmic Mg^{2+} concentration below a certain threshold (**Figure 1**).

The *mgtA* leader utilizes multiple mechanisms to terminate transcription within its leader region when the cytoplasmic Mg^{2+} concentration exceeds a certain value. First, the *mgtA* leader adopts a conformation (29, 60) that favors loading of the transcription termination factor

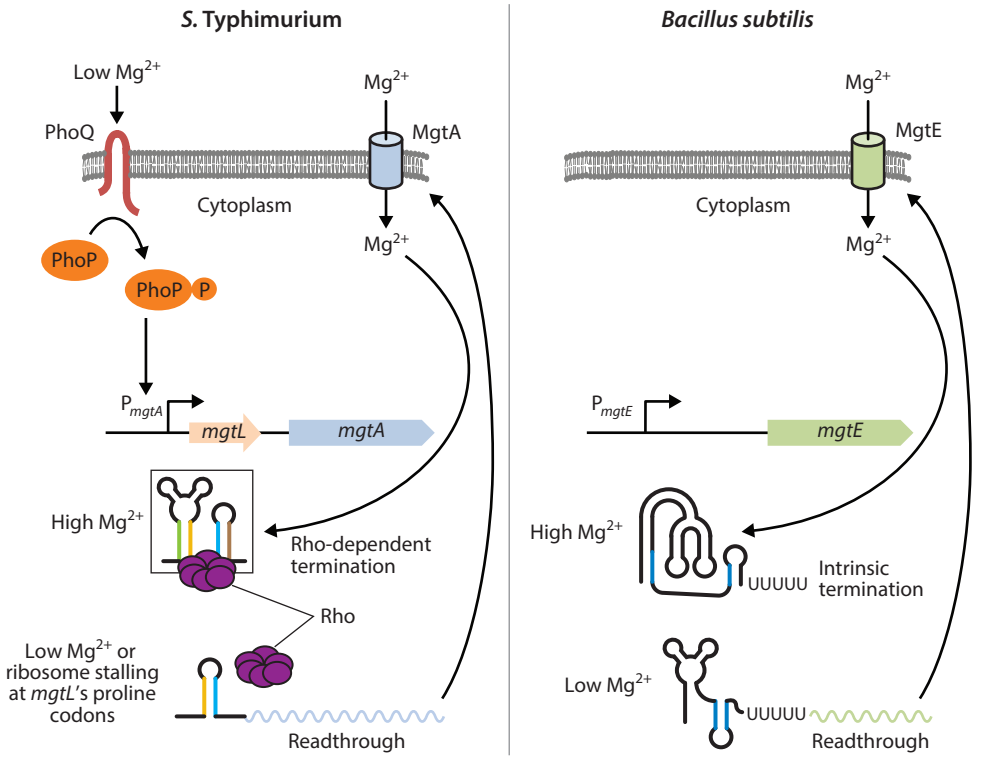

Figure 1

Regulation of the Mg^{2+} transporters MgtA and MgtE in gram-negative and gram-positive bacteria. (*Left*) The regulator PhoP promotes transcription from the *mgtA* promoter when activated by the sensor PhoQ in response to specific signals, including low periplasmic Mg^{2+} in *Salmonella enterica* serovar Typhimurium. The leader region of the Mg^{2+} transporter mRNA *mgtA* contains a Mg^{2+}-responding riboswitch and the *mgtL* open reading frame, which control transcription elongation into the *mgtA* coding region in response to cytoplasmic signals. When cytoplasmic Mg^{2+} levels are high or *mgtL* is fully translated, the *mgtA* leader RNA adopts a conformation that favors transcription termination within the *mgtA* leader region by factor Rho. When levels of Mg^{2+} in the cytoplasm fall below a particular threshold or the ribosome stalls at *mgtL*'s proline codons, the *mgtA* leader RNA adopts an alternative conformation that hinders Rho-mediated termination, allowing transcription to continue into the *mgtA* coding region. (*Right*) Transcription of the *Bacillus subtilis* Mg^{2+} transporter gene *mgtE* is governed by a Mg^{2+}-sensing riboswitch. When levels of Mg^{2+} in the cytoplasm are high, Mg^{2+} binds to the *mgtE* leader region and promotes compaction of the RNA into a structure that includes an intrinsic transcription terminator. This drives transcription termination within the *mgtE* leader region. When cytoplasmic Mg^{2+} is low, the RNA adopts an alternative conformation that prevents formation of the intrinsic terminator and thus permits transcription elongation into the *mgtE* coding region. In contrast to *mgtA*, transcription initiation at the *mgtE* promoter does not respond to extracytoplasmic Mg^{2+}. Colors of vertical lines correspond to mRNA stretches that form different stem structures depending on the cytoplasmic Mg^{2+} concentration. Adapted from Reference 48.

Rho onto the transcript (53). And second, high cytoplasmic Mg^{2+} promotes an unusually long transcriptional pause that is necessary for Rho-dependent transcription termination within the *mgtA* leader (54). The Mg^{2+}-stimulated transcription termination in the *mgtA* leader has been reconstituted in vitro with purified RNA polymerase, Rho, nucleotides, and the corresponding DNA template (53), arguing against additional factors being essential for this regulation to take place in vivo. In addition, the RNA structure promoted when the Mg^{2+} concentration is high (29, 60) renders the *mgtA* leader susceptible to degradation by RNase E (110). The effect of

RNase E on expression of the *mgtA* coding region is much smaller than that of Rho-dependent transcription termination (110). In sum, the *mgtA* leader mRNA is a Mg^{2+} sensor that prevents expression of the Mg^{2+} importer MgtA when cytoplasmic Mg^{2+} is above a certain concentration.

The *mgtA* leader mRNA harbors a short ORF termed *mgtL*, in which 4 of 17 sense codons are proline codons (82). Signals that reduce *mgtL* translation promote transcription elongation into the *mgtA* coding region. For instance, hyperosmotic stress results in transcription elongation into the *mgtA* coding region in a manner dependent on *mgtL*'s proline codons (53, 82). Hyperosmotic stress is proposed to cause ribosome stalling at *mgtL*'s proline codons because proline is an osmoprotectant (30) and thus not available to charge $tRNA^{Pro}$ when bacteria experience hyperosmotic stress. Ribosome stalling at *mgtL*'s proline codons favors a conformation in the *mgtA* leader that hinders Rho access to the *rut* (Rho utilization) site, resulting in transcription of the *mgtA* coding region (53, 82). By contrast, when proline is available to charge $tRNA^{Pro}$, full *mgtL* translation favors an alternative conformation in the *mgtA* leader region that is conducive to Rho loading and transcription termination in the *mgtA* leader region (82). This regulatory mechanism is analogous to classical transcription attenuation governing expression of amino acid biosynthetic operons in that the uncoupling of transcription and translation results in a transcription termination event (51).

The *mgtA* leader was proposed to detect a decrease in cytoplasmic Mg^{2+} by a reduction in *mgtL* translation rather than by changes in the *mgtA* leader RNA structure resulting from direct Mg^{2+} binding. One group hypothesized that translation of *mgtL*'s proline codons is particularly sensitive to a decrease in cytoplasmic Mg^{2+}, thereby mediating the control of transcription termination within the *mgtA* leader region (38). Likewise, another group proposed that acidic residues in *mgtL* cause "intrinsic ribosome destabilization" when the Mg^{2+} concentration is low, which would somehow favor transcription of the *mgtA* coding region (17). Inactivation of the *rpmE*-encoded ribosomal protein bL31 antagonized intrinsic ribosome destabilization (17) and promoted expression of the *mgtA* coding region (38). However, a ribosome profiling experiment showed no ribosome stalling at *mgtL*'s proline codons in bacteria experiencing low cytoplasmic Mg^{2+} (8), arguing against the two proposed models.

A third group hypothesized that *mgtL* translation is required solely to transduce the Mg^{2+} signal (i.e., it plays no role in the response to proline-charged $tRNA^{Pro}$) (136). According to this hypothesis, low cytoplasmic Mg^{2+} would promote a structure in the *mgtA* leader in which ribosome access to *mgtL*'s Shine-Dalgarno sequence (or ribosome binding site) is hindered by an anti-Shine-Dalgarno sequence that sequesters *mgtL*'s Shine-Dalgarno sequence (136). Oddly, the putative anti-Shine-Dalgarno sequence is located downstream of *mgtL*'s Shine-Dalgarno sequence, putting it at a kinetic disadvantage to prevent ribosome access to *mgtL*'s Shine-Dalgarno sequence (136).

Several additional lines of evidence argue that low cytoplasmic Mg^{2+} and proline limitation are sensed independently by the *mgtA* leader: (*a*) Replacement of *mgtL*'s proline codons by other sense codons abolishes the response to proline limitation but does not eliminate the response to low Mg^{2+} (82, 136) (although it does decrease it); (*b*) a strain with an *mgtL* variant with the proline codon at the third position replaced by a leucine codon retains a normal response to proline limitation but is defective in the response to low cytoplasmic Mg^{2+} (82); and (*c*) low Mg^{2+} and low proline-charged $tRNA^{Pro}$ act synergistically on the *mgtA* leader to stimulate transcription of the *mgtA* coding region (82).

Despite the lack of sequence and structure similarity with the *mgtA* leader mRNA, the leader region of the *mgtCBRUcigR* operon also responds to a decrease in cytoplasmic Mg^{2+} by promoting transcription elongation into the associated coding region (29, 110). Moreover, the *mgtCBRUcigR* leader mRNA includes two ORFs—*mgtM* and *mgtP*—that respond to an increase in ATP concentration (67) and a decrease in proline-charged $tRNA^{Pro}$, respectively (66, 68), by promoting transcription of the associated coding region. The response to ATP is independent of

the response to proline-charged $tRNA^{Pro}$, which requires two of three consecutive proline codons in the 16-sense-codon-long *mgtP* (68). As discussed above for the *mgtA* leader (53, 54), Rho terminates transcription in the *mgtCBRUcigR* leader in the absence of inducing conditions (106).

The *corA* leader mRNA harbors the short ORF *corL*, translation of which determines whether transcription proceeds into the *corA* coding region or terminates within the *corA* leader. That is, poor *corL* translation favors transcription elongation. Rho is also required to terminate transcription in the *corA* leader under noninducing conditions (61, 62), as it does in the leader regions of the other two Mg^{2+} transporter–specifying transcripts (53, 54, 106).

What is the reason for *S.* Typhimurium favoring transcription of all three Mg^{2+} transporters when translation decreases? One possibility is that Mg^{2+} is essential for translation and a reduction in translation of the ORFs located in the leader regions of the Mg^{2+} transporter–specifying transcripts serves as a signal that Mg^{2+} must be imported to sustain protein synthesis. In agreement with this notion, Mg^{2+} removal from the growth medium results in loss of ribosomes, halt in protein synthesis, and growth arrest in *E. coli*, all of which are reversed upon restoration of Mg^{2+} to the medium (75). It is notable that the 5′ leader region of the mammalian Mg^{2+} channel–kinase TRPM7 harbors a short ORF that inhibits *TRPM7* translation when the Mg^{2+} concentration is high (79). Therefore, the regulatory strategy of controlling Mg^{2+} transporters in response to translation of an ORF(s) in the leader region appears to be conserved in evolution.

What is the reason for *S.* Typhimurium utilizing Rho to terminate transcription in the leader regions of all three Mg^{2+} transporter genes when the vast majority of transcription attenuators harbor intrinsic transcription terminators? One possibility is that Rho enforces polarity in gram-negative bacteria. Therefore, conditions that uncouple transcription and translation typically result in Rho-dependent transcription termination (63), and a decrease in cytoplasmic Mg^{2+} below a certain threshold compromises translation before it impacts transcription (92).

In addition to the transcription elongation and mRNA stability control mechanisms operating in leader regions, the Mg^{2+} transporters of *S.* Typhimurium are subjected to regulation by Mg^{2+}. First, the MgtA protein acts as a Mg^{2+} sensor hypothesized to hinder Mg^{2+} uptake when cytoplasmic Mg^{2+} is high because high Mg^{2+} inhibits MgtA's ATPase activity (111), which is essential for Mg^{2+} uptake (48). And second, the promoters of the *mgtA* (86, 126) and *mgtB* (86, 137) genes (but not that of *corA*) are positively controlled by PhoP, a transcriptional regulator activated by multiple signals, including low periplasmic Mg^{2+} detected by PhoP's cognate sensor, PhoQ (40). The *mgtA* promoter is active even in bacteria experiencing a physiological concentration of Mg^{2+} (i.e., 1 mM) (28), which activates the PhoP protein only mildly (40). However, transcription of the *mgtA* coding region and synthesis of the MgtA protein come about only when bacteria face cytoplasmic Mg^{2+} limitation (28, 29). This is why the *mgtA* coding region is expressed in strains with nonsense mutations early in *mgtL* in organisms experiencing >1 mM Mg^{2+} (38, 82).

In the gram-positive bacterium *B. subtilis*, a Mg^{2+}-responding riboswitch controls expression of the Mg^{2+} transporter gene *mgtE* (32). This riboswitch—referred to as the M box—responds to an increase in the Mg^{2+} concentration by adopting a compact structure that hinders transcription elongation into the *mgtE* coding region by sequestering an antiterminator sequence that can otherwise prevent formation of an intrinsic transcription terminator structure (32) (**Figure 1**). The M box is also found in the leader regions of (*a*) an *mgtE* sequelog (118) in the gram-negative *Serratia marcescens* (9), (*b*) genes specifying Mg^{2+} transporters other than MgtE in gram-positive bacteria (32, 108), and (*c*) genes of unknown function in both gram-negative and gram-positive bacteria (96).

In sum, Mg^{2+}-responding leader mRNAs enable bacteria to promote expression of Mg^{2+} transporters when the concentration of free cytoplasmic Mg^{2+} drops below a certain value, and when translation is disrupted, even if the extracytoplasmic Mg^{2+} concentration remains high.

MOUNTING A RESPONSE: IMPORTING Mg^{2+}, REDUCING ATP AMOUNTS, AND INCREASING PhoP ABUNDANCE

S. Typhimurium responds to low cytoplasmic Mg^{2+} in three major ways: by furthering Mg^{2+} uptake into the cytoplasm, by decreasing the ATP concentration, and by increasing the amount of the active form of the transcriptional regulator PhoP (**Figure 2**). These processes are interconnected because PhoP is a direct transcriptional regulator of the *mgtCBRUcigR* operon, which specifies the Mg^{2+} transporter MgtB and the ATP-decreasing protein MgtC (48), and also because MgtC protects PhoP from degradation by the ATP-dependent protease ClpAP (133). Moreover, the response reflects that NTPs and ribosomes chelate approximately 50% and 25% of the total cytoplasmic Mg^{2+}, respectively, and that DNA, nonribosomal RNAs, proteins, and membranes bind the remaining 25% (90).

The response to low cytoplasmic Mg^{2+} enables *S.* Typhimurium to carry out protein synthesis, albeit at a reduced rate; to decrease regulated proteolysis by ATP-dependent proteases while continuing proteolysis of certain proteins destined for degradation by the very same proteases; and to alter the amounts of PhoP-regulated proteins that help bacteria cope with cytoplasmic Mg^{2+} limitation (**Figure 2**).

Critically, cytoplasmic Mg^{2+} may become limited if the amounts of certain Mg^{2+}-chelating anionic metabolites increase beyond a certain threshold. For example, mutations in polyphosphate kinase that increase polyphosphate levels are toxic to *E. coli* because they cause Mg^{2+} starvation (100). Likewise, a large increase in ATP amounts without a concomitant increase in the cytoplasmic Mg^{2+} concentration impairs bacterial growth (90).

Mg^{2+} Uptake

The Mg^{2+} transporters MgtA and MgtB are P-type ATPases that utilize the energy derived from ATP hydrolysis to import Mg^{2+} into the cytoplasm against an electrochemical gradient (48). MgtA and MgtB share 50% amino acid identity and topology but differ in multiple properties, including the cations that inhibit Mg^{2+} uptake (Co^{2+} and Zn^{2+} for MgtA, Co^{2+} and Mn^{2+} for MgtB); temperature at which they operate (20°C and 37°C for MgtA, only 37°C for MgtB); virulence role

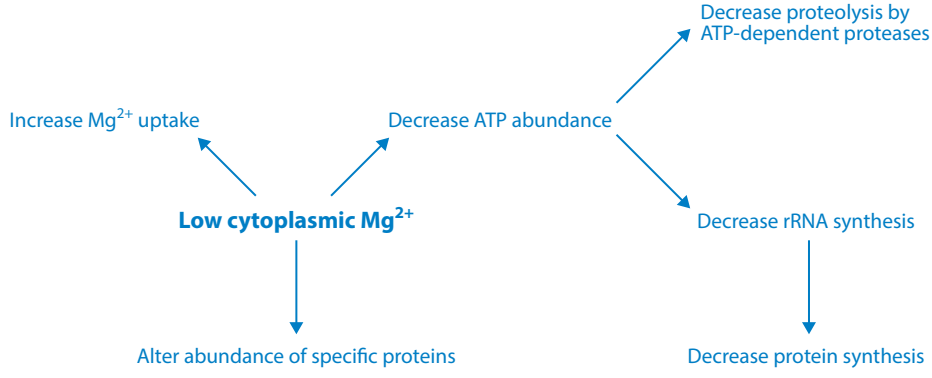

Figure 2

The cellular response to low cytoplasmic Mg^{2+}. Microbes respond to low cytoplasmic Mg^{2+} by increasing Mg^{2+} uptake; altering the abundance of specific proteins that help them cope with stress caused by low Mg^{2+}; and decreasing ATP synthesis, which decreases both proteolysis by ATP-dependent proteases and synthesis of rRNA, thereby decreasing the number of ribosomes, which, in turn, results in a decreased rate of protein synthesis.

(dispensable for MgtA, required for MgtB); phylogenetic distribution within the family *Enterobacteriaceae* (broad for MgtA, narrow for MgtB); and properties of their mRNA leader regions that enable their differential expression. In addition, MgtB enables bacterial survival under prolonged Mg^{2+} starvation whereas MgtA does not (132).

Under normal growth conditions, bacterial cells are negatively charged on the cytoplasmic side of the inner membrane and positively charged on the periplasmic side of the inner membrane. This membrane potential favors Mg^{2+} movement from the periplasm to cytoplasm through the channel CorA, abundance of which is not controlled by PhoP. However, low Mg^{2+} activates PhoP (40), which reverses membrane potential (5), thereby preventing Mg^{2+} importation via CorA and creating the need for ATP-dependent Mg^{2+} importation by MgtA and MgtB.

PhoP promotes MgtA-dependent Mg^{2+} importation in two additional ways. First, it promotes an increase in the concentration of cardiolipin (27), a negatively charged phospholipid in the inner membrane (where MgtA is located) that is proposed to act as a Mg^{2+} chaperone for MgtA (111). And second, it promotes expression of MgtS, a 31-residue protein that binds and stabilizes MgtA in *E. coli* (122). By contrast, MgtS neither modifies MgtA stability nor is regulated by PhoP in *S.* Typhimurium (132).

Mg^{2+}-promoted conformational changes in the multimeric CorA protein alter CorA gating properties (74). Both CorA and MgtE are Mg^{2+} channels that also operate as cytoplasmic Mg^{2+} sensors by regulating the gating properties of the ion-conducting pore. Low cytoplasmic Mg^{2+} favors dissociation of Mg^{2+} from the channels and Mg^{2+} importation into the cytoplasm (78, 85).

B. subtilis specifies proteins belonging to the MgtE, MgtA, and CorA families of Mg^{2+} transporters. MgtE appears to be the Mg^{2+} transporter operating under normal laboratory conditions, because an *mgtE* mutant displayed defective growth in rich media, and this defect was corrected by Mg^{2+} supplementation (121). In addition, the transporter CitM imports citrate along with divalent cations, allowing importation of Mg^{2+} under conditions that promote CitM expression (121).

Decreasing ATP Amounts

MgtC is an integral membrane protein that decreases ATP amounts in two ways. First, MgtC binds to the AtpB protein of the multi-subunit F_1F_o ATPase, inhibiting ATP synthesis (69). The F_1F_o ATPase is the machine responsible for making the majority of ATP in the cell (105). And second, MgtC decreases ATP amounts even in an *atpB* null mutant under some growth conditions (81), indicating the existence of one or more additional MgtC targets. MgtC reduces the cytoplasmic phosphate concentration by inhibiting phosphate uptake (16), thus reducing ATP synthesis. By decreasing ATP abundance, MgtC decreases the amount of Mg^{2+} needed to neutralize ATP, the most abundant NTP in the cell.

The physiological connection between Mg^{2+} and ATP exists even in organisms that lack MgtC. For example, *E. coli* decreases ATP amounts when experiencing low cytoplasmic Mg^{2+}, despite the absence of *mgtC* from its genome (92). In a *B. subtilis* strain defective in all known Mg^{2+} transporters, growth in low Mg^{2+} was restored by deletion of the genes specifying F_1F_o ATPase subunits (121). Moreover, several subunits of the F_1F_o ATPase and MgtE co-immunoprecipitated with the protease YqgP in wild-type *B. subtilis* (11). Furthermore, the F_1F_o ATPase-specifying *atp* operon from *Bacillus pseudofirmus* OF4 includes a gene that mediates Mg^{2+} transport (52). Thus, there is a strong association between the ATP-generating F_1F_o ATPase and Mg^{2+} transport.

Increasing the Abundance of Active PhoP

The transcriptional regulator PhoP forms a two-component regulatory system with its cognate sensor kinase, PhoQ, which responds to activating signals by promoting the active (i.e.,

phosphorylated) state of PhoP (PhoP-P) (107). These signals include low Mg^{2+} (40) and particular antimicrobial peptides in the periplasm (7) and a mildly acidic pH in the cytoplasm (22, 93). In addition to the signals acting on PhoQ, the PhoP-activated MgtA and MgtC proteins increase PhoP-P amounts in response to signals acting on the *mgtA* and *mgtC* mRNA leaders (83, 133).

MgtA increases PhoP phosphorylation in a manner dependent on PhoQ's ability to respond to periplasmic Mg^{2+} (83). It is proposed that by importing Mg^{2+} into the cytoplasm—and thus lowering the Mg^{2+} concentration in the periplasm—MgtA reduces the negative signal for PhoQ (40). The MgtA-dependent increase in PhoP-P amounts favors transcription of genes regulated by promoters that need higher amounts of PhoP-P for changes in transcription to take place (83).

MgtC prevents proteolysis of PhoP and PhoP-P by sequestering them away from ClpS (133), the protease adaptor necessary for degradation of these proteins by ClpAP (39). As with MgtA, the MgtC-dependent increase in PhoP-P amounts enables transcription of genes that need high PhoP-P amounts for DNA binding and control of the corresponding promoters (133).

PhoP-P reduces its own proteolysis by two additional mechanisms that reduce the amount of active ClpS protein. First, PhoP represses transcription of the *clpS* gene directly (127). PhoP repression of *clpS* transcription is *mgtA*- and *mgtC*-dependent (127), indicating a requirement for high PhoP-P amounts achieved when the cytoplasmic Mg^{2+} concentration drops below the threshold that triggers production of the MgtA and MgtC proteins. By repressing *clpS* transcription and being proteolyzed in a ClpS-dependent manner, PhoP forms a double negative-feedback loop with ClpS that stabilizes PhoP under low cytoplasmic Mg^{2+} conditions (127).

And second, PhoP is a transcriptional activator of the *qad* gene (129), which specifies an acetyl-CoA-binding protein necessary for acetylation of the HspQ protein (128). Acetylation favors HspQ accumulation because it renders HspQ resistant to proteolysis by the Lon protease (128). Acetylated and nonacetylated HspQ proteins bind to ClpS, preventing ClpS from delivering its substrates, including PhoP, to the protease ClpAP (128). As discussed above for repression of *clpS* transcription (127), activation of *qad* transcription is *mgtA*- and *mgtC*-dependent (129). By promoting *qad* transcription, PhoP creates a positive-feedback loop on PhoP amounts because Qad increases the amounts of HspQ, a ClpS antagonist.

PhoP variants with specific single–amino acid substitutions display defective binding to ClpS (39), which prevents their proteolysis and renders their abundance MgtC-independent (133). The PhoP variants retain a wild-type ability to regulate gene transcription (133). Therefore, by selecting for a degradable PhoP protein, evolution has enabled bacteria to tune wild-type PhoP abundance not only to the signals detected by PhoQ, which impact the positive transcriptional feedback that PhoP-P exerts on the *phoP* promoter (109), but also to signals that control PhoP (and PhoP-P) abundance and stability, such as those acting on the *mgtA* and *mgtC* leaders (127, 128, 133).

THE PHENOTYPIC CONSEQUENCES OF THE RESPONSE TO LOW CYTOPLASMIC Mg^{2+}

Lowering ATP Amounts Decreases Regulated Proteolysis by ATP-Dependent Proteases

Many cellular processes rely on the energy derived from ATP hydrolysis, including proteolysis by ATP-dependent proteases. These proteases are composed of an ATPase domain or subunit that unfolds the substrate and a peptidase domain or subunit that cleaves the unfolded polypeptide (72). Proteolysis is a physiological mechanism that enables cells to control protein quantity and quality. Protein quantity control typically involves degradation of functional proteins to achieve preferred concentrations of specific proteins when conditions change. Protein quality control, by contrast, entails degradation of misfolded, nonfunctional, or aggregated proteins that must be disposed of

to avoid cellular toxicity (72). Substrates may be recognized directly by a protease or indirectly via an adaptor that recognizes the substrate but is not itself degraded by its partner protease (10).

The reduction in ATP concentration taking place when *S.* Typhimurium experiences low cytoplasmic Mg^{2+} causes a systemic decrease in proteolysis by the ATP-dependent proteases ClpAP, ClpXP, Lon, and FtsH (130) (the fifth ATP-dependent protease in *S.* Typhimurium, HslV/HslU, has not been investigated) (**Figure 2**). The decreased proteolysis is due specifically to low ATP amounts, because protease activity can be restored by increasing ATP amounts in organisms experiencing low cytoplasmic Mg^{2+} (130). Given that the rate of protein synthesis decreases when cytoplasmic Mg^{2+} is low, limiting systemic proteolysis minimizes degradation of an already reduced protein pool (**Figure 2**). In addition, by targeting the energy source necessary for substrate unfolding rather than protease adaptors, bacteria simultaneously reduce proteolysis of both adaptor-dependent and adaptor-independent substrates of a given protease.

Numerous substrates that would normally be degraded by ATP-dependent proteases are subsequently stabilized when cytoplasmic Mg^{2+} is low (130). These substrates have been implicated in a variety of physiological processes, including virulence, stress responses, and metabolism. As a result, a reduction in protease activity is likely to have effects on multiple pathways that collectively contribute to the response to low Mg^{2+}, which are further discussed below. Substrate preservation during Mg^{2+} starvation conditions helps bacteria emerge from a slow-growth, quiescent state once Mg^{2+} becomes available (130).

Importantly, degradation of misfolded and nonfunctional proteins by ATP-dependent proteases continues unabated during cytoplasmic Mg^{2+} limitation (130), and this is anticipated to preserve proteome quality. Consistent with this notion, many bacteria possess methods of preserving translation fidelity under stress conditions (70), raising the question of whether these methods also apply during cytoplasmic Mg^{2+} limitation.

Maintaining Protein Synthesis by Reducing rRNA Transcription

When cytoplasmic Mg^{2+} is not limited, the rate of protein synthesis is directly related to the number of ribosomes present in the cell. The number of ribosomes, in turn, is dependent on the amount of rRNA because rRNA synthesis is the rate-limiting step in ribosome biosynthesis. When *S.* Typhimurium experiences low cytoplasmic Mg^{2+}, it reduces rRNA production to avoid the accumulation of ribosome precursors that cannot assemble into functional ribosomes (92). This reduction takes place even when bacteria have sufficient amino acids and ATP to carry out protein synthesis (92). That is to say, the abundance of Mg^{2+}, the cation necessary for ribosome assembly in living cells, supersedes control of rRNA synthesis by substrate abundance and energy availability (92). In agreement with the notion that bacteria adjust cytoplasmic Mg^{2+} levels according to their needs, the cytoplasmic Mg^{2+} concentration is lower in engineered *B. subtilis* strains lacking multiple rRNA-expressing *rrs* operons than in wild-type *B. subtilis* with the full complement of *rrs* operons (3).

S. Typhimurium produces rRNA from the P1 promoter in each of its seven *rrs* operons. Transcription initiation from the P1 promoter is regulated by intracellular levels of the initiating NTP (iNTP) and the second messenger guanosine pentaphosphate/tetraphosphate [(p)ppGpp] (37, 117). The RNA polymerase promoter open complex is unstable at P1 promoters until adequate levels of the iNTP are reached, at which time the iNTP binds the complex, stabilizes it, and promotes expression of rRNA-encoding genes. By contrast, (p)ppGpp decreases the stability of the complex and represses rRNA synthesis (117).

ATP is the iNTP for all seven P1 promoters in *S.* Typhimurium. During growth in low Mg^{2+}, transcription of rRNA decreases, reflecting a decrease in intracellular ATP levels caused by the MgtA, MgtB, and MgtC proteins (92). Besides reducing ATP levels, low cytoplasmic Mg^{2+}

promotes (p)ppGpp accumulation (92). In an adaptation known as the stringent response, (p)ppGpp is produced during nutrient-limited conditions and modifies the expression of a large number of genes (50). These global changes allow the cell to adjust its metabolism during starvation. Similar to limitation of amino acids and other nutrients, Mg^{2+} limitation promotes (p)ppGpp accumulation (92). The mechanism responsible for this process remains to be characterized but appears to be MgtC-dependent, as an *mgtC S.* Typhimurium mutant experiencing low cytoplasmic Mg^{2+} had approximately fourfold-lower (p)ppGpp amounts than wild-type *S.* Typhimurium with low cytoplasmic Mg^{2+} (92). By simultaneously decreasing ATP amounts and promoting (p)ppGpp accumulation, *S.* Typhimurium compromises the stability of the RNA polymerase–promoter open complex at P1 promoters when cytoplasmic Mg^{2+} is low. Indeed, rRNA transcription is tenfold lower during growth in 10 μM Mg^{2+} than during growth in 10 mM Mg^{2+} (92).

In addition to regulating rRNA synthesis, Mg^{2+} directly assists with the folding and compaction of rRNA during ribosome assembly (58). rRNA makes up two-thirds of the ribosome and contains multiple negative charges due to phosphate groups present in its backbone (65). Mg^{2+} neutralizes these charges and forms bridges that stabilize the secondary structures (65). Thus, during Mg^{2+} limitation, the production of functional ribosomes is slowed by both repressed rRNA synthesis and reduced availability of Mg^{2+} to enable proper ribosome assembly. Ribosomes utilize a significant amount of Mg^{2+} for stability and catalytic activity; each *E. coli* ribosome is estimated to contain >170 Mg^{2+} ions (104). By reducing the number of ribosomes present in the cell, *S.* Typhimurium frees up Mg^{2+} ions that can then be reallocated to other essential processes in the cytoplasm. And because Mg^{2+} is required for the association between 30S and 50S ribosomal subunits, a failure to decrease rRNA synthesis results in the inability to assemble functional ribosomes from the ribosomal components that are produced (42). Thus, reducing overall ribosome biosynthesis allows translation to continue, albeit at a slower rate, and both protein synthesis and Mg^{2+} homeostasis are achieved during stress caused by low cytoplasmic Mg^{2+}. *E. coli* also responds to low cytoplasmic Mg^{2+} by reducing rRNA synthesis even though it lacks the *mgtC* and *mgtB* genes, which are required for *S.* Typhimurium to achieve this task (92). Therefore, the discussed adaptation appears to be widespread even where organisms differ in the genes responsible for mediating such an adaptation.

Sparing Specific Substrates from Proteolysis

The *S.* Typhimurium PhoP protein controls expression of proteins that modify the specificity of the ClpAP, ClpXP, Lon, and FtsH proteases (**Figure 3**). This control is in addition to the systemic effects that PhoP exerts on the activity of these ATP-dependent proteases by decreasing ATP abundance (130).

First, PhoP-P represses *clpS* transcription (127) (**Figure 3**). Rather than eliminating *clpS* expression, PhoP-P reduces ClpS amounts approximately eightfold (133), thereby creating a hierarchy among ClpS-dependent substrates of ClpAP (127). The phenotypic consequences of this ClpS reduction include increased resistance to the antibiotic kanamycin, which is mediated by the ClpSAP substrate Oat, and increased abundance of RNA corresponding to genes regulated by the ClpSAP substrate SirA (UvrY in *E. coli*) (127). By contrast, proteolysis of the ClpSA substrate FtsA remains unaffected (127).

Second, the HspQ protein of *S.* Typhimurium is an antiadaptor for ClpS that reduces proteolysis by ClpS-dependent substrates of ClpAP (128). HspQ, in turn, is degraded by the Lon protease, except when HspQ is acetylated (128). The HspQ antagonism of ClpS is also PhoP-dependent because HspQ acetylation was identified in *S.* Typhimurium experiencing low Mg^{2+} (128), which is a PhoP-activating condition (40), and also because it requires the PhoP-activated *qad* gene, which specifies an acetyl-CoA-binding protein that is necessary for the acetyl transferase Pat to acetylate

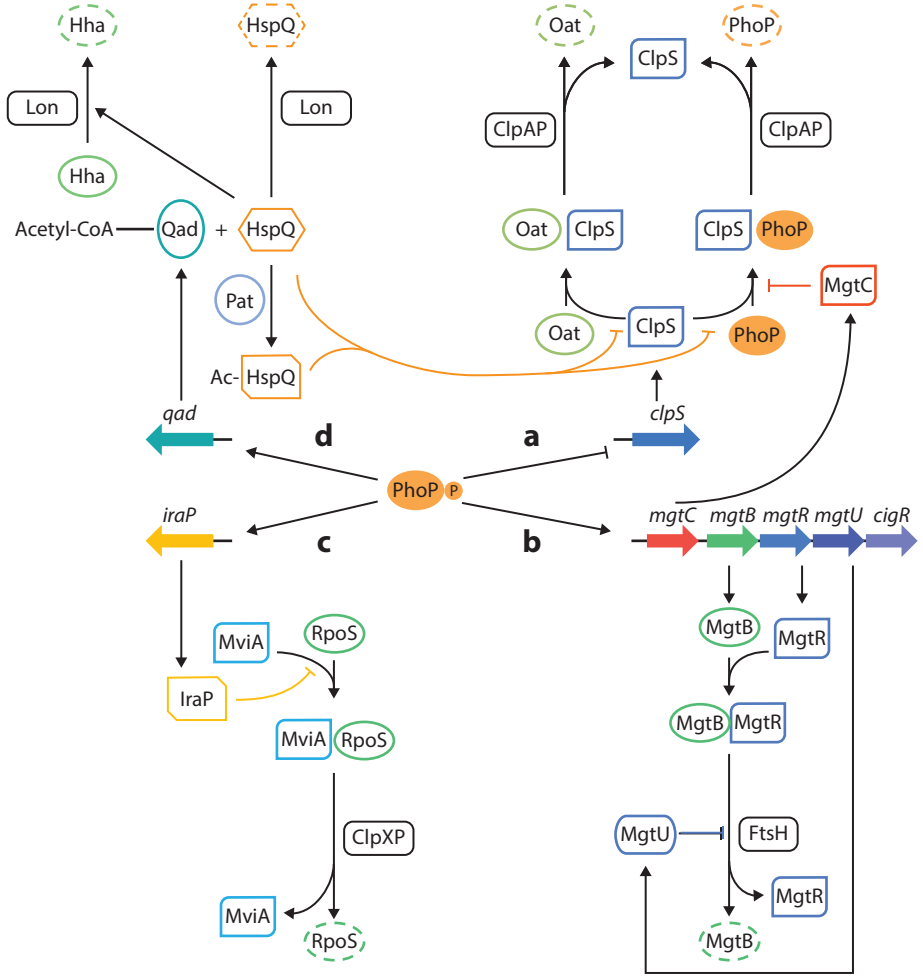

Figure 3

The transcriptional regulator PhoP controls proteolysis by the ATP-dependent proteases ClpAP, ClpXP, Lon, and FtsH in *Salmonella enterica* serovar Typhimurium. (*a*) PhoP represses transcription of *clpS*, a gene specifying a protease adaptor that delivers substrates PhoP and Oat to ClpAP, but is not itself degraded by ClpAP. (*b*) PhoP is a transcriptional activator of the *mgtCBRUcigR* operon. The MgtC protein is an adaptor competitor that sequesters PhoP away from ClpS, preventing ClpS from delivering PhoP to ClpAP. MgtR is a protease adaptor that delivers the MgtB protein to FtsH. MgtU is a substrate protector that binds to MgtB and prevents its degradation by FtsH, even in the absence of MgtR. (*c*) PhoP is a transcriptional activator of *iraP*, a gene specifying an antiadaptor of the adaptor MviA, which delivers the RpoS protein to ClpXP. (*d*) PhoP is a transcriptional activator of *qad*, a gene specifying an acetyl-CoA-binding protein required for acetylation of the HspQ protein by the protein acetyl transferase Pat. HspQ is a Lon substrate that enhances the proteolysis of the Lon substrate Hha. Both HspQ and acetylated HspQ (Ac-HspQ) are antiadaptors of the adaptor ClpS. Though not shown in the figure, the protease adaptor MgtR delivers the MgtC and MgtA proteins to FtsH for degradation. The dashed outlines represent degraded proteins. Ovals represent protease substrates, except for Qad, which is not a protease substrate. A hexagon represents a protease substrate that is also a protease enhancing factor. Rounded rectangles are used for the proteases ClpAP, ClpXP, Lon, and FtsH, and sharp-cornered rectangles represent substrates. Other shapes represent protease adaptors, antiadaptors, adaptor competitors, or substrate competitors. The circle represents an enzyme promoting protein acetylation.

the HspQ protein (129). In addition, low Mg^{2+} favors protein acetylation in *E. coli* (26). Acetylation of HspQ prevents HspQ from stimulating the proteolysis of the Lon substrate Hha (128).

Third, PhoP promotes transcription of genes that specify antiadaptors for MviA (RssB or SprE in *E. coli*), which is the adaptor that delivers the alternative sigma factor RpoS to the protease ClpXP. In *S.* Typhimurium, PhoP promotes transcription of the antiadaptor-specifying *iraP* gene (115) (**Figure 3**). The deduced amino acid sequence of the *S.* Typhimurium *iraP* gene shares 83% amino acid identity with the deduced amino acid sequence of the *E. coli iraP* gene, which also functions as an antiadaptor (13). However, *S.* Typhimurium and *E. coli* differ in that (*a*) PhoP does not promote *iraP* transcription in *E. coli*; (*b*) PhoP promotes transcription of *iraM*, an *E. coli*–specific gene that specifies an antiadaptor for MviA despite sharing no amino acid similarity with IraP (13); and (*c*) the *S.* Typhimurium *iraP* mRNA harbors an unusually long leader region that is absent from the *E. coli iraP* leader. That is, *E. coli* and *S.* Typhimurium rely on different antiadaptors to stabilize RpoS under low Mg^{2+} conditions.

And fourth, the PhoP-activated *mgtCBRUcigR* operon is a unique example of an operon that specifies a substrate, an adaptor required for proteolysis of that substrate, and a protein that protects that very same substrate from proteolysis (132) (**Figure 3**). The protease adaptor MgtR enables the inner membrane protease FtsH to proteolyze MgtB (132), as well as MgtC (4) and MgtA (21), the latter being encoded somewhere else in the genome. MgtU is a small protein that protects MgtB, but neither MgtC nor MgtA, from FtsH (132). Although the *mgtU* gene is located immediately downstream of the *mgtR* gene, inactivation of *mgtU* results in lower MgtB amounts than in the wild-type strain after 24 h in low-Mg^{2+} conditions but not after 6 h; by contrast, inactivation of *mgtR* results in higher MgtB amounts than in the wild-type strain at both times (132). This behavior may reflect the abundance and affinity of MgtR and MgtU for their targets as well as the abundance and affinity of the targets for other proteins. For example, MgtC binds to the MgtR (4), PhoP (133), CigR (131), and MgtB (69) proteins. Thus, under very low Mg^{2+} conditions, Mg^{2+} uptake is strictly dependent on MgtB because MgtA is degraded (21) and CorA is not operational due to the reversal of membrane potential (5). This is why cultures of *mgtB* and *mgtU* mutants reach a lower optical density than wild-type or *mgtA* mutant bacteria upon extended incubation in low Mg^{2+} conditions (132).

As discussed above, MgtS is a PhoP-activated protein that increases the cytoplasmic Mg^{2+} concentration by stabilizing MgtA in *E. coli* (122). PhoP does not activate MgtS in *S.* Typhimurium, preventing the MgtA stabilization seen in *E. coli* (132). *Salmonella* might have done away with the PhoP control of MgtS expression upon horizontal acquisition of the *mgtB*, *mgtR*, and *mgtU* genes, which enables it to utilize two different Mg^{2+}-importing ATPases, MgtA and MgtB, depending on the degree of Mg^{2+} limitation (132) and presence of additional signals (48).

Lon proteolyzes the gene-silencing protein H-NS (heat-stable, nucleoid structuring) in a PhoP-dependent manner (23). In vitro, Lon does not degrade H-NS when H-NS is bound to DNA (23). By displacing H-NS from DNA, PhoP renders H-NS a Lon substrate (23). For reasons that are presently unclear, H-NS proteolysis in vivo takes place when *S.* Typhimurium experiences a mildly acidic pH but not low Mg^{2+} even though both conditions result in a similar activation of the PhoP protein (23).

Growth with Very Low Cytoplasmic Mg^{2+} Concentration and Bacterial Survival Inside Slc11a1 Macrophages

Slc11a1 is a macrophage protein that localizes to the phagosomal membrane and confers resistance to different intracellular pathogens that remain within a mildly acidic phagosome (123). While Slc11a1 is believed to be a divalent cation transporter, direct biochemical evidence for the identity of the transported cation(s) and the direction of transport (i.e., from inside the

phagosome to the cytoplasm or in the opposite direction) is missing. Slc11a1 causes Mg^{2+} starvation in *S.* Typhimurium and promotes expression of the Mg^{2+} transporter MgtB when *S.* Typhimurium is in host tissues (31).

The Slc11a1 protein may operate as a Mg^{2+} transporter or control trafficking in the macrophage in ways that impact Mg^{2+} availability to the pathogen present inside a phagosome. In support of the first possibility, two genes from the bacterium *Clostridium acetobutylicum* that specify proteins distantly related to Slc11a1 support growth when expressed in a *B. subtilis* mutant in which all known Mg^{2+} transporters have been deleted (108). In addition, high Mg^{2+} represses expression of these two genes in *C. acetobutylicum* (108).

The *mgtB* gene is required for *S.* Typhimurium virulence in mice (20) and survival inside macrophages with a functional Slc11a1 protein, and for growth in very low-Mg^{2+} laboratory media (132). The small protein MgtU binds to the Mg^{2+} transporter MgtB and protects it from degradation by FtsH (132). Thus, MgtU is also necessary for survival inside macrophages with a functional Slc11a1 protein and for growth in very low-Mg^{2+} laboratory media (132), like MgtB. The acquisition of the *mgtB* and *mgtU* genes enables *S.* Typhimurium to survive in hosts with a functional *Slc11a1* gene (12).

Changing the Form of Motility

PhoP inhibits flagellum-mediated motility by reducing flagellar abundance (1). By contrast, PhoP promotes a flagellum-independent form of motility when *S.* Typhimurium experiences low cytoplasmic Mg^{2+} (84). This motility requires the *mgtA*- and *mgtC*-dependent, *Salmonella*-specific *pagM* gene (84). PagM is an inner membrane protein that promotes group motility and is necessary for *S.* Typhimurium replication in semisolid agarose media of low Mg^{2+} but dispensable in low-Mg^{2+} liquid media (84). The PagM-mediated motility may allow *S.* Typhimurium to scavenge nutrients when experiencing environments that cause low cytoplasmic Mg^{2+}.

RECRUITMENT OF OTHER REGULATORY SYSTEMS IN RESPONSE TO CYTOPLASMIC Mg^{2+}

Activation of the PhoB/PhoR System When Cytoplasmic Mg^{2+} Concentration Is Low

Classical studies on the low cytoplasmic Mg^{2+} response in gram-negative bacteria have historically focused on regulon control by the PhoP/PhoQ system (45), inactivation of which hinders growth in low-Mg^{2+} media (40). The identification and early characterization of PhoP-regulated genes suggested a connection between PhoP/PhoQ and phosphate homeostasis. The first-identified PhoP-activated gene—*phoN*—specifies a periplasmic nonspecific acid phosphatase (57), and the second one that was identified—*psiD*, later renamed *pmrC* (47)—was originally said to be transcriptionally induced upon phosphate starvation (36). We now know that bacteria experiencing low cytoplasmic Mg^{2+} activate PhoB/PhoR, a two-component system that mediates the response to phosphate limitation in several gram-negative bacteria (41).

The PhoB/PhoR system consists of the membrane-bound sensor PhoR and its cognate regulator, PhoB. The latter is a direct transcriptional activator of genes mediating uptake and scavenging of phosphate; uptake and metabolism of alternative phosphorus sources, such as phosphonates; and uptake of organophosphates, such as *sn*-glycerol-3-phosphate (41). Despite lacking a large extracytoplasmic domain, PhoR was believed to respond to low extracytoplasmic phosphate (55). However, PhoR is likely to respond to a cytoplasmic signal, because the PhoB regulon is activated when bacteria experience a decrease in cytoplasmic Mg^{2+} below a certain threshold even in the presence of millimolar concentrations of phosphate (89).

The PhoB/PhoR activation taking place when cytoplasmic Mg^{2+} is low has been ascribed to a reduction in the number of functional ribosomes dramatically decreasing ATP consumption. This is because protein synthesis is directly correlated with the number of ribosomes and the cellular activity that utilizes most energy; thus, a reduction in ribosomes decreases the rate of protein synthesis, thereby decreasing the amount of free cytoplasmic phosphate (89). Low cytoplasmic Mg^{2+} activates PhoB/PhoR only transiently, lasting until the MgtA and MgtB proteins are made and start importing Mg^{2+} so that some ribosomes can be assembled, protein synthesis returns, ATP is consumed, and free phosphate is generated. MgtC, by contrast, activates PhoB/PhoR by inhibiting phosphate import by a yet to be identified phosphate transporter (16), thereby reducing the cytoplasmic phosphate concentration (89).

The PhoB/PhoR activation triggered when cytoplasmic Mg^{2+} is low may reflect that cells coordinate the metabolism of phosphate and Mg^{2+} with ribosome activity. This physiological response signifies that Mg^{2+} is the counterion used to neutralize the negative charge conferred by phosphates to different biological molecules, including ATP, GTP, and the rRNA backbone. That PhoB/PhoR activation is due to decreased protein synthesis lowering phosphate liberation from NTPs is further supported by the following: (*a*) Protein synthesis inhibitors activate PhoB/PhoR in both *S.* Typhimurium and *E. coli* even in the presence of millimolar phosphate concentrations (89); (*b*) increases in adenine nucleotide pools trap phosphate as ATP, thereby activating PhoB/PhoR even when phosphate is abundant in the growth medium (125); and (*c*) (p)ppGpp prevents PhoB/PhoR activation by inhibiting adenine biosynthesis when environmental phosphate is low (102), which alleviates the consumption of free cytoplasmic phosphate (89).

It was reported that MgtC directly activates PhoR even when phosphate is not limited, resulting in increased phosphate uptake and creating a population of nonreplicating bacteria when *S.* Typhimurium is inside macrophages (24). These results are puzzling given that phosphate is highly toxic to bacteria experiencing cytoplasmic Mg^{2+} starvation (16), a condition that triggers MgtC expression (29, 110). In addition, PhoP/PhoR activation precedes MgtC expression in bacteria experiencing cytoplasmic Mg^{2+} starvation (16, 89), and, as discussed above, MgtC inactivates PhoB/PhoR (16, 89).

The physiological connection between Mg^{2+} and phosphate is supported by several independent findings. First, bacterial genes involved in the importation or metabolism of phosphate are often found near those participating in Mg^{2+} homeostasis (89), which is anticipated to favor their coacquisition during horizontal gene transfer events. And second, PhoP-induced MgtS has been shown to bind to the PitA phosphate symporter in *E. coli* (134). MgtS binding to PitA is proposed to hinder Mg^{2+} leakage via PitA in bacteria experiencing low cytoplasmic Mg^{2+} (134). Because PhoP does not promote *mgtS* expression in *S.* Typhimurium (132), PitA may not be stabilized when cytoplasmic Mg^{2+} is low in this species.

In sum, the exceptionally far-reaching nature of cellular adaptations elicited during cytoplasmic Mg^{2+} starvation (e.g., slowdown of translation) requires adjustments to a regulatory system—PhoB/PhoR—that does not respond directly to Mg^{2+}. By contrast, low phosphate does not trigger a response to low cytoplasmic Mg^{2+}. That is, low cytoplasmic Mg^{2+} activates both the PhoP/PhoQ and PhoB/PhoR systems, but low phosphate activates only PhoB/PhoR (89).

Activation of General and Specific Stress Systems When Cytoplasmic Mg^{2+} Concentration Is Low

RpoS is an alternative sigma factor that accumulates when bacteria experience a variety of unfavorable nutritional and growth conditions, allowing it to reprogram RNA polymerase and modify expression of ~500 genes in *E. coli* and related bacteria (44, 103). The output, termed the general stress response, confers resistance to numerous stresses regardless of the nature of the initial input.

Low cytoplasmic Mg^{2+} stabilizes RpoS by promoting the PhoP-dependent expression of IraP in *S.* Typhimurium (115) (**Figure 3**) and IraM in *E. coli* (13). These proteins are antiadaptors for MviA, the adaptor that delivers RpoS to the ClpXP protease for degradation (44). The resulting RpoS accumulation taking place when cytoplasmic Mg^{2+} is low activates the RpoS-dependent general stress response, which is manifested in multiple ways, including resistance to H_2O_2 mediated by the RpoS-activated catalase gene *katE* (115).

SirA is a response regulator that forms a two-component phosphorelay system with the sensor BarA (135). SirA promotes expression of the *csrB* and *csrC* genes, which specify highly similar regulatory RNAs that titrate the RNA-binding protein CsrA, thereby preventing CsrA from regulating (some of) its targets (98). SirA also activates the response regulator RcsB by controlling expression of the *rcsDB* operon and via the sRNAs CsrB and CsrC (101).

Low cytoplasmic Mg^{2+} stabilizes the response regulator SirA by decreasing the amounts of the protease adaptor ClpS in a *phoP*-dependent manner (127) (**Figure 3**). The SirA stabilization results in increased amounts of both CsrB and CsrC (127). Given the large number of CsrA targets (98), SirA stabilization is anticipated to cause genome-wide effects.

In sum, there are several potential mechanisms by which low cytoplasmic Mg^{2+} can increase RpoS abundance. First, PhoP increases the amount of the antiadaptor IraP in *S.* Typhimurium and IraM in *E. coli*, which sequester the adaptor MviA, thereby decreasing RpoS proteolysis by ClpXP. Second, PhoP stabilizes SirA by decreasing ClpS amounts, which may stabilize RpoS by increasing the amount of IraD. This is because the SirA-activated CsrB and CsrC RNAs titrate CsrA, which is an inhibitor of *iraD* translation (80). And third, SirA activates RcsB, a direct transcriptional activator of the *rprA* gene, which specifies a regulatory RNA that stimulates *rpoS* translation (73). These mechanisms are in addition to the PhoP-dependent decrease in ATP concentration, which hinders proteolysis of RpoS because ClpXP is an ATP-dependent protease (130).

Potential Regulation of Metabolite-Responding Riboswitches

Physiological concentrations of Mg^{2+} have been shown to modify the in vitro conformation of riboswitches that exhibit exquisite specificity for several metabolites, including adenosyl-cobalamine (25), *S*-adenosyl-methionine (99), and lysine (76). These findings raise the possibility of changes in cytoplasmic Mg^{2+} concentrations impacting metabolic pathways by altering expression of genes controlled by these riboswitches. Such genes would respond in vivo to a combination of Mg^{2+} and metabolite amounts.

DIFFERENT MICROBES USE CONSERVED RESPONSES TO LOW CYTOPLASMIC Mg^{2+}

Key aspects of the response to low cytoplasmic Mg^{2+} are conserved across microbial species. This conservation likely reflects the widespread use of ATP as energy currency and of Mg^{2+} as a stabilizer of ribosomes and other cellular components. In addition, the PhoP/PhoQ system is involved in the Mg^{2+} starvation response in multiple gram-negative bacteria, both within and outside of the *Enterobacteriaceae* family (45). These include *E. coli*, *Shigella flexneri*, *Yersinia enterocolitica*, and *Yersinia pestis*. In all these species, the PhoP/PhoQ system is subject to positive autoregulation, controls the expression of one or more Mg^{2+} transporter–specifying genes, and is critical for growth in low Mg^{2+} conditions (46, 87). Although the identity of the Mg^{2+} transporter activated by PhoP often differs across bacterial species, PhoP invariably controls one or more Mg^{2+} transporter genes in species with a PhoQ protein competent in Mg^{2+} sensing (87, 91).

In *B. subtilis*, the Mg^{2+} channel MgtE is degraded by the intramembrane proteases YqgP and FtsH when YqgP detects toxic metals such as Mn^{2+} in the cytoplasm (11). By degrading MgtE in

Table 1 Proposed mobilization of Mg^{2+} from storage sites to the cytoplasm in bacteria and yeasts

Organisms	Storage cite	Mg^{2+} transporter(s)
Gram-negative bacteria	Lipopolysaccharide	MgtA, MgtB, CorA
Gram-positive bacteria	Teichoic acids	MgtE, CorA
Yeasts	Intracellular vacuoles	Mnr2

low Mg^{2+} conditions, *B. subtilis* avoids the uptake of toxic metals that are otherwise outcompeted by Mg^{2+} when this cation is in high abundance (11). A different strategy is used to achieve the same goal in *S.* Typhimurium, which uses the PhoP-dependent reversal of membrane potential (5) to avoid toxicity by metal ions internalized by the Mg^{2+} channel CorA (18). This is the reason why *corA* inactivation overcomes the Fe^{2+} hypersusceptibility of a *phoP* mutant experiencing low Mg^{2+} (18).

The mechanisms of maintaining Mg^{2+} homeostasis extend far beyond the bacterial domain. The yeast *Saccharomyces cerevisiae* is one of the best-studied eukaryotic systems with respect to Mg^{2+} homeostasis. While a significant amount of cellular Mg^{2+} is found in the cytoplasm of *S. cerevisiae*, the highest Mg^{2+} concentrations are inside organelles, allowing for the development of Mg^{2+} response pathways not found in prokaryotes (88). Under non-Mg^{2+}-limited conditions, *S. cerevisiae* sequesters and stores excess Mg^{2+} in intracellular vacuoles that accumulate Mg^{2+} to a concentration fourfold higher than that of the cytoplasm (88). When cytoplasmic Mg^{2+} is low, Mg^{2+} is released from the vacuoles via the Mg^{2+} transporter Mnr2, a sequelog of the CorA transporter found in bacteria and archaea, to replenish the Mg^{2+} ion pool. In a similar manner, the anionic metabolite L-lactate acid has recently been shown to promote the release of Mg^{2+} from endoplasmic reticulum stores, allowing Mg^{2+} importation by mitochondria in several mammalian cells (33). These mechanisms bear similarities to those operating in gram-negative and gram-positive bacteria, in which covalent modifications in the cell wall are proposed to release Mg^{2+} to be transported into the cell in order to support functions in the cytoplasm (**Table 1**). Therefore, while the participating proteins and sources of liberated Mg^{2+} differ, accessing extracytoplasmic reservoirs of Mg^{2+} is a shared adaptation for maintaining cytoplasmic Mg^{2+} homeostasis in both prokaryotic and eukaryotic microorganisms.

Likewise, phosphate acquisition systems are transcriptionally induced in both bacteria and yeasts upon pharmacological inhibition of protein synthesis (89). Because Mg^{2+} is essential for ribosome assembly in both prokaryotes and eukaryotes, yeasts are likely to induce phosphate acquisition systems if the cytoplasmic Mg^{2+} concentration decreases below the threshold where ribosome assembly becomes compromised, as established in *S.* Typhimurium and *E. coli* (89).

CONCLUDING REMARKS

Maintaining the cytoplasmic Mg^{2+} concentration at ~1 mM enables microbes to carry out protein synthesis. Thus, a decrease in Mg^{2+} amounts that compromises ribosome assembly results in cellular adaptations aimed at maintaining the proteome. This occurs by reducing ATP-dependent proteolysis systemically and by promoting the expression of Mg^{2+} importers, which, by tapping into Mg^{2+} reservoirs, allow some ribosome assembly and protein synthesis to take place (**Figure 2**). In this vein, proteins that participate in the folding of misfolded proteins may also participate in the adaptation to low cytoplasmic Mg^{2+}.

Although Mg^{2+} starvation has multiple detrimental consequences, it can alter metabolic pathways in ways that favor the production of metabolites of commercial interest. For instance, Mg^{2+}

starvation of an engineered strain of *E. coli* favors the accumulation of malonyl-CoA, which is used in the synthesis of a precursor of plant flavonoids incorporated into food and cosmetics (114). Therefore, understanding the cellular adaptations to low Mg^{2+} also provides strategies to improve human well-being.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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